Prostacyclin Analogs Inhibit Fibroblast Contraction of Collagen Gels through the cAMP-PKA Pathway

Koichiro Kamio, Xiangde Liu, Hisatoshi Sugiura, Shinsaku Togo, Tetsu Kobayashi, Shinsaku Kawasaki, Xingqi Wang, Lijun Mao, Youngsoo Ahn, Cory Hogaboam, Myron L. Toews, and Stephen I. Rennard

Departments of Pulmonary and Critical Care Medicine and of Pharmacology, University of Nebraska Medical Center, Omaha, Nebraska; Immunology Program, Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan; Third Department of Internal Medicine, Wakayama Medical University, Wakayama; Third Department of Internal Medicine, Mie University Graduate School of Medicine, Mie, Japan; and Department of Rheumatology, The Third Hospital of Peking University, Beijing, China

Prostacyclin is an arachidonic acid metabolite that modulates vascular tone within the lung. The current study evaluated the hypothesis that prostacyclin can also modulate tissue remodeling by affecting fibroblast-mediated contraction of extracellular matrix. To accomplish this, fibroblasts were cultured in three-dimensional native type I collagen gels in the presence of prostacyclin analogs: carbaprostacyclin, iloprost, and beraprost. All three analogs significantly inhibited contraction of the three-dimensional collagen gels mediated by three different fibroblasts. All three analogs significantly inhibited fibronectin release and reduced fibroblast fibronectin mRNA expression. Addition of exogenous fibronectin restored the contractile activity to fibroblasts incubated in the presence of all three analogs. Iloprost and beraprost significantly activated cAMP-dependent protein kinase-A (PKA), and an action through this pathway was confirmed by blockade of the inhibitory effect on contraction and fibronectin release with the PKA inhibitor KT-5720. In contrast, carbaprostacyclin, which is not as selective for the prostacyclin (IP) receptor, did not activate PKA, and its effects on contraction and fibronectin release were not fully blocked by KT-5720. Finally, the cAMP analogs N⁶-Benzoyl- (6-Bnz-) cAMP and dibutyryl-cAMP inhibited contraction, and this contrasted with the activity of an Epac selective agonist 8-pCPT-2'-O-Me-cAMP, which had no effect. Taken together, these results indicate that prostacyclin, acting through the IP receptor and by activating PKA, can lead to inhibition of fibronectin release and can subsequently inhibit fibroblast-mediated collagen gel contraction. The ability of prostacyclin to modulate fibroblast function suggests that prostacyclin can contribute to tissue remodeling.

Keywords: prostacyclin; fibroblasts; tissue remodeling; fibronectin

Alterations in tissue structure are characteristically present in many chronic disorders. Prominent among these alterations is fibrosis, an accumulation of mesenchymal cells and collagenous extracellular matrix produced by these cells. Importantly, fibrotic tissues are contracted, which can lead to disruption of architectural relationships within tissues and compromise of organ function. The ability of fibroblasts to contract extracellular matrix, which is believed to play a role in normal wound healing such as in resolution of granulation tissue (1), likely also plays a crucial pathogenic role in many diseases. Importantly, it has been suggested to contribute to the airway narrowing characteristic of asthma and chronic obstructive pulmonary disease (COPD), the

vascular narrowing that characterizes pulmonary hypertension, and to tissue distortion present in interstitial pulmonary fibrosis (2–4).

The ability of fibroblasts to contract extracellular matrix can be modulated by diverse exogenous mediators. Recent studies have suggested that prostacyclin, a potent vasodilator that is used therapeutically in the treatment of primary pulmonary hypertension, can also modulate fibroblast chemotactic recruitment (5). This action on fibroblasts suggests that prostacyclin, in addition to regulating vascular tone, may also contribute to tissue remodeling. The current study was conducted to determine if prostacyclin can also modulate contraction of extracellular matrix by lung fibroblasts. Because prostacyclin itself is relatively unstable, prostacyclin analogs were used to evaluate this question in an *in vitro* model system assessing fibroblast contraction of extracellular matrix. The signaling pathway and the mechanism by which prostacyclin mediates its effect on contraction were also defined.

MATERIALS AND METHODS

Materials

Native type I collagen (rat tail tendon collagen [RTTC]) was extracted from rat-tail tendons by a previously published method (6, 7). Commercially available reagents were obtained as follows: Dulbecco's Modified Eagle's Medium (DMEM) and fetal calf serum (FCS) were from Invitrogen Life Technologies (Grand Island, NY); carbaprostacyclin, iloprost, and beraprost were from Cayman Chemical (Ann Arbor, MI); monoclonal anti-human fibronectin antibody (Clone FN-15), polyclonal anti-human fibronectin antibody, phenylmethylsulfonyl fluoride (PMSF), 8-pCPT-2'-O-Me-cAMP, and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide (MTT) were from Sigma (St. Louis, MO); horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody was from Rockland (Gilbertsville, PA); human fibronectin was from Roche Applied Science (Indianapolis, IN); KT-5720, 6-Bnz-cAMP, and dibutyryl-cAMP were from Calbiochem (San Diego, CA).

Cell Culture

Human fetal lung fibroblasts (HFL-1; lung, diploid, human) were obtained from the American Type Culture Collection (#CCL-153; Rockville, MD). Human lung fibroblasts were isolated from normal-appearing alveolar lung tissue removed at surgery for suspected malignancy under a protocol approved by the Human Studies Committee of the University of Michigan. Human bronchial fibroblasts from healthy adults were initiated from endobronchial biopsy at the University of Nebraska Medical Center under an Institutional Review Board-approved protocol. The cells were cultured on 100-mm tissue culture dishes (Falcon; Becton-Dickinson Labware, Lincoln Park, NJ) with DMEM supplemented with 10% FCS, 100 µg/ml penicillin, 250 µg/ml streptomycin sulfate (penicillin-streptomycin; Invitrogen Life Technologies), and 2.5 µg/ml amphotericin B (Geneva Pharmaceuticals, Inc., Dayton, NJ). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and passaged every 3–5 d at a 1:4 ratio. In all experiments, cells between the 14th and 18th passage were used. To evaluate mediator production in monolayer culture, cells (2 × 10⁵ cells/well) were

(Received in original form January 11, 2007 and in final form February 22, 2007)

Funded by the Larson Endowment, University of Nebraska Medical Center and NIH Grant #HL64088 to S.I.R.

