The endothelins are a family of endothelium-derived peptides that possess a variety of biological activities, including potent vasoconstriction. Endothelin-1 (ET-1) is up-regulated during tissue repair and pulmonary fibrosis. Here, we use genome-wide expression array analysis to show that the addition of ET-1 (100 nM, 4 h) to normal lung fibroblasts directly induces expression of matrix and matrix-associated genes, including the pro-fibrotic protein CCN2 (connective tissue growth factor, or CTGF). ET-1 induces the MEK/ERK MAP kinase pathway in fibroblasts. Blockade of the MEK/ERK kinase pathway with U0126 abrogates the ability of ET-1 to induce expression of matrix and matrix-associated mRNAs and the CCN2 protein. The CCN2 promoter possesses an ET-1 response element, which maps to the previously identified basal control element-1 (BCE-1) site. Our results suggest that ET-1 induces a program of matrix synthesis in lung fibroblasts and that ET-1 may play a key role in connective tissue deposition during wound repair and in pulmonary fibrosis.

As a response to environmental insults or a consequence of local inflammatory processes, structural damage to tissue can occur, triggering a wound-healing response. This response consists of an integrated series of biochemical, immunological, and structural changes that result in the de novo synthesis of a new epithelium, blood vessels, and connective tissue (1). The proper repair of connective tissue requires the synthesis and organization of new ECM1 components, such as collagen and fibronectin (2).

A growing body of evidence implicates the vasoconstrictive peptide endothelin-1 (ET-1) as a key mediator of tissue repair (3). Each of the three known endothelin isoforms (1, -2, and -3) arise by proteolytic processing of large precursors (~200 amino acid residues). Intermediates, termed big ET-1, -2, and -3 (38~41 amino acids), are excised from pre-propeptides at sites containing paired basic amino acids. Big endothelins, which have little or no biological activity (4), are cleaved at Trp-21-Val/ile-22 to produce mature, 21-residue, biologically active peptides (5, 6). The enzyme responsible for the specific cleavage at Trp-21 has been termed the endothelin-converting enzyme (7, 8). Injury and the wound-healing response lead to stabilization of endothelin-converting enzyme-1 mRNA and to the generation of bioactive endothelin (9). Elevated levels of ET-1 have been shown in patients with fibrotic disease, suggesting that ET-1 may play a key role not only in normal wound repair but also in the pathogenesis of fibrosis (10~14).

ET-1 demonstrates a wide range of biological properties, including significant mitogenic activity toward a number of cell types such as smooth muscle cells and fibroblasts (15). ET-1 also promotes the contractile ability of normal fibroblasts (16), which is essential for wound closure and reconstitution of the dermis (17). In addition, ET-1 modifies extracellular matrix metabolism (15, 18~20). For example, ET-1 enhances collagen types I and III and decreases matrix metalloproteinase-1 (MMP-1) mRNA and protein expression in dermal fibroblasts (18, 20). However, the signal transduction mechanism and transcription factors through which ET-1 affects gene expression is largely unknown. Furthermore, it is unclear to what extent ET-1 might contribute to wound repair or fibrogenic responses.

In this report, we investigate the functional and mechanistic contribution of ET-1 to matrix synthesis in primary human lung fibroblasts. Our results provide new insights into ET-1 biology and suggest a role for ET-1 in enhancing matrix expression and organization during tissue repair and fibrogenesis.

MATERIALS AND METHODS

Cell Culture—Primary human lung fibroblasts were grown from macroscopically and histologically normal lung resection specimens by explant culture as described previously (21) using DMEM with 10% fetal bovine serum (Invitrogen). Cells were used between passages 2 and 5.

Gene Array Analysis—Lung fibroblasts were serum starved for 18 h and treated with 100 nM ET-1 for 4 h. At the end of the treatment period, total RNA was harvested (Trizol; Invitrogen) and quantified, and integrity was verified by denaturing gel electrophoresis. Equal amounts of identically treated RNA were pooled and reverse tran-
ET-1 Induces Matrix Synthesis in Fibroblasts via MEK/ERK

