Opposing Effects of Protein Kinase Cα and Protein Kinase Cε on Collagen Expression by Human Lung Fibroblasts Are Mediated via MEK/ERK and Caveolin-1 Signaling*

The roles of MEK, ERK, the ε and α isoforms of protein kinase C (PKC), and caveolin-1 in regulating collagen expression were studied in normal lung fibroblasts. Knocking down caveolin-1 gave particularly striking results. A 70% decrease caused a 5-fold increase in MEK/ERK activation and collagen expression. The combined data reveal a branched signaling pathway. In its central portion MEK activates ERK, leading to increased collagen expression. Two branches converge on MEK/ERK. In one, increased PKCε leads to MEK/ERK activation. In another, increased PKCα induces caveolin-1 expression, which in turn inhibits MEK/ERK activation and collagen expression. Lung fibroblasts from scleroderma patients with pulmonary fibrosis showed altered signaling. Consistent with their overexpression of collagen, scleroderma lung fibroblasts contain more activated MEK/ERK and less caveolin-1 than normal lung fibroblasts. Because cutaneous fibrosis is the hallmark of scleroderma, we also studied dermal fibroblasts. As in lung, there was more activated MEK/ERK in cells from scleroderma patients than in control cells, and MEK inhibition decreased collagen expression. However, the distinctive levels of PKCε, PKCα, and caveolin-1 in lung and dermal fibroblasts from scleroderma patients and control subjects indicate that the links between these signaling proteins and MEK/ERK must function differently in the four cell types. Finally, we confirmed the relevance of these signaling cascades in vivo. The combined results demonstrate that a branched signaling pathway involving MEK, ERK, PKCε, PKCα, and caveolin-1 regulates collagen expression in normal lung tissue and is perturbed during fibrosis.

The normal regulation of collagen expression in lung tissue is disrupted in many diseases including scleroderma (systemic sclerosis), leading to the overexpression of collagen, pulmonary fibrosis, and frequently death. Despite its importance, relatively little is known about the signaling mechanisms that regulate collagen expression in normal lung tissue and how these mechanisms are altered in fibrotic lung tissue. The current studies focus on three families of signaling proteins, MEK/ERK, PKC, and caveolin, whose activities are likely to be integrated given that caveolin-1 can serve as a scaffold to which MEK, ERK, PKCα, and PKCε bind (1).

The family of mitogen-activated protein kinases (MAPKs) consists of three known subfamilies, MEK1 and -2 (the MAPK/ERK kinases), ERK1 and -2 (the extracellular signal-regulated kinases), the JNKs (c-Jun N-terminal kinases), and the p38 MAPKs (2, 3). ERK1 and ERK2 are activated when they are phosphorylated and then translocated from the cytoplasm to membranes, the cytoskeleton, or the nucleus (9). The family of serine-threonine kinases forms three subfamilies based on structural homologies and sensitivity to activators. Conventional PKCs (α, β1, β2, γ) are calcium-dependent and are activated by phosphatidylserine, diacylglycerol, or phorbol esters; the novel PKCs (δ, ε, η, ζ) differ in that they are calcium-independent; the atypical PKCs (λ, η) are also calcium-independent and additionally are insensitive to diacylglycerol and phorbol esters (6, 7). All PKC isoforms contain catalytic and regulatory domains. The regulatory domain contains a pseudosubstrate region that binds to the catalytic cleft thereby inhibiting the kinase activity of PKC. Members of the conventional subfamily contain four homologous domains (C1, C2, C3, and C4) interspersed with isoencezyme-unique variable or V domains. Members of the novel subfamily have a modified C2 domain; members of the atypical subfamily lack both the C2 and half of the C1 domain (8). Upon activation, PKC isoforms translocate from the cytoplasm to membranes, the cytoskeleton, or the nucleus (9).

Two of the novel PKCs, δ and ε, play roles in scleroderma. PKCδ is present at elevated levels in scleroderma dermal fibroblasts and promotes collagen expression (10). In contrast, PKCε functions abnormally in scleroderma lung fibroblasts.
PKC/MEK/ERK/Caveolin-1 Signaling and Collagen Expression

(Caveolae). Depleting PKCe in normal lung fibroblasts (NLF) inhibits its expression of the extracellular matrix protein tenascin-C, whereas depleting PKCe in SLF promotes tenascin-C expression (11). SLF are deficient in PKCe and are sensitive to curcumin-induced apoptosis, whereas NLF are insensitive to curcumin (12). The importance of PKCe in regulating the sensitivity of cells to curcumin is demonstrated by the observations that 1) increasing PKCe expression in SLF provides protection against curcumin and 2) decreasing PKCe expression or activity in NLF causes the cells to become sensitive to curcumin (12). PKCe is associated with the cytoskeleton in SLF but not in NLF (12, 13). Thus, there may be more active PKCe in SLF than in NLF, despite the fact that there is less total PKCe.

