Prostaglandin E₂ inhibits collagen expression and proliferation in patient-derived normal lung fibroblasts via E prostanoid 2 receptor and cAMP signaling

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Huang S, Wetlaufer SH, Hogaboam C, Aronoff DM, Peters-Golden M. Prostaglandin E₂ inhibits collagen expression and proliferation in patient-derived normal lung fibroblasts via E prostanoid 2 receptor and cAMP signaling. Am J Physiol Lung Cell Mol Physiol 292: L405–L413, 2007. First published October 6, 2006; doi:10.1152/ajplung.00232.2006.—Uncontrolled fibroblast activation is one of the hallmarks of fibrotic lung disease. Prostaglandin E₂ (PGE₂) has been shown to inhibit fibroblast migration, proliferation, collagen deposition, and myofibroblast differentiation in the lung. Understanding the mechanisms for these effects may provide insight into the pathogenesis of fibrotic lung disease.

Previous work has focused on commercially available fibroblast cell lines derived from tissue whose precise origin and histopathology are often unknown. Here, we sought to define the mechanism of PGE₂ inhibition in patient-derived fibroblasts from peripheral lung verified to be histologically normal. Fibroblasts were grown from explants of resected lung, and proliferation and collagen I expression was determined following treatment with PGE₂ or modulators of its receptors and downstream signaling components. PGE₂ inhibited fibroblast proliferation by 33% and collagen I expression by 62%. PGE₂ resulted in a 15-fold increase in intracellular cAMP; other cAMP-elevating agents inhibited collagen I in a manner similar to PGE₂. These effects were reproduced by butaprost, a PGE₂ analog selective for the cAMP-coupled E prostanoid (EP) 2 receptor, but not by selective EP3 or EP4 agonists. Fibroblasts expressed both major cAMP effectors, protein kinase A (PKA) and exchange protein activated by cAMP (Epac-1), but only a selective PKA agonist was able to appreciably inhibit collagen I expression. Treatment with okadaic acid, a phosphatase inhibitor, potentiated the effects of PGE₂. Our data indicate that PGE₂ inhibits fibroblast activation in primary lung fibroblasts via binding of EP2 receptor and production of cAMP, inhibition of collagen I proceeds via activation of PKA.

E prostanoid receptor; protein kinase A; exchange protein activated by cAMP-1; okadaic acid; pulmonary fibrosis

PULMONARY FIBROBLASTS PLAY a critical role in regulating the homeostasis of the extracellular matrix in the lung. They synthesize the main matrix component, collagen I, as well as the enzymes involved in collagen degradation, including the family of matrix metalloproteinases. Although fibroblast recruitment, proliferation, and collagen synthesis are important in normal wound healing following lung injury, abnormal or excessive fibroblast activation can lead to pulmonary fibrosis. Indeed, idiopathic pulmonary fibrosis, a disease characterized by excessive fibroblast activation without a known cause, carries a median survival of only 3 years from the time of diagnosis (1, 12). The pathogenesis and factors that influence progression of this disease are still poorly understood, but regulation of fibroblast activation appears to play a key role (39).

Prostaglandins are lipid mediators formed from arachidonic acid via the cyclooxygenase pathway, which affect the proliferative, migratory, and differentiative capacity of numerous cell and tissue types. Prostaglandin E₂ (PGE₂) is the major prostanoid synthesized by lung fibroblasts (48) and neighboring alveolar epithelial cells (3, 40). PGE₂ signals via four different E prostanoid (EP) receptors, EP1-EP4. The EP receptors are a family of G protein-coupled receptors: EP1 signals through Gαi, leading to increased Ca²⁺; EP2 and EP4 signal through Gₛ, leading to increased cAMP; and EP3 primarily signals through Gₛ, leading to decreased cAMP. Protein kinase A (PKA) is the classic effector of cAMP, and its activation traditionally leads to alteration in cellular function by either direct phosphorylation of transcription factors or indirect modulation of other signaling pathways. Cellular phosphatases dephosphorylate many of these same target proteins, providing a brake in signaling (18). Recent studies suggest that phosphatase regulation may play as important a role in regulating cellular response as kinase activation (27, 52, 53). Furthermore, other cAMP effectors, including exchange protein activated by cAMP (Epac), have been recognized to affect diverse signaling pathways involved in differentiation and cell migration. Depending on the specific cell line investigated, PGE₂ has been observed to both stimulate (14, 37, 44, 51) and suppress (4, 6, 23, 26, 28) fibroblast proliferation, differentiation, and growth factor expression. Such heterogeneity may reflect varying expression profiles of EP receptors or downstream effectors within a given fibroblast population.

