cAMP-elevating agents and adenyl cyclase overexpression promote an antifibrotic phenotype in pulmonary fibroblasts

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Liu, Xiaohui, Rennolds S. Ostrom, and Paul A. Insel. cAMP-elevating agents and adenyl cyclase overexpression promote an antifibrotic phenotype in pulmonary fibroblasts. Am J Physiol Cell Physiol 286: C1089–C1099, 2004.—Pulmonary fibroblasts are recruited to sites of lung injury, where they are activated to produce extracellular matrix proteins and to facilitate repair. However, these cells become dysregulated in pulmonary fibrosis, producing excess collagen at sites of injury and forming fibrotic loci that impair lung function. In this study, we used WI-38 human lung fibroblasts and evaluated the ability of G protein-coupled receptor agonists to increase cAMP production and regulate cell proliferation and collagen synthesis. WI-38 cells increase cAMP in response to the β-adrenergic agonist isoproterenol (Iso), prostaglandin E2 (PGE2), certain prostanoid receptor-selective agonists (beraprost, butaprost), an adenosine receptor agonist, and the direct adenyl cyclase activator forskolin (Fsk). Responses to Iso, PGE2, and Fsk were studied in more detail. Each induced a dose-dependent inhibition of serum-stimulated cell proliferation (as measured by 3H)proline incorporation) and collagenase-sensitive collagen synthesis from ATP, and cyclic nucleotide phosphodiesterases (protein kinase A). cAMP levels are regulated by the activity of Gs-AC overexpression promotes an antifibrotic phenotype in pulmonary fibroblasts. These results suggest that therapies that raise cAMP levels may prove useful in the treatment of pulmonary fibrosis.

β-adrenergic receptors; prostaglandin; prostanoid receptors; pulmonary fibrosis; extracellular matrix

PULMONARY FIBROBLASTS, a predominant cell type in the lung, play a critical role in the homeostasis of extracellular matrix (ECM). Recruitment, accumulation, and activation of pulmonary fibroblasts contribute to normal wound healing and to the development of interstitial lung diseases, such as idiopathic pulmonary fibrosis (IPF) (43). IPF, which has limited effective therapy and a 5-year survival rate of only 30%, is characterized by the irreversible and excessive growth of connective tissue that leads to impaired lung function (20, 24, 46). Pulmonary fibrosis has been viewed traditionally as a consequence of inflammation, but this interpretation has been questioned in recent years because clinical measures of inflammation do not correlate well with disease progression and because anti-inflammatory therapy does not appear to affect clinical outcome (8). For these reasons, recent attention has focused on the mechanisms of wound healing and remodeling, including the production of ECM proteins, the activity of ECM homeostatic enzymes, such as matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP), and the activation and differentiation of pulmonary fibroblasts (20, 43, 44). Differentiation of fibroblasts to myofibroblasts, the cell type associated with collagen synthesis and scar formation, and the persistence of the myofibroblast phenotype may be key elements in the progression of IPF (29, 40). Myofibroblast differentiation is stimulated by cytokines such as transforming growth factor (TGF)–β1, tumor necrosis factor (TNF)–α, and platelet-derived growth factor (43). However, much less is understood about the signals that inhibit fibroblast proliferation, differentiation, and activation (10, 28, 50).