**Table I**

| GenBank™ no. | Affymetrix no. | Fold increase* | Gene name |
|-------------|----------------|----------------|-----------|
| NM_003474   | 202952_s_at    | 3.1            | A disintegrin and metalloproteinase domain 12 |
| NM_003474   | 213700_at      | 4.4            | A disintegrin and metalloproteinase domain 12 |
| NM_020305   | 210765_at      | 3.2            | A disintegrin and metalloproteinase domain 19 |
| NM_000385   | 203713_at      | 11.5          | Collagen, type III, α1 |
| NM_004385   | 204619_s_at    | 1.8            | Chondroitin sulfate proteoglycan 2 (vesicular) |
| NM_001845   | 211980_at      | 1.6            | collagen, type IV, α1 |
| NM_000093   | 212485_at      | 1.6            | collagen, type V, α1 |
| NM_000094   | 204136_at      | 3.9            | collagen, type VII, α1 |
| NM_001901   | 209010_at      | 4.8            | CCN2 (connective tissue growth factor) |
| NM_002006   | 204421_s_at    | 5.0            | Fibroblast growth factor 2 (basic) |
| NM_002006   | 204422_s_at    | 4.5            | Fibroblast growth factor 2 (basic) |
| NM_002203   | 205032_at      | 2.3            | Integrin, α3 |
| NM_002203   | 201389_at      | 1.5            | Integrin, α5 |
| NM_000212   | 204627_s_at    | 4.4            | Integrin, β3 |
| NM_002421   | 204475_at      | 2.5            | Matrix metalloproteinase 1 |
| NM_002422   | 205028_at      | 4.1            | Matrix metalloproteinase 3 |
| NM_000935   | 202619_s_at    | 2.4            | Procollagen-lysine (lysine hydroxylase) 2 |
| NM_002997   | 201297_s_at    | 1.3            | Syndecan 2 |
| NM_003246   | 201109_s_at    | 2.5            | Thrombospondin 1 |
| NM_000362   | 201148_s_at    | 2.1            | Tissue inhibitor of metalloproteinase 3 |
| NM_000362   | 201150_s_at    | 2.1            | Tissue inhibitor of metalloproteinase 3 |

* Mean of two pairs of array experiments; gene selection criteria included a 1.5-fold increase and an “increased” call by Affymetrix algorithm (p < 0.01) in both sets.

scribed (Invitrogen) into cDNA, which was then in vitro transcribed into biotinylated cRNA. The target cRNA was then fragmented and hybridized to the Affymetrix human U133A array following Affymetrix (Santa Clara, CA) protocol. Hybridization of cRNA to Affymetrix human U133A chips, signal amplification, and data collection were performed using an Affymetrix fluidics station and chip reader. Chip files were scaled to an average intensity of 100 per gene and analyzed using the Affymetrix version 5.0 (MAS5) comparison analysis software. Experiments were performed twice, and the fold changes presented in Table I are an average of these independent studies. Criteria indicated by Affymetrix were used to determine robust changes in gene expression. Briefly, transcripts were defined as up-regulated by ET-1 only when identified as “present” (ET-1-treated chips) by the Affymetrix detection algorithm and “significantly increased,” as determined by the Affymetrix change algorithm, with a change p value of <0.01. The fold change between treated and untreated samples had to be at least 1.5-fold to identify a transcript as being altered. These criteria had to be met in both sets of experiments. We have entered one experiment (ET-1 untreated/treated) in the NCBI Gene Expression Omnibus (GEO) as samples GSM15500 and GSM15501, and the other experiment has been identified as GSM17262 and GSM17263. The GEO series, including all samples GSM15500 and GSM15501, and the other experiment has been uploaded to the NCBI Gene Expression Omnibus (GEO) as accession number GSE1081 (www.ncbi.nlm.nih.gov/geo).

**Western Blot Analysis**—Lung fibroblasts were grown to confluence in DMEM with 10% fetal bovine serum and cultured in DMEM and 0.5% bovine serum albumin. ET-1 was added to cells for up to 16 h. When appropriate, treatment. To assess the effect of ET-1 on the stimulation of signaling pathways, ET-1 was added to cells for up to 16 h. When appropriate, cells were incubated with an inhibitor for 45 min prior to the addition of ET-1. The activities of inhibitors were verified by examining their ability to block phosphorylation of Akt or by assessing their ability to block induction of promoters responsive to particular signaling cascades (Clontech). Cell layer lysates were subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes. CCN2 protein was detected using an anti-CCN2 (CTGF) antibody (Santa Cruz Biotechnology), and the glyceraldehyde-3-phosphate (GAPDH) protein was detected using an anti-GAPDH antibody (Santa Cruz Biotechnology). Akt, phospho-Akt, p38, phospho-p38, p42/44 MAPK, and phospho-p42/44 MAPK were detected using antibodies as described by the manufacturer (Cell Signaling Technology, Beverly, MA). Blots were then hybridized with an appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories), and protein was detected using chemiluminescence (Amersham Biosciences).