Caveolae were identified 40 years ago as flask-shaped invaginations on the surface of epithelial and endothelial cells. Caveolae play roles in the transport of macromolecules and in signal transduction (14, 15). The caveolin family of caveolae coat proteins contains at least three members. Caveolin-1 and -2 are abundantly expressed in adipocytes, endothelial cells, and fibroblasts; caveolin-3 is muscle-specific (1, 16). Caveolin-1 may modulate signal transduction by linking signaling molecules to caveolae and regulating their activity. In particular, caveolin-1 binds to a variety of kinases including PKC and MEK/ERK and thereby inhibits their catalytic activity (17–19).

Caveolin-1 has been linked strikingly to lung fibrosis. In caveolin-1 null mice, the diameter of alveolar spaces is reduced, alveolar walls are thickened and hypercellular, and the deposition of extracellular matrix is significantly increased (20, 21). Similarly, caveolin-1 levels are strikingly decreased in lung tissue induced to become fibrotic by irradiation (22). Nevertheless, to the best of our knowledge, the current report is the first to demonstrate signaling mechanisms involved in the regulation of collagen expression by caveolin-1.

In the current studies, we have used specific inhibitors and constructs to alter the expression and function of MEK/ERK, PKC, and caveolin-1 in NLF and SLF. These studies have revealed that under normal conditions a branched signaling pathway regulates collagen expression. In the central portion of the pathway MEK activates ERK, leading to increased collagen expression. Two branches converge on MEK/ERK. In one, increased PKCe expression leads to MEK and ERK activation. In the other, increased PKCe expression induces caveolin-1 expression which, in turn, inhibits MEK/ERK activation and collagen expression. Consistent with their overexpression of collagen, SLF contained much more activated MEK and ERK and much less caveolin-1 than NLF. Moreover, because MEK and ERK are constitutively hyperactivated in SLF, treatments that strongly increase MEK/ERK activation and collagen expression in NLF do not affect MEK/ERK activation and collagen expression in SLF. Similar signaling pathways appear to regulate collagen expression in vivo as evidenced by the fact that fibrotic lung tissue from bleomycin-treated mice contains very high levels of activated MEK and ERK and very low levels of PKCa and caveolin-1. Although MEK/ERK signaling also regulates collagen expression in dermal fibroblasts, the other branches of this signaling pathway must differ in dermal and lung fibroblasts given that the relative levels of expression of PKCe, PKCa, and caveolin-1 are very different in NLF, SLF, normal dermal fibroblasts (NDF), and scleroderma dermal fibroblasts (SDF). In summary, these results reveal a complex signaling pathway that regulates collagen expression in the lung under normal conditions and that is perturbed when collagen is overexpressed in both in vitro and in vivo models of lung fibrosis.

**Experimental Procedures**

**Cell Culture**

Fibroblasts were derived from lung tissue obtained at autopsy from scleroderma patients (SLF) and from age-, race-, and sex-matched normal subjects (NLF) and cultured as previously described (12). Fibroblasts were also obtained by punch biopsy from the skin of scleroderma patients and age-, race-, and sex-matched normal subjects and cultured as previously described (23). The harvesting of tissue was approved by the Medical University of South Carolina IRB for Human Research.

Perturbation of Signaling Protein Expression/Activity

**MEK Activity**—To inhibit MEK activity, cells were treated with U0126 (Cell Signaling, Beverly, MA). When cell cultures became nearly confluent, they were shifted to serum-free medium (Dulbecco’s modified Eagle’s medium/10% fetal calf serum containing 25 mg/ml gentamicin sulfate). The next morning the medium was again replaced with serum-free medium alone or containing 10 μM U0126. After an additional 3-h incubation, the culture medium and cell layer were harvested.

**PKC Activity**—To inhibit the expression of various isoforms of PKC, cells were treated with appropriate antisense oligodeoxynucleotides (ODN) and control sense ODN for 48 h as described previously (11) and then shifted to serum-free medium. The next morning the culture medium and cell layer were harvested. Phosphorothioated ODN were synthesized in the Oligonucleotide Synthesis Facility at the Medical University of South Carolina.

