cAMP Inhibits Transforming Growth Factor-β-Stimulated Collagen Synthesis via Inhibition of Extracellular Signal-Regulated Kinase 1/2 and Smad Signaling in Cardiac Fibroblasts

Xiaoqiu Liu, Shu Qiang Sun, Aviv Hassid, and Rennolds S. Ostrom

Department of Pharmacology and the Vascular Biology Center of Excellence, University of Tennessee Health Science Center, Memphis, Tennessee

ABSTRACT

Cardiac fibroblasts produce and degrade extracellular matrix and are critical in regulating cardiac remodeling and hypertrophy. Cytokines such as transforming growth factor-β (TGF-β) play a fundamental role in the development of tissue fibrosis by stimulating matrix deposition and other profibrotic responses, but less is known about pathways that might inhibit fibrosis. Increased cAMP formation inhibits myofibroblast differentiation and collagen production by cardiac fibroblasts, but the mechanism of this inhibition is not known. We sought to characterize the signaling pathways by which cAMP-elevating agents alter collagen expression and myofibroblast differentiation. Treatment with 10 μM forskolin or isoproterenol increased cAMP production and cAMP response element binding protein (CREB) phosphorylation in cardiac fibroblasts and inhibited serum- or TGF-β-stimulated collagen synthesis by 37% or more. These same cAMP-elevating agents blunted TGF-β-stimulated expression of collagen I, collagen III, and α-smooth muscle actin. Forskolin or isoproterenol treatment blocked the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) induced by TGF-β despite the fact that these cAMP-elevating agents stimulated ERK1/2 activation on their own. cAMP-elevating agents also attenuated the activation of c-Jun NH₂-terminal kinase and reduced binding of the transcriptional coactivator CREB-binding protein 1 to transcriptional complexes containing Smad2, Smad3, and Smad4. Pharmacological inhibition of ERK completely blocked TGF-β-stimulated collagen gene expression, but expression of an active mutant of MEK was additive with TGF-β treatment. Thus, cAMP-elevating agents inhibit the profibrotic effects of TGF-β in cardiac fibroblasts largely through inhibiting ERK1/2 phosphorylation but also by reducing Smad-mediated recruitment of transcriptional coactivators.

Cardiac remodeling is characterized by a structural rearrangement of components of the normal chamber wall that involves cardiomyocyte hypertrophy, cardiac fibroblast proliferation, fibrosis, and cell death. Fibrosis is a disproportionate accumulation of fibrillar collagen. Accumulation of type I collagen, the main fibrillar collagen found in cardiac fibrosis,

stiffens the ventricles and impedes both contraction and relaxation. Thus, there is considerable interest in understanding the mechanisms that stimulate collagen deposition in the heart and defining approaches to limit these processes.

In vitro, the ability of TGF-β to stimulate the proliferation of and extracellular matrix production by cultured fibroblasts is well-documented (Ignotz and Massague, 1986; Raghow et al., 1987). TGF-β is a locally generated cytokine that has been implicated as a major contributor to tissue fibrosis in various organ systems. TGF-β activates cardiac fibroblasts, causing their differentiation into myofibroblasts that pro-

ABBREVIATIONS: TGF-β, transforming growth factor-β; CREB, cAMP response element binding protein; CBP-1, cAMP response element binding protein binding protein 1; MEK, mitogen-activated protein kinase kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; caMEK, constitutively active form of mitogen-activated protein kinase kinase; CTGF, connective tissue growth factor; ANOVA, analysis of variance; ERK, extracellular regulated kinase; PBS, phosphate-buffered saline; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; Fsk, forskolin; Iso, isoproterenol; PKA, protein kinase A; PGE₂, prostaglandin E₂; 8-Br-cAMP, 8-bromo-cAMP; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinyl phenyl)-5-(4-pyridyl)-1H-imidazole; PD98059, 2'-amino-3'-methoxyflavone; SP600125, anthra(1,9-cd)pyrazol-6(2H)-one 1,9-pyrazoloanthron; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio)butadiene.

duce matrix proteins such as collagen I, III, V, and fibronectin (Manabe et al., 2002). This observation is consistent with in vivo studies implicating TGF-β in tissue fibrogenesis in a variety of organs, including the heart (Seeland et al., 2002; Schnaper et al., 2003). Smads are key intracellular nuclear effectors of TGF-β receptors, and many of the tissue responses induced by TGF-β are mediated by Smad proteins (Feng and Derynck, 2005). Recent studies suggest that there is an interaction between angiotensin II and TGF-β/Smad signaling in fibroblasts (Dixon et al., 2000). Other studies have shown that cardiac Smad2, -3, and -4 proteins were significantly increased in border and scar tissue (Hao et al., 1999) and that the phenotypic and functional changes associated with TGF-β-induced fibroblast differentiation are regulated by Smad proteins (Evans et al., 2003). However, other evidence has suggested that TGF-β can also signal independently of Smads (Derynck and Zhang, 2003). TGF-β can activate mitogen-activated protein (MAP) kinase signaling pathways, including c-Jun N-terminal kinase (JNK), extracellular-regulated kinase (ERK), and p38, but the specific pathways activated by this cytokine seems to vary with the cell type (Derynck and Zhang, 2003). TGF-β/Smad signaling is controlled in some cells by MAP kinase signaling cascades (Yu et al., 2002). In untransformed epithelial cells, mesangial cells, and fibroblasts, the addition of TGF-β results in a transient activation of the MEK/ERK cascade (Hartsough et al., 1996). In some cell types, MEK/ERK is required for the ability of TGF-β to induce the transcription of effector genes (Watanabe et al., 2001), and in others, it enhances TGF-β-dependent responses (Leask and Abraham, 2004).