Studies of PGE₂ in lung fibroblasts have mostly focused on commercially available cell lines that may or may not mimic the human adult lung fibroblast population. In many instances, these have been of rodent or fetal lung origin. In others, the lung compartment or histology from which they are derived is unknown. Such variables may influence the response to PGE₂, as even primary fibroblasts obtained from pulmonary airways vs. peripheral parenchyma differ phenotypically (24). These observations underscore the importance of studying primary cells from defined anatomic compartments and of known histology to determine the biological significance of PGE₂ at the local tissue level. The response to PGE₂ and relevant...
receptor signaling pathways in primary adult parenchymal lung fibroblasts have not been described.

We sought to study the effects of PGE2 on primary human adult lung fibroblasts by culturing fibroblasts from histologically normal lung tissue of patients undergoing surgical lung resection. In these primary cells, we sought to characterize the effects of PGE2 on fibroblast proliferation and collagen synthesis and to define the machinery that transduces these effects, including specific EP receptors, cAMP effectors (PKA and Epac), and phosphatases. Understanding the effects of PGE2 on fibroblast function may provide insight into its role in the pathogenesis of fibrotic lung disease and also highlight its therapeutic potential.

MATERIALS AND METHODS

Materials. DMEM, penicillin/streptomycin, fungizone, and trypsin 0.05% in 0.53 mM EDTA were purchased from Invitrogen (Carlsbad, CA). The serum-free growth medium SFM4MegaVir and FBS were purchased from HyClone (Logan, UT). PGE2 and the prostacyclin analog iloprost were purchased from Cayman Chemical (Ann Arbor, MI) and dissolved in DMSO. The direct adeny cyclase activator forskolin was purchased from Calbiochem (San Diego, CA). The EP2 receptor agonist, butaprost free acid, was obtained from Cayman, whereas the EP4 antagonist, ONO-AE3-208, and the specific EP3 and EP4 agonists, ONO-AE3-248 and ONO-AE1-329, respectively, were provided as generous gifts from Ono Pharmaceuticals (Osaka, Japan). EP4 agonists, ONO-AE3-248 and ONO-AE1-329, respectively, were provided as generous gifts from Ono Pharmaceuticals (Osaka, Japan). The EP2 antagonist, AH-6809, was obtained from BIOMOL (Plymouth Meeting, PA). The PKA-specific cAMP analog, 6-Bnz-cAMP, and the Epac-1-specific cAMP analog, 8-pCPT-2′-O-Me-cAMP, were obtained from Axxora (San Diego, CA). The phosphatase inhibitor okadaic acid was purchased from BIOMOL. The phosphodiesterase inhibitor IBMX and [3H]thymidine were purchased from GE Healthcare (Piscataway, NJ). TRIZol was purchased from Invitrogen, and primer and probe oligonucleotides for semiquantitative real-time RT-PCR were obtained from Integrated DNA Technologies (Corvallis, IA). Protease cocktail inhibitor was obtained from Roche (Indianapolis, IN). Primary antibody for collagen I was obtained from Cedarlane Laboratories (Ontario, Canada) and that for α-tubulin was from Sigma (St. Louis, MO). Antibody for EP2 was obtained from Cayman and that for EP4 was from US Biological (Swampscott, MA). Antibodies for various regulatory and catalytic subunits of PKA (PKA-R1α, PKA-R1β, PKA-R1ββ, and PKA-Cα) were obtained from BD Biosciences (San Jose, CA), whereas that for PKA-R1β was obtained from Chemicon (Temecula, CA) and that for Epac-1 and protein phosphatase 2A (PP2A) catalytic subunit were obtained from Upstate (Charlottesville, VA). Primary antibodies for cAMP response element binding protein (CREB) and Ser133 phospho-CREB were obtained from Cell Signaling (Danvers, MA).

