Plasmin Overcomes Resistance to Prostaglandin E₂ in Fibrotic Lung Fibroblasts by Reorganizing Protein Kinase A Signaling*

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Collagen deposition by fibroblasts contributes to scarring in fibrotic diseases. Activation of protein kinase A (PKA) by cAMP represents a pivotal brake on fibroblast activation, and the lipid mediator prostaglandin E₂ (PGE₂) exerts its well known anti-fibrotic actions through cAMP signaling. However, fibroblasts from the lungs of patients with idiopathic pulmonary fibrosis, or of mice with bleomycin-induced fibrosis, are resistant to the normal collagen-inhibiting action of PGE₂. In this study, we demonstrate that plasminogen activation to plasmin restores PGE₂ sensitivity in fibrotic lung fibroblasts from human and mouse. This involves amplified PKA signaling resulting from the promotion of new interactions between AKAP9 and PKA regulatory subunit II in the perinuclear region as well as from the inhibition of protein phosphatase 2A. This is the first report to show that an extracellular mediator can dramatically reorganize and amplify the intracellular PKA-A-kinase anchoring protein signaling network and suggests a new strategy to control collagen deposition by fibroblasts.

The intracellular second messenger cAMP serves as an important brake on the activation of mesenchymal cells, including mesenchymal stem cells (1), smooth muscle cells (2), and fibroblasts (3), that drive diseases characterized by tissue remodeling. The classical cAMP effector is protein kinase A (PKA). PKA is a tetramer composed of two regulatory (R)² and two catalytic subunits. There are two different R subtypes, RI and RII, and each subtype exists as α and β isoforms (RIα and RIβ and RIIα and RIIβ). Each PKA-R isoform can mediate either redundant or nonredundant functions based on differences in their intracellular localization, affinity for catalytic subunits, and interaction with a family of scaffold proteins termed A-kinase anchoring proteins (AKAPs) (4, 5). RI is mainly present in the cytosol, whereas RII is predominantly anchored via AKAPs to specific cellular structures and organelles (6). Because they anchor not only R subunits but also other PKA-related proteins (e.g. G protein-coupled receptors, phosphatases, and phosphodiesterases), AKAPs serve to compartmentalize CAMP-PKA signal transduction and thereby dictate its functional consequences (5). Although components of AKAP complexes are subject to post-translational modifications that can dictate changes in their composition or localization, the functional consequences to PKA signaling and the physiologic relevance of their dynamic regulation are poorly understood. In addition, nothing is currently known about the roles of specific PKA-R isoforms or AKAPs in regulating the function of fibroblasts, the cells primarily responsible for the elaboration of extracellular matrix (ECM) proteins such as collagen that compose the scars in fibrotic disease processes.

The lipid mediator prostaglandin E₂ (PGE₂) is one of the most abundant and important endogenous substances acting through cAMP. PGE₂ suppresses virtually all relevant functions of activated fibroblasts, including collagen synthesis, upon binding to its G protein-coupled receptor EP2 and activating adenylyl cyclase (3). However, the PGE₂ axis is dysregulated in idiopathic pulmonary fibrosis (IPF), the most common form of pulmonary fibrosis and one that lacks effective therapy and has a median survival of only 3 years (7). First, lung levels of PGE₂ (8) as well as lung fibroblast PGE₂ biosynthesis (9) are diminished in this disorder. Second, lung fibroblast lines obtained from the majority of IPF patients (10) as well as from mice with bleomycin-induced pulmonary fibrosis (11) are resistant to the normal collagen-inhibiting action of PGE₂. Fibroblast resistance to PGE₂ is also observed in other fibrotic diseases (12, 13), motivating efforts to restore the anti-fibrotic efficacy of deficient PGE₂ as a therapeutic approach in such disorders.

The plasminogen activation (PA) system, in which plasminogen (PA) in plasma is cleaved to plasmin by tissue-type plasminogen activator (tPA), is regulated by multiple feedback mechanisms (14). Plasminogen activator inhibitor (PAI), the endogenous inhibitor of tPA, is a protein that mediates the effect of tPA and plasminogen on PAI (14) and is impaired in IPF (15, 16). We have recently established that plasmin-mediated up-regulation of COX-2 and PGE₂ synthesis in the lung is important for the anti-fibrotic actions of PA (17). Whether the PA system also influences PGE₂-cAMP-PKA signaling is unknown.
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In this study, we demonstrate that PA and plasmin itself can reconstitute the ability of PGE₂ to suppress collagen expression in fibrotic lung fibroblasts obtained from humans and mice. This is mediated by an amplification of PKA signaling that depends on enhanced interactions between PKA-RII and AKAP9. Our findings demonstrate for the first time that the intracellular PKA-AKAP system can be dynamically reorganized and functionally potentiated by an extracellular mediator, plasmin. They also provide a novel approach to restraining activated fibroblasts that promote scarring in fibrotic diseases of the lung and other organs.