Despite TGF-β being recognized as a positive regulator of myofibroblasts, factors that negatively regulate myofibroblast activation are less well-defined. Previous studies have identified cAMP-elevating agents as potent inhibitors of myofibroblast differentiation in pulmonary fibroblasts (Liu et al., 2004). Furthermore, overexpression of adenylyl cyclase can inhibit cardiac myofibroblast differentiation and collagen synthesis (Swaney et al., 2005). Taken together, these observations suggest that cAMP-elevating agents are antifibrotic factors counteracting profibrotic actions of cytokines such as TGF-β in fibroblasts. Nonetheless, the signaling mechanisms through which cAMP acts to inhibit fibrotic responses remain unknown. In the present study, we demonstrate that cAMP markedly blocks TGF-β-mediated activation of rat cardiac fibroblasts via the inhibition of ERK1/2 and JNK phosphorylation and the reduction of CREB-binding protein 1 (CBP1) recruitment to Smad transcriptional complexes.

Materials and Methods

Materials. Primary antibodies for collagen I, collagen III, CBP, CREB, Smad3, and Smad4 and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). p-CREB antibody was from Chemicon International Inc. (Temecula, CA). pERK, ERK, p-Smad2, p38, JNK, and pJNK antibodies were obtained from Cell Signaling Technology (Danvers, MA). Forskolin, 8-bromo-cAMP (8-Br-cAMP), SB203580, PD98059, SP600125, and U0126 were obtained from Calbiochem (San Diego, CA). All other chemicals and reagents were obtained from Sigma (St. Louis, MO).

Preparation and Culture of Adult Rat Cardiac Fibroblasts. Cardiac fibroblasts were prepared from adult male Sprague-Dawley rat hearts weighing 250 to 300 g using a method described by Gustafsson and Brunton (2000). After rapid excision of the hearts,

the ventricles were isolated, minced, pooled, and placed in a solution of 100 U/ml collagenase I and 0.1% trypsin. Sequential 10-min periods of digestion were performed with constant stirring at 37°C. After discarding the eluate from the first digestion period, digested fibroblasts from nine more digestion periods were pooled, pelleted, and resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin, streptomycin, Fungizone, and 10% fetal bovine serum (FBS; Gemini Bio-Products, Irvine, CA). After a 30-min period of attachment to uncoated culture plates, cells that were weakly attached or unattached were rinsed free and discarded. After 2 to 3 days, confluent cultures were amplified by trypsinization and seeding onto new dishes. For signaling assays, only early passage (≤ 3) cells grown to 80 to 90% confluence were used. The purity of these cultures was greater than 95% cardiac fibroblasts as determined by positive staining for vimentin and negative staining for smooth muscle actin and von Willebrand factor.

Measurement of cAMP Accumulation. Cells were plated on 24-well plates to attain 70 to 80% confluence and then washed three times with serum and NaHCO$_3$-free DMEM supplemented with 20 mM HEPES, pH 7.4 and equilibrated for 30 min. Assay for cAMP accumulation was performed by incubation with drugs of interest or vehicle and 0.2 mM 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, for 10 min. To terminate reactions, assay medium was aspirated, and 200 μl of lysis buffer (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was added. cAMP content of the extract was quantified using the Biotrak enzyme immunoassay kit (GE Healthcare), as described previously (Liu et al., 2005). Data were normalized to the amount of protein per sample, as determined using a dye-binding protein assay (Bio-Rad Laboratories, Hercules, CA).

Assays of Collagen Synthesis. Cells were plated as described above and incubated in serum-free DMEM for 24 h. DMEM was then supplemented with 2.5% FBS (except for unstimulated conditions, in which 0.25% FBS was used) for 24 h along with 0.5 mCi of [^{3}H]proline/well and drugs of interest or vehicle, as described previously (Liu et al., 2005). Isoproterenol (Iso) was solubilized in 2 mM ascorbic acid to prevent degradative oxidation. For TGF-β-stimulated collagen synthesis experiments, 10 ng/ml TGF-β was added instead of 2.5% FBS. Medium was removed, and the cells were washed with ice-cold PBS and the incubated with 7.5% trichloroacetic acid for 1 h at 4°C. trichloroacetic acid-precipitated counts were determined by liquid scintillation counting.