**PKC activity** and **PKC expression** were also perturbed by overexpressing constitutively active (c-a) and dominant-negative (d-n) forms of PKCe and PKCa in cells. Cells were incubated for 48 h with PuGENE 6 transfection reagent (Roche Diagnostics) according to manufacturer’s instructions (1 μg of each plasmid/well in 6-well plates) as described previously (12) and then shifted to serum-free medium. The next morning the culture medium and cell layer were harvested. When U0126 treatment and PKC construct transfection were combined, an additional 3-h incubation in fresh serum-free medium alone or containing 10 μM U0126 was performed prior to the harvesting of the medium and cell layer. PKCe constructs eA159E (c-a) and Kn-eA159E (d-n) in the SRD vector were a kind gift of Dr. Shigeo Ohno, Yokohama City University School of Medicine, Japan (24). c-a and d-n PKCs constructs in the pMT2 vector were a kind gift of Dr. Peter Parker, Imperial Cancer Research Fund, UK (25). Empty vectors were used in control experiments.

Caveolin-1 Expression—Caveolin-1 expression was inhibited using short inhibitory RNA (siRNA) synthesized as double-stranded oligoribonucleotides by MWG Biotech (High Point, NC). siRNA was designed to target the human caveolin-1 sequence 5’-AACGATGACGTGTTCAAGATT. Cells in 6-well plates at 50–60% confluency were incubated with 2 μl of Dulbecco’s modified Eagle’s medium, 10% fetal calf serum lacking antibiotics and containing 2 μg/l of Oligonucleotides in the presence or absence of 40 pmol of caveolin-1 siRNA. After 48 h, the cells were shifted to serum-free medium. The next morning the culture medium and cell layer were harvested.

**Western Blot Analyses**

Cell layers were extracted with boiling SDS-PAGE sample buffer. Concentrated SDS-PAGE sample buffer was added to the culture medium. Aliquots of culture medium or of cell layer extract representing material derived from the same number of cells were probed using the following primary antibodies and appropriate secondary antibodies: culture medium, goat anti-human collagen I (AB758P) from Chemicon International (Temecula, CA); rabbit polyclonal anti-PKCα (sc-208), anti-PKCδ (sc-214), and anti-caveolin-1 (sc-864) from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal anti-ERK 1/2 (9102), anti-activated ERK 1/2 (9106), anti-MEK 1/2 (9122), and anti-activated MEK 1/2 (9121) from Cell Signaling; and mouse monoclonal anti-actin (MAB1501) from Chemicon International.

**Immunocytochemistry and Laser Confocal Microscopy**

NLF and SLF were cultured in 4-well glass chamber slides (Nalge Nune International, Naperville, IL) and labeled as described previously (12) using rabbit anti-caveolin-1 (see above) as the primary antibody. Nuclei were stained using 0.01% 7-aminoactinomycin D (Molecular Probes, Eugene, OR). For Fig. 6, images were captured using a Zeiss 510SMI Laser Confocal Microscope, excitation at 488 nm, and an oil-immersion objective (63×/1.4).
ERK Activation Regulates Collagen Expression by Both NLF and SLF

ERK1 and -2 (p42 and p44) are kinases that are activated when they are phosphorylated by either of two related kinases, MEK1 and MEK2. Despite the fact that ERK1/2 play central roles in signaling in a vast number of biological systems, there are few, if any, reports relating ERK signaling to collagen type I (hereafter referred to as collagen) expression in NLF and examining whether the overexpression of collagen by SLF may be related to ERK signaling. To directly test the idea that ERK activation regulates collagen expression, cells were treated with the MEK1/2 inhibitor, U0126. This treatment caused a parallel inhibition in ERK phosphorylation and in collagen accumulation in both NLF and SLF cultures (Fig. 1), demonstrating that the activation of ERK by MEK is an upstream regulator of collagen expression in both types of cells.

Endogenous Levels of Activated MEK and ERK Are Much Higher in SLF than in NLF

To evaluate whether the overexpression of collagen by SLF may be related to altered ERK signaling, we compared ERK levels and activation (phosphorylation) in NLF and SLF (Fig. 2). We found that phosphorylated ERK levels are nearly twice as high in SLF than in NLF, even though total ERK levels are only ~60% as high in SLF as in NLF. Thus ERK polypeptides are about three times as likely to be active in SLF than in NLF. Because MEK is a kinase that activates ERK, we also compared MEK levels and activation in NLF and SLF (Fig. 2). Although total MEK levels were slightly lower in SLF than in NLF, phosphorylated MEK levels were more than twice as high in SLF than in NLF. In summary, we find that the MEK/ERK signaling pathway is hyperactivated in SLF resulting in the overexpression of collagen by these cells.