Isolation and culture of pulmonary fibroblasts. Fibroblasts were isolated from lung tissue specimens obtained from the periphery of adult lung resections. In these primary cells, we sought to characterize the effects of PGE2 on fibroblast proliferation and collagen synthesis and to define the machinery that transduces these effects, including specific EP receptors, cAMP effectors (PKA and Epac), and phosphatases. Understanding the effects of PGE2 on fibroblast function may provide insight into its role in the pathogenesis of fibrotic lung disease and also highlight its therapeutic potential.

Proliferation assay. Cells were plated at a concentration of 2 × 10^4 cells/well in a 96-well plate with DMEM plus 10% FBS and allowed to adhere for 8 h at 37°C in 5% CO_2. The medium was then removed, and cells were serum starved in DMEM overnight. After serum starvation, medium was removed and replaced with the serum-free growth medium SFM4MegaVir ± PGE2 at indicated concentrations with the addition of 10 μl of 0.1 mCi/ml [3H]thymidine to each well. The cells were incubated at 37°C for 16–18 h. The cells were then harvested, and the incorporated [3H]thymidine was measured in scintillation fluid using a beta-scintillation counter. Proliferation was also validated by direct cell counts. Cells (4 × 10^5/well) were allowed to adhere for 8 h at 37°C in six-well plates with DMEM plus 10% FBS before being serum starved in DMEM overnight. Medium was then replaced with SFM4MegaVir ± PGE2, and cells were incubated for 18 h. Cells were detached with 0.05% trypsin in 0.53 mM EDTA and counted via microscopy using a hemocytometer.

Collagen I expression. Cells were plated in six-well dishes at 7 × 10^5 cells/well and allowed to adhere for 8 h. They were then serum starved in DMEM overnight. Before treatment, medium was changed to the serum-free growth medium SFM4MegaVir before experimental agents were added. Cells were then cultured at 37°C with 5% CO_2 for 20 h before harvesting. Whole cell lysates were obtained by scraping cells in lysis buffer (phosphate-buffered saline containing 1% Nonident P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM orthovanadate, and protease cocktail inhibitor; Roche). Equal amounts of lysate protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Protran B83, Whatman) by electroblotting. Membranes were blocked in 7% milk/TBST overnight at 4°C. Bound primary antibody was conjugated to horseradish peroxidase and developed with enhanced chemiluminescence reagent (Roche). Equal amounts of lysate protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Protran B83, Whatman) by electroblotting. Membranes were blocked in 7% milk/TBST overnight at 4°C. Bound primary antibody was visualized with appropriate secondary antibody conjugated to horse-radish peroxidase (GE Healthcare) and developed with enhanced chemiluminescence reagent (GE Healthcare). Quantification of chemiluminescent signal was performed by densitometric analysis on all bands using Scion Image (NIH, Frederick, MD). Densitometry of collagen I bands was normalized to α-tubulin, and levels of collagen I protein were expressed as percent of untreated control.

PKA, Epac-1, PP2A, CREB, and phospho-CREB expression. Expression of PKA, Epac-1, PP2A, CREB, and phospho-CREB was assessed by immunoblot. Cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes as described above. For PKA and Epac-1 subunits, membranes were blocked in 5% BSA/TBST and probed with primary antibodies: PKA-R1α (1:10,000), PKA-R1β (1:1,000), PKA-R1ββ (1:10,000), PKA-Cα (1:10,000), PKA-R1ββ (1:5,000), or Epac-1 (1:500). Primary antibodies for CREB and Ser133 phospho-CREB were used at 1:500 and 1:1,000, respectively. PP2Ac subunit was probed at 1:1,000. All primary antibodies were incubated overnight at 4°C. Bound primary antibodies were visualized with appropriate secondary antibody conjugated to horseradish peroxidase and developed with enhanced chemiluminescence reagent. Densitometry analysis for CREB and phospho-CREB was performed in a manner similar to that described above.

Semiquantitative real-time PCR. Cells were plated at 1 × 10^6 cells/well in six-well plates in DMEM plus 10% FBS and left to adhere for at least 8 h. The medium was then removed and replaced with DMEM without serum. After serum starvation for 20 h, the medium was removed, and the RNA solubilized in 1 ml of TRIZol and was extracted according to the manufacturer’s instructions. RNA was quantitated on a spectrophotometer at a wavelength of 260 nm, and the RNA of interest was amplified by semiquantitative real-time RT-PCR performed on an ABI Prism 7000 Thermocycler (Applied Biosystems). Gene-specific primers and probes were designed using Primer Express software (PerkinElmer/Applied Biosystems) and are
RESULTS

