

Susceptibility of Cyclooxygenase-2-Deficient Mice to Pulmonary Fibrogenesis

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The cyclooxygenase (COX)-2 enzyme has been implicated as an important mediator of pulmonary fibrosis. In this study, the lung fibrotic responses were investigated in COX-1 or COX-2-deficient (-/-) mice following vanadium pentoxide (V₂O₅) exposure. Lung histology was normal in saline-instilled wild-type and COX-deficient mice. COX-2^{-/-}, but not COX-1^{-/-} or wild-type mice, exhibited severe inflammatory responses by 3 days following V₂O₅ exposure and developed pulmonary fibrosis 2 weeks post-V₂O₅ exposure. Western blot analysis and immunohistochemistry showed that COX-1 protein was present in type 2 epithelial cells, bronchial epithelial cells, and airway smooth muscle cells of saline or V₂O₅-exposed wild-type and COX-2^{-/-} mice. COX-2 protein was present in Clara cells of wild-type and COX-1^{-/-} terminal bronchioles and was strongly induced 24 hours after V₂O₅ exposure. Prostaglandin (PG) E₂ levels in the bronchoalveolar lavage (BAL) fluid from wild-type and COX-1^{-/-} mice were significantly up-regulated by V₂O₅ exposure within 24 hours, whereas PGE₂ was not up-regulated in COX-2^{-/-} BAL fluid. Tumor necrosis factor- α was elevated in the BAL fluid from all genotypes after V₂O₅ exposure, but was significantly and chronically elevated in the BAL fluid from COX-2^{-/-} mice above wild-type or COX-1^{-/-} mice. These findings indicate that the COX-2 enzyme is protective against pulmonary fibrogenesis, and we suggest that COX-2 generation of PGE₂ is an important factor in resolving inflammation. (*Am J Pathol* 2002, 161:459-470)

Prostaglandins (PG) are important mediators of normal tissue homeostasis, yet their aberrant production may be linked to the pathobiology of a variety of inflammatory diseases. Two distinct enzymes termed cyclooxygenase

(COX)-1 and COX-2 are encoded by genes (*Pgbs-1* and *Pgbs-2*) located on different chromosomes. COX-1 and COX-2 convert arachidonic acid (AA) to PGH₂, which is acted on by various prostaglandin synthases to give rise to the individual bioactive PG species.¹ COX-1 is constitutively expressed in a variety of tissues including the lung. COX-2 is also constitutively expressed in lung, but is highly inducible and up-regulated by several proinflammatory cytokines.^{1,2} While the role of COX-1 in inflammation is largely unknown, COX-2 appears to play a role in the inflammatory process and has been implicated as a mediator of various inflammatory diseases.

Pulmonary fibrosis is a disease characterized by the proliferation of lung fibroblasts and subsequent collagen deposition by these cells. COX-2 has been implicated as a potentially important mediator in the fibrogenic process. Cultured lung fibroblasts isolated from patients with pulmonary fibrosis have a diminished capacity to express COX-2 and these fibrotic fibroblasts synthesize less PGE₂.^{3,4} We previously showed that platelet-derived growth factor (PDGF)-induced proliferation of lung myofibroblasts was inhibited by PGE₂, and this was due in part to PGE₂-stimulated down-regulation of the PDGF α -receptor subtype.⁵ PGE₂ has also been reported to inhibit transforming growth factor- β (TGF- β)-stimulated increases in α 1-collagen and connective tissue growth factor (CTGF) mRNAs.^{6,7} Accordingly, PGE₂ has been described as an anti-fibrotic factor. Nevertheless, the relative importance of COX-2 and PGE₂ to the progression of pulmonary fibrosis has not been clearly established.

Mice with disrupted *Pgbs-1* or *Pgbs-2* genes have been generated using gene-targeting strategies,^{8,9} and the characteristics of these mice have been reviewed.¹⁰ COX-1-deficient (COX-1^{-/-}) mice exhibit reduced AA-induced ear inflammation, whereas COX-2^{-/-} mice had normal inflammatory responses to AA.^{8,9} Recently, Gavett and co-workers² investigated the allergic lung responses in COX-1^{-/-} and COX-2^{-/-} mice following ovalbumin challenge. They reported that allergen-induced pulmonary inflammation and airway hyper-responsiveness were greater in COX-deficient mice compared to wild-type (WT) mice, although the COX-1^{-/-} mice had a greater inflammatory response than COX-2^{-/-} mice.

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In this study, we investigated the inflammatory and fibrotic responses of COX-deficient mice following a single intratracheal instillation of vanadium pentoxide (V_2O_5), a transition metal released from the industrial burning of fuel oil that causes bronchitis and airway remodeling in humans and rats.^{11,12} In wild-type mice, V_2O_5 caused a lung inflammatory response that resolved within days after exposure. COX-1^{-/-} mice also resolved lung inflammation following V_2O_5 exposure. In contrast, COX-2^{-/-} mice did not resolve lung inflammation in response to V_2O_5 , and fibrotic lesions developed within two weeks following exposure. PGE₂ levels in the bronchoalveolar lavage (BAL) fluid from wild-type and COX-1^{-/-} mice were significantly up-regulated by V_2O_5 exposure, whereas PGE₂ in BAL fluid from COX-2^{-/-} mice were not significantly elevated. Tumor necrosis factor- α (TNF- α) levels in the BAL fluid of V_2O_5 -exposed COX-2^{-/-} mice were significantly higher than in COX-1^{-/-} or wild-type mice. These data suggest that COX-2 has important anti-inflammatory functions that protect against pulmonary fibrosis and that the susceptibility of COX-2^{-/-} mice to lung fibrosis correlates with increased TNF- α expression.

Materials and Methods

Experimental Animals

All animal studies were conducted in accordance with principles and procedures outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the NIEHS Animal Care and Use Committee. Pathogen-free wild-type, COX-1^{-/-}, and COX-2^{-/-} mice were obtained from the breeding colony at NIEHS. They were housed under identical conditions and fed NIH 31 rodent chow (Agway, St. Mary, OH) *ad libitum*. All mice were of a hybrid C57BL/6J \times 129/Ola genetic background, intercrossed for at least twenty generations. Mice were genotyped by PCR using DNA isolated from tail pieces as described.^{8,9}

Experimental Design for Intratracheal Instillation of V_2O_5

Male and female mice, 6 to 8 months old, weighing 20 to 35 g, were used. Within each experimental group, the sex ratio was approximately equal. V_2O_5 suspensions (10 mg/ml) were vortexed thoroughly, then sonicated for 30 minutes at 25°C before instillation. Mice were instilled with 50 μ l of saline alone or 1 mg/kg V_2O_5 in saline.¹³ At days 1, 3, 6, or 15 days following instillation, the lungs were lavaged for collection of BAL fluid as described below, then removed *en bloc* and inflated with formalin for histopathology. In some experiments, the left lung was ligated and removed for hydroxyproline assay or COX immunoblotting as described below.

Lung Histopathology

Evaluation of histopathology was done on lungs that were not lavaged. Lungs were perfusion fixed with 10% neutral

buffered formalin, processed routinely, and embedded in paraffin. Serial sections (5–6 μ m) were stained with either hematoxylin and eosin or Masson's trichrome. The histopathology scoring system was based on the quantitative method previously published by Cherniack and co-workers.¹⁴ Using this system, scales ranging from 0 to 5 were used to describe the two different components of the pathological process. The parameter defined as *q* expressed the numbers of different types of inflammatory cells (polymorphonuclear cells, lymphocytes, eosinophils, monocytes/macrophages, and multinucleated giant cells) infiltrating the alveoli. The evaluation was based on the following arbitrary grades of severity: 0 = no inflammatory cell infiltration; 1 = 1–2 cells per alveolus; 2 = 3–4 cells per alveolus; 3 = 5–8 cells per alveolus; 4 = 9–11 cells per alveolus; 5 = more than 12 cells per alveolus.

The parameter defined as *a* expressed the proportion, or relative area, of the lung tissue showing inflammation and included these grades: 0 = no damage detected; 1 = 1–3% of total lung area; 2 = 4–15% of total lung area; 3 = 16–40% of total lung area; 4 = 41–75% of total lung area; 5 = 76–100% of total lung area.

The scoring was done by blind evaluation without knowing the genotype or treatment.