Correspondence and requests for reprints should be addressed to Stephen I. Rennard, M.D., University of Nebraska Medical Center, 985885 Nebraska Medical Center, Omaha, NE 68198-5885. E-mail: sreennard@unmc.edu

Am J Respir Cell Mol Biol Vol 37, pp 113–120, 2007

Originally Published in Press as DOI: 10.1165/rcmb.2007-0009OC on March 15, 2007

Internet address: www.atsjournals.org

seeded in 6-well tissue culture plates (Falcon). At 90% confluence, medium was changed to DMEM without FCS (serum-free [SF]-DMEM) for 6 h and then treated with various concentrations of prostacyclin analogs in SF-DMEM. The supernatants from monolayer cultures were harvested 48 h later and stored at -80°C until assayed.

Collagen Gel Contraction Assay

Collagen gel contraction assays were conducted in the absence of FCS. Confluent fibroblasts were detached with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA-4Na; GIBCO, Grand Island, NY) and resuspended in SF-DMEM containing soybean trypsin inhibitor (Sigma). The cell number was then counted with a Coulter Counter (Beckman Coulter, Inc., Fullerton, CA). Collagen gels were prepared by mixing the appropriate amount of RTTC, distilled water, 4 $\times$ concentrated DMEM, and cell suspension so that the final mixture resulted in 0.75 mg/ml of collagen, 3×10^5 fibroblasts/ml gel, and a physiologic ionic strength of 1 $\times$ DMEM, and a pH of 7.4 (7). Fibroblasts were always added last to minimize damage during the preparation of collagen gels. A 500- μl portion of the gel solution was then cast into each well of a 24-well tissue culture plate with a 2-cm² growth area (Falcon). Gelation occurred in 20 min at room temperature, after which the gels were released from the surface of the culture well using a sterile spatula. They were then transferred into 60-mm tissue culture dishes (three gels in each dish) containing 5 ml of SF-DMEM with or without designated reagents and incubated at 37°C , 5% CO_2 for 3 d. The ability of the fibroblasts to contract the floating gels was determined by quantifying the area of the gels daily using an Optomax V image analyzer (Optomax, Burlington, MA). Data are expressed as the percentage of gel area compared with the original gel size; contraction is then expressed as a decrease in gel area.

Measurement of Fibronectin by Enzyme-Linked Immunosorbent Assay

Fibronectin in the media from cells in collagen gel culture or monolayer culture was determined by enzyme-linked immunosorbent assay (ELISA). Plates were coated with monoclonal anti-fibronectin antibody at 4°C overnight. After washing three times, standards and samples were added and incubated at room temperature for 2 h. After washing, bound antigen was detected by adding polyclonal anti-human fibronectin antibody (1:2,000 dilution) at room temperature for 1 h. After washing, HRP-conjugated anti-rabbit IgG antibody (1:10,000 dilution) was added at room temperature for 1 h, and then, after a final wash, bound HRP was detected with 0.1 mg/ml *o*-phenylenediamine (Fisher Scientific, Pittsburgh, PA). The reaction was stopped with 8 M H_2SO_4 , and the product was quantified at 490 nm with a microplate reader (BioRad Laboratories, Inc., Hercules, CA).

Data are expressed as ng fibronectin produced per day per 10^5 cells. For monolayer culture, the cell number was counted at the end of the experiment. For three-dimensional collagen gel culture, the number of cells cast into the gel was used.

RNA Preparation and Complementary DNA Synthesis

To determine whether changes in fibronectin mRNA levels were present, quantitative real-time polymerase chain reaction (PCR) was performed. Cells were cultured until confluence and serum-starved for 4 h. Then cells were exposed to 1×10^{-6} M of carbaprostacyclin, iloprost, and beraprost for 5 h. Total RNA was isolated by a single-step guanidinium-thiocyanate-phenol-chloroform extraction procedure designed by Chomczynski (8) and the total amount of RNA was quantified spectrophotometrically (Shimadzu Scientific Instruments, Inc., Columbia, MD). One microgram of total RNA was treated with RNase-free DNase I following the manufacturer's instructions (Invitrogen) for 15 min at room temperature to remove possible contaminating genomic DNA. The reaction was then stopped with 25 mM EDTA by heating at 65°C for 10 min, followed by 95°C for 5 min. For cDNA synthesis, ~ 400 ng of DNase-treated RNA was transcribed with cDNA transcription reagents (Applied Biosystems, Foster City, CA) by using random hexamers, and the cDNA was used for quantitative real-time PCR.

Quantitative Real-Time PCR

Gene expression was measured with the use of an ABI Prism 7500 Sequence Detection System (Applied Biosystems) as described pre-

viously (9). TaqMan Gene Expression Assays, which include pre-designed primers and probes for the detection of fibronectin mRNA (Hs01549962_m1), were purchased from Applied Biosystems. Probes were labeled at the 5' end with the reporter dye molecule 6-carboxy-fluorescein (FAM) and at the 3' end with the quencher dye molecule 6-carboxytetramethyl-rhodamine (TAMRA). rRNA was simultaneously tested using TaqMan Ribosomal RNA Control Reagents (Applied Biosystems). Quantitative real-time PCRs were then conducted in a total volume of 50 μl with 1 $\times$ TaqMan Universal Master Mix (Applied Biosystems) and primers at 900 nM and probes at 250 nM.

Thermal cycler parameters were 2 min at 50°C , 10 min at 95°C , and 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Data were normalized to the amount of rRNA quantified spectrophotometrically from the same preparations and are expressed as fold of control.

Effect of Exogenous Fibronectin on the Inhibition of Three-Dimensional Gel Contraction by Prostacyclin Analogs

To evaluate modulation of three-dimensional gel contraction by fibronectin, 50 $\mu\text{g}/\text{ml}$ of human fibronectin was added to the collagen solution before gels were made. Gels were then cast and suspended in the media with 25 μg fibronectin and cultured. The areas of the gels were measured as described in COLLAGEN GEL CONTRACTION ASSAY.