**Promoter Assays**—CCN2 promoter/promoter-secreted alkaline phosphatase (SEAP) reporter constructs were as described previously (25, 26). Promoter/reporter constructs were transfected into lung fibroblasts using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. Promoter/reporter plasmids were cotransfected with pCMV-βGal (Clontech), which was used to adjust for differences in transfection efficiencies between samples. Following transfection, cells were incubated in DMEM with 0.5% fetal bovine serum for 18 h. Media were changed, and cells were incubated in the presence or absence of inhibitors for 45 min and cultured for an additional 24 h in the presence or absence of ET-1 (100 nM). Media were taken for SEAP assays. Fibroblasts were rinsed once with phosphate-buffered saline, and cellular protein was extracted using 200 μl of reporter lysis buffer (Promega Corp, Madison, WI). Reporter gene activity was measured by luminometry (Turner Designs, Sunnyvale, CA) using SEAP and β-galactosidase assays (Tropix Inc, Bedford, MA) according to the manufacturer’s instructions. Values given are mean ± S.E. of triplicate assays from three individual experiments.

**Reverse Transcription PCR**—Lung fibroblasts were serum-starved for 18 h and treated with 100 nM ET-1 for 2, 4, or 12 h. Total RNA was isolated using Triozl (Invitrogen), and the integrity of the RNA was verified by gel electrophoresis. Total RNA (10 μg) was reverse transcribed in a 20-μl reaction volume containing an oligonucleotide (dT18) and random decamers (dN10), using M-MLV reverse transcriptase (Promega) for 1 h at 37 °C. The cDNA was diluted to 100 μl with diethylpyrocarbonate-treated water, and the target was measured by real time PCR FastStart DNA Master SYBR Green (Roche Applied Science) according to the manufacturer’s instructions. Triplicate samples were run, transcripts were measured in picograms, and expression values were standardized to values obtained with control 28 S RNA primers. Primers (Sigma Genosys) were as follows: COL1A1, 5′-ATAGGTTTTC-CCAGGCGACG3′ (forward) and 5′-CCAGCGCTCTTCCTTCAATCTC3′ (reverse); TSP1, 5′-CAAGCTGAAATGGTGTCCTG-3′ (forward) and 5′-CTGGGCATGTTCGACACCCT-3′ (reverse); TIMP3, 5′-GGCAAGAGCAAGATAGACCTC-3′ (forward) and 5′-GTCTGGTCTCCAGACCTGAC-3′ (reverse); CCN2, 5′-CTCCGGCCTGGTACCCGACT-3′ (forward) and 5′-GCAGTCTGAACTCAGCCG-3′ (reverse); MMP1, 5′-TCCACAGGTTCTGAGGTTCAAGG-3′ (forward) and 5′-GGATGACCATCAATTCATCAATCG-3′ (reverse).

**RESULTS**

**Global Expression Profiling in Response to ET-1**—To identify transcripts induced by ET-1, we treated primary lung fibroblasts for 4 h in the presence or absence of 100 nM ET-1. After this period of incubation, we harvested total RNA. Affymetrix U133A gene arrays were hybridized to these labeled cRNAs derived from the total RNA. Gene array analysis revealed that a 4-h treatment of primary lung fibroblasts with ET-1-modified expression of a cluster of transcripts encoding proteins that would be expected to promote matrix deposition and remodeling (Table I). ET-1 treatment induced expression of the matrix protein collagen types IV, V, and VII (27). ET-1 treatment also induced expression of the collagen-modifying enzyme lysyl ox-
anti-phospho Akt antibodies.