EXPERIMENTAL PROCEDURES

Reagents Used—The two-chain active form of human urokinase-type plasminogen activator (uPA) was purchased from American Diagnostica, and human plasminogen (Plg) and human plasmin were purchased from Calbiochem. The non-selective COX inhibitor aspirin (acetylsalicylic acid (ASA)) was purchased from Sigma. Primary antibodies (Abs) for immunoblot analysis were obtained from the following suppliers: anti-human and anti-mouse Abs against type I collagen both from CedarLane Laboratories; anti-GAPDH from Santa Cruz Biotechnology; anti-PKA-Cα, total and phosphorylated cAMP response element-binding protein (CREB), and phosphorylated vasodilator-stimulated phosphoprotein (VASP) from Cell Signaling Technologies; anti-AKAP1 and -AKAP9 from Bethyl; anti-protein phosphatase 2A (PP2A) catalytic subunit from Millipore; anti-PKA-RIα from BD Biosciences; anti-PKA-RIβ from Chemicon International; anti-PKA-RIα from Santa Cruz Biotechnology; anti-PKA-RIIβ from BD Biosciences for immunoblotting and from Santa Cruz Biotechnology for immunoprecipitation; anti-human EP2 from Cayman Chemical; and anti-α-tubulin from Sigma. Secondary anti-rabbit and anti-murine Abs for immunoblotting were from Cell Signaling Technologies. Nonspecific control rabbit, mouse, and goat IgGs for immunofluorescence microscopy (IFM) or immunoprecipitation were obtained from Santa Cruz Biotechnology. Protein A-Sepharose for immunoprecipitation was from GE Healthcare. Bleomycin, myristoylated PKA inhibitory peptide 14–22 (PKI), and sodium orthovanadate were purchased from Sigma. PGE₂ was obtained from Cayman Chemicals and dissolved in DMSO. The AKAP-PKA-RI-binding disruptor (RI-anchoring disruptor, RIAD) and the protease-activated receptor-1 (PAR-1) blocking peptide FLLRN were purchased from AnaSpec. The AKAP-PKA-RII-binding disruptor Ht31, its control peptide Ht31c, and serine/threonine phosphatase assay system were obtained from Promega. Okadaic acid and PKA inhibitor KT5720 were from Biomol. cAMP analogs 8-PIPCAMP, 6-MBC-cAMP, 2-CI-8-MA-cAMP, and (S₅)–5,6-DCI-cBIMPS were from Biolog. Control siRNA and siRNAs targeting PKA-RIα, RIIB, AKAP1, and AKAP9 were ON-TARGET plus SMART pool products from Thermo Scientific. OptimEM I reduced serum medium, RNAiMAX for siRNA transfection, and Prolong Gold AntiFade Reagent with DAPI mounting media for cover glasses on glass slides were purchased from Invitrogen. The PGE₂ ELISA kit and the Direct cAMP Correlate ELISA kit were obtained from Assay Designs. Blocking antibody against the hepatocyte growth factor (HGF) receptor c-Met and HGF ELISA kit were obtained from R&D Systems. The c-Met kinase inhibitor PHA-665752 was purchased from Tocris Bioscience. Protease inhibitor was purchased from Roche Applied Science. Phosphatase inhibitor mixture set I was from Calbiochem-Novabiochem.

Murine Lung Fibroblast Purification—C57BL/6 mice were purchased from The Jackson Laboratory. Animals were approved by the University of Michigan Committee on the Use and Care of Animals. PAI-1−/− mice on a C57BL/6 background, as described previously (18), were bred in-house. Bleomycin was dissolved in PBS and instilled intratracheally at a dose of 0.0135 units/g mouse in a volume of 50 µl, and fibroblasts were isolated from lungs of mice on day 14 or 21 post-saline or bleomycin, as described previously (17).

Human Adult Lung Fibroblasts—As described previously (10), primary adult nonfibrotic human lung fibroblasts were isolated from the margins of lung tissue resected from patients with suspected lung cancer that displayed normal lung histology, and IPF fibroblasts were cultured from lung biopsy specimens of patients diagnosed with IPF whose tissue histopathology showed usual interstitial pneumonia. Both groups of patients were of similar age, and specimens from both groups were obtained with written informed consent under a University of Michigan IRB-approved protocol.

Cell Culture and Treatment—Primary fibroblasts were cultured in DMEM + 10% FBS + antibiotics (100 units/ml penicillin/streptomycin) at 37 °C in 5% CO₂ and used for experimentation at passage 6–10 (human) or 3–4 (mouse). No apparent change in fibroblast responsiveness to PGE₂ was noted during cell passage. Human fibroblasts were plated in Falcon 6-well plates (BD Biosciences) at 5 × 10⁵ cells per well or at 2–3 × 10⁶ cells per well for RNA silencing. Mouse fibroblasts were plated at 3–5 × 10⁵ cells per well. They were allowed to adhere for 8 h in serum-containing DMEM and then cultured in serum-free DMEM for 18–24 h. Then, after removal of medium, cells were pretreated for 24 h ± human Plg (150 milliunits/ml, which is equivalent to 13.6 µg/ml), human uPA (15 IU/ml, which is equivalent to 167 ng/ml), Plg alone, uPA alone, or human plasmin (150 milliunits/ml, which is equivalent to 9.5 µg/ml), along with or without 200 µM ASA in serum-free DMEM. These reagent concentrations were based on preliminary experiments and our previous experience (17). In some experiments, cells received additional pretreatment with PAR-1 antagonist FLLRN (500 µM), the HGF receptor c-Met kinase inhibitor PHA-66575 (0.1 µM), or anti-c-Met blocking Ab (20 µg/ml) 30 min prior to Plg + uPA addition. In previous papers, these concentrations for FLLRN (19), PHA-66575 (20), and c-Met blocking Ab (17) were established as effective. In some experiments, PKA inhibitors KT5720 (100 nM) or PKI (10 µM) were added 30 min prior to Plg + uPA addition or 4 h before PGE₂ addition, respectively. These time courses and concentrations for PKA inhibitors were based on our preliminary experiments and previous papers (21, 22). In some experiments, cells received additional pretreatment with Ht31, Ht31c (both at 100 µM), or RIAD (50 µM) 30 min prior to Plg + uPA addition. A previous report (23) demonstrated that RIAD and Ht31 at these concentrations selectively disrupt binding of AKAPs to PKA-RI and PKA-RII, respectively, in intact cells. In
some experiments, cells were pretreated with ASA ± okadaic acid at the indicated concentrations for 4 h. After the specified pretreatment period, medium was removed, and cells were newly treated ± PGE₂ at 500 nM, unless otherwise indicated, or with CAMP analogs at indicated concentrations in serum-free DMEM. 18 h later, or as otherwise specified in the figure legends, cells were lysed in lysis buffer consisting of PBS with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1× protease inhibitor, and 1:100 dilution of phosphatase inhibitor mixture set I, and 0.2 m sodium orthovanadate as described previously (17).