Reduction of tissue distortion due to fibrosis, a key therapeutic goal in interstitial lung diseases, may be attained by enhancing signals that inhibit fibroblast proliferation and collagen synthesis. CAMP is a ubiquitous second messenger that influences growth, death, and differentiated cell functions primarily through its ability to increase phosphorylation of proteins via the activation of CAMP-dependent protein kinase (protein kinase A). CAMP levels are regulated by the activity of both the enzyme adenyl cyclase (AC), which catalyzes its synthesis from ATP, and cyclic nucleotide phosphodiesterases (PDEs) that catalyze its degradation. Prostacycins analogs, prostaglandin (PG)E2, and PGD3 inhibit lung fibroblast migration, proliferation, and collagen synthesis (7, 25, 27). Because these agents are capable of activating receptors that couple to Gi and the stimulation of AC, CAMP appears to be a negative regulator of fibroblast function. The importance of the Gs-AC-CAMP pathway in the progression of IPF is further implied by evidence that this system is compromised after experimental pulmonary fibrosis induced by bleomycin (21) and that fibroblasts from IPF patients have a diminished capacity to generate PGE2 (48). Moreover, PGE2 and CAMP elevation inhibit the differentiation of pulmonary fibroblasts into myofibroblasts (28). Thus CAMP appears to attenuate pulmonary fibroblast differentiation and collagen synthesis. However, the effects of CAMP on MMP and TIMP expression and the ability of other CAMP-elevating agonists or AC itself to inhibit fibroblast function are not known. It is also not known whether increased AC expression might enhance the antifibrotic effects of such CAMP-elevating agonists.
In the present study, we have used WI-38 cells, a human pulmonary fibroblast cell line, to test the hypotheses that cAMP-elevating agonists inhibit cell proliferation and collagen synthesis in pulmonary fibroblasts and that increased expression of AC enhances the activity of these agonists. We find that forskolin (Fsk), isoproterenol (Iso), PGE₂, prostacyclin analogs, or overexpression of AC6 (an endogenously expressed isoform of AC) inhibits pulmonary fibroblast cell proliferation and total collagen synthesis. We also find that increased cellular cAMP levels are associated with decreased expression of mRNA for collagen types 1α(II) and 5α(I) and increased expression of MMP-2 and TIMP-1. MMP-2 activity was also increased by 24-h treatment with cAMP-elevating agents, but TIMP-1 protein was paradoxically reduced. We conclude that although the effects of cAMP on proteins involved in ECM synthesis and degradation are complex, approaches that enhance cAMP formation or block its degradation inhibit cell proliferation and net collagen synthesis of pulmonary fibroblasts. These results suggest that increasing cellular cAMP levels may provide a means to blunt pulmonary fibrosis.

**MATERIALS AND METHODS**

**Materials and cell culture.** Primary antibodies for MMP-1 and MMP-2 were obtained from Chemicon. Primary antibodies for TIMP-1 and TIMP-2 were obtained from Oncogene Research Products. All other antibodies were obtained from Santa Cruz Biotechnology. Radiolabeled chemicals were obtained from Perkin Elmer. Other chemicals and reagents were obtained from Sigma. WI-38 cells were obtained from American Type Culture Collection and were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin and kept in a 37°C incubator with 5% CO₂. Cells were used between 30% and 80% confluence, and synchronized by serum deprivation in MEM containing 0.25% FBS for 2 h followed by serum deprivation in MEM containing 0.25% FBS for 24 h. MEM was then supplemented with 2.5% FBS (except for unstimulated conditions, where 0.25% FBS was used) for 24 h along with 0.5 μCi of [³H]proline/well and drugs of interest. Medium was then replaced, and the cells were washed with ice-cold PBS and then incubated with 7.5% TCA for 1 h at 4°C. TCA-precipitated counts were determined by liquid scintillation counting. In some studies, collagenase-sensitive [³H]proline incorporation was assayed as previously described (37). Briefly, cells were removed by trypsinization and protein was precipitated in 20% TCA. Pellets were washed three times with 1.0 ml of 5% TCA-0.01% proline and then dissolved with 0.2 M NaOH and titrated to neutral pH. Samples were incubated with collagenase (2 mg/ml; Worthington Biochemical) in Tris-Cl-N-ethylmaleimide buffer for 1 h at 37°C and then precipitated with 10% TCA for 1 h on ice. Samples were centrifuged at 14,000 rpm for 10 min, and the collagenase-sensitive [³H]proline in the supernatant was determined by liquid scintillation counting.

**Quantification of human procollagen type I C-peptide.** Type I collagen derives from a larger protein, type I procollagen, which has propeptide extensions at both ends of the molecule. Specific enzymes remove these propeptides before the collagen molecules are assembled into fibers. The sequence removed from the carboxy terminus, procollagen type I C-peptide (PICP), is secreted by cells, and its level reflects the amount of synthesis of type I collagen. For PICP determination, cells were incubated under the same conditions as described for collagen synthesis. The medium in each well was then collected and frozen until being assayed with a PICP enzyme immunoassay (Takara) following the manufacturer’s instructions.

