Prostacyclin Prevents Pulmonary Endothelial Cell Apoptosis Induced by Cigarette Smoke

S. Patrick Nana-Sinkam^{1*}, Jong Deog Lee^{2*}, Sylk Sotto-Santiago³, Robert S. Stearman³, Robert L. Keith⁴, Qamrul Choudhury³, Carlyne Cool³, Jane Parr³, Mark D. Moore³, Todd M. Bull³, Norbert F. Voelkel³, and Mark W. Geraci³

¹Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, The Ohio State University, Columbus, Ohio; ²College of Medicine, Gyeongsang National University, Chinju, South Korea; ³Division of Pulmonary Sciences and Critical Care Medicine and COPD Center, University of Colorado Health Science Center, Denver, Colorado; and ⁴Department of Veterans Affairs Medical Center, Denver, Colorado

Rationale: Impaired endothelial cell-dependent vasodilation, inflammation, apoptosis, and proliferation are manifestations of endothelial dysfunction in chronic obstructive pulmonary disease (COPD). Prostacyclin (PGI₂) is a major product of the cyclooxygenase pathway with potent vasodilatory and antimitogenic properties and may be relevant to endothelial dysfunction in COPD.

Objectives: To determine if PGI₂ expression is altered in smoking-related lung disease and if it may be protective in COPD-associated endothelial dysfunction.

Methods: We evaluated, by immunohistochemistry, Western blotting, and polymerase chain reaction, human emphysema tissue compared with normal tissue for expression of prostacyclin synthase (PGI₂S). We examined the effects of cigarette smoke extract (CSE) and aldehyde components on eicosanoid expression in primary human pulmonary microvascular endothelial cells. Finally, we used a murine model of lung-specific PGI₂S overexpression and *in vitro* studies to determine if PGI₂ expression has protective effects on cigarette smoke-induced endothelial apoptosis.

Measurements and Main Results: Human emphysema lung tissue exhibited lower PGI₂S expression within the pulmonary endothelium than in normal lung. *In vitro* studies demonstrated that CSE, and in particular the α,β unsaturated aldehyde acrolein, suppressed PGI₂S gene expression, whereas CSE significantly induced the upstream mediators COX-2 and cytosolic phospholipase A2 in human pulmonary microvascular endothelial cells. Mice with lung-specific PGI₂S overexpression exhibited less endothelial apoptosis after chronic smoke exposure. *In vitro*, iloprost exhibited protective effects on CSE-induced apoptosis.

Conclusions: PGI₂ has protective effects in the pulmonary vasculature after acute and chronic cigarette smoke exposure. An imbalance in eicosanoid expression may be important to COPD-associated endothelial dysfunction.

Keywords: eicosanoids; emphysema; inflammation; vascular

Chronic obstructive pulmonary disease (COPD) can be characterized as a destruction of small airways and parenchyma resulting in a progressive impairment in pulmonary function (1). Cigarette smoke is the major pathogenic factor implicated in

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Prostacyclin seems to be biologically relevant in endothelial dysfunction; however, the role of eicosanoids in smoking-related lung disease remains unclear.

What This Study Adds to the Field

Prostacyclin expression is decreased in chronic obstructive pulmonary disease. Prostacyclin may have protective effects in the pulmonary endothelium after cigarette smoke exposure.

COPD, and pulmonary hypertension develops in approximately 6% of smokers with COPD (1). The interaction between parenchymal disease and the vasculature is often clinically evident by the observation that patients with severe COPD often have mild or moderate pulmonary hypertension at rest (2). Histopathologically and microscopically, the pulmonary vasculature in COPD is characterized by intimal thickening, with smooth muscle deposition (2) and loss of alveolar septal structures and microvasculature (3). In COPD, alveolar septal and endothelial cells undergo apoptosis, which is likely important to the pathogenesis of disease (4). The etiology of pulmonary hypertension in COPD has been considered to be chronic hypoxemia, but pulmonary hypertension is evident in milder forms of COPD where hypoxemia is absent. This finding suggests that other mechanisms are responsible for the vascular changes in COPD (2).

Cigarette smoke is considered to be a major risk factor in the development of COPD, and its effects on the lung epithelium have been well characterized (5–7). The alveolar septae and microvasculature are affected in COPD. Because endothelial dysfunction has been implicated in the pathogenesis of several chronic diseases, including coronary artery disease, peripheral vascular diseases, diabetes, and renal failure, impaired endothelial function of pulmonary arteries has been described in small pulmonary arteries from patients with COPD (8–11). Endothelial cell apoptosis has been implicated in smoking-related endothelial dysfunction (12). Researchers have also demonstrated that cigarette smoke induces necrosis of epithelial and endothelial cells (13, 14).

The role of eicosanoid expression in COPD and other smoking-related lung disease remains unclear. Previous studies have identified elevated levels of markers of oxidative stress, such as the prostaglandin (PG)F₂ α analog 8-isoprostane in the exhaled breath condensate of patients with COPD (15). Proinflammatory

(Received in original form May 30, 2006; accepted in final form January 19, 2007)

Supported by grants from the National Heart, Lung, and Blood Institute Mentored Scientist Award HL077717 (S.P.N.S.), a SPOR in Lung Cancer Career Development Award (S.P.N.S.), a FAMRI CIA Award (R.S.S.), a Veterans' Affairs Merit Award (R.L.K.), and the SPOR in Lung Cancer (CA58187) Project 3 (M.W.G.).

* These authors contributed equally to this work.