PKA Activity Assay

The PKA activity assays were performed using a nonradioactive, protein kinase assay kit purchased from Calbiochem following the manufacturer's instructions. Briefly, cells were grown to confluence in 100-mm tissue culture dishes. After treatment with prostacyclin analogs, cells were washed twice with ice-cold PBS and then harvested in 500 μl of cell lysis buffer (50 mM Tris-HCl, 50 mM β -mercaptoethanol, 10 mM EGTA, 10 mM benzamide, 5 mM EDTA and 1 mM PMSF, pH 7.5). After sonication four times for 10 s each, they were centrifuged at $100,000 \times g$ for 60 min at 4°C and the supernatants were collected. The protein concentration in each sample was determined by Bio-Rad Protein Assay kit (Bio-Rad Laboratories), which is based on the method of Bradford. Twelve microliters of samples plus 108 μl of reaction mixture with 1 mM ATP were applied to plates that were precoated by the peptide pseudosubstrate, which is phosphorylated by PKA. Plates were incubated for 20 min at 25°C in a water bath and the reaction was stopped. After washing five times, 100 μl of biotinylated monoclonal antibody for the phosphorylated form of the peptide pseudosubstrate was added and incubated at 25°C for 60 min. After washing five times, 100 μl of HRP-conjugated streptavidin were added and incubated at 25°C for 60 min. After a final wash, bound HRP was detected with *o*-phenylenediamine. After the reaction was stopped, the product was quantified at 492 nm with a microplate reader.

Cell Viability Assay

Cell viability was evaluated by MTT assays using previously described methods (10). Briefly, on Day 3, fibroblast-populated gels were incubated with MTT solution (0.25 mg/ml in SF-DMEM, 0.5 ml/gel) for 12 h. Gels were washed once with PBS. Formazan crystals were then dissolved with dimethyl sulfoxide (0.5 ml/gel) by shaking 6 h at room temperature, and then absorbance at wavelength of 540 nm was determined with a microplate reader.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Experiments with multiple comparisons were evaluated by one-way ANOVA followed by *post hoc* analysis by pair-wise comparisons of values with Tukey's test to adjust for multiple comparisons. Probability values < 0.05 were considered significant.

RESULTS

Effect of Prostacyclin Analogs on Fibroblast-Mediated Contraction of Three-Dimensional Collagen Gels

To investigate prostacyclin modulation of collagen gels, HFL-1 cells cast into collagen gels were floated in media with various concentrations of three prostacyclin analogs, carbaprostacyclin,

iloprost, and beraprost. Three-dimensional gel contraction was inhibited in a concentration-dependent manner (1×10^{-9} M to 1×10^{-6} M) by all the three analogs (Figure 1). This inhibition of collagen gel contraction was observed throughout the experimental period. Using analogs at the concentration of 1×10^{-6} M, inhibition was statistically significant on Days 2 and 3 and showed a trend toward inhibition already at Day 1 (Figure 2). On Day 3, carbaprostacyclin-treated gels were $69 \pm 2\%$ of initial size, iloprost-treated gels were $69 \pm 2\%$ and beraprost-treated gels were $67 \pm 3\%$, in contrast with control gels, which were $54 \pm 4\%$ ($P < 0.05$, all comparisons, Figure 2). None of the reagents affected cell viability evaluated by MTT assay (data not shown).

Because fibroblasts may be functionally heterogeneous, we also investigated the effect of prostacyclin analogs on the inhibition of three-dimensional gel contraction mediated by human lung parenchymal and bronchial fibroblasts from healthy adults. Prostacyclin analogs inhibited three-dimensional gel contraction mediated by both adult lung parenchymal and bronchial fibroblasts in a concentration-dependent manner (1×10^{-9} M to 1×10^{-6} M) (data not shown). All three of the prostacyclin analogs at the concentration of 1×10^{-6} M significantly inhibited three-dimensional gel contraction mediated by normal adult lung fibroblasts at Days 2 and 3 ($P < 0.05$), and showed a trend toward inhibition at Day 1 (Figure 3A). Carbaprostacyclin also inhibited bronchial fibroblasts to a similar degree (Figure 3B). Iloprost significantly inhibited bronchial fibroblasts, but had quantitatively less effect than did carbaprostacyclin. In bronchial fibroblasts, beraprost demonstrated a trend toward inhibition that did not achieve statistical significance ($P = 0.06$ at Day 3).

Effect of Prostacyclin Analogs on Fibronectin Release by Human Fibroblasts

Fibronectin is a multifunctional macromolecule produced by fibroblasts that can modulate fibroblast-mediated collagen gel contraction. To determine whether prostacyclin analogs could alter fibronectin production, fibronectin release was evaluated by ELISA. Prostacyclin analogs (1×10^{-9} M to 1×10^{-6} M) decreased fibronectin release in a concentration-dependent manner both in the three-dimensional gel ($P < 0.05$ at 1×10^{-6} M; Figure 4A) and monolayer cultures ($P < 0.05$ at 1×10^{-6} M; Figure 4B).

Figure 1. Effect of prostacyclin analogs on three-dimensional collagen gel contraction mediated by HFL-1 cells. HFL-1 cells were cast into collagen gels and cultured in SF-DMEM with or without three prostacyclin analogs (carbaprostacyclin [diamonds], iloprost [triangles], and beraprost [circles]) in varying concentrations (1×10^{-9} M to 1×10^{-6} M). Vertical axis: gel size as % of initial area on Day 2; horizontal axis: concentration of prostacyclin analog (M). The data presented are mean $\pm$ SEM from three separate experiments, each of which included triplicate gels for each condition. * $P < 0.05$ compared with control.

Figure 2. Effect of prostacyclin analogs on contraction of collagen gels mediated by HFL-1 cells, time course. HFL-1 cells were cast into collagen gels and cultured in SF-DMEM with or without prostacyclin analogs (1×10^{-6} M). Gel size was measured daily. Vertical axis: gel size as % of initial area; horizontal axis: time after release (days). The data presented are mean $\pm$ SEM from three separate experiments, each of which included triplicate gels for each condition. Squares, control; diamonds, carbaprostacyclin; triangles, iloprost; circles, beraprost. * $P < 0.05$ compared with control.