Prepared and subjected to Western blot analysis with anti-Akt and 

/H9262;1 0

Brotic protein that induces collagen and acts with TGF

—

CCN2 (CTGF) is an important profi-

ment, we sought to explore the notion that ET-1 might contrib-

17 (Fig. 4,

ments of the CCN2 promoter spanning nucleotides

moter. To perform this experiment, we transfected a full-length 

CCN2 promoter/SEAP reporter construct, spanning nucleo-

244 to 

/H11002

et al. (29, 34). Because our array 

alysis, we confirmed that ET-1 induced MMP-1, CCN2, collagen IV, TSP-1, and TIMP-3 mRNAs (Fig. 

Induction of MMP-1, CCN2, TSP-1, and TIMP-3 seemed to be direct, showing potent induction 2 h after the application of 

To determine whether ET-1 induced these transcripts via a MEK/ERK-dependent mechanism, cells were pre-incubated 

for 45 min with or without the MEK/ERK MAP kinase inhibitor 

U0126 (10 

M) and the MEK inhibitors U0126 (U; 10 

M) and 

PD98059 (PD; 30 

M). SB203580 (SB; 30 

M), wortmannin (W; 10 

M), and 

LY294002 (Ly; 10 

M) had no appreciable effect on CCN2 expres-

sion. Bottom panel, to verify specificity of inhibitors used, cells were 

treated for ET-1 for the time indicated in the presence or absence of 

inhibitors as denoted in the top panel. Protein extracts were then 

prepared and subjected to Western blot analysis with anti-Akt and 

anti-phospho Akt antibodies. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

idase (28) and the pro-adhesive and pro-fibrotic gene CCN2 

(CTGF) (29). ET-1 also induced thrombospondin-1, a multi-

functional matricellular protein that promotes the function of 

many molecules involved with the wound-healing response, 

including transforming growth factor-β (TGFβ) (30), matrix 

metalloproteinases (31), and focal adhesions (32). In addition, 

ET-1 induced expression of the tissue inhibitor of matrix met-

alloproteinases-3 (TIMP-3) and MMP-1 and -3, which adjust 

and control levels of matrix deposition (33). Other than MMP-1, 

all genes revealed by our transcriptional profiling are novel 

targets of ET-1 in fibroblasts (Table I).

**ET-1 Activates the CCN2 Promoter through the BCE-1 Ele-

ment—**After establishing that ET-1 induced the appearance of 

CCN2 protein and mRNA in lung fibroblasts, we sought to 

investigate further the mechanism through which ET-1 per-

forms this function. For these experiments, we assessed 

whether an ET-1 response element existed in the CCN2 pro-

moter. To perform this experiment, we transfected a full-length 

CCN2 promoter/SEAP reporter construct, spanning nucleo-

tides −805 to +17 of the CCN2 promoter (Fig. 4, −805; Ref. 26), 

into lung fibroblasts. After an 18-h serum starvation step, cells 

were cultured in the presence or absence of 100 nM ET-1 for an 

additional 24 h. Consistent with our data showing that ET-1 

induced CCN2 protein and mRNA expression, we found that 

ET-1 induced CCN2 promoter activity (Fig. 4). To further de-

line the ET-1 response element in the CCN2 promoter, we 

transfected two additional CCN2 promoter/SEAP reporter con-

structs into fibroblasts. These new constructs contained seg-

ments of the CCN2 promoter spanning nucleotides −244 to 

+17 (Fig. 4, −244) or −86 to +17 (Fig. 4, −86; Ref. 26). We 

found that, although the fragment of the CCN2 promoter be-

tween −244 to +17 could respond to ET-1, the removal of 

nucleotides −244 to −86 abolished the ability of the CCN2 

promoter to respond to ET-1 (Fig. 4). Thus, the ET-1 response 

element in the CCN2 promoter lay between nucleotides −244 

and −86.

Previously, we had analyzed the transcription factor binding 
sites lying between nucleotides −244 and −86 of the CCN2 

promoter and identified a Smad binding element, which is 

required for the TGFβ-induction of CCN2, and a basal control 

element-1 (BCE-1) site, which is required for basal CCN2 ex-

pression but is not involved with the TGFβ-induction of CCN2 

(25). To assess the roles of Smad and BCE-1 elements in the 

ET-1 induction of the CCN2 promoter, we transfected into lung 

dress the contribution of these pathways to the ET-1 induction 

of CCN2, we used Western blot analysis with an anti-CCN2 

antibody to show that a 45-min pre-incubation with the MEK/ERK inhibitors PD98059 and U0126 (35, 36), prior to the 