PP2A Activity Assay—Serine/threonine phosphatase assay system was used to measure PP2A activity in cell lysates per the manufacturer’s instructions with the specific buffer for PP2A as reported previously (24).

Specific Stimulation of PKA-RI or -RII—The combination of 8-PIP-cAMP and 2-Cl-8-MA-cAMP was used for specific PKA-RI stimulation, and the combination of 8-PIP-cAMP and 6-MBC-cAMP was used for specific PKA-RII stimulation (25). Each compound was used at the same concentration (at 1:1 ratio) as indicated in Fig. 5, B and C.

RNA Silencing—Cells were incubated with 50 nM of each targeting or control siRNA (total 100 nM for treatment with two different siRNAs) plus 5 μl of RNAiMAX in 2 ml of Opti-MEM I reduced serum medium without antibiotics. 72 h later, medium was changed to serum-free DMEM with antibiotics, and cells were pretreated with ASA ± Plg + uPA for 24 h, followed by 18 h treatment ± PGE₂ at 0.5 μM as described above.

Immunoblotting—20–25 μg of protein in scraped cell lysates, which contain both intracellular and extracellular proteins, was loaded for electrophoresis, and subsequent immunoblot analysis and densitometric analysis were performed as described previously (17), using the primary antibodies indicated. Densitometric values of bands for all proteins except for AKAP9 were normalized to GAPDH or α-tubulin detected in the same membrane, and relative levels of each protein were expressed as defined in legends. Except for AKAP9, 8 or 10% acrylamide gels were used. Because of its high molecular weight, 4.5% acrylamide gel was used for AKAP9 as reported previously (26). For normalization of AKAP9 to GAPDH, an identical amount of lysate protein as employed for AKAP9 immunoblotting was loaded on a 10% acrylamide gel for electrophoresis, and GAPDH was detected by subsequent immunoblotting. For normalization of pCREB to total CREB, an identical amount of lysate protein as employed for immunoblotting for pCREB and GAPDH was loaded on another parallel 10% acrylamide gel, and total CREB was detected by subsequent immunoblot analysis.

Immunoprecipitation—For PKA-RIIβ immunoprecipitation, cell lysates were harvested in detergent-free buffer containing 25 mM Tris-Cl, 150 mM NaCl, and 5 mM EDTA with the same protease inhibitor and phosphatase inhibitors, as described above, and were disrupted by sonication. Then cell lysates were incubated overnight at 4°C with anti-PKA-RIIβ Ab or control goat IgG (both at 2.5 μg/ml). Protein A-Sepharose was added and incubated for 3–4 h with rotation at 4°C, and immunoprecipitates were isolated and subjected to electrophoresis, as described previously (27). 200–400 μg of total protein was used for immunoprecipitation, and immunoprecipitates were subjected to electrophoresis and immunoblotting to detect both PKA-RIIβ and PKA-Cα. Anti-PKA-RIIβ Abs from goat and from mouse were used for immunoprecipitation and immunoblotting, respectively.

**RESULTS**

**PA Overcomes PGE₂ Resistance in Fibrotic Lung Fibroblasts from Both Mouse and Human**—Intrapulmonary administration of bleomycin is commonly employed to model pulmonary fibrosis. We have previously reported that lung fibroblasts from bleomycin-injured mice are resistant to the inhibitory effects of PGE₂ (11). Moreover, plasminogen activator inhibitor (PAI)-1 gene null (PAI-1⁻/⁻) mice are protected from experimental pulmonary fibrosis, and this reflects enhanced proteolytic activity of uPA in the absence of its inhibitor PAI-1 (14). To determine whether endogenous PA modulates responsiveness to PGE₂ in fibrotic fibroblasts, we compared the effects of PGE₂ on synthesis of collagen I in fibroblasts isolated from the lungs of bleomycin-treated WT or PAI-1⁻/⁻ mice on day 14 post-bleomycin administration. Collagen I is the predominant type of collagen produced by lung fibroblasts (30). Levels of collagen...
in the ECM are dictated by transcription of the procollagen gene, post-translational modification of the protein, and its degradation by matrix metalloproteinases. We chose to measure total collagen I levels by immunoblot as an index of this net balance. It should be noted, however, that the anti-collagen I Ab utilized here detects a single or a predominant band at a molecular mass between 150 and 250 kDa, which actually corresponds to the immature form of collagen I termed procollagen I. Although PGE\textsubscript{2} was unable to suppress collagen I levels in fibroblasts from fibrotic WT mice, as demonstrated previously (11), this prostanoid significantly suppressed collagen production in fibroblasts from PAI-1\textsuperscript{-/-} mice (Fig. 1A). We recently reported that PAI-1\textsuperscript{-/-} lung fibroblasts produce more PGE\textsubscript{2} than do WT lung fibroblasts (17). To exclude the possibility that increased endogenous PGE\textsubscript{2} production contributed to the enhanced collagen suppression in the presence of exogenous PGE\textsubscript{2} in PAI-1\textsuperscript{-/-} cells, we pretreated cells with the COX inhibitor ASA for 24 h prior to addition of exogenous PGE\textsubscript{2}. Under these conditions, responsiveness to PGE\textsubscript{2}, as judged by its ability to suppress collagen I levels, was likewise observed only in PAI-1\textsuperscript{-/-} fibroblasts and not in WT fibroblasts (Fig. 1B). Interestingly, basal collagen production was consistently and significantly greater in fibroblasts from PAI-1\textsuperscript{-/-} than WT animals only after ASA pretreatment, suggesting that the enhanced generation of endogenous PGE\textsubscript{2} restrains basal collagen production in PGE\textsubscript{2}-sensitive PAI-1\textsuperscript{-/-} fibroblasts, although it cannot do so in PGE\textsubscript{2}-resistant WT fibroblasts. A similar enhancement of PGE\textsubscript{2} responsiveness in PAI-1\textsuperscript{-/-} fibroblasts compared with WT fibroblasts was observed in cells isolated on day 21 post-bleomycin (data not shown). To examine the effect of exogenous PA on responsiveness to PGE\textsubscript{2}, we pretreated day 14 post-bleomycin WT fibroblasts with or without reagent human Plg and uPA for 24 h, and we included ASA to eliminate the contribution of up-regulated endogenously produced PGE\textsubscript{2}. Pretreatment with Plg \textsuperscript{+} uPA enhanced the ability of PGE\textsubscript{2} to inhibit collagen I levels in PGE\textsubscript{2}-resistant fibrotic fibroblasts during a subsequent 18-h treatment (Fig. 1C). Because human uPA cannot bind to mouse uPA receptors (31), and because pretreatment with human plasmin also enhanced responsiveness to PGE\textsubscript{2} in WT mouse fibroblasts (data not shown) as it did in human fibrotic fibroblasts (see below), this enhancement of PGE\textsubscript{2} responsiveness elicited by Plg \textsuperscript{+} uPA pretreatment appears to be mediated through the proteolytic actions of plasmin.