Hydroxyproline Assay

The procedure for quantitation of lung hydroxyproline has been described elsewhere.¹⁵ Whole lung tissue was washed in phosphate-buffered saline (PBS) and hydrolyzed for 18 hours in 6N HCl at 110°C (~40 ml/6 g of tissue). 1 drop of 1% phenolphthalein in ethanol was added to each sample and the pH adjusted to 6.0 with NaOH titration. Two ml from each sample was centrifuged 5 minutes at 1500 rpm and the pellet oxidized with 1 ml of 0.6 mol/L Chloramine-T for 30 minutes. Each sample then received 1 ml of 7.5% *p*-dimethylaminobenzaldehyde and was incubated at 65°C for 15 minutes. The absorbance was measured at 560 nm on a spectrophotometer. Lung hydroxyproline was quantitated against a standard curve set up with purified hydroxyproline (Sigma, St. Louis, MO) and values were corrected for total lung wet weight.

Western Blotting

Analysis of COX-1 and COX-2 protein levels in lung homogenates was performed by Western blot analysis as described previously.² Whole lung lysates were prepared from frozen lung tissues by homogenization in a buffer containing 50 mmol/L Tris-HCl (pH 7.4), 1% Triton X-100, 150 mmol/L NaCl, 1 mmol/L EGTA, 0.25% sodium deoxycholate, 1 mmol/L NaF, 0.25 mol/L PMSF, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mg/ml pepstatin, and 100 mmol/L Na₃VO₄. Goat anti-mouse COX-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and rabbit anti-mouse COX-2 (Cayman Chemical Co., Ann Arbor, MI) were specific for their respective COX isoforms and used according to the manufacturers' instructions. Recombi-

nant COX-1 and COX-2 protein standards were prepared as described by Chulada et al and were used to confirm that anti-COX-1 only recognized recombinant COX-1 and anti-COX-2 only recognized recombinant COX-2.¹⁶ For immunoblotting, proteins were resolved by electrophoresis in 10% SDS (w/v) polyacrylamide gels (Novex, San Diego, CA) and transferred to nitrocellulose membranes. Membranes were immunoblotted using the primary COX-1 or COX-2 antibodies (1:1000 dilution) and then either goat anti-rabbit or rabbit anti-goat IgG conjugated to horseradish peroxidase (1:2000 dilution) (Bio-Rad Laboratories, Richmond, CA). Western blots were developed using the ECL Western Blotting Detection System (Amersham International, Buckinghamshire, UK). Densitometry of the COX-1 and COX-2 protein bands was performed using the NIH Image Program (National Institutes of Health, Bethesda, MD).

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded lung tissue. Six- μ m lung sections were de-paraffinized with xylene, progressively re-hydrated in decreasing percentages of ethanol, and immersed in 3% hydrogen peroxide to degrade any endogenous peroxidases. Antigen sites were retrieved by heating the sections on slides in 0.01 mol/L sodium citrate in a microwave oven and cooling for 20 minutes to room temperature. Sections were placed in a humidity chamber and incubated in a blocking solution (anti-goat IgG, Vectastain Elite Kit) for 1 hour at room temperature. All antibodies described here after were diluted in 1X automation buffer (Biomedica Corp., Foster City, CA) containing 1% bovine serum albumin (Sigma). Sections were incubated overnight with primary antibody (rabbit anti-mouse COX-2 polyclonal antibody, 1:5000, or rabbit anti-mouse COX-1 polyclonal antibody, Cayman Chemical). A streptavidin-biotin affinity system (Vectastain Elite ABC Kit, Rabbit IgG, Vector Laboratories, Burlingame, CA) was used as the detection system. Tissues were incubated with biotinylated secondary rabbit IgG at room temperature for 30 minutes, washed three times with 1X automation buffer and incubated with ABC complex for 30 minutes. COX-1 or COX-2 were visualized by the addition of 3,3'-diaminobenzidine for 5 minutes. Tissues were counterstained with hematoxylin. Immunohistochemistry was also used to verify cell types staining for COX-1 or COX-2. Type 2 cells were identified using a goat anti-SP-A (surfactant protein A, C-20) polyclonal antibody at a dilution of 1:10 (Santa Cruz Biotechnology), Clara cells were identified using a goat anti-CC10 (Clara cell 10 kd protein, T-18) polyclonal antibody at a dilution of 1:50 (Santa Cruz Biotechnology), and macrophages were identified with a rat anti-mouse antibody against F4/80 (a 160-kd glycoprotein expressed by murine macrophages) at a dilution of 1:50 (Serotec, Raleigh, NC). The methodology for detection of cell-specific markers was essentially the same as that for COX-1 and COX-2 immunostaining, except the secondary antibodies used for SP-A and CC10 immunostaining was donkey anti-goat

IgG at a dilution of 1:500 (Jackson ImmunoResearch Laboratories, West Grove, PA), and the secondary antibody used for F4/80 immunostaining was goat anti-rat IgG (Serotec).

Collection of Bronchoalveolar Lavage Fluid and Immunoassays for Prostanoids and Cytokines

Bronchoalveolar lavage fluid was collected from wild-type and COX null lungs at 1, 3, 6, and 15 days postinstillation. Lungs were lavaged with three 1-ml aliquots of sterile saline. Approximately 90% of the total injected volume was consistently recovered. The BAL fluid was placed on ice and centrifuged at $360 \times g$ for 10 minutes at 4°C. BAL cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) and counted using a hemocytometer. An aliquot of the suspension was taken for preparation of differential slides of BAL cells (Cytospin 3, Shandon, Pittsburgh, PA), which were then stained with Leuko-Stat (Fisher Scientific, Pittsburgh, PA), and differentiated using conventional morphological criteria for macrophages/monocytes, lymphocytes, neutrophils, and eosinophils. Aliquots of BAL fluid were assayed for prostanoids (PGE₂, PGD₂, and LTB₄) by enzyme immunoassay (Cayman Chemical Co.), or assayed for cytokines using commercially available ELISA kits (TNF- α , interleukin (IL)-8, TGF- β 1, PDGF-BB, IL-13). TNF- α ELISA was purchased from Endogen, Inc. (Woburn, MA). All other ELISA kits were purchased from R & D Systems (Minneapolis, MN).

Statistical Analysis

All values are expressed as mean \pm SEM. Data were analyzed by analysis of variance using SYSTAT software (SYSTAT Inc., Evanston, IL). When *F* values indicated that a significant difference was present, Fisher's LSD test for multiple comparisons was used. Values were considered significantly different if *P* was less than 0.05.

Results

COX-2^{-/-} Mice Exhibit Increased Lung Inflammatory Responses Following V₂O₅ Instillation

The lungs from wild-type, COX-1^{-/-}, and COX-2^{-/-} mice were histologically normal after saline instillation (Figure 1A, C, and E). V₂O₅ instillation caused a mild inflammatory response within the lung parenchyma and peribronchiolar regions of either wild-type or COX-1^{-/-} mice characterized primarily by the presence of mononuclear cells (Figure 1, B and D). In contrast to wild-type and COX-1^{-/-} mice, a marked inflammatory response was observed in the lungs of COX-2^{-/-} mice 3 days following V₂O₅ exposure characterized by infiltration of mononuclear cells and multinucleated giant cells (Figure 1F).

A histopathological examination of the formalin-fixed, paraffin-embedded lung sections from wild-type and