Bleomycin-induced Lung Fibrosis

The following procedure was approved by the Medical University of South Carolina Institutional Animal Care and Use Committee. 10-week-old male CD-1 mice (Charles River, Boston, MA) were anesthetized and received bleomycin (Calbiochem, La Jolla, CA) or saline solution by intratracheal instillation as described previously (12). After 21 days, mice were sacrificed, and their lungs removed, fixed, and sectioned for immunohistochemical analysis using rabbit anti-caveolin-1 and for histochemical analysis using Masson’s trichrome stain or sectioned for immunohistochemical analysis using antibodies against ERK 1/2, activated MEK 1/2, PKC and Western blotted using the antibodies against ERK 1/2, activated ERK 1/2, MEK 1/2, activated MEK 1/2, PKCα, and caveolin-1 as described above.

RESULTS

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Opposing Roles for PKCα and PKCe in Collagen Accumulation

To extend the signaling cascade linking MEK, ERK, and collagen expression to kinases further upstream, we examined several PKC isoforms. In these experiments, we inhibited PKC expression using antisense oligonucleotides and read out the effects of these treatments on the expression of the targeted PKC isoform and on the expression of collagen. The ability of antisense, but not sense, oligonucleotides to inhibit the expression of PKCα, PKCδ, and PKCe in NLF is shown in Fig. 3. When the level of collagen secretion into the medium in these cultures was examined (Fig. 3), we found that depletion of PKCα increased collagen accumulation, depletion of PKCe inhibited collagen accumulation, and depletion of PKCδ had no effect. Similar results were obtained with both NLF (Fig. 3) and SLF (data not shown).

![Image](image-url)
To confirm and extend these observations, NLF were transfected with vectors encoding d-n and c-a PKCa and PKCe and the effects of these treatments on PKC expression, ERK expression and activation, and collagen expression were determined (Fig. 4). In all cases, the overexpression of d-n or c-a PKCa or PKCe was apparent (Fig. 4, A–C). Consistent with the results obtained using antisense oligonucleotides, overexpression of d-n PKCa increased collagen accumulation in the culture medium, whereas overexpression of c-a PKCa inhibited collagen accumulation in the culture medium (Fig. 4D). Consistent with the idea that ERK activation promotes collagen expression, d-n PKCa increased ERK phosphorylation, whereas c-a PKC inhibited ERK phosphorylation (Fig. 4). Neither of these treatments affected total ERK levels. Similar results using c-a and d-n PKCa to affect collagen accumulation and ERK activation were obtained with both NLF (Fig. 4A) and SLF (data not shown).

Also consistent with results obtained using antisense oligonucleotides, overexpression of d-n PKCe inhibited collagen accumulation in both NLF (Fig. 4B) and SLF (Fig. 4C). Further supporting the idea that ERK activation promotes collagen expression, d-n PKCe inhibited total ERK expression and ERK phosphorylation in both NLF (Fig. 4B) and SLF (Fig. 4C). In contrast, NLF and SLF responded differently to overexpression of c-a PKCe. Although overexpression of c-a PKCe increased both ERK phosphorylation and collagen expression in NLF cultures (Fig. 4B), this treatment did not affect the already high levels of both ERK phosphorylation and collagen expression observed in SLF cultures (Fig. 4C). The overexpression of c-a PKCe had no effect on total ERK expression in either NLF or SLF. In summary, these observations further support the idea that ERK phosphorylation and collagen expression are tightly linked. In accord with our previous studies (11, 12), these observations also suggest that the PKCe signaling pathway functions aberrantly in SLF.

Mek and Erk Are Located Downstream from PkCa and PkCe

To evaluate the possibility that both the overexpression of PKCe and the inhibition of the function of PKCa increase collagen expression by activating the MEK/ERK signaling pathway, NLF were transfected with empty vector, c-a PKCes and d-n PKCes and treated with U0126 to block the activation of ERK by MEK (Fig. 4D). U0126 treatment completely blocked the increase in ERK phosphorylation and the increase in collagen expression caused by either c-a PKCes or d-n PKCes, proving that ERK activation is downstream from PKCe and PKCa in the regulation of collagen accumulation in NLF.