**Reverse transcriptase-PCR.** Reverse transcriptase-PCR (RT-PCR) for AC isoforms was performed with the primer pairs described by Xu et al. (49). Total RNA was extracted from WI-38 cells grown to 80–90% confluency on 10-cm plates with Trizol reagent (Invitrogen) and an RNeasy RNA isolation kit (Qiagen). A DNase reaction was performed to eliminate DNA contaminants, and the RNA was reverse transcribed with Superscript II (Invitrogen) and poly(dT) primer. PCR reactions with each primer pair were performed on cDNA, genomic DNA (positive control), and minus RT (negative control) templates. PCR products were analyzed by agarose gel electrophoresis and visualized under UV light with ethidium bromide. For real-time quantitative PCR studies, RT-PCR was performed initially to confirm that single PCR products resulted from reactions with each primer pair. Suitable primers were then used in real-time PCR reactions with Sybr Green (Applied Biosystems). The primers were designed based on GenBank sequences and are listed in Table 1. The thermal profile for all real-time PCR reactions was 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60–62°C for 1 min. Fluorescence data from each sample were analyzed with the 2⁻ΔΔCt method with the vehicle-treated control as the calibrator: fold induction = 2⁻ΔΔCt, where ΔΔCt = [Ct G1 (unknown sample) – Ct β-actin (unknown sample)] – [Ct G1 (calibrator sample) – Ct β-actin (calibrator sample)], G1 is the gene of interest, and Cβ-actin is the cycle threshold (the cycle number where the fluorescent signal crosses an arbitrary intensity threshold).

**Immunoblot analysis.** Whole cell lysates were obtained from WI-38 cells by scraping cells in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, plus mammalian protease inhibitor cocktail) and homogenizing by sonication. Equal protein amounts of the lysates were separated by SDS-polyacrylamide gel electrophoresis (7.5% acrylamide and 0.1% SDS) and transferred to polyvinylidene difluoride membranes (Millipore) by electroblotting. Membranes were blocked in 20 mM phosphate-buffered saline (PBS) with 3% nonfat dry milk and incubated with primary antibody (see Materials and cell culture).
Table 1. Primer sequences used for RT-PCR studies

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Collagen type I α1</td>
<td>5’-GTGCTAAAGTGGCGGATGAGT-3’</td>
</tr>
<tr>
<td>Collagen type I α2</td>
<td>5’-ACGGGTTGAGTCGGGAGG-3’</td>
</tr>
<tr>
<td>Collagen type III α1</td>
<td>5’-CGGAGTGGCTGAGGAGG-3’</td>
</tr>
<tr>
<td>Collagen type V α1</td>
<td>5’-GGCTAAGGAGGAGG-3’</td>
</tr>
<tr>
<td>Collagen type VI α1</td>
<td>5’-GAGGATGGCCAGGAGG-3’</td>
</tr>
<tr>
<td>Collagen type VI α2</td>
<td>5’-TCCTCTGCTGCTGAGG-3’</td>
</tr>
<tr>
<td>Collagen type VI α3</td>
<td>5’-CTGGCCAGACATGACATTTG-3’</td>
</tr>
<tr>
<td>MMP1</td>
<td>5’-GAAGCTTCTGCCGATGTTTTTGGCA-3’</td>
</tr>
<tr>
<td>MMP2</td>
<td>5’-TGAGCTTCTGCCGATGTTTTTGGCA-3’</td>
</tr>
<tr>
<td>TIMP1</td>
<td>5’-GAGCTTCTGCCGATGTTTTTGGCA-3’</td>
</tr>
<tr>
<td>TIMP2</td>
<td>5’-GAGCTTCTGCCGATGTTTTTGGCA-3’</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>5’-GCTTTCGATGTTTTTGGCA-3’</td>
</tr>
<tr>
<td>Laminin</td>
<td>5’-CAATCCAGGAGGAAATCTCTC-3’</td>
</tr>
<tr>
<td>Elastin</td>
<td>5’-CGGCTTACGATGTTTTTGGCA-3’</td>
</tr>
<tr>
<td>Decorin</td>
<td>5’-GAGTCAAGGAAATCTCAGTTTTTGGCA-3’</td>
</tr>
<tr>
<td>Fibrillin-1</td>
<td>5’-GAGTCAAGGAAATCTCAGTTTTTGGCA-3’</td>
</tr>
<tr>
<td>Fibrillin-2</td>
<td>5’-GAGTCAAGGAAATCTCAGTTTTTGGCA-3’</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5’-GAGTCAAGGAAATCTCAGTTTTTGGCA-3’</td>
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MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.

overnight at 4°C. Bound primary antibodies were visualized with appropriate secondary antibody with conjugated horseradish peroxidase (Santa Cruz Biotechnology) and enhanced chemiluminescence reagent (Pierce). Most primary antibodies recognized multiple nonspecific species; only the band representing the appropriately sized molecule is shown here. The amount of protein per sample was determined with a dye-binding protein assay (Bio-Rad).