**FIGURE 1.** Endogenous and exogenous PA sensitizes otherwise resistant murine fibrotic fibroblasts to the collagen-inhibiting actions of PGE\textsubscript{2}. On day 14 after bleomycin administration, fibroblasts were obtained from lungs of WT or PAI-1\textsuperscript{-/-} mice. A and B, enhanced PGE\textsubscript{2} responsiveness in lung fibroblasts from PAI-1\textsuperscript{-/-} mice. After 24 h of incubation without (A) or with (B) ASA (200 \textmu M), cells were treated with or without human Plg and uPA for 24 h, and we included ASA to eliminate the contribution of up-regulated endogenously produced PGE\textsubscript{2}. Pretreatment with Plg \textsuperscript{+} uPA enhanced the ability of PGE\textsubscript{2} to inhibit collagen I levels in PAI-1\textsuperscript{-/-} fibroblasts, although it cannot do so in PGE\textsubscript{2}-resistant WT fibroblasts. A similar enhancement of PGE\textsubscript{2} responsiveness in PAI-1\textsuperscript{-/-} fibroblasts compared with WT fibroblasts was observed in cells isolated on day 21 post-bleomycin (data not shown). To examine the effect of exogenous PA on responsiveness to PGE\textsubscript{2}, we pretreated day 14 post-bleomycin WT fibroblasts with or without reagent human Plg and uPA for 24 h, and we included ASA to eliminate the contribution of up-regulated endogenously produced PGE\textsubscript{2}. Pretreatment with Plg \textsuperscript{+} uPA enhanced the ability of PGE\textsubscript{2} to inhibit collagen I levels in PGE\textsubscript{2}-resistant fibrotic fibroblasts during a subsequent 18-h treatment (Fig. 1C). Because human uPA cannot bind to mouse uPA receptors (31), and because pretreatment with human plasmin also enhanced responsiveness to PGE\textsubscript{2} in WT mouse fibroblasts (data not shown) as it did in human fibrotic fibroblasts (see below), this enhancement of PGE\textsubscript{2} responsiveness elicited by Plg \textsuperscript{+} uPA pretreatment appears to be mediated through the proteolytic actions of plasmin.
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Next, we asked if pretreatment with human Plg + uPA enhances PGE₂ responsiveness in lung fibroblast lines obtained from patients with IPF (IPF fibroblasts) that have previously been demonstrated to manifest resistance to the collagen-inhibiting actions of PGE₂ (10, 32). As described above for murine fibroblasts, IPF fibroblasts were pretreated with ASA in the absence or presence of human Plg + uPA for 24 h and subsequently treated for 18 h with PGE₂ at 0.25 or 0.5 μM. We confirmed that ASA pretreatment succeeded in further reducing the already reduced levels (9) of endogenously generated PGE₂ in these IPF cells (255.9 ± 36.6 pg/ml without ASA and 139.1 ± 3.0 pg/ml with ASA; n = 3 experiments, p < 0.05). As expected based on our previous report (10), IPF fibroblasts were inherently resistant to PGE₂ suppression of collagen I levels following control pretreatment; however, they acquired sensitivity to PGE₂ suppression when pretreated with Plg + uPA (Fig. 2A). Although plasmin lacks intrinsic collagenase activity (33), it has been reported to promote degradation of collagen by enhancing activity of collagenase matrix metalloproteinase-1 (34). Nevertheless, in the absence of subsequent PGE₂ treatment, we found little effect of Plg + uPA pretreatment on collagen I expression in IPF fibroblasts. Enhancement of PGE₂ responsiveness by Plg + uPA pretreatment was confirmed in PGE₂-resistant fibroblast lines established from three different IPF patients (Fig. 2B) and was a stable characteristic that persisted through cell passages 6–10. Further mechanistic studies were performed primarily in fibroblasts from a single IPF patient unless otherwise indicated.