Caveolin-1 and MEK/ERK Signaling

Caveolin-1 can inhibit MEK/ERK signaling by acting as an endogenous kinase inhibitor (18, 19). To determine whether caveolin-1 might be a participant in the regulation of MEK/ERK signaling and collagen expression in NLF, we used siRNA technology to inhibit caveolin-1 expression. Strikingly, an siRNA treatment that inhibited caveolin-1 expression by 70% caused a 5-fold increase in MEK phosphorylation, ERK phosphorylation, and collagen accumulation (Fig. 5, A and B). These data demonstrate the remarkable ability of caveolin-1 to regulate MEK/ERK signaling and collagen accumulation.

Caveolin-1, MEK/ERK Hyperactivation, and Collagen Overexpression in SLF

To determine whether caveolin-1 might contribute to the hyperactivation of MEK/ERK signaling and the overexpression of collagen observed in SLF, we first compared caveolin-1 levels in NLF and SLF (Fig. 5, A and B). Consistent with the idea that low levels of caveolin-1 lead to the hyperactivation of MEK/ERK signaling, caveolin-1 levels were only 46% as high in SLF as in NLF. When we next compared caveolin-1 in NLF and SLF by confocal microscopy (Fig. 6), we confirmed that caveolin-1 is present at much higher levels in NLF than in SLF. These results are consistent with the idea that the decreased expression of caveolin-1 in SLF plays a role in the MEK/ERK hyperactivation and collagen overexpression observed in these cells.

To determine whether further decreasing the already low level of caveolin-1 in SLF might further activate MEK/ERK signaling and enhance collagen expression, SLF were treated with siRNA targeting the expression of caveolin-1. Although this treatment did inhibit caveolin-1 expression (Fig. 5, A and B), it had little effect on the already high level of MEK/ERK and collagen expression in these cells. This result stands in
sharp contrast to the effect of the same treatment on NLF in which the inhibition of caveolin-1 expression causes a 5-fold increase in MEK/ERK activation and collagen expression (Fig. 5, A and B).

Caveolin-1 and PKC Signaling

To determine whether caveolin-1 might be a participant in the regulation by PKC of MEK/ERK signaling and collagen expression in NLF, we first evaluated whether using siRNA to inhibit caveolin-1 expression affected the expression of PKCα or PKCe. This treatment had no effect on either PKCα or PKCe levels in either NLF or SLF (Fig. 5C). We next tested the possibility that regulating the expression of PKCα or PKCe might affect caveolin-1 protein levels. Whereas transfection of NLF with either c-a or d-n PKCe had no effect on caveolin-1 expression, transfection with c-a PKCα promoted caveolin-1 expression and transfection with d-n PKCα inhibited caveolin-1 expression (Fig. 7). In these experiments, similar results were obtained with both NLF and SLF. These observations strongly suggest that the enhanced expression of caveolin-1 in cells transfected with c-a PKCα leads to the inhibition of MEK/ERK activation and to the inhibition of collagen expression that we have observed in these cells.

Regulation of Collagen Expression in Dermal Fibroblasts

Because the hallmark of scleroderma is the overexpression of collagen in the dermis, we compared the expression of PKCα, PKCα, ph-MEK, ph-ERK, and caveolin-1 in NDF and SDF to their expression in NLF and SLF (Fig. 8, A and B). Although ph-MEK and ph-ERK were substantially up-regulated in both SLF and SDF as compared with their normal counterparts, the other signaling molecules examined were differently regulated in lung and dermis. In particular, although PKCα is present at similar levels in NLF and NDF, it is increased in expression in SDF (as compared with NDF), even though it is decreased in expression in SLF (as compared with NLF). In contrast, PKCα is present at much lower levels in NDF than in NLF and increased in expression in SDF (as compared with NDF), even though there is no difference in PKCα expression between NLF and SLF. Finally, caveolin-1 is expressed at much higher levels in NDF than in NLF, and its expression increases further in SDF (as compared with NDF), even though its expression is much lower in SLF than in NLF.