PGE<sub>2</sub> inhibits fibroblast proliferation and collagen expression. Previous work had shown that PGE<sub>2</sub> inhibits fibroblast proliferation and collagen synthesis in IMR-90 (11) and WI-38 (28), both human embryonic lung fibroblast cell lines. We sought to examine the effect of PGE<sub>2</sub> on fibroblast proliferation and collagen expression in primary adult lung fibroblasts. Cells were treated overnight with PGE<sub>2</sub> at varying concentrations and assessed for proliferation, as measured by [<sup>3</sup>H]thymidine incorporation (Fig. 1A) and collagen expression, as determined by immunoblot analysis (Fig. 1B). Inhibition of proliferation was also verified by direct cell counts (465,000 cells counted after initial plating; 912,500 cells after 18 h without PGE<sub>2</sub>; 635,000 cells after 18 h with 500 nM PGE<sub>2</sub>). PGE<sub>2</sub> inhibited proliferation and collagen I expression starting at concentrations of 1 nM and 10 nM, respectively. A dose-dependent response was seen, with peak effects at 100–1,000 nM for both proliferation and collagen I. Inhibitory effects of PGE<sub>2</sub> were observed in all cell lines (n = 7), and PGE<sub>2</sub> at 500 nM maximally inhibited proliferation by a mean of 33% (Fig. 1B) and collagen I expression by a mean of 62% (Fig. 1D); this concentration was thus chosen for all subsequent experiments. Increased cAMP is a key mediator in the inhibition of collagen expression by PGE<sub>2</sub>. Previous studies in fetal lung fibroblasts suggest that the suppressive effects on proliferation and collagen synthesis by PGE<sub>2</sub> are mediated by increased cAMP (4, 28). To determine whether this correlation occurs in primary lung fibroblasts, we measured cAMP levels after

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**Fig. 1.** PGE<sub>2</sub> inhibits fibroblast proliferation and collagen synthesis. Fibroblasts in serum-free SFM4MegaVir growth medium were treated with indicated concentrations of PGE<sub>2</sub> overnight (20 h). A: proliferation was determined by [<sup>3</sup>H]thymidine incorporation (results are from a representative experiment, performed in triplicate wells, expressed as means ± SE). B: collagen I expression was assayed by immunoblot analysis and densitometry (results from a representative experiment are shown). A mean of 10 nM maximally inhibited proliferation by a mean of 33% (Fig. 1C) and collagen I expression by a mean of 62% (Fig. 1D); this concentration was thus chosen for all subsequent experiments. In all patient-derived cell lines (n = 7). Results are expressed as means ± SE. *P < 0.05 compared with untreated control.
treatment with PGE2 for 15 min. Treatment with PGE2 resulted in a 15-fold increase in cAMP levels (Fig. 2A). This elevation was further amplified by the addition of a phosphodiesterase inhibitor, IBMX (50 µM), which inhibits the degradation of cAMP. In the presence of IBMX, the degree of cAMP increase seen with PGE2 treatment was comparable to that seen with forskolin, a direct adenyl cyclase activator.

To corroborate the importance of cAMP in mediating the effects of PGE2, we investigated whether other cAMP-elevating agents produced the same biological effects as PGE2. Forskolin, a direct adenyl cyclase activator, and iloprost, a prostacyclin analog that binds to the Gs-coupled I prostanoid receptor (49), both inhibited collagen I expression in a manner similar to PGE2 (Fig. 2B). These results suggest that suppression of collagen production can be accomplished by diverse cAMP-elevating pathways. These findings are consistent with inhibition of collagen synthesis in fetal lung fibroblasts by treatment with a β-adrenergic agonist (36), phenylbutyrate (34), and forskolin (4), and by overexpression of adenyl cyclase (28), all of which increase cAMP.