Next, we examined the effect of PGE₂ at lower concentrations on collagen I expression in IPF fibroblasts pretreated in the absence or presence of Plg + uPA, and we confirmed that Plg + uPA pretreatment strongly potentiated PGE₂ suppression of collagen I in IPF fibroblasts. PGE₂, at a dose of only 3 nM achieved 50% suppression of collagen I expression in IPF fibroblasts pretreated with Plg + uPA, although a similar effect of PGE₂ could not be achieved at even a 30-fold higher dose in IPF fibroblasts without Plg + uPA pretreatment (Fig. 2C).

To identify the roles of individual PA components in this Plg + uPA effect, IPF fibroblasts were pretreated with ASA alone or with ASA together with Plg + uPA, Plg, uPA, or plasmin prior to treatment with PGE₂. A degree of enhancement in PGE₂ responsiveness similar to that promoted by Plg + uPA was elicited by plasmin, the proteolytic product of uPA action on Plg, but not by uPA alone (Fig. 2D). A more modest effect was seen following pretreatment with Plg, presumably reflecting its conversion to plasmin by endogenous fibroblast uPA. Again, as was the case for Plg + uPA pretreatment alone without subsequent PGE₂ treatment, plasmin itself had little effect on collagen I expression in IPF fibroblasts (Fig. 2E). Even in nonfibrotic patient-derived lung fibroblasts that are sensitive to the collagen-inhibiting actions of PGE₂, Plg + uPA modestly enhanced PGE₂ responsiveness (Fig. 2F), and once again, plasmin but not uPA alone had an effect comparable with that of Plg + uPA (Fig. 2G). Plg + uPA pretreatment enhanced responses to PGE₂ doses as low as 31.3 nM for nonfibrotic lung fibroblasts (Fig. 2H).

Plasmin can proteolytically activate PAR-1 (35). However, an antagonist of PAR-1, FLLRN, failed to abrogate the effect of Plg + uPA (Fig. 3A). It should also be noted that thrombin, the other proteolytic activator of PAR-1, failed to enhance PGE₂ actions in IPF fibroblasts (data not shown). These data suggest that plasmin-mediated enhancement of PGE₂ actions is independent of PAR-1, a conclusion consistent with substantial evidence that PAR-1 promotes, rather than opposes, fibrogenesis (36). The proteolytic release of HGF from ECM has previously been shown to play an important role in the anti-fibrotic actions of PA in vivo (17, 37). We therefore considered the possibility that released HGF could be responsible for PA enhancement of PGE₂ sensitivity. We confirmed that HGF was released from ECM by Plg + uPA treatment and that exogenously administered HGF significantly enhanced responsiveness to PGE₂ in IPF fibroblasts (data not shown). However, neither the c-Met kinase inhibitor PHA-665752 (Fig. 3B) nor c-Met-neutralizing Ab (Fig. 3C) abolished the sensitizing effect of Plg + uPA. Thus, although HGF is capable of enhancing responsiveness to PGE₂ in IPF fibroblasts, it does not seem to account for the actions of Plg + uPA.

Taken together, these results demonstrate that PA significantly enhanced suppression of collagen I expression by PGE₂ in both murine and human lung fibroblasts. This is especially evident in PGE₂-resistant fibroblasts obtained from fibrotic lungs in both species, and it appears to involve a proteolytic effect mediated by plasmin.