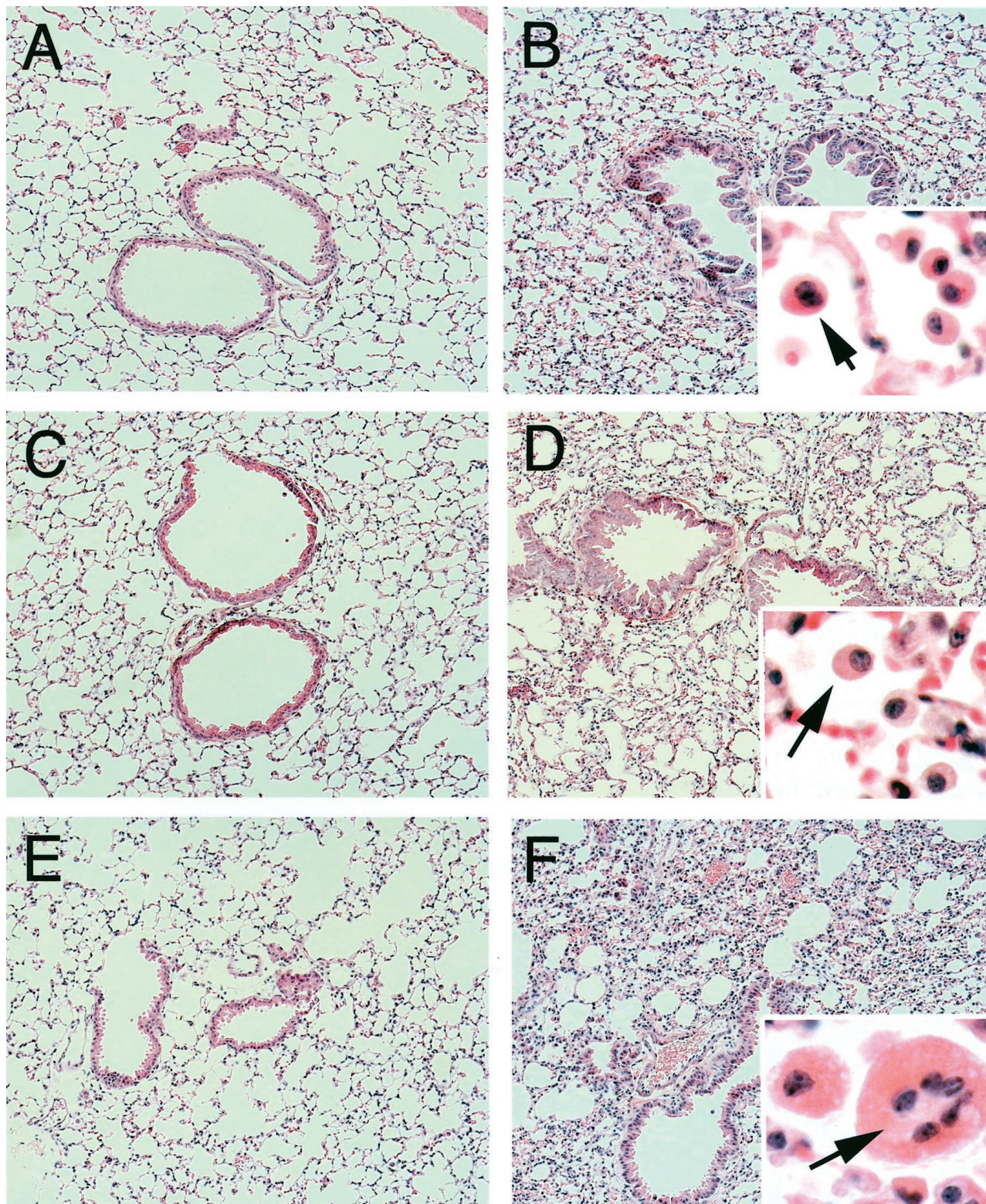


Figure 1. Lung histopathology showing increased injury and inflammation in COX-2^{-/-} mice relative to wild-type and COX-1^{-/-} mice 3 days following intratracheal instillation of V₂O₅. **A:** Saline-instilled wild-type. **B:** V₂O₅-instilled wild-type. **Inset** shows mononuclear cell infiltration (**arrow**). **C:** Saline-instilled COX-1^{-/-}. **D:** V₂O₅-instilled COX-1^{-/-}. **Arrow in inset** indicates mononuclear cell. **E:** Saline-instilled COX-2^{-/-}. **F:** V₂O₅-instilled COX-2^{-/-}. **Arrow in inset** indicates multinucleated giant cell amid numerous mononuclear cells. All sections were stained with hematoxylin and eosin. Magnification, ×80, except for insets which are ×400.

Table 1. Lung Inflammatory Scores in Saline- and V₂O₅-Instilled WT, COX-1^{-/-}, and COX-2^{-/-} Mice

Lesion	Saline			V ₂ O ₅		
	WT (4)	COX-1 ^{-/-} (5)	COX-2 ^{-/-} (6)	WT (7)	COX-1 ^{-/-} (5)	COX-2 ^{-/-} (6)
PMN leukocytes	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.85 ± 0.49*	0.00 ± 0.00	1.16 ± 0.40*
Mononuclear cells	1.25 ± 0.25	1.60 ± 0.25	1.60 ± 0.44	2.42 ± 0.46*	1.80 ± 0.00	3.50 ± 0.22*
Multinucleated giant cells	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.14 ± 0.15	0.00 ± 0.00	0.50 ± 0.34*
Total cellular score (q)	1.25 ± 0.25	1.60 ± 0.25	1.60 ± 0.44	3.44 ± 0.77*	1.80 ± 0.38	5.00 ± 0.69*†
% of alveoli affected (a)	2.00 ± 0.41	1.60 ± 0.25	2.00 ± 0.35	2.57 ± 0.29	2.00 ± 0.32	5.00 ± 0.78*†

Histopathological scoring system (0 to 5, where 5 is greatest inflammation) was based on types of inflammatory cells per alveoli (q) and % of alveoli affected in the lung tissue (a) as described in Materials and Methods. Data show the mean ± SEM. Numbers in parentheses below group name indicate number of animals in that group.

*P < 0.05, compared to saline control.

†P < 0.05, compared to WT or COX-1^{-/-}.

COX null mice was performed in a blinded fashion. This evaluation showed that the majority of inflammatory cells occupying the alveolar spaces following V₂O₅ exposure were mononuclear cells, with the remaining cells identified as neutrophils (Table 1). A small but consistent number of multinucleated giant cells were observed in COX-2^{-/-} mice 3 days after V₂O₅ exposure (Figure 1F, Table 1). In wild-type mice and COX-1^{-/-} mice, V₂O₅ instillation caused as much as a twofold increase in the overall histopathology score, which accounted for all of the parameters shown in Table 1. V₂O₅ instillation caused a three- to fourfold increase in the inflammatory score in COX-2^{-/-} mice.

COX-2^{-/-} Mice Develop Pulmonary Fibrotic Lesions and Have Increased Total Lung Hydroxyproline Following V₂O₅ Instillation

Wild-type mice and COX-1^{-/-} mice did not develop pulmonary fibrosis within the lung parenchyma (Figure 2, A–D). In contrast, trichrome staining showed that COX-2^{-/-} mice developed severe pulmonary fibrosis at 15 days post-V₂O₅ lung injury (Figure 2, E and F). Total lung collagen was increased twofold above saline-instilled counterparts only in the COX-2^{-/-} mice (Figure 3).

COX-2 Protein Levels Are Increased Following V₂O₅ Instillation, Whereas COX-1 Levels Were Not Increased

Western blot analysis of whole lung protein was performed to demonstrate a lack of the appropriate COX protein in null mice and to measure inducible COX protein at day 1 following V₂O₅ exposure. The specificity of COX antibodies was confirmed by using recombinant COX-1 or COX-2 proteins in Western blots (data not shown). Both COX-1 and COX-2 were detected in saline-treated wild-type mice (Figure 4). COX-2 protein was up-regulated threefold at day 1 after V₂O₅ exposure in wild-type mice, and was increased about twofold by V₂O₅ in COX-1^{-/-} mice. COX-1 was not increased by V₂O₅ in wild-type or COX-2^{-/-} mice. COX-1 and COX-2

were not detected in COX-1^{-/-} and COX-2^{-/-} mice, respectively (Figure 4).

Immunohistochemical Localization of COX-1 and COX-2 Following V₂O₅-Induced Lung Injury

Immunohistochemistry was performed at day 1 post-V₂O₅ instillation to determine which cell types were expressing COX-1 and COX-2 following V₂O₅-induced lung injury. In both saline- and V₂O₅-instilled wild-type mice, abundant COX-1 immunostaining was observed in type 2 epithelial cells, airway smooth muscle cells, and bronchiolar epithelial cells (Figure 5, A and B). The identity of type 2 cells was confirmed by immunostaining for SP-A (surfactant protein-A) (Figure 5A). No COX-1 immunostaining was observed in saline or V₂O₅-instilled COX-1^{-/-} mice (Figure 5, C and D). Saline-instilled COX-2^{-/-} mice had a similar pattern of COX-1 immunostaining compared to that of wild-type mice. The lungs of V₂O₅-exposed COX-2^{-/-} mice exhibited COX-1 staining within early inflammatory lesions and COX-1 was localized to type 2 cells, bronchial epithelial cells, and airway smooth muscle cells (Figure 5, E and F). In these V₂O₅-induced inflammatory lesions, some lung macrophages (identified by immunostaining for F4/80 antigen) contained both SP-A and COX-1 (Figure 5F). COX-2 immunostaining was weak in saline-instilled wild-type and COX-1^{-/-} mice and localized to Clara cells of the terminal bronchioles (Figure 6, A and C). The identity of COX-2 positive Clara cells was confirmed by immunostaining for CC10 (Clara cell 10 kd protein) (Figure 6, B and D). Following V₂O₅ instillation, intense COX-2 immunostaining was observed in Clara cells of wild-type and COX-1^{-/-} mice (Figure 6, B and D). No COX-2 immunostaining was observed in the lungs of COX-2^{-/-} mice (Figure 6, E and F).