Correspondence and requests for reprints should be addressed to Patrick Nana-Sinkam, M.D., The Ohio State University, Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, 201 Davis Heart and Lung Research Institute, 473 West 12th Avenue, Columbus, OH 43210. E-mail: patrick.nana-sinkam@osumc.edu

Am J Respir Crit Care Med Vol 175, pp 676–685, 2007

Originally Published in Press as DOI: 10.1164/rccm.200605-724OC on January 25, 2007
Internet address: www.atsjournals.org

molecules, such as leukotriene B₄ and prostaglandin E₂, are elevated in the exhaled breath condensate of individuals with COPD (16).

Prostacyclin (PGI₂) has potent vasodilatory and antimitogenic properties and is one of the main therapies proven to improve survival in patients with severe idiopathic pulmonary arterial hypertension (17). PGI₂ production occurs primarily in pulmonary vascular smooth muscle and endothelium via the cyclooxygenase prostaglandin H synthase pathway. Subsequent conversion to PGI₂ is mediated by prostacyclin synthase (PGI₂S), a member of the group of cytochrome P450 enzymes. Our laboratory previously identified decreased expression of PGI₂S in the lungs from patients with severe idiopathic pulmonary arterial hypertension (18). Furthermore, transgenic mice with lung specific PGI₂S overexpression were protected from hypoxia-induced pulmonary hypertension (19).

Cigarette smoke may contribute to endothelial dysfunction and the development of cardiovascular disease through the inhibition of PGI₂ release from the endothelium (20, 21). The mecha-

nism of inhibition of PGI₂ release by cigarette smoke is unclear. Researchers have implicated arachidonic acid mobilization and downstream enzymes as potential targets of cigarette smoke (20, 21).

We hypothesized that PGI₂ expression may be reduced in other smoking-related diseases, such as COPD. In addition, we hypothesized that reduced PGI₂ expression may be important to the pathogenesis of the disease, and, given the decreased survival among patients with COPD who have concomitant pulmonary hypertension, PGI₂ expression may be protective to the pulmonary vasculature. We examined the pulmonary endothelium of patients with emphysema for PGI₂S expression and the direct effects of cigarette smoke and acrolein, a potent $\alpha\beta$ unsaturated aldehyde found in cigarette smoke, on PGI₂S expression in the pulmonary microvasculature. Finally, we determined whether PGI₂ has protective effects on the pulmonary endothelium (*in vivo* and *in vitro*) after cigarette smoke exposure. Some of the results of these studies have been previously reported in the form of an abstract (22).

Figure 1. Prostacyclin synthase (PGI₂S) expression in human emphysema and nondiseased lung. We performed immunohistochemistry for PGI₂S in emphysema (n = 4) and nondiseased lung (n = 3). There is a reduction in PGI₂S staining (arrow) of arteriolar pulmonary endothelium from a patient with emphysema (A) compared with nondiseased lung (B). In small and medium-sized vessels, staining was similar in the endothelium from a patient with emphysema (C) compared with nondiseased lung (D) (arrow) (original magnification: $\times 40$). Sections were scored by a pathologist in blinded fashion (number of PGI₂S-positive endothelial cells/100 endothelial cells per case) for PGI₂S staining and expressed as a ratio in capillaries, small/medium arteries, and arterioles. The differences in PGI₂S staining were statistically significant in pulmonary arteriolar endothelium (E) (0.53 ± 0.086 vs. 0.97 ± 0.015 ; $p < 0.01$) but not in small/medium-sized vessels (F) (0.74 ± 0.12 vs. 0.97 ± 0.005 ; $p = 0.16$).

METHODS

Cell Culture and Materials

Human pulmonary microvascular endothelial cells (HPMVECs, passage 4–6) and endothelial growth medium-2-microvascular (EGM-2-MV) medium (containing 5% fetal bovine serum, hydrocortisone, human recombinant vascular endothelial growth factor, recombinant human fibroblast growth factor-B, recombinant insulinlike growth factor-1, human recombinant epidermal growth factor, ascorbic acid, gentamycin, and amphotericin-B) were purchased from Clonetics Corporation (Baltimore, MD). Cells were grown to confluence at 37°C in a humidified atmosphere of 21% O₂, 5% CO₂. N-acetylcysteine (NAC) and diphenyleneiodonium chloride (DPI) were obtained from Sigma-Aldrich (St. Louis, MO). N^ω-nitro-L-arginine methyl ester (L-NAME) was obtained from Cayman Chemical (Ann Arbor, MI). Z-Asp2,6-dichlorobenzoylmethylketone (Caspase Family Inhibitor IV) was obtained from Alexis Biochemicals (San Diego, CA). A superoxide dismutase (SOD) mimetic and catalase were received as a generous gift from Russell Bowler, M.D. (National Jewish Medical Center, Denver CO). Acrolein and crotonaldehyde ($\alpha\beta$ unsaturated aldehydes) and saturated aldehydes (acetaldehyde, propionaldehyde) were purchased from Sigma-Aldrich.

Preparation of Cigarette Smoke Extract

Cigarette smoke extract (CSE) was prepared by a modification of a previously published method (23). Briefly, one nonfiltered Camel cigarette (R.J. Reynolds, Winston-Salem, NC) was passed through 10 ml of phosphate-buffered saline (PBS) using a vacuum pump. This 100% CSE was adjusted to a pH of 7.4 and filtered through a 0.22- μ m pore filter (Fisher, Hampton, NH) to remove particles and bacteria, and the CSE was diluted to the appropriate concentration and added to endothelial cells within 10 minutes of preparation.