**Central importance of EP2 in mediating PGE2 inhibition of proliferation and collagen expression.** Both EP2 and EP4 couple to Gi, which stimulates adenyl cyclase, resulting in increased cAMP. To determine which EP receptor(s) were candidates to mediate the ability of PGE2 to inhibit proliferation and collagen expression, we first performed real-time RT-PCR on primary lung fibroblast mRNA for analysis of EP receptor profiles. Relative mRNA expression for EP2 was almost 4-fold greater than EP3 or EP1 and 160-fold greater than EP4 (Fig. 3A). Both EP2 and EP4 receptor protein expression were confirmed by immunoblot analysis (Fig. 3A, insert). We then measured cAMP levels after cells were treated with either the specific EP2 agonist butaprost free acid or PGE2 in the presence or absence of the relatively selective EP2 and EP4 antagonists, AH-6809 and ONO-AE3-208, respectively. Treatment with butaprost free acid resulted in increased cAMP to levels even greater than that seen with PGE2 (Fig. 3B). The EP2 antagonist was able to inhibit the ability of PGE2 to increase cAMP, whereas the EP4 antagonist had no inhibitory effect. We next treated cells with EP-specific agonists and measured collagen I expression by immunoblot. The EP2-specific agonist butaprost free acid suppressed collagen expression to similar levels as PGE2, whereas the EP3 agonist ONO-AE3-248 had little effect. The EP4 agonist ONO-AE1-329 also suppressed collagen, but to a lesser degree compared with PGE2 or butaprost free acid (Fig. 3C). The combination of the EP4 agonist with the EP2 agonist did not suppress collagen to a greater degree than did the EP2 agonist or PGE2 alone (data not shown). The EP agonists also produced a similar pattern of response on fibroblast proliferation (data not shown).

Since the EP4 agonist produced mild effects on collagen and proliferation, we sought to determine whether the EP4 receptor plays a role in PGE2 inhibition of fibroblasts. We pretreated fibroblasts with the EP4 receptor antagonist ONO-AE3-208 for 30 min before the addition of PGE2. The EP4 antagonist was unable to inhibit the suppression of collagen (Fig. 3D) or proliferation (Fig. 3E) by PGE2. Together with the cAMP data, these results suggest that although pharmacological activation of the EP4 receptor can result in mild inhibition of collagen expression and proliferation, PGE2 effects are largely mediated by the cAMP-coupled EP2 receptor, independently of EP4 activation.

**PKA expression and activity.** PKA is the classic intracellular effector of cAMP. PKA exists as a tetramer of two regulatory subunits and two catalytic subunits; multiple isoforms exist for each regulatory (RⅢa, RⅢβ, RIⅢα, and RIⅢβ) and catalytic (CⅢ, CB, Cγ) subunit. Catalytic activity occurs when cAMP binds to the regulatory subunits and releases the catalytic subunit. As shown in Fig. 4A, all four regulatory subunits (RⅢα, RⅢβ, RIⅢα, and RIⅢβ) as well as the catalytic subunit PKA-CⅢ are expressed in primary adult lung fibroblasts. The transcription factor CREB is a key downstream phosphorylation target and effector of PKA. CREB can be activated by PKA-dependent phosphorylation at Ser133, and its phosphorylation often serves as a marker for intact cell PKA activity. In primary adult human fibroblasts, stimulation with PGE2 resulted in CREB phosphorylation within minutes (Fig. 4B). By 2 h, phosphorylation was no longer detectable.

**PKA is the dominant effector of cAMP that inhibits collagen expression.** Epac-1 is a guanine nucleotide exchange factor that is directly activated by cAMP and activates the GTPase Rap1.

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**Fig. 2.** cAMP production and collagen suppression by other cAMP-elevating agents. A: primary lung fibroblasts (n = 3) were treated for 15 min with PGE2 (500 nM) or forskolin (100 µM) in the presence or absence of IBMX (50 µM), a phosphodiesterase inhibitor that prevents degradation of cAMP; cAMP levels were measured as described in MATERIALS AND METHODS. Results are expressed as means ± SE. *P < 0.05 relative to no treatment control, ANOVA. B: treatment with cAMP-elevating agents forskolin (100 µM, n = 4) and iloprost (500 nM, n = 6) decreased collagen I protein expression in primary lung fibroblasts in a manner similar to PGE2. Results are expressed as means ± SE. *P < 0.05 relative to no treatment control, ANOVA.
Rap1, in turn, can activate other growth factor signaling pathways, including ERK1/2 (7, 20). The Epac-1 pathway is distinct from the traditional cAMP-mediated PKA pathway but is thought to have an increasingly important role in many cellular processes. As seen in Fig. 4A, expression of Epac-1 was observed for the first time in primary lung fibroblasts. Furthermore, we verified its functional activity by demonstrating that treatment with the specific Epac-1 agonist 8-pCPT-2′-O-Me-cAMP increased active GTP-bound Rap1, whereas the specific PKA agonist 6-Bnz-cAMP did not (Fig. 5). To distinguish which cAMP-dependent pathway mediates the inhibitory effects of PGE2 on collagen, we measured collagen I expression following overnight incubation with either a specific PKA or Epac-1 agonist. Collagen expression, measured by immunoblot, was inhibited by the specific PKA agonist 6-Bnz-cAMP, whereas the specific Epac-1 agonist 8-pCPT-2′-O-Me-cAMP had no effect (Fig. 5). The combination of 6-Bnz-cAMP and 8-pCPT-2′-O-Me-cAMP resulted in collagen suppression similar to 6-Bnz-cAMP alone (data not shown). This suggests that PKA is the dominant effector of cAMP in the ability of PGE2 to inhibit collagen.