Differential Production of PGE₂ in the Lungs of Wild-Type and COX-Deficient Mice Following V₂O₅ Instillation

BAL fluid from saline or V₂O₅-instilled wild-type and COX-2^{-/-} mice was analyzed for several prostanoids (PGE₂,

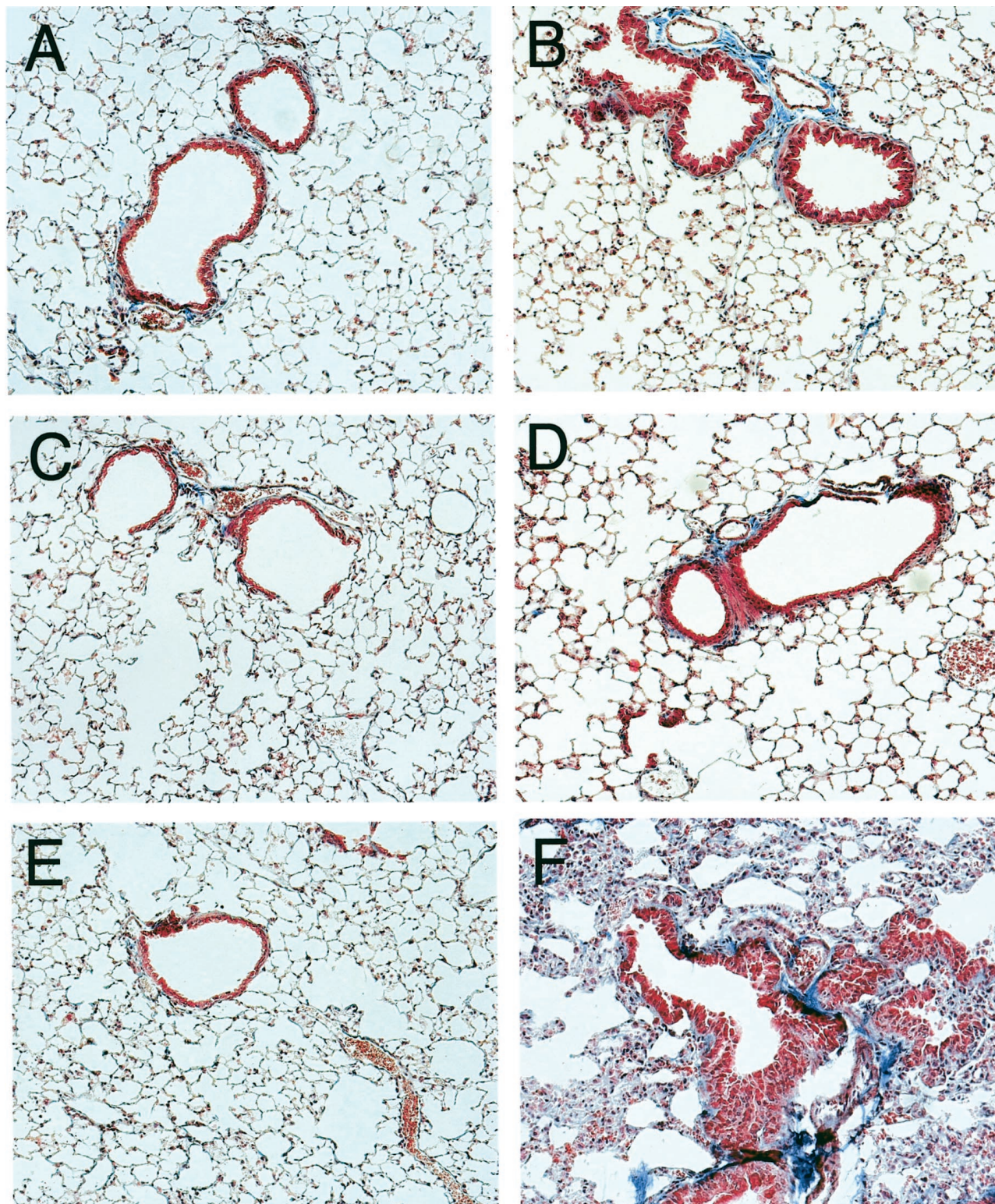


Figure 2. Trichrome staining of lung sections showing enhanced fibrosis in $COX-2^{-/-}$ mice relative to wild-type and $COX-1^{-/-}$ mice 15 days following intratracheal instillation of V_2O_5 . The magnification of each panel is $\times 80$. **A:** Saline-instilled wild-type. **B:** V_2O_5 -instilled wild-type. **C:** Saline-instilled $COX-1^{-/-}$. **D:** V_2O_5 -instilled $COX-1^{-/-}$. **E:** Saline-instilled $COX-2^{-/-}$. **F:** V_2O_5 -instilled $COX-2^{-/-}$.

PGD_2 , LTB_4) to gain mechanistic insight regarding the susceptibility of $COX-2^{-/-}$ to the fibrotic effects of V_2O_5 . PGD_2 and LTB_4 levels in BAL were increased by V_2O_5 instillation but were not statistically different among ge-

notypes (data not shown). The PGE_2 level in the BAL from saline-instilled wild-type mice was ~ 500 pg/ml and increased approximately twofold 24 hours after V_2O_5 instillation (Figure 7). However, V_2O_5 instillation did not cause

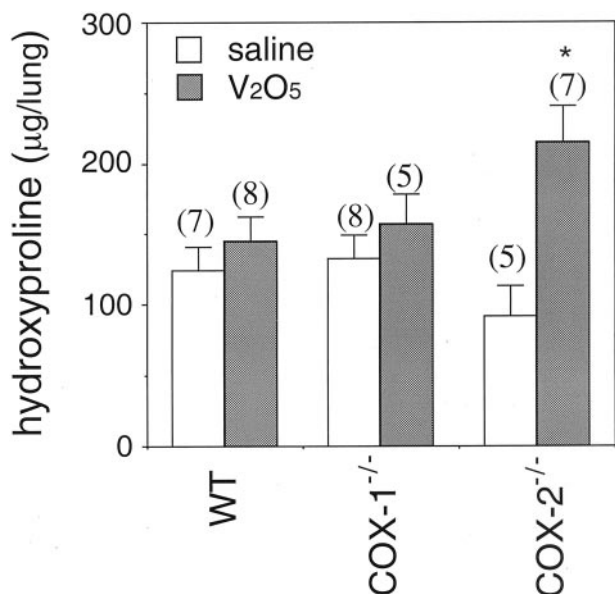


Figure 3. Hydroxyproline content in lungs of wild-type, COX-1^{-/-}, and COX-2^{-/-} mice instilled with saline or V₂O₅. Lungs were harvested 15 days after instillation and digested for hydroxyproline content using the colorimetric assay described in Materials and Methods. Numbers in parentheses above each bar indicate number of animals in that group. *, *P* < 0.05 compared to saline.

significant increases in BAL PGE₂ at days 3, 6, and 15 post-instillation. This was consistent with Western blot analyses of total lung protein that showed induction of COX-2 at 24 hours post-V₂O₅ instillation (Figure 4). The

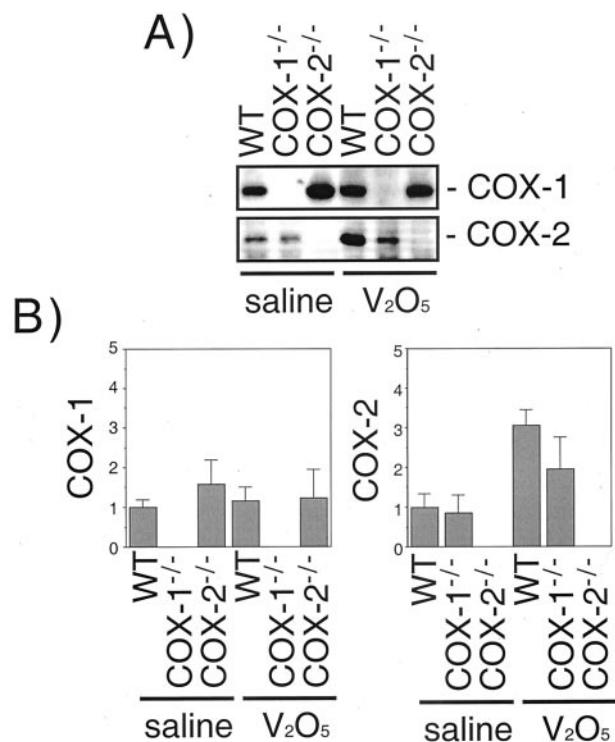


Figure 4. COX-1 and COX-2 protein expression 24 hours following saline and V₂O₅ instillation in wild-type, COX-1^{-/-}, and COX-2^{-/-} mice. The abundance of COX-1 and COX-2 protein in the lungs was determined by Western blotting using immunospecific antibodies as described in Materials and Methods. **A:** Representative Western blot. **B:** Scanning densitometry of COX-1 and COX-2 expression. Data are the mean ± range of two separate experiments.