We also measured fibronectin release into three-dimensional gel culture media mediated by human parenchymal and bronchial fibroblasts from healthy adults. All three analogs decreased fibronectin release into three-dimensional gel culture media by adult lung fibroblasts at a concentration of 1×10^{-6} M (Figure 5A). In bronchial fibroblasts, only the effect of carbaprostacyclin was significant, although both iloprost and beraprost showed trends toward inhibition ($P = 0.090$ and $P = 0.096$ at 1×10^{-6} M, respectively).

Effect of Prostacyclin Analogs on Fibronectin mRNA Expression by Human Lung Fibroblast

To determine whether the changes in fibronectin release were associated with changes in fibronectin gene expression, quantitative real-time PCR assays were performed. Consistent with the effect on protein release assessed by ELISA, prostacyclin analogs markedly inhibited fibronectin mRNA expression in a concentration-dependent manner (Figure 6). Fibronectin mRNA was 14 ± 9 , 16 ± 3 , and $28 \pm 1\%$ fold of control after incubation

Figure 3. Effect of prostacyclin analogs on three-dimensional gel contraction mediated by human adult lung parenchymal and bronchial fibroblasts. Fibroblasts were cast into collagen gels and cultured in SF-DMEM with or without prostacyclin analogs (1×10^{-6} M). (A) Adult human lung parenchymal fibroblasts. (B) Adult human bronchial fibroblasts. Gel size was measured daily. Vertical axes: gel size as % of initial area; horizontal axes: time after release (days). The data presented are mean $\pm$ SEM from three separate experiments, each of which included triplicate gels for each condition. Squares, control; diamonds, carbaprostacyclin; triangles, iloprost; circles, beraprost. * $P < 0.05$ compared with control.

Figure 4. Effect of prostacyclin analogs on fibronectin release into (A) three-dimensional gel and (B) monolayer culture media. (A) HFL-1 cells were cast into collagen gels and maintained in floating culture medium in the presence of varying concentrations of prostacyclin analogs. (B) Fibroblasts were cultured until 90% confluence in 6-well tissue culture plates, after which medium was changed to SF-DMEM with varying concentrations of prostacyclin analogs. After 2 d of incubation, both three-dimensional gel culture

and monolayer culture media were harvested and assayed for fibronectin by ELISA. *Vertical axes:* fibronectin production (ng produced/day/10⁵ cells); *horizontal axes:* concentration of prostacyclin analog (M). The data presented are mean $\pm$ SEM from four separate experiments, each performed in triplicate. *Diamonds,* carbaprostacyclin; *triangles,* iloprost; *circles,* beraprost. * $P < 0.05$ compared with the values of control.

with 1×10^{-6} M by carbaprostacyclin ($P < 0.01$), iloprost ($P < 0.01$), and beraprost ($P < 0.01$), respectively (Figure 6).

Effect of Exogenous Fibronectin on the Inhibition of Three-Dimensional Collagen Gel Contraction by Prostacyclin Analogs

Since prostacyclin analogs reduced fibronectin mRNA levels and protein release, we addressed the question of whether a decrease

Figure 5. Effect of prostacyclin analogs on fibronectin release into three-dimensional gel culture media mediated by human adult lung parenchymal and bronchial fibroblasts. Fibroblasts were cast into collagen gels and cultured in SF-DMEM with varying concentrations of prostacyclin analogs. After 2 d of incubation, media were harvested and assayed for fibronectin by ELISA. (A) Adult human lung parenchymal fibroblasts. (B) Adult human bronchial fibroblasts. *Vertical axes:* fibronectin production (ng produced/day/10⁵ cells); *horizontal axes:* concentration of prostacyclin analog (M). The data presented are mean $\pm$ SEM from three separate experiments, each performed in triplicate. *Diamonds,* carbaprostacyclin; *triangles,* iloprost; *circles,* beraprost. * $P < 0.05$ compared with the values of control.

in fibronectin may contribute to the inhibition of collagen gel contraction caused by prostacyclin analogs. To evaluate this possibility, the effect of exogenously added fibronectin was investigated. HFL-1 fibroblasts were cast into gels with or without exogenous fibronectin (50 μ g/ml) and then incubated with 1×10^{-6} M prostacyclin analogs in culture medium that was supplemented with 25 μ g fibronectin. Exogenous fibronectin slightly stimulated three-dimensional gel contraction (Figure 7). In the presence of exogenous fibronectin, all three prostacyclin analogs failed to inhibit collagen gel contraction.

Figure 6. Effect of prostacyclin analogs on fibronectin mRNA expression. HFL-1 fibroblasts were cultured in 100-mm dishes until confluence. Media were changed to DMEM without serum for 4 h, after which the cells were incubated with varying concentrations of prostacyclin analogs for 5 h. Extracted RNA was quantified by Taqman RT-PCR. *Vertical axis:* mRNA expression level normalized to the amount of rRNA and expressed as fold of control; *horizontal axis:* concentration of prostacyclin analog (M). The data presented are mean $\pm$ SEM from three separate experiments, each performed in duplicate. *Diamonds,* carbaprostacyclin; *triangles,* iloprost; *circles,* beraprost. * $P < 0.05$, ** $P < 0.01$ compared with control.

in fibronectin may contribute to the inhibition of collagen gel contraction caused by prostacyclin analogs. To evaluate this possibility, the effect of exogenously added fibronectin was investigated. HFL-1 fibroblasts were cast into gels with or without exogenous fibronectin (50 μ g/ml) and then incubated with 1×10^{-6} M prostacyclin analogs in culture medium that was supplemented with 25 μ g fibronectin. Exogenous fibronectin slightly stimulated three-dimensional gel contraction (Figure 7). In the presence of exogenous fibronectin, all three prostacyclin analogs failed to inhibit collagen gel contraction.