The phosphatase inhibitor okadaic acid potentiates the ability of PGE2 to suppress collagen synthesis. Serine-threonine phosphatases dephosphorylate many of the targets of PKA, thereby providing a brake on PKA signaling (52). Okadaic acid is a serine-threonine phosphatase inhibitor that has varying specificity for different phosphatases depending on the dose. At doses less than 1 μM, okadaic acid has relative selectivity for inhibiting PP2A vs. PP1 (9). Since PKA is the major signaling pathway for PGE2 in lung fibroblasts, we expected that blocking PP2A may amplify the ability of PGE2 to suppress collagen expression. We first verified the expression of the catalytic subunit of PP2A in primary fibroblasts (Fig. 6). We next treated the fibroblasts with okadaic acid (40 nM) for 30 min before the addition of PGE2 and incubated them for an additional 20 h before harvesting. Okadaic acid not only suppressed collagen expression by itself but further amplified the effects of PGE2 (Fig. 6). Incubation with okadaic acid at 40 nM for 20 h with or without PGE2 did not result in significant cell toxicity. Additional experiments showed no such effect with the alternative phosphatase inhibitor, calyculin A (data not shown), at concentrations favoring...
PP1 inhibition, suggesting that this biological effect of okadaic acid is PP2A specific. In the presence of okadaic acid, CREB phosphorylation was detectable at 20 h (Fig. 6) compared with its dephosphorylation within 2 h when cells were treated with PGE2 in its absence (Fig. 4B).

**DISCUSSION**

PGE2 can exert either stimulatory or inhibitory effects on fibroblasts, depending on the specific cell line or tissue of origin. PGE2 stimulated proliferation in NIH/3T3 cells (44) and 3T6 cells (37), glycosaminoglycan synthesis in cervical fibroblasts (50), and expression of early growth response factor-1, a transcription factor that regulates cell growth and differentiation, in synovial fibroblasts (8). On the other hand, lung fibroblasts, PGE2 inhibited migration (22, 47), proliferation (6, 26), collagen synthesis (10, 11, 23), and myofibroblast differentiation (23). Much of this work has been accomplished in either rodent cells or commercially available human cell lines, many of which are either fetal in origin or are derived from immortalized cell lines.

**Fig. 4.** Protein kinase A (PKA) subunit and exchange protein activated by cAMP-1 (Epac-1) expression and cAMP response element binding protein (CREB) phosphorylation. A: immunoblot analysis for PKA subunits and Epac-1 was performed on primary human lung fibroblasts as described in MATERIALS AND METHODS. Results from a representative experiment are shown. B: total CREB and phospho-CREB (P-CREB; Ser133) were measured by immunoblot after treatment in the presence or absence of PGE2 (500 nM) for the indicated time points. Data in the graph represent the densitometric ratio of phospho-CREB to total CREB, with the untreated control taken as 1. Results from a representative experiment are shown.

**Fig. 5.** Effect of PKA and Epac agonists on collagen I expression. Primary lung fibroblasts were treated with PGE2 (500 nM), the specific PKA agonist 6-Bnz-cAMP (500 μM), or the specific Epac-1 agonist 8-pCPT-2’-O-MecAMP (500 μM) for 15 min, and GTP-Rap1 was isolated by affinity purification as described in MATERIALS AND METHODS. Total and active GTP-Rap1 were assayed by immunoblot analysis as shown. Primary lung fibroblasts were treated with the specific PKA agonist 6-Bnz-cAMP (500 μM) or the specific Epac-1 agonist 8-pCPT-2’-O-MecAMP (500 μM) in serum-free SFM4MegaVir growth medium overnight (20 h), and collagen I expression was measured by immunoblot (n = 5). Results are expressed as means ± SE. *P < 0.05 relative to no treatment control.