Because MEK/ERK signaling is up-regulated in both SLF and SDF, we treated NDF and SDF with the MEK inhibitor U0126 (Fig. 8C) to determine whether MEK/ERK signaling regulates collagen expression in dermal fibroblasts as we have shown above for lung fibroblasts (Fig. 1). As expected, in the absence of U0126 more collagen was secreted by SDF than by NDF. In accord with the idea that MEK/ERK signaling regulates collagen expression in dermal fibroblasts, U0126 treatment almost completely blocked the expression of ph-ERK and of collagen by both NDF and SDF. In summary, this comparison...
FIG. 6. Subcellular distribution of caveolin-1 is different in NLF and SLF. Cells were cultured in 4-well slides, fixed, permeabilized, labeled with antibodies against caveolin-1 and appropriate secondary antibodies and with the nuclear stain 7-aminoactinomycin D, and examined by laser confocal microscopy. A and D, caveolin-1 staining. B and E, 7-aminoactinomycin D staining. C and F, merged images. Note that in NLF (A–C), caveolin-1 is present at high levels in a punctate pattern both in the nuclear/perinuclear region and throughout the cell. In contrast, in SLF (D–F) caveolin-1 is present at much lower levels. All panels are at the same magnification. Bar in C, 20 μm.

FIG. 7. c-a and d-n PKCα, but not c-a and d-n PKCε, affect caveolin-1 expression in NLF and SLF. Cells were transfected with the indicated constructs, then serum-starved overnight. The levels of the targeted PKC isoform, caveolin-1, and actin (loading control) in the cell layer were determined by Western blotting. Representative data are shown from one of three independent experiments performed using distinct matched strains of NLF and SLF. Quantification of the data showed no more than a 20% difference between experiments.

Discussion

We have demonstrated above that MEK/ERK and caveolin-1 signaling are altered in an in vitro model system for studying lung fibrosis, i.e. fibroblasts grown from the lung tissue of patients with scleroderma lung disease differ from fibroblasts grown from the lung tissue of normal subjects. In addition, we have shown that the opposing effects of PKCα and PKCε on collagen expression are mediated via MEK/ERK and caveolin-1 signaling pathways. In previous studies (12) we demonstrated that PKCε levels are decreased in both the in vitro model system and an in vivo model system in which mice treated with bleomycin develop lung fibrosis. To determine whether MEK, ERK, PKCα, and caveolin-1 expression are also altered in the in vivo model, lung tissue from bleomycin-treated mice and control, saline-treated mice was harvested 21 days after treatment (when lung fibrosis is most severe in bleomycin-treated mice) and performed Western blot analyses using the appropriate antibodies. These experiments revealed major differences in the expression and activation of MEK, ERK, PKCα, and caveolin-1 (Fig. 9). Densitometric analyses of Western blots showed that activated MEK levels were >4-fold higher in bleomycin-treated lung tissue even though total MEK levels were only 40% as high. Similarly, activated ERK levels were 2.5-fold higher in bleomycin-treated lung tissue even though total ERK levels were only 50% as high. Thus the proportion of MEK and ERK molecules that are activated is 5–10-fold higher in the lungs of bleomycin-treated mice than in the lungs of control saline-treated mice. In the case of PKCα, densitometric analyses of Western blots showed a 70% decrease in its expression in bleomycin-treated lung tissue. In the case of caveolin-1, densitometric analyses of Western blots showed a 70% decrease in its expression in bleomycin-treated lung tissue. In addition, this decrease in caveolin-1 expression was confirmed by immunohistochemistry (Fig. 10). In the alveoli of saline-treated mice, caveolin-1 staining (brown reaction product) was associated with multiple cell types, including type I and type II pneumocytes and endothelial cells. Little or no collagen was observed (blue reaction product with Masson’s Trichrome stain). In contrast, in the alveoli of bleomycin-treated mice little or no caveolin-1 staining was detected in any cell type, even when the alveoli had a relatively normal morphology and only moderate blue staining with Masson’s Trichrome (Fig. 10, Bleomycin Mild Fibrosis). In regions of extensive fibrosis, little or no caveolin-1 staining was detected, and high levels of collagen were detected (Fig. 10, Bleomycin Severe Fibrosis). In summary, these results strongly suggest that several alterations in signaling protein expression that we have observed in vitro are relevant to lung fibrosis in human patients, given that we observe the same alterations in an in vivo animal model of lung fibrosis.

**DISCUSSION**

The results of the current study support the model shown in Fig. 11 depicting the signaling cascades that regulate collagen expression in normal human lung tissue. This model can be divided into three modules. In the central module MEK activates ERK leading to enhanced collagen expression. In a second module (Fig. 11, top) the activation of PKCε leads to MEK activation and, therefore, to ERK activation and enhanced collagen expression. In a third module (Fig. 11, right) the activation of PKCα increases the expression of caveolin-1, which in turn inhibits MEK, ERK, and kinases that activate MEK. Thus overexpression of active PKCε promotes collagen expression, whereas overexpression of active PKCα inhibits collagen expression. PKCα and PKCε have been reported to have opposing functions in other
systems, including glioma cell migration (26) and basolateral membrane dynamics in intestinal epithelia (27). However, the current study is the first to demonstrate that PKC/H9251 and PKC/H9280 can oppose one another through their opposite effects on the MEK/ERK signaling cascade.