Immunohistochemistry

Paraffin-embedded sections of human lung tissue (obtained from the University of Colorado Tissue Bank), four emphysema and three non-diseased, were deparaffinized and rehydrated with xylene and ethanol. Antigen retrieval was performed using the microwave method with citrate buffer for 20 minutes. Avidine and biotin block was performed, and endogenous peroxidase was quenched by 3% hydrogen peroxide. After blocking with 5% normal goat serum, rabbit anti-human PGI₂S antibody (1:25) (a generous gift from D. Dewitt, Ph.D., Michigan State University) was applied overnight at 4°C. The sections were washed

with PBS with 0.05% Tween and incubated with biotinylated goat anti-rabbit IgG. After washing, sections were stained with ABC Vectastain reagents (PK-6101; Vector Labs, Burlingame, CA) and DAB (SK4100; Vector Labs). Sections were then scored by a pathologist in blinded fashion (number of PGI₂S-positive endothelial cells/100 endothelial cells per case) for PGI₂S staining in capillaries, small/medium arteries, and arterioles.

Apoptosis Assay

In vitro, the rate of apoptosis in primary human microvascular endothelial cells was analyzed by the Vybrant Apoptosis Assay Kit (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's protocol. After treatment with CSE, the cells were washed and trypsinized. Cells (1×10^6) were suspended in 100 μ l of annexin-binding buffer and fluorescein isothiocyanate annexin-V/propidium iodide. After 15 minutes of incubation, samples were analyzed by flow cytometry measuring the fluorescence emission at 530 nm and greater than 575 nm. In addition, apoptosis of HPMVECs was assessed by a second method (caspase 3/7 assay; Promega, Madison, WI). Immunohistochemical staining of PGI₂S transgenic mice and wild-type littermates was conducted using the manufacturer's protocol with cleaved caspase 3 antibody (Cell Signaling Technology, Danvers, MA) to assess apoptosis. In a blinded fashion, small, medium, and large pulmonary arteries were scored for endothelial caspase staining (total of six vessels per mouse).

Western Blotting

A total of 20 μ g of protein was separated on NuPAGE Novex 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% nonfat dry milk in PBS containing 0.05% Tween (PBST) for 1 hour. After washing with PBST, the membrane was incubated with rabbit polyclonal PGI₂S antibody (1:1,000) overnight at 4°C. The membrane was washed four times and incubated with horseradish peroxidase-conjugated secondary antibody (anti-rabbit, 1:3,000; Santa Cruz, Santa Cruz, CA) for 1 hour. Film was developed by chemiluminescence (Perkin Elmer, Boston, MA).

Real-time Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. RNA (1 μ g) was reverse-transcribed using random primer and MultiScribe RT (High-Capacity cDNA Archive Kit; Applied Biosystems, Foster City, CA).

Figure 2. Prostacyclin synthase (PGI₂S) expression and 6-keto-PGF1 α measurements in whole lung lysates from emphysema (n = 4) and nondiseased lung (n = 4). The expression of PGI₂S measured by Western blotting is decreased in patients with emphysema (A). 6-Keto-PGF1 α metabolite level assessed by ELISA is decreased in patients with emphysema (B) (p < 0.05). PGI₂S gene expression measured by reverse transcriptase–polymerase chain reaction is decreased in patients with emphysema (C) (p < 0.05).

Assay-on-demand gene expression probes for PGI₂S:Hs00168766, cyclooxygenase (COX)-1:Hs00377721, COX-2:Hs00153133, cytosolic phospholipase A2 (cPLA2):Hs00233352, and B-actin:Hs999999903 were obtained from Applied Biosystems. Polymerase chain reactions were performed in 20- μ l volumes containing 9 μ l of cDNA, 10 μ l of TaqMan Master Mix (Applied Biosystems), and 1 μ l of assay-on-demand primer and probe. Real-time polymerase chain reaction (PCR) was conducted on the GeneAmp 5700 sequence Detection System (Applied Biosystems), and signal was detected by the GeneAmp 5700 SDS software (Applied Biosystems). All values were reported relative to β -actin expression.

6-Keto-PGF1 α Measurements

We used competitive ELISA to measure 6-keto-PGF1 α . Human whole lung lysates, antibody (mouse anti-PGE or rabbit anti-6-keto-PGF1 α), and tracer were added to wells on plates coated with anti-rabbit (6-keto-PGF1 α) antibodies. The tracer is 6-keto-PGF1 α linked to acetylcholinesterase. After overnight incubation at 4°C, plates were washed, and Ellman's reagent was added (acetylthiocholine iodide and 55'-dithiobis-[2-nitrobenzoic acid] in a 1-M phosphate buffer). The samples were read in a spectrophotometer at 405 nm. Antibodies and tracer were obtained from Cayman Chemicals.

Murine Model of PGI₂S Overexpression

Transgenic mice were developed using a construct consisting of the human surfactant protein C promoter and full-length rat prostacyclin synthase cDNA (24). The surfactant protein C promoter allows targeted expression to alveolar and distal airway epithelial cells (25). We conducted genotyping of animals by performing PCR on genomic DNA isolated from tails as described previously. Each line was propagated

as heterozygotes. Transgenic mice were bred with wild-type FVB/N (Jackson Laboratories, Bar Harbor, ME) mice to produce the experimental transgenic mice and transgenic-negative littermates, which were used as controls in all of the experiments. Transgenic (n = 3) and wild-type (n = 3) mice were exposed to 6 months of mainstream cigarette smoke. All animal studies were conducted under an approved protocol from our local Institutional Animal Care and Use Committee (IACUC).