DNA dot blot hybridization. We used the GEArray Q Series Human Extracellular Matrix & Adhesion Molecules Gene arrays (SuperArray) to assess gene expression with total RNA prepared by Trizol reagent (Invitrogen). cDNA probes were synthesized from two total RNA samples with the Probe Synthesis Kits and GEArray primer mixes (SuperArray). The cDNA probes were then hybridized to the gene-specific cDNA fragments spotted on the membranes according to the manufacturer’s instructions. The expression level of each gene on the membrane was determined by quantifying the chemiluminescent signal with digital image analysis and then normalizing to the level of GAPDH expression.

Gelatin zymography. Media samples from treated cells and control cells were subjected to in-gel zymography with gelatin substrate. NuPAGE zymographic gels (Invitrogen) were used according to the manufacturer’s instructions. Briefly, samples were mixed with SDS sample buffer, and SDS-PAGE was run with Tris-glycine running buffer. Gels were incubated in renaturing buffer followed by two incubations in developing buffer for 4 h at 37°C. Gelatin gels were stained with Coomassie blue for 30 min and then destained in 30% methanol-10% acetic acid. Bands caused by degradation of the enzyme substrate in the gels indicate the presence of substrate-degrading proteinases. The identity of the proteinases was confirmed by their molecular weight, as estimated from molecular weight standards. Enzymatic activity was quantified by digitalizing the zymographs and then analyzing them with the NIH Image program. The product of the pixel density and the surface area digested was calculated to determine the gelatinase activity in each sample.

Data analysis and statistics. Data are presented as means ± SE of at least three separate experiments. Statistical comparisons (t-tests and 1-way analysis of variance) and graphics were performed with GraphPad Prism 4.0 (GraphPad Software).

RESULTS

Regulators of cAMP production in pulmonary fibroblasts. To characterize the responsiveness of WI-38 cells to agonists that activate G protein-coupled receptors that couple to Ga, we evaluated cAMP production in response to several such agonists in the presence of a nonselective PDE inhibitor, IBMX (Fig. 1A). Significant (P < 0.05 compared with basal) stimulation was measured in response to the direct activator of AC

Fig. 1. cAMP production in WI-38 cells. cAMP accumulation in WI-38 cells was measured in the presence of maximal concentrations of the indicated drugs (A). Fsk, forskolin; PGE_2, prostaglandin E_2, Iso, isoproterenol; NECA, 5’-(N-ethylcarboxamido)adenosine; CGRP, calcitonin gene-related peptide. cAMP production was also measured in response to various concentrations of Fsk, PGE_2, or Iso (B). Each point or bar represents the mean ± SE of at least 6 experiments. *P < 0.05 compared with basal by paired t-test.
cAMP INHIBITS PULMONARY FIBROBLASTS

Fsk (1 μM), the β-adrenergic receptor agonist Iso (1 μM), PGE2 (1 μM), the prostacyclin analog beraprost (1 μM), the EP2-selective agonist butaprost (1 μM) and the adenosine receptor agonist 5′-(N-ethylcarboxamido)adenosine (NECA; 10 μM). Calcitonin gene-related peptide (CGRP; 0.1 μM) did not increase cAMP production over basal levels. Fsk, Iso, PGE2, beraprost, and butaprost also each stimulated cAMP production in the absence of a PDE inhibitor (data not shown). Iso stimulated cAMP production with an EC50 of 0.4 μM and a maximum response of 115 ± 6.8 pmol cAMP/mg protein (Fig. 1B). PGE2 stimulated cAMP production with similar potency but higher efficacy, exhibiting an EC50 of 0.7 μM and a maximum response of 156 ± 8.7 pmol cAMP/mg protein. Fsk was less potent than either Iso or PGE2, with an EC50 of 7.3 μM but a higher maximum response, 227 ± 10.1 pmol cAMP/mg protein.