PA Amplifies PKA Signaling and Suppresses PP2A Activity—Previous work has established that inhibition of collagen levels by PGE₂ in normal lung fibroblasts proceeds via an EP2-adenyl cyclase-cAMP pathway (3). Neither EP2 protein expression (Fig. 4A) nor PGE₂-induced cAMP production (Fig. 4B) was increased by Plg + uPA pretreatment, suggesting that PA enhancement of PGE₂ responsiveness reflects an action exerted downstream of cAMP. We have previously demonstrated that PGE₂ inhibition of lung fibroblast collagen expression is mediated by PKA, although its inhibition of cellular proliferation is mediated by the alternative cAMP effector, exchange protein activated by cAMP-1 (22). Unlike its effect on collagen expression, Plg + uPA pretreatment failed to potentiate PGE₂ suppression of cell proliferation in IPF fibroblasts (4.30 ± 5.1% inhibition by 0.5 μM PGE₂ in control and 3.10 ± 2.6% inhibition in Plg + uPA-pretreated cells; n = 3 experiments). Moreover, the ability of Plg + uPA to enhance PGE₂ suppression of collagen I was substantially abrogated by coincubation with the PKA inhibitors KT-5720 (Fig. 4C) and PKI peptide (Fig. 4D). These results suggest that the ability of PA to sensitize IPF fibroblasts to the collagen-inhibitory effect of PGE₂ involves an effect exerted on the PKA axis and are consistent with previous reports of impaired PKA signaling in IPF fibroblasts (10, 38). Indeed, PGE₂-induced phosphorylation of the well known PKA substrates VASP (Fig. 4E) and CREB (Fig. 4F) was significantly increased in IPF fibroblasts pretreated for 24 h with Plg + uPA as compared with that in IPF fibroblasts without Plg + uPA pretreatment, supporting the conclusion that PKA signaling is amplified by Plg + uPA pretreatment. We next examined the effect of Plg + uPA pretreatment on the catalytic activity of PP2A, which can dephosphorylate substrates phosphorylated by PKA (39). Plg + uPA pretreatment significantly reduced PP2A activity without changing PP2A protein expression in IPF.
FIGURE 2. Exogenous PA enhances responsiveness to PGE₂ in human fibroblasts. Fibroblasts from IPF (A–E) or nonfibrotic (F–H) human lungs were pretreated for 24 h with or without Plg + uPA in addition to ASA and then treated for 18 h with PGE₂ at indicated concentrations. Collagen I levels were examined by immunoblotting. A, left panel, immunoblot from one representative experiment. Right panel, after normalization to GAPDH, collagen I was expressed relative to that in control (without either Plg + uPA or PGE₂) in each experiment. Data were obtained from five experiments using fibroblasts from a single IPF patient. B, mean data for collagen I levels after 18 h of treatment with PGE₂ (0.5 μM) in cells from three different IPF patients. C, values from two different nonfibrotic lung fibroblast cell lines. D, dose-response curve for PGE₂ at lower concentrations in fibroblasts from one IPF lung in the presence (○) or absence (△) of Plg + uPA pretreatment. Data were obtained from two different experiments. D, E, and G, effect of individual PA components. Fibroblasts from one IPF (D and E) or nonfibrotic (G) lung were pretreated with or without Plg (150 milliunits/ml) + uPA (15 IU/ml), Plg alone (150 milliunits/ml), uPA alone (15 IU/ml), or plasmin (150 milliunits/ml) for 24 h in addition to ASA, followed by PGE₂ (0.5 μM) treatment for 18 h. Data are obtained from five (D and E) or two (G) different experiments. After normalization to GAPDH, collagen I levels after PGE₂ treatment were expressed relative to those in fibroblasts without PGE₂ in each treatment group in each experiment in B–D, F, and G. E, collagen I expression in IPF fibroblasts pretreated with plasmin followed by subsequent incubation for 18 h without PGE₂ was expressed relative to that in control (without either plasmin or PGE₂). *, p < 0.05; **, p < 0.01; ***, p < 0.001. Data in A, B, D, and F represent the mean ± S.E. Data in C represent mean ± half-range from two different experiments. Each symbol indicates the results from a separate cell line in F and the results from a separate experiment with a single cell line in G. H, effect of Plg + uPA pretreatment on sensitivity to low dose PGE₂ in nonfibrotic adult lung fibroblasts. A representative immunoblot from two different experiments is shown. Cont, control.
neither PAR-1 nor HGF plays a critical role in PA enhancement of PGE₂ responses in IPF fibroblasts. IPF fibroblasts were pretreated with ASA ± Plg + uPA for 24 h, followed by treatment with PGE₂ (0.5 μM) for 18 h. A, effect of PAR-1 antagonist on PA-enhanced PGE₂ responsiveness. The PAR-1 antagonist peptide FLLRN (500 μM) was added where indicated 30 min prior to Plg + uPA addition. Collagen I levels in cell lysates were determined by immunoblot, and an immunoblot representative of three different experiments is shown. B and C, effect of c-Met inhibition on PA-enhanced PGE₂ responsiveness. c-Met receptor kinase inhibitor PHA-665752 (0.1 μM, B) or anti-c-Met neutralizing Ab (20 μg/ml, C) was added where indicated 30 min prior to Plg + uPA addition. An immunoblot representative of three different experiments is shown.

**PA Enhances PKA-RII Signaling**—Next, we sought to clarify the role of each PKA-R isoform in PA-enhanced PGE₂ responsiveness. We first examined the effect of Plg + uPA on protein expression of each PKA-R isoform, and we found that Plg + uPA increased the ratio of PKA-RII to RI, causing a modest decrease in PKA-RIα protein expression while increasing PKA-RIIβ expression (Fig. 5A); PKA-RIβ protein was detected at a very low but similar level in both groups (data not shown). We next examined the effect of Plg + uPA pretreatment on the collagen-inhibiting actions of cAMP analogs that selectively activate either PKA-RI or PKA-RII. PA enhanced responses to PKA-RII-specific stimulation (Fig. 5B) but had only a minimal effect on responses to PKA-RI-specific stimulation (Fig. 5C) in IPF fibroblasts. Because PKA-Co must be released from PKA-R to manifest its kinase activity (4), we determined the effect of Plg + uPA pretreatment on PGE₂-induced PKA-Co release from PKA-RII in IPF fibroblasts. After 24 h of pretreatment in the presence or absence of Plg + uPA, IPF fibroblasts were treated with or without PGE₂ for 30 min. The coimmunoprecipitation was then performed on cell lysates using an antibody against PKA-RIIβ (the subtype whose protein expression was significantly increased by Plg + uPA, as shown in Fig. 5A), and the immunoprecipitate was immunoblotted for PKA-Co in addition to PKA-RIIβ. PKA-Co release from PKA-RIIβ 30 min after PGE₂ addition was significantly enhanced by 24 h of pretreatment with Plg + uPA (Fig. 5D). By contrast, immunoprecipitation using control IgG demonstrated no such change in the PKA-Co/PKA-RIIβ ratio among these four different treatment groups (data not shown). These results indicate that PA enhances PKA-RII signaling with enhanced PKA-RII protein expression and PKA-Co release from PKA-RIIβ.

**Enhancement of AKAP9 Interaction with PKA-RII Is Required for PA to Overcome PGE₂ Resistance**—AKAPs comprise a family of scaffold proteins that spatially focus and modulate PKA signaling, and the majority of such proteins bind preferentially to PKA-RII over PKA-RI (5). To clarify the role of AKAP binding to PKA-RI or PKA-RII in the actions of PA, we examined the effect of the RIAD peptide, which disrupts AKAP-RI binding, or Ht31 peptide, which disrupts AKAP-RII binding (23), on the enhanced PGE₂ effects elicited by Plg + uPA pretreatment in IPF fibroblasts. Addition of Ht31 30 min prior to addition of Plg + uPA almost completely abolished the ability of Plg + uPA to enhance PGE₂ suppression of collagen I in IPF fibroblasts (Fig. 6, A and B), although neither the inactive control peptide for Ht31 (Ht31c) nor RIAD interfered with this action of Plg + uPA (Fig. 6B). Of note, addition of Ht31 either 30 min (Fig. 6C) or 24 h (Fig. 6D) before addition of PGE₂ alone did not interfere with the suppressive effects of the prostanoid on collagen levels in PGE₂-responsive nonfibrotic lung fibroblasts. These results indicate that an interaction between PKA-RII and AKAP(s) is critical for PA to overcome PGE₂ resistance in IPF fibroblasts, although such an interaction is not necessary for the direct inhibitory actions of PGE₂ alone on collagen in nonfibrotic lung fibroblasts.