PGE₂ level in the BAL fluid from COX-1^{-/-} mice was extremely low (~10 pg/ml), yet increased 25-fold within 24 hours after V₂O₅ instillation. PGE₂ in the BAL fluid from saline-instilled COX-2^{-/-} mice was ~200 pg/ml and was not significantly increased following V₂O₅ instillation.

Increased Levels of TNF-α in the BAL Fluid from COX-2^{-/-} Mice

To gain further mechanistic insight into the susceptibility of COX-2^{-/-} mice to the fibrogenic effects of V₂O₅, we analyzed BAL fluid for a variety of cytokines that have been implicated in the pathogenesis of fibrosis, including IL-8, TNF-α, IL-13, PDGF-BB, and TGF-β1. IL-8 was detectable in BAL and up-regulated by V₂O₅ but was not significantly different among genotypes (data not shown). IL-13, PDGF-BB, and TGF-β1 were not detectable in the BAL fluid in any of the genotypes using the commercially available ELISA kits, even when the BAL fluid was acid-activated to liberate TGF-β1 from putative latent complexes. It may be that these cytokines play an important role in the pathogenesis of vanadium-induced fibrosis; however, these factors were below the detection range of the commercially available ELISAs used in this study. TNF-α was consistently detectable in the BAL fluid from all genotypes and significant differences were observed following V₂O₅ instillation among the various genotypes (Figure 8). TNF-α in the BAL fluid of wild-type mice or COX-1^{-/-} increased two to sixfold between 1 and 15 days post-instillation. However, there were no significant differences in TNF-α levels among V₂O₅-exposed wild-type and COX-1^{-/-} mice. In contrast, TNF-α in the BAL fluid from V₂O₅ exposed COX-2^{-/-} mice was elevated by as much as 10-fold above saline controls, and these levels were significantly higher than either wild-type or COX-1^{-/-} at days 1, 3, and 6 post-V₂O₅ instillation. However, by day 15, there were no significant differences in TNF-α levels among genotypes in the V₂O₅-instilled groups.

Discussion

A variety of studies have implicated COX enzymes and their eicosanoid products in the pathogenesis of pulmonary fibrosis. However, to date no studies have established a definitive role for either COX-1 or COX-2 enzymes during lung fibrogenesis. Our investigation has revealed a central role for the COX-2 enzyme in protecting the lung against fibrosis. V₂O₅ exposure caused a significantly higher inflammatory score in COX-2^{-/-} mice as compared to COX-1^{-/-} or wild-type mice. COX-2^{-/-} mice subsequently developed pulmonary fibrosis within the lung parenchyma and a significant increase in total lung hydroxyproline, whereas COX-1^{-/-} and wild-type did not. These observations suggest that eicosanoid products of the COX-2 enzyme mediate a protective effect in the development and/or resolution of inflammation. Other studies have reported increased inflammatory responses following ovalbumin challenge to the lung² or

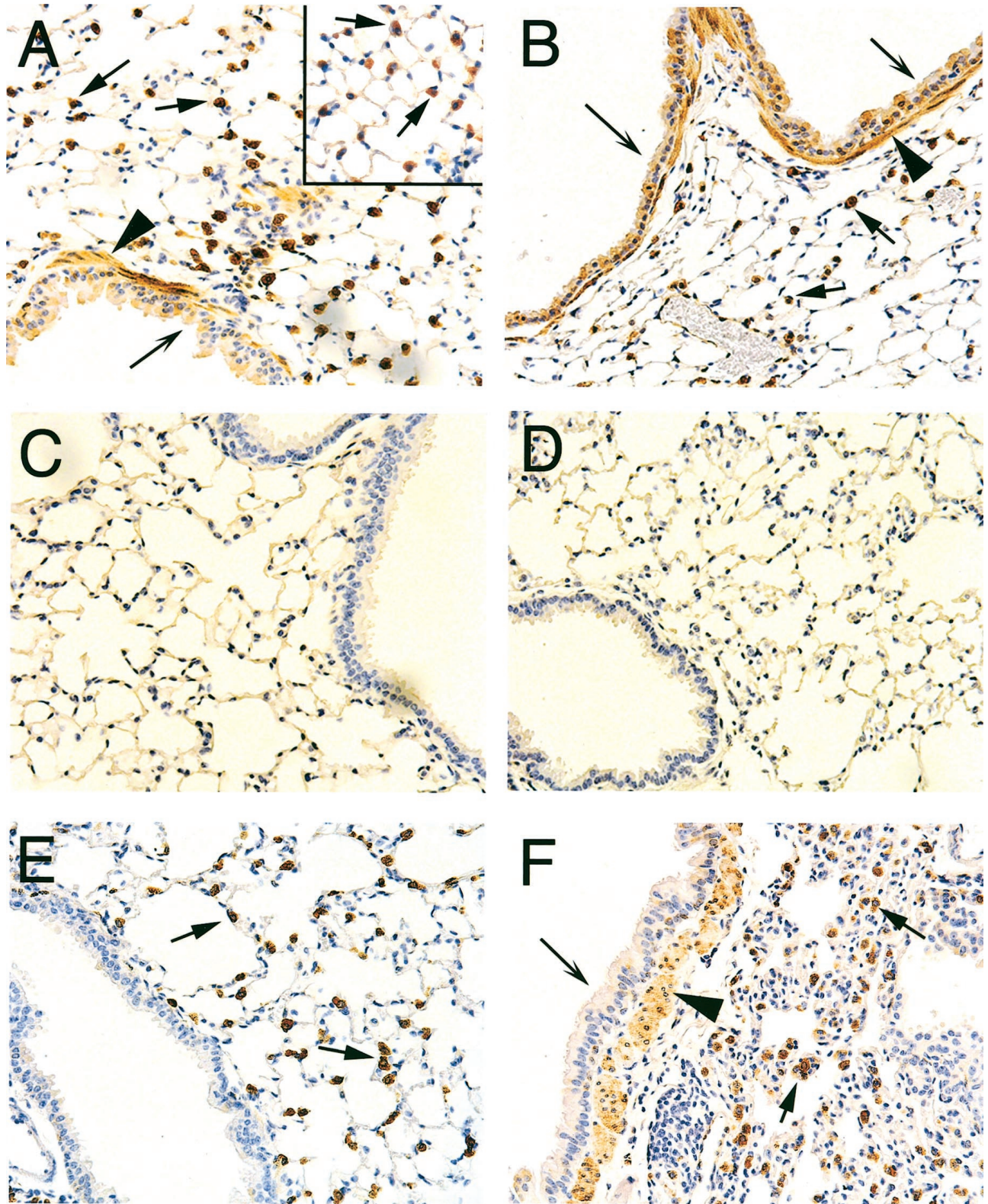


Figure 5. Immunohistochemistry showing COX-1 localization in type II epithelial cells, airway smooth muscle cells, and airway epithelium. **A:** Saline-instilled wild-type showing numerous COX-1 positive cells residing within the alveolar walls of the lung parenchyma (arrows); the inset panel shows the results of SP-A immunostaining that confirmed these cells as type II pneumocytes. Airway epithelial cells (barbed arrow) and airway smooth muscle cells (arrowhead) were also positive for COX-1. **B:** V_2O_5 -instilled wild-type showing COX-1 localized in type II epithelial cells (arrows), airway epithelium (barbed arrows), and airway smooth muscle (arrowhead). **C:** Saline-instilled COX-1 null. **D:** V_2O_5 -instilled COX-1 null. **E:** Saline-instilled COX-2 null showing COX-1 localized in type II cells (arrows) but not in Clara cells of terminal bronchiole. **F:** V_2O_5 -instilled COX-2 null showing COX-1 localized in airway epithelium (barbed arrow), airway smooth muscle (arrowhead), and type II cells (arrows). Note the absence of COX-1 immunostaining in the COX-1 null mice (C and D). Magnification of all panels $\times 160$.