**cAMP-elevating agents inhibit pulmonary fibroblast proliferation.** Pulmonary fibroblasts stimulated with proinflammatory cytokines differentiate into myofibroblasts, a process that can be inhibited by cAMP and that may relate to a decrease in cell proliferation (28). Thus we measured [3H]thymidine incorporation as an index of DNA synthesis in WI-38 cells that had been serum starved for 24 h in 0.25% FBS and then grown for 24 h with 2.5% FBS (a maximally effective concentration; data not shown) and either vehicle or various concentrations of cAMP-elevating agonists. Fsk and PGE2 inhibited FBS-stimulated [3H]thymidine incorporation in a concentration-dependent manner, with Fsk and PGE2 inducing a maximum of 86 ± 5% and 56 ± 3% inhibition, respectively (Fig. 2). Iso (1 μM) or IBMX (0.2 mM) had effects similar to that of PGE2, each inducing a maximum of 60–75% inhibition (data not shown). None of the above treatments changed cell number over a 24-h period. Thus numerous approaches for increasing cAMP levels inhibit pulmonary fibroblast proliferation.

**cAMP-elevating agents inhibit pulmonary fibroblast collagen synthesis.** Stimulation of prostanoid or β-adrenergic receptors (β-ARs) can inhibit collagen synthesis in pulmonary fibroblasts (5, 28, 32). Thus we examined whether the cAMP-elevating agonists identified above could inhibit collagen synthesis in WI-38 cells. We measured [3H]proline incorporation in cells that had been serum starved for 24 h in 0.25% FBS and then activated for 24 h with 2.5% FBS and either vehicle or various concentrations of cAMP-elevating agonists. Because collagen consists of 33% proline, [3H]proline incorporation is a semiselective measure of collagen synthesis (13). Fsk, Iso, and PGE2 inhibited FBS-stimulated [3H]proline incorporation in a concentration-dependent manner, with Fsk and PGE2 inducing a >100% inhibition (high concentrations reduced [3H]proline incorporation below that of unstimulated cells; Fig. 3). Iso was nearly as efficacious as Fsk in inhibiting FBS-stimulated [3H]proline incorporation, maximally inhibiting this response 93 ± 4%. IBMX (0.2 mM) reduced basal and FBS-stimulated [3H]proline incorporation (data not shown) and therefore was not included in assays of [3H]proline incorporation in response to agents that stimulate cAMP synthesis. Thus cAMP-elevating agents inhibit both proliferation and [3H]proline incorporation by pulmonary fibroblasts.

To confirm that the observed effects of cAMP-elevating agents on [3H]proline incorporation are specific to the synthesis of collagen (versus that of other proline-containing proteins or the degradation of collagen containing [3H]proline), we measured the levels of PICP, a peptide that is cleaved from the carboxy terminus of procollagen type I during posttranslational processing into collagen fibers (47). Treatment of serum-starved cells with 2.5% FBS increased PICP levels in the culture media nearly threefold; addition of Fsk or PGE2 together with FBS decreased PICP levels (Fig. 3D). Inclusion of a general MMP inhibitor (ilomastat; 1 μM) did not alter basal or FBS-stimulated PICP levels and had no effect on the inhibition by Fsk or PGE2 (data not shown). These data indicate that collagen synthesis, as opposed to collagen cleavage or degradation, is inhibited by agents that increase cAMP production in WI-38 cells.

**Effect of FSK and PGE2 on specific ECM and ECM-regulatory proteins.** An antifibrotic phenotype would be expected to result from a reduced synthesis of ECM protein, an increased degradation of ECM, or both. Therefore, we used RT-PCR to examine how increased cellular cAMP levels alter expression of specific ECM and ECM-regulatory genes. mRNA was isolated from vehicle-treated (control) cells and cells incubated with Fsk (10 μM) plus IBMX (0.2 mM) for 24 h. Semiquantitative RT-PCR was used to compare expression of several ECM genes, including laminin, fibronectin,
elastin, decorin, fibrillin types 1 and 2, and collagen types Iα1, Iα2, IIIα1, Vα1, VIα1, VIα2, and VIα3. We detected changes in mRNA levels of fibronectin, laminin, and collagen types Iα1, Iα2, IIIα1, Vα1, and VIα3 (data not shown). The level of expression of these genes was then quantified more precisely with real-time quantitative RT-PCR. Laminin and collagen type IIIα1 mRNA levels increased 3.3 ± 0.3-fold and 3.2 ± 0.5-fold, respectively, in Fsk + IBMX-treated cells, whereas collagen type Iα2 and type Vα1 decreased by 42 ± 9% and 43 ± 5%, respectively; fibronectin and collagen type Iα1 and VIα3 mRNA levels were not statistically different from those in control cells (Fig. 4A). The lack of effect of cAMP-elevating agents on fibronectin expression is consistent with previous studies of WI-38 cells (30). Although overall collagen synthesis measured by [3H]proline incorporation decreased on treatment with cAMP-elevating agents (Fig. 3), these data indicate that expression of only certain collagen genes decreased whereas expression of other, presumably less predominant, ECM genes increased (Fig. 4A).