Effect of Prostacyclin Analogs on PKA Activity in Fibroblasts

Prostacyclin is known to act, at least in part, through the cAMP-PKA pathway that is activated by prostacyclin binding to the G_s-coupled prostacyclin receptor followed by subsequent activation of adenylyl cyclase (11). To investigate whether prostacyclin analogs increase PKA activity in human lung fibroblasts, confluent cells were incubated with the three prostacyclin analogs at the concentration of 1×10^{-6} M for 15, 30, and 60 min. Cell lysates were harvested and subjected to PKA assay. Iloprost and beraprost stimulated PKA activity, which was maximal by 15 min after stimulation, the earliest time assessed (Figures 8B and 8C, respectively); however, stimulation of PKA by carbaprostacyclin was not observed (Figure 8A).

Effect of the PKA Inhibitor of KT-5720 on Inhibition of Three-Dimensional Gel Contraction by Prostacyclin Analogs

To determine whether the effect of prostacyclin analogs on three-dimensional gel contraction is mediated by cAMP activation of PKA, we incubated the collagen gels with the PKA inhibitor KT-5720 at the concentration of 1×10^{-7} M for 1 h (12, 13) before the addition of prostacyclin analogs. KT-5720 alone had minimal effect on three-dimensional gel contraction (Figure 9). In contrast, KT-5720 significantly blocked the effects of iloprost and beraprost on three-dimensional gel contraction by 75% and 83%, respectively; KT-5720 was less effective in blocking the effect of carbaprostacyclin, reducing it by 28%, which did not achieve statistical significance ($P = 0.087$).

Figure 7. Effect of exogenously added fibronectin on the inhibition of three-dimensional gel contraction by prostacyclin analogs. Fibroblasts were cast into collagen gels with (solid bars) or without (open bars) fibronectin (50 μ g/ml) and incubated with or without prostacyclin analogs (1×10^{-6} M). Vertical axis: gel size as % of initial area on Day 3; horizontal axis: conditions. The data presented are mean $\pm$ SEM from three separate experiments, each of which included triplicate gels for each condition. * $P < 0.05$ compared with fibronectin-untreated groups.

Effect of cAMP Analogs

To further assess the role of PKA in mediating inhibition of collagen gel contraction, we used the PKA-specific cAMP analog, 6-Bnz-cAMP, the guanine nucleotide exchange factor for the Ras-like small GTPases (Epac) selective agonist 8-pCPT-2'-O-Me-cAMP, and the nonselective agonist dibutyryl-cAMP. Fibroblasts contraction of collagen gels was significantly inhibited by both of the PKA activators (Figure 10). In contrast, the selective activator of Epac had no effect on three-dimensional gel contraction (Figure 10).

Effect of KT-5720 on the Inhibition of Fibronectin Release by Prostacyclin Analogs

To further confirm the role of PKA-mediated signaling in prostacyclin analog inhibition of three-dimensional gel contraction, we investigated whether KT-5720 blocks prostacyclin analog inhibition of fibronectin release. Fibroblasts in both three-dimensional gel and monolayer cultures were preincubated with KT-5720 (1×10^{-7} M) for 1 h before the addition of prostacyclin analogs (1×10^{-6} M). When assessed after 2 d of incubation, KT-5720 significantly prevented the decrease in fibronectin release caused by iloprost and beraprost; however, as with contraction, the effect of KT-5720 on carbaprostacyclin-treated cells was minimal and not statistically significant (Figure 11).

DISCUSSION

The current study demonstrates that prostacyclin analogs inhibit human lung fibroblast-mediated contraction of three-dimensional collagen gels. The prostacyclin analogs also inhibit the release of fibronectin from cultured fibroblasts. The addition of exogenous fibronectin restored fibroblast-mediated contractions, suggesting that the inhibitory effect of the prostacyclin analogs was mediated, at least in part, through the inhibition of fibronectin release. The effect of the prostacyclin analogs on both fibronectin release and gel contraction was inhibited by the protein kinase-A inhibitor KT-5720. PKA-specific cAMP analogs inhibited three-dimensional gel contraction, but an Epac activator was without effect, consistent with an action through

Figure 8. PKA activation by prostacyclin analogs, time course. HFL-1 fibroblasts were cultured in 100-mm dishes until confluence. Three prostacyclin analogs were applied at the concentration of 1×10^{-6} M and incubated for 15, 30, and 60 min. Cell lysates were harvested and assayed for PKA. (A) Carbaprostacyclin. (B) Iloprost. (C) Beraprost. Vertical axes: relative PKA activity expressed as fold of control; horizontal axes: time. The data presented are mean $\pm$ SEM from three separate experiments, each performed in duplicate. * $P < 0.05$ compared with control.

the PKA pathway. Taken together, these results suggest that prostacyclin analogs acting through cyclic AMP and protein kinase-A modulation of fibronectin release can modify fibroblast-mediated contraction of extracellular matrix.

Prostacyclin is an arachidonic acid-derived mediator that acts on a specific G protein-coupled receptor, the IP receptor. Prostacyclin has potent effects as a vasodilator, and prostacyclin agonists are used in the treatment of primary pulmonary hypertension (4). Primary pulmonary hypertension is characterized not only by vasoconstriction and increases in pulmonary blood pressure, but also by remodeling of vascular tissue (14). Treatment with prostacyclin analogs not only reduces vascular pressure but also appears to modulate the remodeling processes. The current study supports the concept that prostacyclin may be able to affect remodeling processes by actions on fibroblasts in addition to actions on vascular smooth muscle.

The current study used fibroblasts from both fetal and adult lung tissues, which behaved similarly. The exact origin of these cells is not known, but the similarity of response suggests that the responsiveness to prostacyclin is a property of lung fibroblasts and is not specific to a single strain. Interestingly, the normal airway fibroblasts were slightly less responsive, particularly to iloprost and beraprost which, in contrast to carbaprostacyclin, are relatively more selective for the IP receptor. This suggests that fibroblasts may be heterogeneous in their receptor expression and sensitivity to prostacyclin.