**Fig. 6.** Effect of PGE2 and the protein phosphatase 2A (PP2A) phosphatase inhibitor okadaic acid on collagen I expression. Expression of the PP2A catalytic subunit was demonstrated by immunoblot (inset, representative of n = 6). Primary lung fibroblasts were treated overnight (20 h) in the presence of PGE2 and/or okadaic acid in serum-free SFM4MegaVir growth medium, and collagen I protein expression and amount of CREB phosphorylation at 20 h were determined by immunoblot. Tubulin was used as a loading control. An immunoblot from a representative experiment is shown. Graphical data represent the densitometric ratio of collagen in experimental treatments relative to no treatment control (n = 2).
from lung tissue of uncertain anatomic compartment or histopathology. The importance of the precise tissue source is exemplified by the recent observation that primary adult human fibroblasts derived from the conducting airways differ markedly in baseline morphology, proliferation, procollagen, and α-smooth muscle actin expression from those derived from parenchymal tissue (24). Variations in the origin of even primary lung fibroblasts may be associated with profound differences in response to PGE2. As our interests center on parenchymal pulmonary fibrosis, we sought, in this investigation, to characterize the biological effects and modes of action of PGE2 on primary fibroblasts obtained from adult lung parenchyma verified to be histologically normal.

We have shown that PGE2 inhibited both proliferation and collagen expression. cAMP is a key mediator of these effects; cells treated with PGE2 had increased cAMP levels, and other cAMP-elevating agents, such as forskolin and iloprost, resulted in decreased collagen expression similar to PGE2. These data are consistent with results seen in forskolin-treated IMR-90 cells (4) and adenyl cyclase-overexpressing WI-38 cells (28), both of fetal lung origin.

Although cAMP is a key mediator in the suppression of proliferation and collagen expression by PGE2, the specific receptor involved in PGE2 signaling had not been identified since both EP2 and EP4 are coupled to Gs and signal via increased cAMP production (13, 32, 33). Previous studies of EP receptor expression in IMR-90 cells used non-quantitative RT-PCR and have shown differing results in regards to relative EP receptor expression (4, 23). We used quantitative real-time RT-PCR in primary lung fibroblasts to show that EP2 is expressed in greater abundance relative to the other EP receptors. To determine which EP receptor might mediate the functional effects of PGE2, we used selective EP agonists and showed that EP2 activation increased intracellular levels of cAMP and suppressed collagen and proliferation to a degree similar to PGE2, whereas the EP3 agonist had no effect, and the EP4 agonist had only a mild effect. The combination of the EP4 and EP2 agonists did not suppress collagen expression more than the EP2 agonist alone, and the EP4 antagonist was unable to inhibit the effects of PGE2 on collagen expression or proliferation. These data confirm the dominant role of EP2 in PGE2 suppression of collagen expression and proliferation, with EP4 having modest effects only under pharmacological conditions. Studies of myofibroblast differentiation (23) and fibroblast migration (47) also support the role of EP2 in PGE2 signaling, suggesting that this receptor mediates many of the inhibitory actions of PGE2 and that these biological responses share a common pathway. The importance of EP2 is further supported by observations that EP2 receptor expression decreases during the development of bleomycin-induced pulmonary fibrosis in wild-type mice and that EP2 knockout mice show an increased susceptibility to bleomycin-induced fibrosis (31).

PKA is the classic effector of cAMP. Activation of PKA is commonly assessed by measuring Ser133 phosphorylation of its classic target, CREB. CREB is a transcription factor that is a key regulator of fibroblasts (17, 29). We showed that PGE2 induced CREB phosphorylation at Ser133 within minutes before gradually decreasing over 2 h. This verifies PKA activation by PGE2 in these cells.