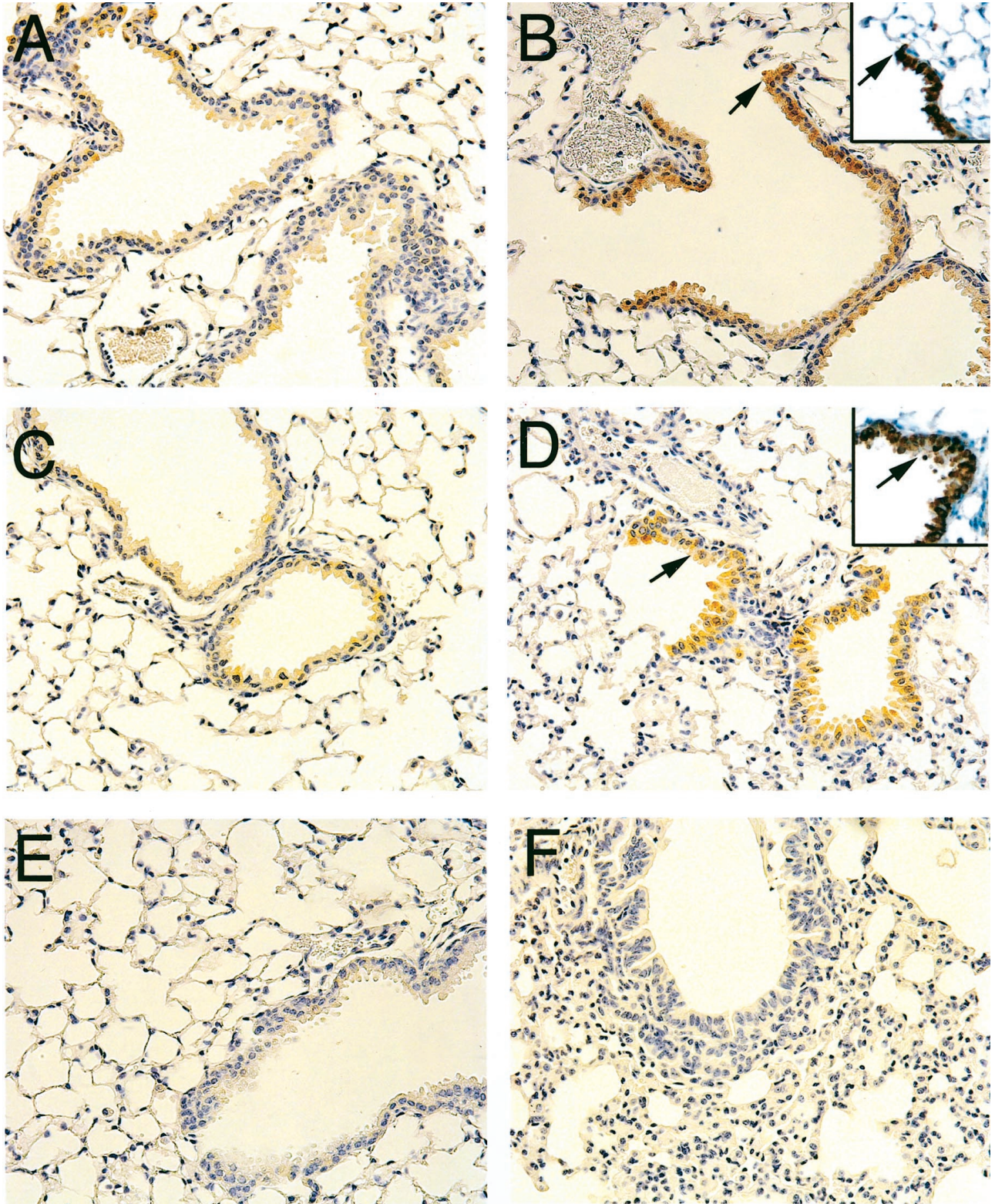


Figure 6. Immunohistochemistry showing COX-2 localization in Clara cells of the distal airways and terminal bronchioles. **A:** Saline-instilled wild-type showing weak COX-2 immunostaining in Clara cells. **B:** V_2O_5 -instilled wild-type showing increased COX-2 immunostaining in epithelial cells of terminal bronchioles that were confirmed as Clara cells by immunostaining with a CC10 antibody (**inset**). **Arrows** indicate the distal end of the terminal bronchiole in serial sections stained for COX-2 or CC10. **C:** Saline-instilled COX-1 null. **D:** V_2O_5 -instilled COX-1 null showing increased COX-2 immunostaining in Clara cells of terminal bronchioles (**inset** shows CC10 immunostaining on a serial section and **arrows** indicate the same bronchiole that is positive for both COX-2 and CC10). **E:** Saline-instilled COX-2 null. **F:** V_2O_5 -instilled COX-2 null. Note the absence of COX-2 immunostaining in the COX-2 null mice. All panels $\times 160$.

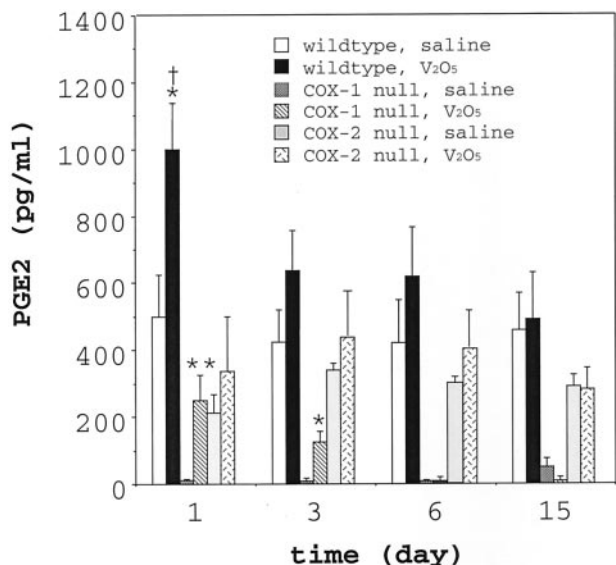


Figure 7. PGE₂ levels in BAL fluid in wild-type, COX-1^{-/-}, and COX-2^{-/-} mice following instillation with saline or V₂O₅. BAL fluid was collected at the indicated time points post-instillation and PGE₂ measured by an enzyme immunoassay. Each V₂O₅ group represents 5 to 6 animals and each saline group represents 3 to 4 animals. *, P < 0.05 vs. all saline-instilled groups. **, P < 0.01 vs. all saline-instilled groups. †, P < 0.05 vs. COX-1^{-/-} or COX-2^{-/-} V₂O₅ group.

following acute colonic injury¹⁷ in both COX-1^{-/-} and COX-2^{-/-} mice. On the other hand, Zeldin et al¹⁸ recently reported that LPS-induced inflammatory responses were not increased in COX-1^{-/-} or COX-2^{-/-} mice compared to wild-type mice. Thus, COX enzymes appear to be important to the development of fibrosis and inflammation in response to certain stimuli, yet COX enzymes may not be required for the development of LPS-induced inflammation.

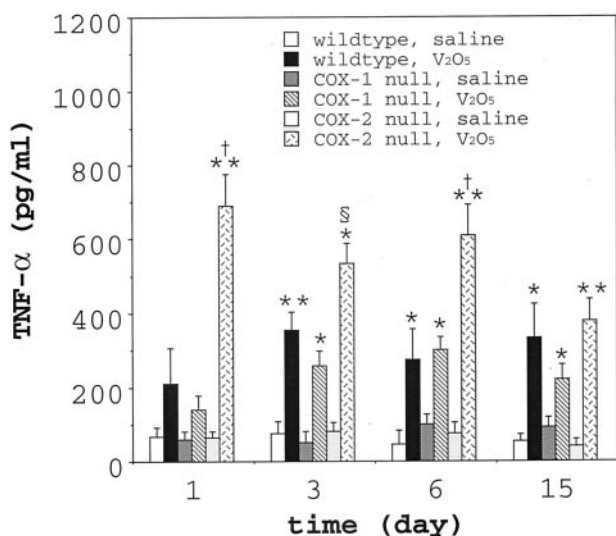


Figure 8. TNF-α levels in BAL fluid in wild-type, COX-1^{-/-} and COX-2^{-/-} mice following instillation with saline or V₂O₅. BAL fluid was collected at the indicated time points post-instillation and TNF-α was measured by ELISA. Each V₂O₅ group represents 5 to 6 animals and each saline group represents 3 to 4 animals. *P < 0.05 vs. all saline-instilled groups. **, P < 0.01 vs. all saline-instilled groups. †, P < 0.05 vs. wild-type V₂O₅ group. ‡, P < 0.01 vs. wild-type V₂O₅ group.

PGE₂ is a major eicosanoid product of both COX-1 and COX-2 enzymes,¹⁹ and has been proposed as an anti-fibrotic mediator in the lung.^{3,6,20} We previously reported that PGE₂ suppresses PDGF-stimulated growth of rat pulmonary myofibroblasts in part by down-regulating the PDGF α receptor subunit.⁵ Other investigators have shown that fibroblasts from patients with lung fibrosis have a diminished capacity to produce PGE₂.^{3,4} Moreover, Ogushi and co-workers reported that PGE₂ synthesis was decreased in lung fibroblasts isolated from rats with bleomycin-induced lung fibrosis.²⁰ Thus, there is abundant evidence to support the idea that PGE₂ is an anti-fibrotic mediator.