Statistical Analysis

All statistical analyses in tissue scoring and *in vitro* were conducted by *t* test ($p < 0.05$).

RESULTS

PGI₂S Protein Expression in Lung Tissue from Patients with Emphysema and Those with Normal Lung

We performed immunohistochemistry for PGI₂S on human lung tissue from four individuals with emphysema and three individuals with normal lung parenchyma. Scoring of endothelial cells for PGI₂S expression as a factor of total endothelial cells revealed that PGI₂S was decreased primarily in the endothelial cell monolayer of arterioles in the lungs from patients with pulmonary emphysema (Figure 1A) compared with normal subjects (Figures 1B and 1E). PGI₂S staining in medium-sized pulmonary vessels in emphysema and nondiseased lung was comparable (Figures 1C, 1D and 1F). Western analysis of whole-lung lysates for PGI₂S (Figure 2A) confirmed that protein expression was decreased (by $\sim 50\%$ by densitometry) in lung tissue extracts

Figure 3. Effect of cigarette smoke extract (CSE) on human pulmonary microvascular endothelial cell eicosanoid expression. Pulmonary microvascular endothelial cells were treated with two concentrations (0.5% and 1%) of CSE and then examined at 24 hours for eicosanoid gene expression. Both 0.5% and 1.0% CSE decreased prostacyclin synthase (PGI₂S) at 24 hours (A) ($p < 0.05$), and cyclooxygenase (COX)-1 gene expression was decreased at 24 hours (B) ($p < 0.05$). CSE increased COX-2 and cytosolic phospholipase A2 (cPLA2) ($p < 0.05$) gene expression 24 hours after CSE exposure (C, D). All studies were conducted in triplicate on separate days.

from the emphysema lungs compared with normal control lung tissue extracts.

6-Keto-PGF1 α (the Stable Metabolite of PGI $_2$) in Emphysema versus Normal Lung

To examine whether the reduced amount of lung tissue PGI $_2$ S protein was associated with decreased amounts of PGI $_2$, we measured (by ELISA) the stable metabolite of PGI $_2$ (6-keto-PGF1 α). The amount of 6-keto-PGF1 α measured per milliliter of lung tissue homogenate was reduced in lung tissue samples from patients with emphysema by 75% (Figure 2B). Because the amount of the enzyme protein and the product of the terminal synthetic reaction were reduced in emphysema lung tissues, we questioned whether the synthesis defect was related to a reduced expression of the PGI $_2$ S gene. We examined lung tissue samples using real-time PCR and detected decreased PGI $_2$ S mRNA expression in the lungs from patients with emphysema when compared with normal lung tissue (Figure 2C).

Effect of CSE on Eicosanoid Gene Expression in HPMVECs

We treated primary HPMVECs (passages 4–6) with CSE (0.5%, 1%), which decreased PGI $_2$ S gene expression in a dose-dependent manner with a maximum effect at 24 hours after incubation (Figure 3A). For the remainder of the experiments, we treated endothelial cells with 0.5% and 1% CSE and examined gene expression at 24 hours. COX-1 gene expression was decreased by 1% CSE (Figure 3B), whereas COX-2 and cPLA2 were significantly increased (tenfold and threefold, respectively) (Figures 3C and 3D).

Effect of Aldehydes on Eicosanoid Gene Expression

We exposed HPMVECs to 1 and 10 μ M of unsaturated aldehydes (acrolein and crotonaldehyde) and saturated aldehydes (acetaldehyde and propionaldehyde). Acrolein caused a statistically significant reduction in PGI $_2$ S gene expression 24 hours after exposure at both concentrations (Figure 4A) and PGI $_2$ S protein expression 48 hours after exposure (Figure 4B). No significant change in COX-2 gene expression was noted after acrolein exposure (Figure 4C). None of three other aldehydes (at 1 and 10 μ M) tested caused a similar decrease in PGI $_2$ S expression (Figure 4D). Propionaldehyde induced PGI $_2$ S gene expression.

Effects of Pretreatment Treatment with Antioxidant Agents on CSE-induced Gene Expression Changes in Eicosanoid Pathway Enzymes

We examined whether experimental antioxidant strategies would protect against the CSE-related decrease of PGI $_2$ S expression in HPMVECs. Four-hour preincubation of HPMVECs with NAC (1 mM) followed by CSE treatment and examination at 24 hours did not prevent down-regulation of PGI $_2$ S gene expression by 1% CSE (Figure 5A). Pretreatment with NAC prevented the CSE-mediated induction of COX-2 gene expression (Figure 5B). Four-hour preincubation of endothelial cells with an SOD mimetic (100 U/ml) (Figures 6A and 6B), catalase (100 U/ml) (Figures 6C and 6D), diphenyleneiodonium chloride (0.1 μ M) (nitric oxide inhibitor) (Figures 6E and 6F), or L-NAME (nitric oxide synthase [NOS] inhibitor) (1 mM) (Figures 7A and 7B) did not affect the CSE changes in PGI $_2$ S and COX-2 gene expression.

Figure 4. Effect of components of cigarette smoke (saturated and unsaturated aldehydes) on prostacyclin synthase (PGI $_2$ S) and COX-2 expression. Human pulmonary microvascular endothelial cells were exposed to acrolein, acetaldehyde, crotonaldehyde, and propionaldehyde at concentrations of 1 and 10 μ M and assessed at 24 hours for eicosanoid expression. Acrolein exposure suppressed PGI $_2$ S mRNA at 24 hours and protein expression at 48 hours (A, B) ($p < 0.01$) while not altering COX-2 mRNA expression (C). Administration of other aldehydes (acetaldehyde, crotonaldehyde, and propionaldehyde) did not result in suppression of PGI $_2$ S mRNA expression. Propionaldehyde resulted in a statistically significant induction in PGI $_2$ S expression (D) ($p < 0.001$). All studies were conducted in triplicate on separate days. A = acrolein; C = control.