Figure 9. Effect of the PKA inhibitor KT-5720 on the inhibition of three-dimensional gel contraction by prostacyclin analogs. Gels were preincubated with KT-5720 (1×10^{-7} M; solid bars) or control (open bars) for 1 h before treatment with prostacyclin analogs (1×10^{-6} M). Vertical axis: gel size as % of initial area on Day 2; horizontal axis: conditions. The data presented are mean $\pm$ SEM from four separate experiments, each of which included triplicate gels for each condition. * $P < 0.05$ compared with KT-5720 untreated groups.

The current study used HFL-1 cells, a widely used normal human lung fibroblast, to characterize and define the pathways for prostacyclin analog action on fibroblast-mediated contraction of collagen gels. In addition to pulmonary hypertension, several other chronic lung diseases are characterized by both pulmonary hypertension and tissue remodeling, including idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease. Treatment of the pulmonary hypertension associated with these conditions by agents such as prostacyclin analogs has been suggested. Several studies have demonstrated that fibroblasts cultured from IPF lung or COPD can differ functionally from fibroblasts from normal tissue (15, 16). Whether fibroblasts present in these conditions will respond to prostacyclin analogs remains to be determined, but the current study supports the concept that these agents may also be able to alter the fibrotic processes that lead to tissue disruption in these conditions.

The current study used fibroblasts cultured in three-dimensional collagen gels as a model of tissue remodeling. Fibroblasts cultured in a gel composed of native collagen attach to the collagen fibers and generate a traction force. If the collagen gel is allowed to float freely, this traction force results in contraction of the gel. This process has been used to model the contraction that

Figure 10. Effect of cAMP analogs on three-dimensional gel contraction. HFL-1 cells were cast into collagen gels and cultured in SFDMEM with or without the PKA-selective cAMP analog, 6-Bnz-cAMP (X symbols), with the Epac selective agonist 8-pCPT-2'-O-Me-cAMP (circles), or with dibutyryl-cAMP (triangles) (all at 5×10^{-4} M). Squares, control. Vertical axis: gel size as % of initial area; horizontal axis: time after release (days). The data presented are mean $\pm$ SEM from three separate experiments, each of which included triplicate gels for each condition. * $P < 0.05$ compared with control.

The data presented are mean $\pm$ SEM from three separate experiments, each of which included triplicate gels for each condition. * $P < 0.05$ compared with control.

Figure 11. Effect of the PKA inhibitor KT-5720 on the inhibition of fibronectin release by prostacyclin analogs in three-dimensional gel and monolayer culture media. (A) Three-dimensional gel culture. Gels were incubated with KT-5720 (1×10^{-7} M) for 1 h before treatment with prostacyclin analogs (1×10^{-6} M). (B) Monolayer culture. Fibroblasts cultured in monolayer were incubated with KT-5720 (1×10^{-7} M) for 1 h before treatment with prostacyclin analogs (1×10^{-6} M). After 2 d of incubation, both three-dimensional gel and monolayer culture media were harvested and assayed for fibronectin by ELISA. Vertical axes: fibronectin production (ng produced/day/ 10^5 cells); horizontal axes: conditions. The data presented are mean $\pm$ SEM from four (three-dimensional gel culture) and three (monolayer culture) separate experiments, each performed in triplicate. Open bars, control; solid bars, KT-5720. * $P < 0.05$ compared with KT-5720 untreated groups.

characterizes healing granulation tissue and fibrosis (1, 17). Several factors that are believed to stimulate healing responses and/or fibrosis are known to stimulate the contraction of fibroblast-populated collagen gels, including TGF- β (18, 19), fibronectin (20), platelet-derived growth factor (21), thrombin (22), lysophosphatidic acid (23), and endothelin-1 (24). Among these factors, fibronectin was found to play a particularly important role in the modulation of gel contraction mediated by prostacyclin analogs. Blockade of fibroblast-mediated collagen gel contraction, therefore, may be regarded as a potential means to prevent the development of fibrotic-contracted tissues. Whether prostacyclin analogs would be able to reverse fibrosis once developed is not addressed by the experimental system used.

Fibronectin is a multifunctional extracellular matrix macromolecule. It can interact with cells through multiple integrin receptors through its RGD domain (25). It can also bind to collagen and other extracellular matrix components and can thereby mediate fibroblast adhesion to collagenous substrates (26). Fibroblasts can adhere to extracellular collagen through other mechanisms. Also, the $\alpha_2\beta_1$ integrin is absolutely required for the direct adhesion of fibroblasts to collagen and then for fibroblast contraction of three-dimensional matrices (27, 28). While fibronectin can, under some circumstances, augment

fibroblast-mediated collagen gel contraction (20), it is not absolutely required for this process to occur, and RGD-containing peptides do not completely block collagen gel contraction (29). This suggests that both fibronectin-dependent and fibronectin-independent mechanisms for collagen gel contraction occur, a concept that is also supported by antibody inhibition experiments (20, 29). Only the fibronectin released into the culture media was measured in the current study. Previous studies have demonstrated that approximately one-third of fibronectin is released into the culture medium in three-dimensional culture collagen gel, and more than half is released into the culture media in monolayer culture (30). Thus the absorption amounts measured do not reflect the total fibronectin production. It is possible that the reduction in fibronectin release observed in response to prostacyclin analogs represents, in part, redistribution between media and matrix. Nevertheless, the restoration of contraction by the addition of exogenous fibronectin establishes this as a mechanism for the inhibited contraction.

The mechanism by which fibronectin leads to augmented contraction of three-dimensional collagen gels, and the receptors involved, remain to be fully defined. The current study demonstrates that fibronectin-dependent contraction is inhibited by prostacyclin analogs through inhibition of fibronectin release. These results resemble previous results with cigarette smoke, which also inhibits contraction by inhibiting fibronectin release (31, 32). In both cases, inhibition of contraction is reversed by exogenous fibronectin. Prostacyclin, therefore, seems to be acting through a similar pathway. Whether inhibition of fibroblast-mediated collagen gel contraction through inhibition of fibronectin plays a pathogenic role in a disease process or represents a normal tissue homeostatic mechanism, we suspect, may depend on the specific *in vivo* context. Interestingly, different effects of cigarette smoke are observed at high cell density. A high density of cells, which produce TGF- β , results in an increased concentration of active TGF- β , resulting in augmented contraction (33). These cell density-dependent effects depend on the paracrine action of released mediators and suggest an important mechanism by which the activity of neighboring cells can be coordinated during repair processes. The ability of prostacyclin analogs to modulate paracrine regulation is consistent with a role for prostacyclin in regulating repair. Finally, prostacyclin analogs have previously been shown to inhibit fibroblast chemotaxis, suggesting that prostacyclin may regulate several aspects of repair and remodeling (5).