The validity of the central module of this model is demonstrated by the fact that the MEK inhibitor U0126 inhibits the phosphorylation of ERK and thereby also inhibits collagen expression (Fig. 1). This module functions in the same manner in NLF and SLF, i.e. U0126 inhibits ERK phosphorylation and collagen expression in both cell types. The link between MEK/ERK activation and enhanced collagen expression is also consistent with the observations that 1) SLF, which express more collagen than do NLF, also contain higher levels of activated MEK and ERK (Fig. 2), and 2) fibrotic lung tissue in vivo contains higher levels of activated MEK and ERK compared with control lung tissue (Fig. 9).

The validity of the second module is supported by the observations that in NLF increasing PKC/H9280 expression increases quantification of relative PKCα, PKCe, ph-MEK, ph-ERK, caveolin-1, and actin levels in NLF, SLF, NDF, and SDF. The level of each protein in NLF was defined as 100 arbitrary units. These data represent the average ± S.D. of three independent experiments performed using distinct matched strains of lung and dermal fibroblasts. Quantification of the data showed no more than a 20% difference between experiments.

Fig. 8. Comparison of the expression and function of MEK/ERK, PKCe, PKCα, and caveolin-1 in lung and dermal fibroblasts. A, the levels of PKCe, PKCα, ph-MEK, ph-ERK, caveolin-1, and actin (loading control) in the cell layers of serum-starved NLF, SLF, NDF, and SDF were determined by Western blotting. B, densitometric quantification of the data showed no more than a 20% difference between experiments.

Fig. 9. Altered signaling in mice with lung fibrosis. Lung fibrosis was induced with a single intratracheal instillation of bleomycin. Control mice received only saline vehicle. A, after 21 days, mice were sacrificed, and a set of lungs from a saline-treated or a bleomycin-treated mouse was homogenized, and 25 μg of protein from each extract was Western blotted using antibodies against PKCα, ph-MEK, MEK, ph-ERK, ERK, and caveolin-1. B, densitometric quantification of Western blots. These data represent the average ± S.D. of three independent experiments similar to the one depicted in A. The level of each protein in the extracts of saline-treated mice was defined as 100 arbitrary units. Quantification of the data showed no more than a 20% difference between experiments.
ERK activation and collagen expression, inhibiting PKCe expression or activity decreases ERK activation and collagen expression and that the MEK inhibitor U0126 blocks both the ERK activation and collagen expression caused by the overexpression of PKCe (Figs. 3 and 4). The ability of U0126 to block the positive effect of PKCe overexpression on collagen expression is particularly informative, because it proves that the activation of MEK and ERK are necessary intermediates in the pathway linking PKCe and collagen.

The functioning of the second module is aberrant in SLF. Decreasing PKCe expression (Fig. 3) or activity (Fig. 4) decreases MEK/ERK activation and collagen expression in both NLF and SLF. However, increasing PKCe expression has no effect on MEK/ERK activation and collagen expression in SLF (Fig. 4), presumably because MEK/ERK are already highly activated, and collagen is already being expressed at high levels in SLF under base-line conditions (Figs. 1 and 2). Because MEK/ERK are highly activated in fibrotic lung tissue in vivo, high levels of collagen expression are achieved despite the fact that PKCe is present at lower levels in SLF than in NLF and in fibrotic lung tissue than in control lung tissue (12).

The validity of the third module is supported by the observations that in NLF inhibiting PKCa expression enhances collagen expression (Fig. 3), inhibiting PKCa activity enhances ERK activation and collagen expression via a U0126-inhibitable mechanism (Fig. 4), increasing PKCa expression increases caveolin-1 expression and inhibits MEK/ERK activation and collagen expression (Figs. 4 and 7), and decreasing caveolin-1 expression increases MEK/ERK activation and collagen expression (Fig. 5). These observations clearly substantiate the link in lung fibroblasts between low levels of PKCa, low levels of caveolin-1, high levels of MEK/ERK activation, and high levels of collagen expression. These links are consistent with the observations that fibrotic lung tissue contains less PKCa and caveolin-1 than control lung tissue (Figs. 9 and 10) and that both Western blotting (Fig. 5) and confocal microscopy experiments (Fig. 6) demonstrate that SLF contain much less caveolin-1 than do NLF. Although increasing PKCe expression had no effect on caveolin-1 expression in lung fibroblasts (Fig. 7), increasing PKCe can increase caveolin-1 in other cell types, e.g., human prostate cancer cells (28).