Figure 5. Effect of pretreatment with N-acetylcysteine (NAC). Human pulmonary microvascular endothelial cells were treated with 1% cigarette smoke extract (CSE) after 4 hours of preincubation with 1 mM of NAC and examined at 24 hours for gene expression. NAC preincubation did not prevent down-regulation of prostacyclin synthase (PGI₂S) by CSE (A) but did prevent up-regulation of COX-2 ($p < 0.05$) (B). All studies were conducted in triplicate on separate days.

Effect of CSE and PGI₂ on HPMVEC Apoptosis

Incubation of HPMVECs with 1 and 2% CSE increased the apoptosis rate at 24 hours from 3% in control cells to 9% (as measured for annexin V by fluorescence) in cells treated with 2% CSE (Figure 8A). We confirmed the induction of apoptosis by annexin V and caspase 3/7 analysis (Figure 8B). Given the research to support that PGI₂ is important to vascular integrity and likely confers protective effects to the vasculature after stressors, we sought to determine whether PGI₂ (analog iloprost) would prevent apoptosis induced by CSE. Pretreatment of HPMVECs with the prostacyclin analog iloprost (1 μ M and 10 μ M) for 30 minutes before 2% CSE exposure significantly reduced endothelial apoptosis as measured by caspase 3/7 activity (Figure 8C).

Effect of Chronic CSE Exposure in PGI₂S Transgenic Animals

We have previously reported that transgenic mice with lung-specific overexpression of PGI₂S were partially protected from chemically and cigarette smoke-induced lung tumors (24). We attempted to determine if lung-specific PGI₂S overexpression conferred protection to the pulmonary endothelium after 6 months of mainstream cigarette smoke exposure. Wild-type (Figure 9A) and PGI₂S transgenic mice (Figure 9B) (FVB background) were exposed to 6 months of cigarette smoke. Immunohistochemistry and subsequent scoring for endothelial cleaved caspase activity per total endothelial cells revealed a significant reduction in caspase 3 expression in transgenic animals (0.3946 ± 0.06583) compared with wild-type littermates (0.6521 ± 0.07224), suggesting decreased apoptosis (Figure 9C).

DISCUSSION

COPD is predominantly related to cigarette smoke exposure. COPD is associated with varying degrees of lung function

Figure 6. Effect of pretreatment with antioxidants. Human pulmonary microvascular endothelial cells were preincubated with superoxide dismutase (SOD) mimetic (100 U/ml) (A, B), catalase (100 U/ml) (C, D), or diphenyleneiodonium chloride (DPI) (0.1 μ M) (E, F) for 4 hours followed by 1% cigarette smoke extract (CSE) exposure. Gene expression at 24 hours revealed that SOD mimetic, catalase, or DPI did not prevent down-regulation of PGI₂S or up-regulation of COX-2 gene expression by CSE. All studies were conducted in triplicate on separate days.

Figure 7. Effect of pretreatment with N^ω-nitro-L-arginine methyl ester (L-NAME). Human pulmonary microvascular endothelial cells were preincubated with L-NAME 1 mM for 4 hours followed by 1% cigarette smoke extract (CSE) exposure. L-NAME pretreatment did not prevent down-regulation of prostacyclin synthase (PGI₂S) at 24 hours (A). There was a trend toward the reversal of induction of COX-2 expression (B). This was not statistically significant. All studies were conducted in triplicate on separate days.

Impaired endothelial cell-dependent vasodilation, inflammation, apoptosis, and proliferation are important to the endothelial dysfunction observed in smoke angiopathy and perhaps in the pathogenesis in emphysema. Endothelial cell dysfunction has been implicated in the pathogenesis of COPD (9). Whether or not cigarette smoke exposure alters lung cell PGI₂ expression has been minimally explored. Previously, researchers demonstrated that cigarette smoke condensate impairs PGI₂ release in human umbilical vein cells (20). In that study, inhibition seemed to occur at the level of arachidonic acid mobilization rather than directly on downstream eicosanoids (20). In our study, we used a comprehensive approach incorporating human tissue analysis, *in vitro* study, and transgenic animal modeling to determine if PGI₂ expression is biologically relevant to the pulmonary vasculature in smoking-related lung disease. We report that pulmonary endothelial PGI₂S expression is decreased in the arteriolar endothelium in emphysema and that, in HPMVECs, cigarette smoke and in particular acrolein suppress PGI₂S expression.

abnormalities and can lead to respiratory failure. However, it is less appreciated that major causes of morbidity and mortality in these patients are pulmonary hypertension and cardiovascular manifestations leading to coronary artery disease and stroke.

Figure 8. Apoptosis in human pulmonary microvascular endothelial cells (HPMVECs) exposed to cigarette smoke extract (CSE) and effect of pretreatment with prostacyclin analog iloprost. HPMVECs exposed to CSE exhibited a concentration-dependent increase in apoptosis rate (by annexin V) from 3 to 9% (A) (control vs. 2% CSE after 24 h) ($p < 0.05$). To confirm, HPMVECs exposed to 2% CSE increased apoptosis at 24 hours by twofold by caspase 3/7 analysis (B) ($p < 0.05$). Thirty-minute preincubation of HPMVECs with iloprost (1 μM and 10 μM) resulted in a statistically significant reduction in CSE-induced apoptosis (C) ($p < 0.05$).