With a similar strategy, we analyzed the isoforms of MMP and TIMP expressed in WI-38 cells and assessed the genes altered by increases in cAMP levels. An initial screen of the MMP and TIMP isoforms was performed with DNA dot-blot hybridization on cDNA arrays that detect 18 MMP isoforms and 3 TIMP isoforms (SuperArray). We found that WI-38 cells express detectible amounts (>5% of GAPDH expression) of MMP-1, MMP-2, MMP-10, MMP-14, MMP-like 1, TIMP-1, and TIMP-2; elevation of cAMP levels (by incubation of cells with 10 μM Fsk + 0.2 mM IBMX for 24 h) increased expression of MMP-2, TIMP-1, and TIMP-2, as determined by real-time quantitative RT-PCR. MMP-2 and TIMP-1 mRNA levels increased three- to fourfold in cells incubated with Fsk plus IBMX for 24 h (Fig. 4B). TIMP-2 mRNA did not increase to levels that were statistically different from control. Expression of MMP-1 mRNA was equivalent in control and untreated cells, consistent with results obtained with cDNA arrays. SDS-PAGE and immunoblot analysis indicated that collagen type III and MMP-2 protein levels increased in whole cell lysates from WI-38 cells incubated with either 100 μM Fsk or 100 μM PGE2 alone for 24 h (Fig. 4, C and D). Immunoblot analyses also indicated that protein levels of collagen type I, TIMP-1, and α-smooth muscle actin (a marker of myofibroblast differentiation) were decreased by treatment with Fsk or PGE2. A discrepancy exists between the TIMP-1 mRNA and protein levels after elevation of cAMP production (Fig. 4, B vs. C and D); similar discrepancies in TIMP-1 expression have been observed by others (12).

To confirm the effect of cAMP-elevating agents on MMP expression, we measured activity of MMP-2 with in-gel zymography of conditioned media. Gelatin zymography revealed that WI-38 cells secrete two gelatinases, a 72-kDa species (expected size of pro-MMP-2) and a 62-kDa species (expected size of pro-MMP-3).
We then compared gelatinase activity in media from cells treated for 24 h with various concentrations of either Fsk or PGE2. Image analysis and densitometry of the 62-kDa bands showed that MMP-2 activity was dose-dependently increased in Fsk- or PGE2-treated cells (Fig. 5). Thus cAMP elevation causes an increase in MMP-2 activity, which would be expected to result in an overall increase in degradation of collagens, particularly types I and III.
IV and V (45), in pulmonary fibroblasts. However, this assay does not account for any change in TIMP expression. AC6 overexpression inhibits pulmonary fibroblast function. Previous studies indicated that AC expression limits the maximal cAMP generation and subsequent cellular response stimulated by Gs-coupled receptors (18, 19, 38). Thus we tested the hypothesis that overexpression of AC would enhance the ability of cAMP-elevating agents to inhibit pulmonary fibroblast proliferation and collagen synthesis. First, we examined the expression of AC isoforms in WI-38 cells with RT-PCR and immunoblot analysis. RT-PCR using primer pairs specific for each of the nine transmembrane isoforms of AC, followed by sequence analysis, indicated expression of AC3, AC5, AC6, AC7, AC8, and AC9 (Fig. 6, top). Immunoblot analysis detected protein for AC5/6 (this antibody cannot distinguish between AC5 and AC6), AC7, AC8, and AC9 (Fig. 6, bottom). AC5/6 immunoreactivity was the most readily detected isoform, whereas no immunoreactivity was detected for AC2, AC3, or AC4.