addition of ET-1, blocked the ability of ET-1 to induce CCN2 protein (Fig. 1, bottom panel). Conversely, the addition of 

the p38 inhibitor SB203580 (37) or the Akt/PI-3-kinase inhibitors 

wortmannin and LY294002 (38, 39) had no discernible effect on 

the ability of ET-1 to modulate CCN2 production. In addition, 

a 45-min preincubation of lung fibroblasts with the dual spec-

ificity ETA/B receptor antagonist bosentan (40) blocked the 

ability of ET-1 to induce CCN2 (Fig. 1). Thus, ET-1 stimulated 

CCN2 protein expression in normal human lung fibroblasts 

through a MEK/ERK-dependent mechanism that required ei-

ther the ETA or the ETB receptor.

**ET-1 Enhances CCN2 Expression through a MEK/ERK-de-

pendent Mechanism—**CCN2 (CTGF) is an important profi-

brotic protein that induces collagen and acts with TGFβ to 

promote sustained fibrosis in vivo (29, 34). Because our array 

analysis showed that CCN2 mRNA was induced by ET-1 treat-

ment, we sought to explore the notion that ET-1 might contrib-

ute to fibrotic responses in vivo through the induction of CCN2. 

Thus, we treated normal lung fibroblasts for 24 h with 100 nM 

ET-1. Cell layers were harvested, and equal amounts of protein 

(20 μg) were subjected to Western blot analysis with an anti-

CCN2 antibody. Confirming our microarray data, we found that 

100 nM ET-1 induced expression of CCN2 protein (Fig. 1).

To begin to probe the signaling mechanism through which 

ET-1 induced CCN2, we performed Western blot analysis of 

protein extracts prepared from lung fibroblasts that had been 

treated with ET-1 for different lengths of time. Using anti-

phospho-ERK, anti-phospho-p38, and anti-phospho-Akt anti-

bodies, we showed that ET-1 activated ERK (p42/p44), p38, and 

PI3-kinase/Akt pathways in lung fibroblasts (Fig. 2). To ad-

Top panel, normal lung fibroblasts were serum-starved for 18 h and then incubated for an additional 24 h with ET-1 (100 nM). Whole cell protein extracts were made, and equal amounts of protein were subjected to SDS/PAGE by Western blot analysis with an anti-CCN2 antibody (see “Materials and Methods”). ET-1 induced CCN2 protein expression, which was completely abol-

ished by co-incubation with the mixed ETA/B receptor antagonist 

bosentan (Bos; 10 μM) and the MEK inhibitors U0126 (U; 10 μM) and 

PD98059 (PD; 30 μM). SB203580 (SB; 30 μM), wortmannin (W; 10 μM), and 

LY294002 (Ly; 10 μM) had no appreciable effect on CCN2 expres-

sion. Bottom panel, to verify specificity of inhibitors used, cells were 

treated for ET-1 for the time indicated in the presence or absence of 
inhibitors as denoted in the top panel. Protein extracts were then 

prepared and subjected to Western blot analysis with anti-Akt and 

anti-phospho Akt antibodies. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
ET-1 Induces Matrix Synthesis in Fibroblasts via MEK/ERK

**Fig. 2.** ET-1 induces p88 MAPK, p42/44 MAPK, and Akt phosphorylation in normal primary lung fibroblasts. Normal lung fibroblasts were cultured, serum-starved for 18 h, and treated with 100 nM ET-1. Whole cell protein extracts were made, and equal amounts of protein were subjected to SDS/PAGE and Western blot analysis with anti-ERK, anti-phospho-ERK, anti-p38, anti-phospho-p38, anti-Akt, and anti-phospho-Akt antibodies. Treatment of lung fibroblasts with ET-1 induces all three pathways tested.

**Fig. 3.** ET-1 induces transcription of matrix genes by a MEK/ERK-dependent mechanism. Reverse transcription PCR was used to detect MMP-1, CCN2, collagen type IV, TSP-1, TIMP-3, and 28 S RNA as indicated. Expression values of ECM genes are expressed as a ratio relative to 28 S RNA. After incubation for 45 min with or without the MEK1/2 inhibitor U0126 (10 µM), fibroblasts were treated with or without 100 nM ET-1 for 2, 4, or 12 h. Total RNA was extracted, purified, and reverse transcribed into cDNA and amplified with specific primers (see “Materials and Methods”) to detect the expression of particular ECM genes. All assays were performed in triplicate. Solid lines, ET-treated cells; dotted lines, untreated cells; dashed lines, ET plus U0126.