We next sought to identify the AKAP(s) that mediate PA enhancement of PGE₂ responsiveness in IPF fibroblasts. Of the >50 possible AKAPs that are recognized, some have been reported to associate with and suppress the activity of protein phosphatases (41). We focused on two, AKAP1 (D-AKAP1, AKAP149) and AKAP9 (CG-NAP, AKAP450), because of their known ability to bind to both PKA-RII and PP2A (5, 42). We first examined the colocalization of these two AKAPs with PKA-RII subtypes using confocal IFM. We employed rabbit antibodies against AKAP1 and -9, which were subsequently detected with a red fluorophore-conjugated secondary antibody, and mouse antibodies against PKA-RIIα and -RIIβ,
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FIGURE 4. PA enhances PGE₂ sensitivity in IPF fibroblasts through amplification of PKA signaling. IPF fibroblasts were pretreated for 4 h with or without Plg + uPA in addition to ASA. A, EP2 protein expression. After 4 h of pretreatment, cell lysates were obtained from IPF fibroblasts, and EP2 levels were determined by immunoblotting. Upper panel, representative immunoblot is shown: the dashed line indicates that the two lanes depicted, although from the same membrane, were not contiguous. Lower panel, after normalization to GAPDH, EP2 was expressed relative to that in fibroblasts without Plg + uPA pretreatment in each experiment. Data are from three experiments and represent the mean ± S.E. B, cAMP production after 15 min PGE₂ treatment. After pretreatment with or without Plg + uPA, IPF fibroblasts were treated with or without PGE₂ (0.5 μM) for 15 min, and cAMP levels in cell lysates were determined using the CAMP ELISA kit. Data were obtained from two experiments, and each symbol depicts the results of a single experiment. C and D, effect of PKA inhibitors on PA enhancement of PGE₂ responses in IPF fibroblasts. IPF fibroblasts were pretreated with ASA ± Plg + uPA for 24 h. PKA inhibitors KT5720 (100 nM) or PKI (10 μM) were added where indicated 30 min prior to Plg + uPA addition or 4 h before PGE₂ addition, respectively. Then PGE₂ (0.5 μM) was added for 18 h. Collagen I levels in the cell lysates were determined by immunoblotting. A representative immunoblot from three (C) or two (D) different experiments is shown. E, phosphorylation of VASP (pVASP) after PGE₂ treatment. After pretreatment, IPF fibroblasts were treated with or without PGE₂ (0.5 μM) for 30 min, and pVASP levels in cell lysates were determined by immunoblotting. Left panel, representative immunoblot. Right panel, results of densitometric analysis from three experiments. After normalization to GAPDH, pVASP levels after 30 min PGE₂ treatment were expressed relative to those in control fibroblasts without Plg + uPA pretreatment. F, phosphorylation of CREB (pCREB) after PGE₂ treatment. After pretreatment, IPF fibroblasts were treated with or without PGE₂ (0.5 μM) for 2 min, and pCREB levels in cell lysates were determined by immunoblotting. G, effect of Plg + uPA on PP2A activity. After 24 h on PP2A activity (expressed relative to the activity in control fibroblasts without Plg + uPA pretreatment; upper) and PP2A protein expression (lower) in cell lysates obtained from IPF fibroblasts. Data are obtained from three experiments and represent the mean ± S.E. *, p < 0.05 versus control fibroblasts without Plg + uPA pretreatment. H, enhancement of PGE₂ responsiveness after pretreatment with the selective PP2A inhibitor okadaic acid. IPF fibroblasts were pretreated with ASA ± okadaic acid at indicated concentrations for 4 h. The cells were then treated with or without PGE₂ (0.5 μM) for 18 h. Collagen I expression in the cell lysates was determined by immunoblotting. A representative immunoblot from two different experiments is shown.
in IPF fibroblasts (Fig. 8, B and C), although basal responses to PGE\(_2\) in control fibroblasts without Plg + uPA treatment tended to be greater in these experiments with siRNAs than in the experiments presented previously. Simultaneous silencing of AKAP9 and PKA-RI\(\beta\) completely abolished the potentiating effect of Plg + uPA on PGE\(_2\) responsiveness in fibroblasts (Fig. 8B), although silencing of either AKAP9 alone, PKA-RI\(\alpha\) alone, PKA-RI\(\alpha \beta\) alone, AKAP9 plus RI\(\alpha\), AKAP1 alone, or AKAP1 plus RI\(\beta\) (Fig. 8C) had little or no impact on the priming effects of Plg + uPA. These results indicate that PKA-RI\(\beta\) plays a more important functional role in the actions of Plg + uPA than does PKA-RI\(\alpha\). This can be explained by the facts that both protein expression (Fig. 5A) and colocalization with

**DISCUSSION**

Here, we report that PA overcomes PGE\(_2\) resistance in lung fibroblasts from both humans with IPF and mouse models of pulmonary fibrosis (Figs. 1 and 2). This action of PA involves enhanced PKA signaling (Fig. 4) that is mainly the consequence of induced interactions between PKA-RI and AKAP9 (Figs. 5–8). PA also modestly increased the protein expression of PKA-RI\(\beta\) (Fig. 5A). A model integrating our findings, which are based on a variety of experimental approaches, including confocal IFM, communoprecipitation, and RNA silencing, is presented in Fig. 9. Although several reports have explored AKAP regulation of PKA signaling in fibroblasts (42, 43), none has addressed a human fibrotic disorder. Moreover, although modulation of PKA-R protein expression (41) or of AKAP localization in response to various extracellular substances (44, 45) has been described in nonfibroblast cells, we are aware of no previous report in which a single treatment exerted such
diverse and functionally profound effects on the AKAP-PKA signaling network in any cell type as we describe herein for PA to plasmin in fibrotic lung fibroblasts.