In the present study, PGE₂ levels were significantly increased early after V₂O₅ exposure (24 hours) in both wild-type and COX-1^{-/-} mice, whereas PGE₂ levels in COX-2^{-/-} mice were not significantly increased at any time point (Figure 7). Both COX-1^{-/-} and COX-2^{-/-} mice had diminished PGE₂ levels in their BAL fluid compared to wild-type mice 24 hours following V₂O₅ exposure. BAL fluid from COX-1^{-/-} mice contained by far the lowest concentrations of PGE₂ (~10 pg/ml) although V₂O₅ exposure elevated the PGE₂ level 25-fold above the saline-instilled group. In contrast, PGE₂ levels in the BAL fluid from COX-2^{-/-} mice were not significantly increased at any time point post-V₂O₅ instillation compared to the saline-instilled group. These data suggest that the increase in PGE₂, rather than differences in the absolute level of PGE₂ among genotypes, may be a significant factor in protecting the lung from an inflammatory response. An alternative hypothesis is that other AA metabolites, including cyclopentanones such as PGD₂ or leukotrienes such as LTB₄, might be differentially induced among these genotype following V₂O₅ exposure. However, while V₂O₅ increased PGD₂ or LTB₄ in BAL fluid by as much as twofold, we observed no significant differences in these mediators among wild-type, COX-1^{-/-}, or COX-2^{-/-} mice.

Our findings suggest that TNF-α could role in susceptibility of the COX-2^{-/-} mice to pulmonary fibrosis. COX-2^{-/-} mice had significantly higher levels of TNF-α in BAL fluid after V₂O₅ exposure (~10-fold increase above saline controls) compared to wild-type or COX-1^{-/-} groups (three- to sixfold increases above saline controls) (Figure 8). TNF-α has been implicated as a central mediator in the progression of pulmonary fibrosis. For example, Piguet and co-workers²¹ reported that neutralizing antibodies against TNF-α block silica-induced pulmonary fibrosis in mice. Sime and colleagues²² demonstrated that over-expression of TNF-α in rat lung through adenoviral transfer of a TNF-α cDNA caused severe pulmonary inflammation and fibrosis with induction of transforming growth factor-β1. Moreover, the spontaneous development of pulmonary fibrosis in viable motheaten mutant mice has been associated with increased TNF-α in the lung and serum.²³ Finally, TNF-α receptor knockout mice are protected from pulmonary fibrosis induced by asbestos inhalation.²⁴ Thus, several different experimental strategies have been used to demonstrate a role for TNF-α in mediating pulmonary fibrosis.

Enhancement of the early inflammatory events (ie, within 24 hours) appear to be critical to the development of lung fibrosis in COX-2^{-/-} mice. In particular, the increased expression of TNF- α in the lungs of V₂O₅-exposed COX-2^{-/-} mice above their wild-type or COX-1^{-/-} counterparts suggests that products of the COX-2 enzyme are important in regulating TNF- α protein levels. Alternatively, it is possible that increased TNF- α is a consequence of COX-2-deficient mice not being able to resolve inflammation and that the increased TNF- α is simply due to the persistent increased burden of inflammatory cells. Nevertheless, V₂O₅ exposure caused significant increases in PGE₂ above saline-exposed mice in both the wild-type and COX-1^{-/-} groups at 24 hours post-instillation, but V₂O₅ exposure caused no significant increases in PGE₂ in the BAL fluid of COX-2^{-/-} mice (Figure 7). While TNF- α increases PGE₂ synthesis by inducing COX-2 expression,²⁵ PGE₂ has been shown to suppress TNF- α production in a variety of different cell types.²⁶ The constitutive levels of PGE₂ in the BAL fluid from COX-1^{-/-} mice were extremely low compared PGE₂ levels in the BAL fluid from wild-type or COX-2^{-/-} mice, yet PGE₂ levels in the COX-1^{-/-} mice were markedly elevated by V₂O₅ instillation. Therefore, we propose that the induction of PGE₂ synthesis, rather than the absolute concentrations of PGE₂, may more important in suppressing TNF- α production in the lung. Suppression of TNF- α via COX-2 expression would require an intracellular "sensor" that could be turned on or turned off to initiate a negative feedback loop to suppress TNF- α synthesis. Indeed, PGE₂ has been reported to suppress NF κ B,²⁷ a transcription factor that has been reported to mediate vanadium-induced TNF- α expression.²⁸ While our data show a correlation between lack of induction of PGE₂ synthesis and increased TNF- α in COX-2^{-/-} mice, it remains unclear whether or not the inability to up-regulate PGE₂ synthesis is causally related to increased TNF- α production. Future research should address this important issue.

Our immunohistochemical staining of formalin-fixed, paraffin-embedded lung sections showed strong expression of COX-1 within type 2 epithelial cells, bronchial epithelial cells, and airway smooth muscle cells (Figure 6). The identity of type 2 cells was verified by SP-A immunostaining, which clearly demonstrated these cells as the COX-1-positive cell type in the lung parenchyma. However, in V₂O₅-exposed COX-2^{-/-} mice wherein a severe inflammatory response ensued, some F4/80 antigen-positive macrophages also possessed immunostaining for both SP-A and COX-1. It is likely that the phagocytosis of apoptotic type 2 cells by macrophages resulted in COX-1 immunostaining, although we cannot rule out that some macrophages may express endogenous COX-1. COX-1 was expressed in both saline and V₂O₅-instilled wild-type and COX-2^{-/-} mice. These data agree with Western blot analysis of whole lung protein that indicated equal expression of COX-1 in the lungs of saline or V₂O₅-exposed wild-type or COX-2^{-/-} mice, but no expression in COX-1^{-/-} mice (Figure 4). In contrast, COX-2 was almost exclusively localized in Clara cells of the terminal bronchioles of wild-type and COX-1^{-/-} mice,

and was strongly induced 24 hours following V₂O₅ exposure (Figure 6). These data were consistent with Western blot analysis of whole lung protein that showed COX-2 in the lungs of wild-type or COX-1^{-/-} mice that was up-regulated by V₂O₅ exposure (Figure 4). Further studies should focus on the significance of the differential localization of COX-1 and COX-2 in these lung cell types.

V₂O₅ was used as a fibrotic agent in the present study rather than more conventional fibrotic stimuli, such as bleomycin. We previously reported that V₂O₅ causes lung inflammation and fibrosis in rats, yet the inflammatory response largely resolves within 2 weeks.^{12,13} We initially postulated that either COX-1 or COX-2 null mice might be susceptible to fibrotic agents, and therefore selected V₂O₅ as a relatively mild fibrotic agent. Surprisingly, the C57BL/6J \times 129/Ola wild-type mice were quite resistant to V₂O₅-induced injury as compared to Sprague-Dawley rats that were used in our earlier studies.¹³ As a result, V₂O₅-induced inflammatory lung lesions resolved in these wild-type mice. Compared with V₂O₅, the intratracheal instillation of bleomycin causes relatively prolonged inflammation that develops into a robust fibrotic response in rats and mice. Keerthisingam and colleagues²⁹ recently presented pathological evidence of enhanced lung fibrosis in COX-2^{-/-} mice 2 weeks following the intratracheal instillation of bleomycin sulfate. While that was the first study to report enhanced lung fibrogenesis in COX-2^{-/-} mice, the authors did not measure inflammatory mediators or provide evidence for a mechanism of COX-2^{-/-} susceptibility to bleomycin-induced fibrosis. However, it is interesting that COX-2^{-/-} mice are susceptible to the fibrogenic effects of a chemotherapeutic drug (bleomycin) as well as a transition metal (V₂O₅). This indicates that the susceptibility of COX-2^{-/-} mice is a general response to diverse fibrogenic stimuli.

COX-2 may also have a protective role in other inflammatory lung diseases. Gavett and co-workers recently reported that COX-2^{-/-} mice have an increased allergic and inflammatory response to ovalbumin challenge compared to WT mice, yet the COX-2^{-/-} mice have approximately the same BAL level of PGE₂ as WT.² Peebles and colleagues demonstrated that mice treated with the COX inhibitor indomethacin had increased production of interleukin-5 and interleukin-13 following ovalbumin challenge.³⁰ Both of these Th-2 cytokines have been implicated in the development of airway inflammation in asthma.³¹ Mice pre-treated with indomethacin also showed increased airway hyperresponsiveness to ovalbumin challenge. Collectively, these studies suggest that COX-1 and/or COX-2 products may be involved in the development of allergic airway inflammation.