Activation of the IP receptor leads to signaling through G_s, activation of adenylyl cyclase and increases in cyclic AMP levels. This can lead to several signaling pathways, including activation of protein kinase-A and activation of Epac (34). In the current study, an inhibitor of PKA, KT-5720, was able to completely block the effects of iloprost and beraprost on fibronectin release and gel contraction, suggesting that PKA signaling mediated this effect. The effect of KT-5720 on carbaprostacyclin-mediated inhibition, in contrast, was only partial. The results of PKA activity assays, in which iloprost and beraprost stimulated PKA activity whereas carbaprostacyclin did not, were consistent with these results. Direct stimulation of PKA by the cAMP analogs, 6-Bnz-cAMP, and dibutyl-cAMP (35), inhibited the contraction of three-dimensional gels. However, the Epac agonist 8-pCPT-2'-O-Me-cAMP was without effect, suggesting that action through Epac was not responsible for any of the observed effects. Together, these findings support the involvement of PKA. In contrast to iloprost and beraprost, carbaprostacyclin is less specific and can act on a number of other related receptors (36, 37). This suggests that some of the inhibitory effect of carbaprostacyclin may be mediated via other receptors and other signaling pathways.

Prostacyclin is relatively unstable, with a half-life in aqueous solution of 3–5 min (38). The synthetic analogs used in the current study are more stable. Carbaprostacyclin and beraprost have half-lives of 30 d and 10 d, respectively, in neutral aqueous solution (39, 40). The serum half-life of iloprost, in contrast, is $\sim$ 25 min (41), although the half-life in aqueous solution may be longer. While the stability of the analogs used in the current study in tissue culture conditions remains to be determined, it is likely that carbaprostacyclin and beraprost were active throughout the several-day experimental incubations, whereas iloprost may have been progressively inactivated. Thus, we cannot determine if the activity of the prostacyclin analogs depends on continual activation of the receptor or if transient activation at the beginning of the incubation is sufficient.

In summary, the current study demonstrates that prostacyclin analogs can modulate fibroblast-mediated contraction of three-dimensional collagen gels. This effect is mediated through PKA inhibition of fibronectin release. Through actions on fibroblasts, prostacyclin can modulate tissue remodeling and repair. The use of prostacyclin analogs, therefore, may have therapeutic potential in diseases characterized by alterations in tissue architecture.

Conflict of Interest Statement: S.I.R. has participated as a speaker in scientific meeting and courses under the sponsorship of AstraZeneca (AZ) and GlaxoSmithKline (GSK). He serves on advisory boards for Altana, AZ, Dey GSK, and Inspire. He has conducted clinical trials for AZ, Centocor, GSK, Pfizer, Roche, and Sanofi. He has served as a consultant for AZ, GSK, Novartis, Pfizer, and Roche. A patent is pending on the use of PDE4 inhibitors in repair; S.I.R. is a co-inventor of the patent owned by the University of Nebraska Medical Center. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors are grateful for the excellent secretarial support of Ms. Lillian Richards.

References

- Grinnell F. Fibroblasts, myofibroblasts and wound contraction. *J Cell Biol* 1994;124:401–404.
- Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, *et al.* The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med* 2004;350:2645–2653.
- Crystal RG, Bitterman PB, Mossman B, Schwarz MI, Sheppard D, Almasry L, Chapman HA, Friedman SL, King TE Jr, Leinwand LA, *et al.* Future research directions in idiopathic pulmonary fibrosis: summary of a national heart, lung, and blood institute working group. *Am J Respir Crit Care Med* 2002;166:236–246.
- Rubin LJ. Pulmonary arterial hypertension. *Proc Am Thorac Soc* 2006;3: 111–115.
- Kohyama T, Liu X, Kim HJ, Kobayashi T, Ertl RF, Wen FQ, Takizawa H, Rennard SI. Prostacyclin analogs inhibit fibroblast migration. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L428–L432.
- Elsdale T, Bard J. Collagen substrata for studies on cell behavior. *J Cell Biol* 1972;54:626–637.
- Mio T, Adachi Y, Romberger DJ, Ertl RF, Rennard SI. Regulation of fibroblast proliferation in three dimensional collagen gel matrix. *In Vitro Cell Dev Biol* 1996;32:427–433.
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidine thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156–159.
- Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2000; 25:169–193.
- Supino R. MTT assays. *Methods Mol Biol* 1995;43:137–149.
- Stitham J, Stojanovic A, Ross LA, Blount AC Jr, Hwa J. Clusters of transmembrane residues are critical for human prostacyclin receptor activation. *Biochemistry* 2004;43:8974–8986.
- Kase H, Iwahashi K, Nakanishi S, Matsuda Y, Yamada K, Takahashi M, Murakata C, Sato A, Kaneko M. K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem Biophys Res Commun* 1987;142:436–440.