Several details of our model await clarification. First, although we have characterized a number of ways in which PA reorganizes the AKAP-PKA-RII network through its product plasmin, we have not identified the actual molecular target with which plasmin interacts to initiate these diverse actions, and further work will be necessary to do so. We did, however, exclude roles for both proteolytically activated PAR-1 (Fig. 3A) and HGF proteolytically released from ECM (Fig. 3, B and C).

Second, the mechanism by which Plg/H11001 uPA treatment suppresses PP2A activity in fibrotic fibroblasts (Fig. 4G) also remains to be determined. Third, in view of the fact that silencing of AKAP9 alone was not sufficient to abolish Plg/H11001 uPA-enhanced PGE2 sensitivity (Fig. 8C), other AKAPs may play specific or perhaps redundant roles. The precise role of specific AKAPs in the sensitizing actions of PA therefore remains to be fully elucidated.

We acknowledge two limitations to our findings. First, they are derived from a small number of IPF patient-derived cell lines, so the ability of PA to overcome PGE2 resistance by the mechanisms reported herein may not be applicable to all patients. Second, it is not possible to extrapolate the relevance of the amounts of PA and plasmin used in our in vitro experiments to those of the IPF lung fibroblast milieu. Although the activity (15) and the level (16) of uPA have been assayed in lung lavage samples of IPF patients, the technique of bronchoalveolar lavage primarily samples the alveolar surface and does not allow an estimation of the relevant activities in the microenvironment of the fibroblast in the interstitial space of the alveolar wall.

Our finding that Ht31 inhibited the suppressive effect of PGE2 only in resistant IPF fibroblasts pretreated with Plg/H11001 uPA (Fig. 6, A and B), but not in normal lung fibroblasts intrinsically sensitive to PGE2 suppression (Fig. 6, C and D), indicates that a pathway for PGE2 signaling independent of AKAP-PKA-RII interactions is utilized in normal fibroblasts. Characterization of this normal pathway is currently underway in our laboratory. Nevertheless, it is remarkable that PA circumvents PGE2 resistance in IPF fibroblasts not by restoring the usual PKA signaling network operative in normal fibroblasts but by establishing a functionally active alternative network dependent on the formation of new AKAP/H11001 PKA-RII complexes.

There is precedent for prostanoid treatment of lung disease, as idiopathic pulmonary arterial hypertension is characterized by deficient production of another prostanoid, prostacyclin, which can be addressed therapeutically by exogenous administration of prostacyclin or its analogs (46). Although IPF is characterized by diminished lung levels of PGE2 (8, 9), PGE2 resistance in lung fibroblasts would appear to be a critical barrier to PGE2 replacement therapy. However, the data presented in this...
Simultaneous silencing of both AKAP9 and PKA-RII completely abolishes Plg + uPA-induced enhancement of PGE2 responsiveness in IPF fibroblasts. Cells were incubated with one siRNA (50 nM), two siRNAs (50 nM each, total 100 nM), or the same concentrations of control siRNA(s) (Cont) for 3 days. In some experiments, cell lysates were obtained at this time point to determine the effect of siRNAs on target protein expression. Thereafter, cells were pretreated for 24 h with ASA + Plg + uPA followed by 18 h of treatment with PGE2 (0.5 μM). Collagen I levels were determined by immunoblotting and, after normalization to GAPDH, were expressed relative to those in fibroblasts without PGE2 treatment in each treatment group. A, effect of siRNAs on expression of target proteins. B, effect of simultaneous silencing of both AKAP9 and PKA-RIIβ or control siRNA (Cont) on PA enhancement of PGE2 suppression of collagen I levels. C, effect of silencing AKAP9, PKA-Rlα, PKA-Rlβ, AKAP9 and Rlα, AKAP1, AKAP1 and Rlβ, or control siRNA (Cont) on Plg + uPA-induced enhancement of PGE2 responsiveness in IPF fibroblasts. Data in B and C represent the mean ± S.E. from three to four experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001. N.S., not significant.
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Plasminogen Activation (PA)

FIGURE 9. Schematic summary. Plasmin produced by uPA cleavage of Plg induces extracellular proteolysis, which enhances binding of AKAP9 to PKA-RII and movement of these proteins to the perinuclear region (1). PA also increases protein expression of PKA-RII (2) and enhances PKA-Cα release from PKA-RIIβ (3). PA suppresses PP2A activity (4), which further potentiates phosphorylation of substrates by PKA. These events promote PKA actions around/in the nucleus, resulting in inhibition of collagen synthesis in the presence of PGE2. Ht31 disrupts AKAP9-PKA-RII binding, abrogating the potentiation by PA of PGE2 inhibition of collagen I synthesis. It remains to be determined whether suppression of PP2A activity is the consequence of AKAP9 interaction with this phosphatase (5).

study demonstrating that PA resensitizes fibrotic fibroblasts to the collagen-inhibiting actions of this prostanoid raise the possibility that combination therapy with PGE2 and PA may represent a promising therapeutic approach in this disorder.

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