**Fig. 4.** ET-1 induces the CCN2 promoter through the BCE-1 element. Fibroblasts were transiently transfected with DNA reporter constructs containing different fragments, as indicated, of the human CCN2 promoter sequences inserted upstream of a SEAP reporter gene (22, 23). Results shown are for three individual experiments with three replicates per experiment. Values shown are means ± S.D. of the CCN2 promoter; −805, a construct containing nucleotides −805 to +17 of the CCN2 promoter; −244, a construct containing nucleotides −244 to +17 of the CCN2 promoter; −86, a construct containing nucleotides −86 to +17 of the CCN2 promoter; −805 Smad, a construct containing a mutation in the Smad binding element of the CCN2 promoter; −805 BCE-1, a construct containing a mutation in the BCE-1 binding element of the CCN2 promoter. These latter two mutations were made in the context of the larger −805 construct.

This study shows that ET-1 promotes gene expression in a fashion that is independent of Smad/TGFβ signaling but dependent on BCE-1. These results suggest that ET-1 promotes gene expression in a manner that is independent of TGFβ.

**DISCUSSION**

In this study, we used primary human lung fibroblasts as an *in vitro* model with which to probe the role of ET-1 in ECM production. Previously, we have shown that ET-1 enhances collagen types I and III and decreases MMP-1 mRNA and protein expression in dermal fibroblasts (18, 20). In this report, we extend these results by showing that ET-1 in itself is capable of inducing the expression of ECM or ECM-associated genes. ET-1 induced collagen type IV, a key basement membrane component (41). ET-1 induced collagen type VII, the major component of anchoring fibrils that attach epidermis and dermis; mutations in the COL7A1 gene encoding type VII collagen cause a severe blistering disease, dystrophic epidermolysis bullosa (42). In addition, ET-1 induces collagen type V, which interacts with collagen type I to regulate the diameters of type I collagen fibrils (43). Thus, by the ability of ET-1 to induce collagens IV, V, and VII, ET-1 promotes the wound-healing response by enhancing reconstitution of the basement membrane and wound closure.

ET-1 induces CCN2, a member of the CCN family of proteins (44, 45). CCN2 is a cysteine-rich, pro-adhesive, matricellular protein that plays an essential role in the formation of blood vessels, bone, and connective tissue (46). Because the expression of this protein is potently induced by TGFβ in a Smad-dependent fashion (22), it has been hypothesized that CCN2 mediates several of the downstream actions of TGFβ (26). Ours is the first report to show that ET-1 induces CCN2 and, thus, that CCN2 could also be a downstream mediator of ET-1 activity. The ET-1 response element of the CCN2 promoter is independent of its Smad element, which is required for the TGFβ-induction of CCN2 (22, 47). Instead, the ET-1 induction of CCN2 occurs through the BCE-1 element of the CCN2 promoter, which is not required for the TGFβ-induction of CCN2 (22). These results suggest that TGFβ and ET-1 might work
49), and thrombospondin-1 activates latent TGF-β 2 h after ET-1 treatment. CCN2 induces type I collagen (29, 48, 50), ET-1 also induces thrombospondin-1 (30), a potent activator of TGFβ, a key inducer of ECM production and fibrotic responses (61, 62).

Independently on the CCN2 promoter to elevate CCN2 expression in vivo and, thus, that TGFβ and ET-1 might cooperate to produce fibrogenic responses in vivo through the coordinate induction of CCN2 (Fig. 5). The identity of the factor(s) binding to the BCE-1 site of the CCN2 promoter is not known but is currently under investigation.