Other effectors of cAMP, including Epac, are increasingly recognized (30). No prior reports have differentiated which downstream effectors of cAMP are responsible for the effects of PGE2 on lung fibroblasts. We show for the first time that primary lung fibroblasts express both Epac-1 and PKA. Epac is a guanine nucleotide exchange factor that traditionally activates Rap1 (5). Rap1 is thought to be important in regulating cell migration (25) and proliferation (38), but its importance in lung fibroblast activation is not fully characterized. We sought to determine the relative roles of PKA and Epac-1 in mediating cAMP effects in primary lung fibroblasts. The selective activation of PKA inhibited collagen to the same degree as PGE2, whereas activation of Epac-1 did not.

A growing body of evidence suggests that phosphatases may play as important a role as kinases in regulating cell signaling (18, 52). Okadaic acid, an inhibitor of the serine/threonine PP2A, has been shown to inhibit collagen type I gene expression in osteoblasts (2), skin fibroblasts (46), and 3T3 cells (21, 43), while stimulating expression of collagenase-1, an enzyme involved in collagen degradation (45). However, no previous work using phosphatase inhibitors alone, or in the presence of PGE2, has been done on primary adult human lung fibroblasts. In patient-derived lung fibroblasts, okadaic acid by itself decreased collagen I expression. More interestingly, it had a synergistic effect with PGE2 in inhibiting collagen expression, which was greater than that seen with maximal concentrations of PGE2 alone. Presumably, this reflects inhibition of cellular phosphatases, especially PP2A, resulting in enhanced phosphorylation of PKA targets. Notably, PGE2-induced CREB phosphorylation at Ser133 was much more persistent (>20 h) in the presence of okadaic acid than with PGE2 alone (2 h).

Our experiments focused on the proximal steps in PGE2 signaling. With four different EP receptors implicated in a host of cellular responses of different cell types, we established that EP2 activation plays a dominant role in the cAMP-dependent inhibitory effects of PGE2 in primary lung fibroblasts. Moreover, over PKA appears to be the cAMP effector responsible at least for the inhibition of collagen expression. PKA can activate other transcription factors and signaling pathways through its kinase activity, and further experiments are needed to elucidate the relevant downstream pathways. Furthermore, specificity in PKA signaling can derive from the actions of specific regulatory subunits. In our primary lung fibroblasts, we were able to see expression of the major PKA catalytic (Cα) and all regulatory (RⅠα, RⅠβ, RⅡα, RⅡβ) subunits. The relative importance of these specific PKA isoforms in PGE2 signaling remains to be determined.

Collagen I is the most abundant collagen type produced by lung fibroblasts (15). The net balance of collagen in the extracellular matrix is dependent on several factors, including the transcription of the procollagen gene, posttranslational modification of the protein, and its degradation by matrix metalloproteinases. As an index of this net balance, we chose to measure total collagen I expression by immunoblot, but the relative importance of these various determinants of total collagen I was not explored. Others have shown that PGE2 downregulates collagen synthesis, as measured by [3H]proline incorporation, while upregulating matrix metalloproteinase 2 (28).

Idiopathic pulmonary fibrosis is a disease whose pathogenesis is unknown, that lacks effective treatment, and whose mean survival is only 50% at 3 years from the time of diagnosis. Our laboratory (48) and several others (19, 41) have shown that PGE2 production by fibroblasts is diminished in...
patients with pulmonary fibrosis. Understanding the effects and mechanism of action of PGE2 on primary lung fibroblasts may yield insight into the pathogenesis of fibrotic lung disease and highlight novel therapeutic targets. The ability of PGE2, EP2 agonists, other cAMP-elevating agents such as iloprost, PKA agonists, and phosphatase inhibitors such as okadaic acid to inhibit fibroblast activation highlights their therapeutic potential. Furthermore, this potential may extend to fibrotic diseases of other organs, including skin, kidney, liver, and arterial vasculature.

In conclusion, we sought to study the effects of PGE2 on primary adult human lung fibroblasts and to define its mechanism of action. We used patient-derived parenchymal fibroblasts to ascertain effects of this prostanoid. We have shown that PGE2 inhibits collagen expression and proliferation and that this occurs through an EP2- and cAMP-dependent process. Furthermore, despite expression of both Epac and PKA subunits, PKA is the dominant effector of cAMP actions, and the inhibitory actions of PGE2 are potentiated with the administration of a PP2A-specific phosphatase inhibitor. Additional studies are needed to define the pathways downstream of PKA and CREB. Nevertheless, our findings in normal primary fibroblasts provide a framework for studying the response to PGE2 in fibroblasts from patients with fibrotic lung disease.

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REFERENCES

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