13. Cabell L, Audesirk G. Effects of selective inhibition of protein kinase C, cyclic AMP-dependent protein kinase, and Ca(2+)-calmodulin-dependent protein kinase on neurite development in cultured rat hippocampal neurons. *Int J Dev Neurosci* 1993;11:357-368.
14. Stenmark KR, Davie NJ, Reeves JT, Frid MG. Hypoxia, leukocytes, and the pulmonary circulation. *J Appl Physiol* 2005;98:715-721.
15. Miki H, Mio T, Nagai S, Hoshino Y, Nagao T, Kitaichi M, Izumi T. Fibroblast contractility: usual interstitial pneumonia and nonspecific interstitial pneumonia. *Am J Respir Crit Care Med* 2000;162:2259-2264.
16. Holz O, Zuhlke I, Jaksztat E, Muller KC, Welker L, Nakashima M, Diemel KD, Branscheid D, Magnussen H, Jorres RA. Lung fibroblasts from patients with emphysema show a reduced proliferation rate in culture. *Eur Respir J* 2004;24:575-579.
17. Bell E, Ivarsson B, Merrill C. Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. *Proc Natl Acad Sci USA* 1979;76:1274-1278.
18. Montesano R, Orci L. Transforming growth factor- β stimulates collagen-matrix contraction by fibroblasts: Implication for wound healing. *Proc Natl Acad Sci USA* 1988;85:4894-4897.
19. Finesmith TH, Broadley KN. Fibroblasts from wounds of different stages of repair vary in their ability to contract a collagen gel in response to growth factors. *J Cell Physiol* 1990;144:99-107.
20. Gillery P, Maquart FX, Borel JP. Fibronectin dependence of the contraction of collagen lattices by human skin fibroblasts. *Exp Cell Res* 1986;167:29-37.
21. Clark RAF, Folkvord JM, Hart CE, Murray MJ, McPherson JM. Platelet isoforms of platelet-derived growth factor stimulate fibroblasts to contract collagen matrices. *J Clin Invest* 1989;84:1036-1040.
22. Fang Q, Liu X, Abe S, Kobayashi T, Wang XQ, Kohyama T, Hashimoto M, Wyatt TA, Rennard SI. Thrombin induces collagen gel contraction partially through PAR1 activation and PKC- ϵ . *Eur Respir J* 2004;24:918-924.
23. Grinnell F, Ho C, Lin Y, Skuta G. Differences in the regulation of fibroblast contraction of floating versus stressed collagen matrices. *J Biol Chem* 1999;274:918-923.
24. Oshita Y, Lee YR, Ogawa H, Yamazaki M, Tsuboi R. Endothelin-1 secreted from keratinocytes stimulates fibroblast-embedded collagen gel contraction in vitro. *J Dermatol Sci* 2006;43:57-59.
25. Takagi J, Strokovich K, Springer TA, Walz T. Structure of integrin $\alpha 5 \beta 1$ in complex with fibronectin. *EMBO J* 2003;22:4607-4615.
26. Welch MP, Odland GF, Clark RA. Temporal relationships of F-actin bundle formation, collagen and fibronectin matrix assembly, and fibronectin receptor expression to wound contraction. *J Cell Biol* 1990;110:133-145.
27. Schiro JA, Chan BMC, Roswit WT, Kassner PD, Pentland AP, Hemler ME, Eisen AZ, Kupper TS. Integrin $\alpha 2 \beta 1$ (VLA-2) mediates reorganization and contraction of collagen matrices by human cells. *Cell* 1991;67:403-410.
28. Klein CE, Dressel D, Steinmayer T, Mauch C, Eckes B, Krieg T, Barkert RB, Weber TL. Integrin $\alpha 2 \beta 1$ is upregulated in fibroblasts and highly aggressive melanoma cells in three-dimensional collagen lattices and mediates the reorganization of collagen 1 fibrils. *J Cell Biol* 1991;115:1427-1436.
29. Gullberg D, Tingstrom A, Thuresson A-C, Olsson L, Terracio L, Borg TK, Rubin K. $\beta 1$ integrin-mediated collagen gel contraction is stimulated by PDGF. *Exp Cell Res* 1990;186:264-272.
30. Adachi Y, Mio T, Striz I, Romberger DJ, Spurzem JR, Rennard SI. Fibronectin production by cultured human lung fibroblasts in three-dimensional collagen gel culture. *In Vitro Cell Dev Biol* 1998;34:203-210.
31. Carnevali S, Nakamura Y, Mio T, Liu X, Takigawa K, Romberger DJ, Spurzem JR, Rennard SI. Cigarette smoke extract inhibits fibroblast-mediated collagen gel contraction. *Am J Physiol Lung Cell Mol Physiol* 1998;274:L591-L598.
32. Liu X, Kohyama T, Kobayashi T, Abe S, Kim HJ, Reed EC, Rennard SI. Cigarette smoke extract inhibits chemotaxis and collagen gel contraction mediated by human bone marrow osteoprogenitor cells and osteoblast-like cells. *Osteoporos Int* 2003;14:235-242.
33. Wang H, Liu X, Umino T, Kohyama T, Zhu YK, Wen FQ, Spurzem JR, Romberger DJ, Kim HJ, Rennard SI. Effect of cigarette smoke on fibroblast-mediated gel contraction is dependent on cell density. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L205-L213.
34. Bos JL. Epac: a new cAMP target and new avenues in cAMP research. *Nat Rev Mol Cell Biol* 2003;4:733-738.
35. Mei FC, Qiao J, Tsygankova OM, Meinkoth JL, Quilliam LA, Cheng X. Differential signaling of cyclic AMP: opposing effects of exchange protein directly activated by cyclic AMP and cAMP-dependent protein kinase on protein kinase B activation. *J Biol Chem* 2002;277:11497-11504.
36. Abramovitz M, Adam M, Boie Y, Carriere M, Denis D, Godbout C, Lamontagne S, Rochette C, Sawyer N, Tremblay NM, et al. The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs. *Biochim Biophys Acta* 2000;1483:285-293.
37. Kiriya M, Ushikubi F, Kobayashi T, Hirata M, Sugimoto Y, Narumiya S. Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells. *Br J Pharmacol* 1997;122:217-224.
38. Nickolson RC, Town MH, Vorbruggen H. Prostacyclin-analogs. *Med Res Rev* 1985;5:1-53.
39. Whittle BJ, Moncada S, Whiting F, Vane JR. Carbacyclin—a potent stable prostacyclin analogue for the inhibition of platelet aggregation. *Prostaglandins* 1980;19:605-627.
40. Melian EB, Goa KL. Beraprost: a review of its pharmacology and therapeutic efficacy in the treatment of peripheral arterial disease and pulmonary arterial hypertension. *Drugs* 2002;62:107-133.
41. Galie N, Manes A, Branzi A. The new clinical trials on pharmacological treatment in pulmonary arterial hypertension. *Eur Respir J* 2002;20:1037-1049.