It is interesting to note that we have shown previously that a 48-h treatment of normal dermal fibroblasts with ET-1 caused the induction of collagen type I mRNA (20). In this report, using normal lung fibroblasts, our transcriptional profiling using the Affymetrix system showed that ET-1 did not induce collagen type I mRNA after 4 h. Using real-time PCR, we confirmed that ET-1 induced type I collagen mRNA commencing 6 h after ET-1 treatment (not shown). Collectively, these results suggest that the ability of ET-1 to induce type I collagen might be indirect. However, in this report we showed appreciable induction of CCN2 and thrombospondin-1 mRNA 2 h after ET-1 treatment. CCN2 induces type I collagen (29, 48, 49), and thrombospondin-1 activates latent TGFβ (30, 50). Thus, the ability of ET-1 to induce collagen type I expression may be indirect, possibly via the induction of CCN2 or by the activation of latent TGFβ (Fig. 5). It is interesting to note that, in endothelial cells, TGFβ induces ET-1 expression via Smad and Ap-1 sites in the ET-1 promoter (51). Although TGFβ has been shown to be a key pro-fibrotic cytokine, its action can be amplified or suppressed by several other cytokines (52). Understanding the interactions among these profibrotic and anti-fibrotic cytokines is likely to have a major impact in understanding wound-healing and fibrotic responses in vivo. Thus, further research elucidating the mechanism by which ET-1, TGFβ and CTGF might interact to promote pro-fibrotic responses is of major importance.

Because ET-1 is markedly up-regulated during tissue repair (9) and in patients with fibrotic disease, these results suggest that ET-1 may play a key role not only in normal wound repair but also in the pathogenesis of fibrosis (10–14). Elevated levels of circulating ET-1 occurred in patients with skin and lung fibrosis, a finding that correlated with the severity of the fibrotic phenotype (53–55). This increase in circulating ET-1 was paralleled by an increase in ET-1 synthesis in vivo (53–55). Thus, ET-1 may be an important therapeutic target in the modulation of fibrogenesis. Indeed, our data suggest that ET-1 is a potent fibrogenic peptide for lung fibroblasts, able to induce matrix production by lung fibroblasts via a MEK/ERK-dependent mechanism requiring either the ETA or the ETB receptors (Fig. 5).

The results presented in this report are consistent with several studies published recently that have linked the p42/p44 (MEK/ERK) MAP kinase signaling cascade and fibrosis. For example, the TGFβ induction of CCN2 requires the ras/MEK/ERK signaling cascade (47, 56, 57). Recently, we showed that the synthetic prostacyclin iloprost, which alleviates symptoms of fibrosis in patients with the fibrotic disease systemic sclerosis (49), works, at least in part, through the antagonism of the MEK/ERK pathway (57). Collectively, our results suggest that the antagonism of MEK/ERK might be an effective anti-fibrotic approach.

In summary, the results presented in our current report emphasize that ET-1 is an important regulator of extracellular matrix biosynthesis by lung fibroblasts and further emphasize the key role of the MEK/ERK cascade in tissue injury and wound healing. Our results further suggest that there may be a potential therapeutic advantage in using MEK/ERK kinase inhibitors or endothelin antagonists to ameliorate the pathological scarring observed in pulmonary fibrosis.

Acknowledgment—We thank Gary Grotendorst (University of Miami) for providing an initial CTGF promoter construct.