The functioning of the third module is also aberrant in SLF. Whereas inhibiting caveolin-1 expression in NLF results in a 5-fold increase in MEK/ERK activation and collagen expression, inhibiting caveolin-1 expression in SLF has little or no effect on MEK/ERK activation and collagen expression (Fig. 5). This difference cannot simply be because of the fact that the base-line levels of MEK/ERK activation and collagen expression are much higher in SLF than in NLF, because after the inhibition of caveolin-1 expression in both cell types, MEK/ERK activation and collagen expression are much higher in NLF than in SLF. An additional difference between SLF and NLF in the third module is suggested by the observation that inhibiting the expression or activity of PKCa increases MEK/ERK activation and collagen expression in both NLF and SLF. In NLF, this effect can be totally ascribed to the accompanying inhibition of caveolin-1 expression (Fig. 7), because directly inhibiting caveolin-1 expression in NLF also increases MEK/ERK activation and collagen expression (Fig. 5). However, in SLF, PKCa must be affecting MEK/ERK activation and collagen expression through an additional or alternative mechanism, because directly inhibiting caveolin-1 expression in SLF does not increase MEK/ERK activation and collagen expression. These observations make it likely that additional differences between NLF and SLF in signaling cascades that regulate collagen expression remain to be discovered.

The hallmark of scleroderma is dermal fibrosis. Therefore, we were interested in determining whether the regulation of collagen expression occurred via similar signaling pathways in lung and dermal fibroblasts. The importance of MEK/ERK signaling in both lung and dermal fibrosis was indicated by the observations that 1) in both tissues, cells from scleroderma patients contain higher levels of activated MEK and ERK than cells from control subjects, and 2) in dermal and lung fibroblasts from both scleroderma patients and control subjects, the MEK inhibitor U0126 inhibits collagen expression (Figs. 1 and 8). In contrast, the very different levels of expression of PKCe, PKCa, and caveolin-1 in NLF, SLF, NDF, and SDF indicate that the links between these signaling molecules and MEK/ERK differ between NLF and SLF, between NDF and NLF, and between SDF and NDF. Therefore, our observations suggest that 1) a major portion of the changes in signaling that occur during dermal fibrosis differ from the changes that occur during lung fibrosis, and 2) distinct novel treatments based on a knowledge of signaling may be required to treat dermal fibrosis and lung fibrosis in scleroderma patients.

The physiological relevance of our observations linking low levels of caveolin-1 expression in lung cells and tissue to high levels of collagen expression are strongly supported by a variety of experiments in vivo. Two independent groups have shown extensive fibrosis in the lungs of caveolin-1 null mice (20, 21). In these mice, the diameter of alveolar spaces is reduced, alveolar walls are thickened and hypercellular, and the deposition of the extracellular matrix is significantly in-
increased. Similarly, caveolin-1 levels are strikingly decreased in lung tissue induced to become fibrotic by irradiation (22) or by bleomycin treatment (Fig. 9). Thus there is a strong correlation between low levels of caveolin-1 and lung fibrosis. These observations also make sense at the cellular level, because myofibroblasts (i.e. SLF) are present in fibrotic lung tissue but not in normal lung tissue (29), secrete large amounts of collagen, and contain little caveolin-1 (Fig. 5). In contrast, type I and type II pneumocytes are more prominent in normal lung tissue and contain relatively large amounts of caveolin-1 (Fig. 10 and Refs. 22, 30, and 31).

In summary, these observations demonstrate that the regulation of collagen expression in lung tissue is a complex process involving interplay among a number of signaling pathways. Thus it is not surprising that multiple alterations in signaling pathways are observed during lung fibrosis in both in vitro and in vivo model systems. Given that no effective therapies exist for most human diseases involving lung fibrosis, including scleroderma, the signaling proteins whose expression and/or function is altered during lung fibrosis need to be evaluated as potential targets for novel therapies designed to treat this debilitating disease.

Acknowledgment—We thank Dr. Russell Harley for advice on the interpretation of tissue sections.

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