Figure 9. Immunohistochemically cleaved caspase 3 staining of the pulmonary endothelium (arrow) of wild-type (A) and transgenic lung-specific prostacyclin synthase (PGI₂S) overexpressing mice (B) after 6 months of cigarette smoke exposure (original magnification: $\times 200$). Results represent the number of caspase-positive endothelial cells per total number of endothelial cells as a ratio. Quantification of cleaved caspase staining of the pulmonary endothelium in PGI₂S-overexpressing transgenic (PGI₂S OE) (0.3946 ± 0.06583) was significantly decreased in comparison to wild-type (WT) (0.6521 ± 0.07224) littermates after 6 months of cigarette smoke exposure (C). Vessel scoring was conducted in blinded fashion in six vessels per mouse (total, 18 vessels) in each experimental group ($p < 0.05$). OE = overexpressor.

Furthermore, we report that PGI₂ partially prevents CSE-induced apoptosis of the pulmonary endothelium *in vitro* and in a murine transgenic model of lung-specific PGI₂S overexpression. The observed decrease in lung tissue PGI₂S gene and protein expression may be multifactorial and may be caused by

oxidant stress, nitric oxide-related suppression of the PGI₂S protein release, and altered transcriptional control (26).

Cigarette smoke contains over 4,000 compounds (27). Researchers have identified acrolein as playing a role in cigarette smoke-induced lung toxicity and potentially in cigarette smoke-induced lung cancer (28–30). Acrolein mediates pulmonary inflammation through the induction of inflammatory cytokines and the inhibition of neutrophil apoptosis (31). These biological changes are likely important to COPD pathogenesis and possibly lung tumorigenesis. In addition, acrolein contributes to endothelial dysfunction through the depletion of glutathione and subsequent oxidative stress (28). Experimentally, we demonstrated that CSE and acrolein reduce the expression of the PGI₂S gene and that CSE increases the expression upstream mediators of the eicosanoid pathway (COX-2 and cPLA2) in HPMVECs.

The induction of oxidative stress by cigarette smoke has been well documented (32). We postulated that the observed imbalance in eicosanoid gene expression may be mediated by oxidative stress. Our first approach was to incubate HPMVECs with NAC, DPI, an SOD mimetic, or a catalase to examine whether the cells would be protected against CSE-related change in PGI₂S gene repression. In addition, we used pharmacologic inhibition of endothelial NOS by preincubating the cells with L-NAME. None of these strategies, whether they were intended to increase intracellular glutathione (NAC) or inhibit the NADPH-oxidoreductase (DPI), prevented the CSE-related repression of the PGI₂S gene. However, NAC did prevent the induction of COX-2 gene expression. Our findings are consistent with previous studies demonstrating that NAC interferes with COX-2 induction in other inflammatory processes (33). Although CSE provides severe oxidative stress to the cultured endothelial cells, a number of antioxidant pretreatment strategies failed to prevent CSE-related decrease in PGI₂S gene expression.

We selected HPMVECs as our experimental system because small-vessel and alveolar capillary endothelial cells undergo apoptosis in human and experimental lung emphysema (1). Using a standardized CSE exposure protocol, we found that CSE differentially and in a dose-dependent manner directly affects PGI₂S gene expression and causes apoptotic cell death in HPMVECs. Although CSE induced a degree of endothelial cell apoptosis, the decrease in PGI₂S mRNA is unlikely the result of apoptotic cell loss. Immunohistochemical analysis suggests that the observed whole lung decrease in PGI₂S expression is the result of decreased endothelial expression rather than fewer numbers of endothelial cells. We demonstrate that pretreatment with the prostacyclin analog iloprost conferred a partial protective antiapoptotic effect. Finally, lung-specific overexpression of PGI₂S resulted in decreased caspase 3 activity after long-term smoke exposure in a transgenic murine model. Our findings are consistent with several recent studies demonstrating that PGI₂ has antiapoptotic effects in other organ systems (34–36).

Ermert and colleagues previously identified decreased expression of PGI₂S in lung tumor-associated vasculature (37). Our laboratory has observed potent antitumorigenic properties of PGI₂S in murine models of lung-specific overexpression, suggesting that PGI₂S may be a tumor suppressor gene (24). In addition, persistent PGI₂S expression in human lung tumors seems to be an independent factor in survival (38). There are many potential mechanisms for the observed decrease expression of PGI₂S, including epigenetic modification and transcriptional regulation. Our findings suggest that acrolein is one key component of cigarette smoke responsible for PGI₂S suppression and may be relevant to COPD and lung tumorigenesis.

In conclusion, PGI₂S gene expression is decreased in lung microvascular endothelial cells by CSE. Furthermore, CSE and acrolein have direct suppressive effects on PGI₂S gene

expression compared with upstream mediators. Whether these experiments reflect the mechanism of decreased PGI₂S in the lungs of chronic smokers is unclear. There are several potential mechanisms for PGI₂S suppression, including transcriptional regulation by methylation, promoter base pair rearrangement by oxidative stress, and alteration in transcriptional binding factors. The observation that acrolein decreased PGI₂S expression, whereas several other aldehydes did not, suggests a mechanism specific to acrolein. The mechanisms by which acrolein suppresses PGI₂S remain unexplored. There are data to suggest that acrolein may interfere with transcriptional regulation of many genes by preferentially binding to CpG sites (29, 39). In addition, acrolein may affect PGI₂S through generation of reactive oxygen species (40). An imbalance in eicosanoid expression may be relevant to the observed vascular toxicity of acrolein. We are currently investigating potential mechanisms of transcriptional regulation of PGI₂S by acrolein.