REFERENCES
1. Werner, S., and Grose, R. (2003) Physiol. Rev. 83, 835–870
2. Badyak, S. F. (2002) Semin. Cell Dev. Biol. 13, 377–383
3. Bull, H. A., and Dowd, P. M. (1993) Dermatology 187, 1–5
4. Yanagisawa, M. (1994) Circulation 89, 1320–1322
5. Anggard, E. E., Botting, R. M., and Vane, J. R. (1990) Blood Vessels 27, 269–281
6. Rubanyi, G. M., and Betelho, L. H. (1991) FASEB J. 5, 2713–2720
7. Ohnaka, K., Takayanagi, R., Nishikawa, M., Haji, M., and Nawata, H. (1993) J. Biol. Chem. 268, 26759–26766
8. Turner, A. J., and Murphy, L. J. (1996) Biochem. Pharmacol. 51, 91–102
9. Shao, R., Shi, Z., Gotwals, P. J., Kotelyansky, V. E., George, J., and Rockey, D. C. (2003) Mol. Biol. Cell 14, 2327–2341
10. Vane, R. J., and Botelho, L. H. (1991) FASEB J. 5, 2713–2720
11. Ohnaka, K., Takayanagi, R., Nishikawa, M., Haji, M., and Nawata, H. (1993) J. Biol. Chem. 268, 26759–26766
12. Turner, A. J., and Murphy, L. J. (1996) Biochem. Pharmacol. 51, 91–102
13. Koteles, P., and Noble, P. W. (2000) Am. J. Respir. Cell Mol. Biol. 23, 7–10
14. Guidry, C., and Hook, M. (1993) J. Cell Biol. 113, 873–880
15. Grinnell, F. (1998) J. Cell Biol. 140, 401–404
16. Grinnell, F. (1998) J. Cell Biol. 140, 401–404
17. Grinnell, F. (1998) J. Cell Biol. 140, 401–404
18. Xu, X., Denton, C. P., Holmes, A., Dashwood, M. R., Abraham, D. J., and Black, C. M. (1998) J. Cardiovasc. Pharmacol. 31, Suppl. 1, S360–S363
19. Szabo, A., Yonezawa, H., Kizumir, T., Morita, T., Tamura, T., Takenaka, T., and Honda, R. (1998) Eur. J. Pharmacol. 349, 123–128
20. Shi-Wen, X., Denton, C. P., Dashwood, M. R., Holmes, A. M., Bou-Gharios, G., and Hook, M. (1993) J. Cell Biol. 120, 401–404
21. Werner, S., and Grose, R. (2003) Physiol. Rev. 83, 835–870
22. Badyak, S. F. (2002) Semin. Cell Dev. Biol. 13, 377–383
23. Bull, H. A., and Dowd, P. M. (1993) Dermatology 187, 1–5
24. Yanagisawa, M. (1994) Circulation 89, 1320–1322
25. Anggard, E. E., Botting, R. M., and Vane, J. R. (1990) Blood Vessels 27, 269–281
26. Rubanyi, G. M., and Betelho, L. H. (1991) FASEB J. 5, 2713–2720
27. Ohnaka, K., Takayanagi, R., Nishikawa, M., Haji, M., and Nawata, H. (1993) J. Biol. Chem. 268, 26759–26766
28. Turner, A. J., and Murphy, L. J. (1996) Biochem. Pharmacol. 51, 91–102
29. Shao, R., Shi, Z., Gotwals, P. J., Kotelyansky, V. E., George, J., and Rockey, D. C. (2003) Mol. Biol. Cell 14, 2327–2341
30. Vane, R. J., and Botelho, L. H. (1991) FASEB J. 5, 2713–2720
31. Ohnaka, K., Takayanagi, R., Nishikawa, M., Haji, M., and Nawata, H. (1993) J. Biol. Chem. 268, 26759–26766
32. Turner, A. J., and Murphy, L. J. (1996) Biochem. Pharmacol. 51, 91–102
33. Koteles, P., and Noble, P. W. (2000) Am. J. Respir. Cell Mol. Biol. 23, 7–10
34. Guidry, C., and Hook, M. (1993) J. Cell Biol. 113, 873–880
35. Grinnell, F. (1998) J. Cell Biol. 140, 401–404
36. Grinnell, F. (1998) J. Cell Biol. 140, 401–404
37. Grinnell, F. (1998) J. Cell Biol. 140, 401–404
38. Xu, X., Denton, C. P., Holmes, A., Dashwood, M. R., Abraham, D. J., and Black, C. M. (1998) J. Cardiovasc. Pharmacol. 31, Suppl. 1, S360–S363
39. Szabo, A., Yonezawa, H., Kizumir, T., Morita, T., Tamura, T., Takenaka, T., and Honda, R. (1998) Eur. J. Pharmacol. 349, 123–128
40. Shi-Wen, X., Denton, C. P., Dashwood, M. R., Holmes, A. M., Bou-Gharios, G., and Hook, M. (1993) J. Cell Biol. 120, 401–404
Endothelin-1 Induces Expression of Matrix-associated Genes in Lung Fibroblasts through MEK/ERK
Xu Shi-wen, Sarah L. Howat, Elisabetta A. Renzoni, Alan Holmes, Jeremy D. Pearson, Michael R. Dashwood, George Bou-Gharios, Christopher P. Denton, Roland M. du Bois, Carol M. Black, Andrew Leask and David J. Abraham

J. Biol. Chem. 2004, 279:23098-23103.
doi: 10.1074/jbc.M311430200 originally published online March 23, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311430200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 55 references, 22 of which can be accessed free at http://www.jbc.org/content/279/22/23098.full.html#ref-list-1