In summary, we report that COX-2^{-/-} mice are susceptible to the development of pulmonary fibrosis following exposure to the transition metal, V₂O₅. In contrast, COX-1^{-/-} mice were not susceptible to the fibrogenic effects of vanadium and their lung inflammatory response resolved. Vanadium-induced lung injury increased the level of PGE₂ in BAL fluid from WT and COX-1^{-/-} mice, but did not cause a significant increase in the level of PGE₂ in the BAL fluid from COX-2^{-/-} mice. The level of

TNF- α in the BAL fluid of vanadium-exposed COX-2^{-/-} mice was significantly higher than in vanadium-exposed COX-1^{-/-} or wild-type mice. These data indicate that COX-2 is protective against pulmonary fibrosis and we suggest that increased expression of TNF- α caused by disruption of the *Pgbs-2* gene could impair resolution of inflammation and result in a fibrotic outcome.

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References

1. Smith WL, Dewitt DL: Prostaglandin endoperoxidase H synthases-1 and -2. *Adv Immunol* 1996, 62:167-215
2. Gavett SH, Madison SL, Scarborough PE, Qu W, Boyle JE, Chulada PC, Tiano HF, Lee CA, Langenbach R, Roggli VL, Zeldin DC: Allergic lung responses are increased in prostaglandin H synthase-deficient mice. *J Clin Invest* 1999, 104:721-732
3. Wilborn J, Crofford LJ, Burdick MD, Kunkel SL, Strieter RM, Peters-Golden M: Cultured lung fibroblasts isolated from patients with idiopathic pulmonary fibrosis have a diminished capacity to synthesize prostaglandin E₂ and to express cyclooxygenase-2. *J Clin Invest* 1995, 95:1861-1868
4. Vancheri C, Sortino MA, Tomaselli V, Mastruzzo C, Condorelli F, Bellistri G, Pistorio MP, Canonico PL, Crimi N: Different expression of TNF- α receptors and prostaglandin-E₂ production in normal and fibrotic lung fibroblasts. *Am J Respir Cell Mol Biol* 2000, 22:628-634
5. Boyle JE, Lindroos PM, Rice AB, Zhang L, Zeldin DC, Bonner JC: Prostaglandin-E₂ counteracts interleukin-1 β -stimulated up-regulation of platelet-derived growth factor α -receptor on rat pulmonary myofibroblasts. *Am J Respir Cell Mol Biol* 1999, 20:433-440
6. Fine A, Goldstein RH: The effect of PGE₂ on the activation of lung fibroblasts. *Prostaglandins* 1987, 33:903-913
7. Ricupero DA, Rishikof DC, Kuang P-P, Poliks CF, Goldstein RH: Regulation of connective tissue growth factor expression by prostaglandin E₂. *Am J Physiol* 1999, 277:L1165-L1171
8. Langenbach R, Morham SG, Tiano HF, Loftin CD, Ghanayem BI, Chulada PC, Mahler JF, Lee CA, Goulding EH, Kluckman KD, Sim HS, Smithies O: Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. *Cell* 1995, 83:483-492
9. Morham SG, Langenbach RL, Loftin CD, Tiano HF, Vouloumanos N, Jennette JC, Mahler JF, Kluckman KD, Ledford A, Lee CA, Smithies O: Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell* 1995, 83:473-482
10. Langenbach R, Loftin C, Lee C, Tiano H: Cyclooxygenase knockout mice: models for elucidating isoform specific functions. *Biochem Pharmacol* 1999, 15:1237-1246
11. Levy BS, Hoffman L, Gottsegen S: Boilermaker's bronchitis: respiratory tract irritation associated with vanadium pentoxide exposure during oil-to-coal conversion of a power plant. *J Occup Med* 1984, 26:567-570
12. Bonner JC, Rice AB, Moomaw CR, Morgan DL: Airway fibrosis in rats induced by vanadium pentoxide. *Am J Physiol* 2000, 278:L209-L216
13. Bonner JC, Lindroos PM, Rice AB, Moomaw CR, Morgan DL: Induction of PDGF receptor- α in rat myofibroblasts during pulmonary fibrogenesis in vivo. *Am J Physiol* 1998, 274:L72-L80
14. Cherniack RM, Colby TV, Flint A, Thurlbeck WN, Waldron J, Ackerson L, King Jr TE: Quantitative assessment of lung pathology in idiopathic pulmonary fibrosis. *Am Rev Respir Dis* 1991, 144:892-900
15. Rice AB, Moomaw CR, Morgan DL, Bonner JC: Specific inhibitors of platelet-derived growth factor or epidermal growth factor receptor tyrosine kinase reduce pulmonary fibrosis in rats. *Am J Pathol* 1999, 155:213-221
16. Chulada PC, Loftin CD, Winn VD, Young DA, Tiano HF, Eling TE, Langenbach R: Relative activities of retrovirally expressed murine prostaglandin synthase-1 and -2 depend on source of arachidonic acid. *Arch Biochem Biophys* 1996, 330:301-313
17. Morteau O, Morham SG, Sellon R, Dieleman LA, Langenbach R, Smithies O, Sartor RB: Impaired mucosal defense to acute colonic injury in mice lacking cyclooxygenase-1 or cyclooxygenase-2. *J Clin Invest* 2000, 105:469-478
18. Zeldin DC, Wohlford-Lenane C, Chulada P, Bradbury JA, Scarborough PE, Roggli V, Langenbach R, Schwartz DA: Airway inflammation and responsiveness in prostaglandin H synthase-deficient mice exposed to bacterial lipopolysaccharide. *Am J Respir Cell Mol Biol* 2001, 25:457-465
19. Brock TG, McNish RW, Peters-Golden M: Arachidonic acid is preferentially metabolized by cyclooxygenase-2 to prostacyclin and prostaglandin-E₂. *J Biol Chem* 1999, 274:11660-11666
20. Ogushi F, Endo T, Tani K, Asada K, Kawano T, Tada H, Maniwa K, Sone S: Decreased prostaglandin E₂ synthesis by lung fibroblasts isolated from rats with bleomycin-induced lung fibrosis. *Int J Exp Pathol* 1999, 80:41-49
21. Piguet PF, Collart MA, Grau GE, Sappino A-P, Vassalli P: Requirement of tumor necrosis factor for development of silica-induced pulmonary fibrosis. *Nature* 1990, 344:245-247
22. Sime PJ, Marr RA, Gauldie D, Xing Z, Hewlett BR, Graham FL, Gauldie J: Transfer of tumor necrosis factor- α to rat lung induces severe pulmonary inflammation and patchy interstitial fibrogenesis with induction of transforming growth factor- β 1 and myofibroblasts. *Am J Pathol* 1998, 153:825-832
23. Thrall RS, Vogel SN, Evans R, Shultz LD: Role of tumor necrosis factor- α in the spontaneous development of pulmonary fibrosis in viable motheaten mutant mice. *Am J Pathol* 1997, 151:1303-1310
24. Liu J-Y, Brass DM, Hoyle GW, Brody AR: TNF- α receptor knockout mice are protected from the fibroproliferative effects of inhaled asbestos fibers. *Am J Pathol* 1998, 153:1839-1847
25. Fournier T, Fadok V, Henson PM: Tumor necrosis factor- α inversely regulates prostaglandin D₂ and prostaglandin E₂ production in murine macrophages. *J Biol Chem* 1997, 272:31065-31072
26. Rola-Pleszczynski M, Thivierge M, Gagnon N, Lacasse C, Stankova J: Differential regulation of cytokine and cytokine receptors by PAF, LT_{B4}, and PGE₂. *J Lipid Med* 1993, 6:175-181
27. D'Acquisto F, Sautebin L, Iuvone T, Di Rosa M, Carnuccio R: Prostaglandins prevent inducible nitric oxide synthase protein expression by inhibiting nuclear factor- κ B activation in J774 macrophages. *FEBS Lett* 1998, 27:76-80
28. Ye J, Ding M, Zhang X, Rojanasakul Y, Nedospasov S, Vallyathan V, Castranova V, Shi X: Induction of TNF- α in macrophages by vanadate is dependent on activation of transcription factor NF- κ B and free radical reactions. *Mol Cell Biochem* 1999, 198:193-200
29. Keerthisingam CB, Jenkins RG, Harrison NK, Hernandez-Rodriguez NA, Booth H, Laurent GJ, Hart SL, Foster ML, McAnulty RJ: Cyclooxygenase-2 deficiency results in a loss of the anti-proliferative response to transforming growth factor- β in human fibrotic lung fibroblasts and promotes bleomycin-induced pulmonary fibrosis in mice. *Am J Pathol* 2001, 158:1411-1422
30. Peebles Jr RS, Dworski R, Collins RD, Jarzecka K, Mitchell DB, Graham BS, Sheller JR: Cyclooxygenase inhibition increases interleukin-5 and interleukin-13 production and airway hyper-responsiveness in allergic mice. *Am J Respir Crit Care Med* 2000, 162:676-681
31. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD: Interleukin-13: central mediator of allergic asthma. *Science* 1998, 282:2258-2261