Our use of CSE as an experimental model is limited in that cigarette smoke exposure of cultured endothelial cells may not adequately represent the actual exposure *in vivo* (41). However, our studies demonstrate reduced PGI₂S expression in the pulmonary endothelium of long-standing smokers with emphysema. To our knowledge, the present study represents the first report that PGI₂ expression is diminished in the pulmonary endothelium in emphysema and that PGI₂ confers antiapoptotic effects to the pulmonary endothelium after acute and chronic cigarette smoke exposure.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank Dr. Brian Freed for his thoughtful review of this manuscript and invaluable discussion during the course of preparation, Dr. David Dewitt (Michigan State) for providing PGI₂S antibody, and Dr. Russ Bowler (National Jewish Medical Center) for providing antioxidant reagents. The authors also thank the University of Colorado lung tissue bank for providing the human lung tissue samples for analysis.

References

- Voelkel NF, Cool CD. Pulmonary vascular involvement in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 2003;46:28s–32s.
- Santos S, Peinado VI, Ramirez J, Melgosa T, Roca J, Rodriguez-Roisin R, Barbera JA. Characterization of pulmonary vascular remodelling in smokers and patients with mild COPD. *Eur Respir J* 2002;19:632–638.
- Liebow AA. Pulmonary emphysema with special reference to vascular changes. *Am Rev Respir Dis* 1959;80:67–93.
- Sakao S, Tatsumi K, Hashimoto T, Igari H, Shino Y, Shirasawa H, Kuriyama T. Vascular endothelial growth factor and the risk of smoking-related COPD. *Chest* 2003;124:323–327.
- Puljic R, Pahl A. Smoke induced changes in epithelial cell gene expression: development of an *in vitro* model for COPD 1. *ALTEX* 2004;21:3–7.
- Hoshino Y, Mio T, Nagai S, Miki H, Ito I, Izumi T. Cytotoxic effects of cigarette smoke extract on an alveolar type II cell-derived cell line. *Am J Physiol Lung Cell Mol Physiol* 2001;281:L509–L516.
- Su Y, Han W, Giraldo C, De LY, Block ER. Effect of cigarette smoke extract on nitric oxide synthase in pulmonary artery endothelial cells. *Am J Respir Cell Mol Biol* 1998;19:819–825.
- Endemann DH, Schiffrin EL. Endothelial dysfunction. *J Am Soc Nephrol* 2004;15:1983–1992.
- Peinado VI, Barbera JA, Ramirez J, Gomez FP, Roca J, Jover L, Gimferrer JM, Rodriguez-Roisin R. Endothelial dysfunction in pulmonary arteries of patients with mild COPD. *Am J Physiol* 1998;274:L908–L913.
- Barbera JA, Peinado VI, Santos S. Pulmonary hypertension in chronic obstructive pulmonary disease. *Eur Respir J* 2003;21:892–905.
- Kasahara Y, Tuder RM, Cool CD, Lynch DA, Flores SC, Voelkel NF. Endothelial cell death and decreased expression of vascular endothelial growth factor and vascular endothelial growth factor receptor 2 in emphysema. *Am J Respir Crit Care Med* 2001;163:737–744.
- Yang YM, Liu GT. Damaging effect of cigarette smoke extract on primary cultured human umbilical vein endothelial cells and its mechanism. *Biomed Environ Sci* 2004;17:121–134.
- Jiao ZX, Ao QL, Xiong M. Cigarette smoke extract inhibits the proliferation of alveolar epithelial cells and induces apoptosis 3. *Sheng Li Xue Bao* 2006;58:244–254.
- Wickenden JA, Clarke MC, Rossi AG, Rahman I, Faux SP, Donaldson K, MacNee W. Cigarette smoke prevents apoptosis through inhibition of caspase activation and induces necrosis. *Am J Respir Cell Mol Biol* 2003;29:562–570.
- Montuschi P, Collins JV, Ciabattini G, Lazzeri N, Corradi M, Kharitonov SA, Barnes PJ. Exhaled 8-isoprostane as an *in vivo* biomarker of lung oxidative stress in patients with COPD and healthy smokers. *Am J Respir Crit Care Med* 2000;162:1175–1177.
- Montuschi P, Kharitonov SA, Ciabattini G, Barnes PJ. Exhaled leukotrienes and prostaglandins in COPD. *Thorax* 2003;58:585–588.
- Barst RJ, Rubin LJ, Long WA, McGoon MD, Rich S, Badesch DB, Groves BM, Tapson VF, Bourge RC, Brundage BH. A comparison of continuous intravenous epoprostenol (prostaglandin) with conventional therapy for primary pulmonary hypertension. The Primary Pulmonary Hypertension Study Group. *N Engl J Med* 1996;334:296–302.
- Tuder RM, Cool CD, Geraci MW, Wang J, Abman SH, Wright L, Badesch D, Voelkel NF. Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension. *Am J Respir Crit Care Med* 1999;159:1925–1932.
- Geraci MW, Gao B, Shepherd DC, Moore MD, Westcott JY, Fagan KA, Alger LA, Tuder RM, Voelkel NF. Pulmonary prostacyclin synthase overexpression in transgenic mice protects against development of hypoxic pulmonary hypertension. *J Clin Invest* 1999;103:1509–1515.
- Reinders JH, Brinkman HJ, van Mourik JA, de Groot PG. Cigarette smoke impairs endothelial cell prostacyclin production. *Arteriosclerosis* 1986;6:15–23.
- Jeremy JY, Mikhailidis DP, Dandona P. Cigarette smoke extracts, but not nicotine, inhibit prostacyclin (PGI₂) synthesis in human, rabbit and rat vascular tissue. *Prostaglandins Leukot Med* 1985;19:261–270.
- Lee JD, Taraseviciene-Stewart L, Keith R, Geraci MW, Voelkel NF. The expression of prostacyclin synthase is decreased in the small pulmonary arteries from patients with emphysema 2. *Chest* 2005;128:575S.
- Carp H, Janoff A. Possible mechanisms of emphysema in smokers: *in vitro* suppression of serum elastase-inhibitory capacity by fresh cigarette smoke and its prevention by antioxidants. *Am Rev Respir Dis* 1978;118:617–621.
- Keith RL, Miller YE, Hudish TM, Girod CE, Sotto-Santiago S, Franklin WA, Nemenoff RA, March TH, Nana-Sinkam SP, Geraci MW. Pulmonary prostacyclin synthase overexpression chemoprevents tobacco smoke lung carcinogenesis in mice. *Cancer Res* 2004;64:5897–5904.
- Korfhagen TR, Glasser SW, Wert SE, Bruno MD, Daugherty CC, McNeish JD, Stock JL, Potter SS, Whitsett JA. Cis-acting sequences from a human surfactant protein gene confer pulmonary-specific gene expression in transgenic mice. *Proc Natl Acad Sci USA* 1990;87:6122–6126.
- Marcelin-Jimenez G, Escalante B. Functional and cellular interactions between nitric oxide and prostacyclin. *Comp Biochem Physiol C Toxicol Pharmacol* 2001;129:349–359.
- Hoffmann D, Hoffmann I, El-Bayoumy K. The less harmful cigarette: a controversial issue. A tribute to Ernst L. Wynder. *Chem Res Toxicol* 2001;14:767–790.
- Misonou Y, Asahi M, Yokoe S, Miyoshi E, Taniguchi N. Acrolein produces nitric oxide through the elevation of intracellular calcium levels to induce apoptosis in human umbilical vein endothelial cells: implications for smoke angiopathy. *Nitric Oxide* 2006;14:180–187.
- Feng Z, Hu W, Hu Y, Tang MS. Acrolein is a major cigarette-related lung cancer agent: Preferential binding at p53 mutational hotspots and inhibition of DNA repair. *Proc Natl Acad Sci USA* 2006;103:15404–15409.
- Hecht SS. Smoking and lung cancer—a new role for an old toxicant? *Proc Natl Acad Sci USA* 2006;103:15725–15726.
- Finkelstein EI, Nardini M, van der Vliet A. Inhibition of neutrophil apoptosis by acrolein: a mechanism of tobacco-related lung disease? *Am J Physiol Lung Cell Mol Physiol* 2001;281:L732–L739.
- van der Vaart H, Postma DS, Timens W, ten Hacken NH. Acute effects of cigarette smoke on inflammation and oxidative stress: a review. *Thorax* 2004;59:713–721.
- Origuchi T, Migita K, Nakashima T, Honda S, Yamasaki S, Hida A, Kawakami A, Aoyagi T, Kawabe Y, Eguchi K. Regulation of cyclooxygenase-2 expression in human osteoblastic cells by N-acetylcysteine. *J Lab Clin Med* 2000;136:390–394.