We then used an adenovirus construct to overexpress AC6 in WI-38 cells and compared cAMP production, cell proliferation, and collagen synthesis with those responses of cells incubated with an adenovirus expressing lacZ (control). AC6-overexpressing cells displayed increased cAMP production under basal, Fsk-, Iso- and PGE2-stimulated conditions (Fig. 7A). cAMP production stimulated by the prostacyclin analog beraprost was also enhanced in AC6-overexpressing cells. Overexpression of AC6 significantly decreased both basal and serum-stimulated [3H]thymidine incorporation (Fig. 7B) and collagenase-sensitive [3H]proline incorporation (Fig. 7C). Basal and 2.5% serum-stimulated PICP levels were also lower in AC6-overexpressing cells (Fig. 7C, inset). Fsk, Iso, and PGE2 each inhibited FBS-stimulated [3H]thymidine and collagenase-sensitive [3H]proline incorporation in cells overexpressing AC6 (Fig. 7, B and C). However, the percent inhibition of FBS-stimulated cell proliferation and collagen synthesis induced by each of these agonists was not enhanced in AC6-overexpressing cells (control cells: Fsk 48.7 ± 9.1%, PGE2 62.3 ± 13.7% inhibition; AC6-overexpressing cells: Fsk 56.0 ± 9.0%, PGE2 61.3 ± 12.3% inhibition). Furthermore, studies examining the effect of multiple concentrations of Fsk

![MMP-2 activity](image-url)

Fig. 5. MMP-2 activity is increased in WI-38 cells treated with cAMP-elevating agents. MMP-2 activity was measured by in-gel zymography of conditioned medium from WI-38 cells treated with vehicle, Fsk, or PGE2 at the indicated concentrations for 24 h. The volume of each band that corresponded to the molecular weight of MMP-2 was quantified with digital imaging analysis. A representative image of a zymograph is shown at top. Quantification of bands is shown in a bar graph (bottom), in which each bar represents the mean ± SE of the integrated optical density (IOD) of 3 experiments. *P < 0.05, **P < 0.01 compared with control by paired t-test.

![AC isoforms](image-url)

Fig. 6. Expression of adenylyl cyclase (AC) isoforms in WI-38 cells. Top: RT-PCR analysis was performed with AC isoform-specific primer pairs and either cDNA, mRNA (no reverse transcriptase negative control; –RT), or genomic DNA (positive control; not shown). Arrows indicate the size of expected PCR product from each primer pair. MW, molecular weight markers. Image is representative of 3 experiments. The identity of all positive bands was confirmed by sequence analysis. Bottom: expression of AC isoforms was assessed by immunoblot analysis of whole cell lysates prepared from WI-38 cells (see MATERIALS AND METHODS). Lysates were separated by SDS-PAGE, transferred to membrane, and probed with antibodies specific for AC2, AC3, AC4, AC5/6, AC7, AC8, and AC9. No immunoreactivity was detected for AC2, AC3, or AC4. Shown are images of AC5/6, AC7, AC8, and AC9 immunoreactivity that are representative of 3 experiments.
indicated that its potency for inhibiting collagen synthesis was not increased in AC6-overexpressing cells. In control cells, Fsk inhibited FBS-stimulated collagenase-sensitive \[^{3}H\]proline incorporation with high-affinity (EC\(_{50} = 18 \pm 8.2\) nM) and low-affinity (EC\(_{50} = 45 \pm 4.5\) μM) components (Fig. 7D). AC6 overexpression reduced the stimulation by serum and virtually eliminated the high-affinity component of the Fsk-induced inhibition. Thus the increase in basal cAMP production that results from overexpressing AC6 (an increase of 6.9 \pm 1.8 pmol cAMP/mg protein; Fig. 7A) appears sufficient to inhibit pulmonary fibroblast function without further requirement for increased AC activity by exogenously added agents. Overall, these data with AC6 overexpression provide further evidence for the ability of increases in cAMP to decrease proliferation and collagen formation of WI-38 cells.

**DISCUSSION**

The present studies provide evidence that the second messenger cAMP is a negative regulator of fibrosis-promoting functions of human pulmonary fibroblasts. We identified several endogenous G protein-coupled receptors in WI-38 cells that signal via Gs, increase cAMP production, decrease cell proliferation, and reduce synthesis of two major forms of collagen (types I and V). The potency and efficacy of these agonists in stimulating cAMP production roughly correlate with their potency and efficacy to inhibit cell proliferation and collagen synthesis. One exception is that Fsk inhibits cell proliferation at concentrations that produce low levels of cAMP production (Figs. 1 and 2). This discrepancy could be explained by the fact that a PDE inhibitor was present in the cAMP assays but not in the assays of cell proliferation because the PDE inhibitor itself was able to blunt cell proliferation. Perhaps WI-38 cells respond to prolonged, slightly elevated levels of cAMP with alteration in "downstream" responses. This idea is supported by results from the assays of PICP levels (Fig. 3D), which indicated that 10-fold higher concentrations of forskolin or PGE2 are required for inhibition of FBS-stimulated PICP levels compared with FBS-stimulated \[^{3}H\]proline incorporation (Figs. 3 and 7). Assay of PICP levels released into the culture medium may be less sensitive to the changes in collagen production than are detected in assays of proline incorporation. In addition, the stimulation of collagen degradation by cAMP-elevating agents may contribute to their ability to inhibit proline incorporation but not PICP levels (3).

Multiple approaches to increasing cAMP levels, including direct stimulation of AC activity with Fsk, overexpression of AC6, or treatment with a PDE inhibitor, all reduce WI-38 cell proliferation and function. These observations thus support the general conclusion that cAMP is an antifibrotic second messenger in pulmonary fibroblasts. This conclusion is consistent...
with previous observations that β-AR or prostanoid receptor activation or PDE inhibition reduces accumulation of collagen in the lung or production of collagen by lung fibroblasts (3–7, 15, 31, 32). The cAMP signaling pathway can also inhibit activation of fibroblasts from heart and liver, suggesting that it is a common mechanism for attenuating fibrosis (14, 26, 33, 37). However, the present studies show that the effects of cAMP are complex, with certain ECM proteins being increased and others decreased on activation of cAMP production.

Published reports support the idea that WI-38 cells are an appropriate model of human pulmonary fibroblasts. WI-38 cells proliferate and synthesize collagen on activation with serum or TGF-β1, and these responses are inhibited by addition of interleukins (2). These characteristics are identical to those of both primary adult and fetal pulmonary fibroblasts (16, 28, 50).

Fibroblasts can differentiate into myofibroblasts on exposure to particular cytokines or growth factors, such as TGF-β1 and TNF-α, that are upregulated after injury (9, 42). These myofibroblasts are critical for scar formation and repair and for secreting ECM proteins but are also prominent features of the fibrotic foci that appear in the lung and other organs during fibrosis (6, 11, 34, 51). Thus fibrotic diseases likely are accompanied by excessive formation of myofibroblasts, increased activation of myofibroblast-mediated collagen synthesis, decreased ECM degradation, or increased persistence of myofibroblasts (or a combination thereof). Although much work has focused on signals that activate fibroblasts and myofibroblasts, less is known about negative regulators of fibroblast differentiation or myofibroblast function. Interleukin-1β and interferon-γ have antifibrotic properties via their ability to regulate myofibroblast persistence by inducing apoptosis. Interleukin-1β or interferon-γ treatment of pulmonary myofibroblasts induces expression of inducible nitric oxide synthase, which increases NO production, guanylyl cyclase activity, and cGMP formation (23, 50). Recent data from Kolodskie et al. (28) suggest that PGF2α-stimulated cAMP production prevents myofibroblast differentiation and inhibits production of ECM by pulmonary fibroblasts. Our data extend those findings by showing that multiple receptors that couple to Gs and the activation of AC activity negatively regulates the fibrotic phenotype of pulmonary fibroblasts. In conclusion, the current data indicate that multiple receptors coupled to Gs and the activation of AC activity negatively regulate the fibrotic phenotype of pulmonary fibroblasts. In addition, increased AC expression or decreased cAMP metabolism via PDE inhibition also inhibits fibroblast function. The cAMP pathway thus appears to be an important regulator of pulmonary fibroblast proliferation and function. Our results imply that interventions that enhance cellular levels of cAMP may prove useful for the treatment of pulmonary fibrosis and its attendant enhancement in fibroblast proliferation and formation of ECM.

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REFERENCES