34. Chen CH, Lin H, Hsu YH, Sue YM, Cheng TH, Chan P, Chen TH. The protective effect of prostacyclin on adriamycin-induced apoptosis in rat renal tubular cells. *Eur J Pharmacol* 2006;529:8–15.
35. Pozner RG, Negrotto S, D'Atri LP, Kotler ML, Lazzari MA, Gomez RM, Schattner M. Prostacyclin prevents nitric oxide-induced megakaryocyte apoptosis. *Br J Pharmacol* 2005;145:283–292.
36. Cutler NS, Graves-Deal R, LaFleur BJ, Gao Z, Boman BM, Whitehead RH, Terry E, Morrow JD, Coffey RJ. Stromal production of prostacyclin confers an antiapoptotic effect to colonic epithelial cells. *Cancer Res* 2003;63:1748–1751.
37. Ermert L, Dierkes C, Ermert M. Immunohistochemical expression of cyclooxygenase isoenzymes and downstream enzymes in human lung tumors. *Clin Cancer Res* 2003;9:1604–1610.
38. Stearman RS, Dwyer-Nield L, Zerbe L, Blaine SA, Chan Z, Bunn PA Jr, Johnson GL, Hirsch FR, Merrick DT, Franklin WA, *et al.* Analysis of orthologous gene expression between human pulmonary adenocarcinoma and a carcinogen-induced murine model. *Am J Pathol* 2005;167:1763–1775.
39. Kehrer JP, Biswal SS. The molecular effects of acrolein. *Toxicol Sci* 2000;57:6–15.
40. Wu CC, Hsieh CW, Lai PH, Lin JB, Liu YC, Wung BS. Upregulation of endothelial heme oxygenase-1 expression through the activation of the JNK pathway by sublethal concentrations of acrolein. *Toxicol Appl Pharmacol* 2006;214:244–252.
41. Shapiro SD. Smoke gets in your cells. *Am J Respir Cell Mol Biol* 2004;31:481–482.