Nuclear Extraction. Cardiac fibroblasts were cultured in 100-cm culture plates in DMEM supplemented with 10% FBS, 1% nonessential minimal amino acids, and 100 U/ml penicillin/0.1 mg/ml streptomycin. Cells (approximately 80% confluent) were then incubated with DMEM containing 0% FBS for 24 h and then treated with 10 μM forskolin (Fsk), 10 μM prostaglandin E$_2$ (PGE$_2$), and 10 μM Iso, respectively, for another 24 h. Vehicle-treated cells were used as negative controls. Cells were lysed using lysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 0.02 mg/ml leupeptin, and 0.02 mg/ml aprotinin), incubated at 4°C for 5 min, and then centrifuged at 510*g* for 10 min. Nuclear extraction buffer (20 mM HEPES, pH 7.9, 20% glycerol, 400 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride 50 mM sodium fluoride, 0.02 mg/ml leupeptin, and 0.02 mg/ml aprotinin) was added to the resulting pellets, and then pellets were incubated at 4°C with rocking for 2 h and centrifuged at 12,800*g* for 10 min. Protein concentrations in each extract were estimated by a dye-binding assay (Bio-Rad).

Gel Shift Assay (Electrophoretic Mobility Shift Assay). Nuclear extracts were incubated with individual biotin-labeled probes in binding buffer for 30 min at 15°C, according to the manufacturer's protocol (Panomics, Fremont, CA). The negative controls consisted of free probe (without nuclear extracts) and excess unlabeled probes incubated with the sample containing nuclear extract and biotin-labeled probe. The samples were then separated on a 6% polyacryl-

amide gel in $0.5\times$ Tris-borate/EDTA, transferred onto a nylon membrane, and fixed on the membrane by UV cross-linking. The biotin-labeled probe was detected with streptavidin-horseradish peroxidase (Pierce, Rockford, IL).

Immunoprecipitation. Cardiac fibroblasts were plated into 15-cm plates, grown to approximately 90% confluence and then starved with 0% FBS medium for 24 h. Cells were then treated with 10 μM Iso or vehicle for 20 min before treatment of 10 ng/ml TGF-β for another 24 h. Cells were washed twice with cold PBS, scraped in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, and mammalian protease inhibitor cocktail), and then homogenized in a Dounce homogenizer. Samples were incubated with primary antibody for the target protein (or IgG control antibody) for 1 to 3 h and then precipitated by incubating with protein A-agarose overnight. Pellets were washed once in lysis buffer followed by three washes in wash buffer consisting of 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.2% Igepal CA-630, and then three washes in wash buffer containing 10 mM Tris-HCl, pH 7.5, and 0.2% Igepal CA-630. Immunoprecipitated proteins were analyzed by immunoblot analysis.

Immunoblot Analysis. Cells were plated as described and synchronized by incubation in serum-free DMEM for 24 h followed by incubation with drugs of interest or vehicle for an indicated time. In some studies, cells were incubated with adenovirus expressing a constitutively active form of MEK for 24 h. In other studies, cells were pretreated with specific inhibitors for 30 min. Cells were then incubated with 10 μM Iso or 10 μM Fsk for 20 min before treatment with 10 ng/ml TGF-β for 1 h. After treatment, cells were washed twice with ice-cold PBS and were extracted with $2\times$ SDS sample buffer and denatured for 10 min at 70°C. For detection of phospho-ERK1/2, cells were lysed in lysis buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 0.02 mg/ml leupeptin and 0.02 mg/ml aprotinin, pH 7.4). Cells were scraped from the plates, and the protein lysates were sonicated for 15 s and centrifuged at 500g at 4°C for 1 min. Supernatants or immunoprecipitates were collected, equal protein amounts of the lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) by electroblotting. Membranes were blocked in PBS-Tween with 5% nonfat dry milk and incubated with primary antibody (see *Materials*) overnight at 4°C. Bound primary antibodies were visualized using appropriate secondary antibody with conjugated horseradish peroxidase (Santa Cruz Biotechnology) and ECL reagent (Pierce). Most primary antibodies recognized multiple nonspecific species. Only the band representing the appropriately sized immunoreactive band is shown. The amount of protein per sample was determined using a dye-binding protein assay (Bio-Rad).

Quantitative Reverse Transcriptase PCR. Total RNA was extracted from rat cardiac fibroblasts grown to 80 to 90% confluence on 10-cm plates with TRIzol reagent (Invitrogen, Carlsbad, CA) and an RNeasy RNA isolation kit (QIAGEN, Valencia, CA). A DNase reaction was performed to eliminate DNA contaminants, and the RNA was reverse-transcribed with Superscript II (Invitrogen) and random hexamer primers. RT-PCR was performed initially to confirm that single PCR products resulted from reactions with each primer pair. Suitable primers were then used in quantitative real-time PCR reactions using iTaq SYBR Green Supermix (Bio-Rad). Primer sequences were as follows: collagen Iα2, 5′-TGTTCGTGGT-TCTCAGGGTAG-3′ and 5′-TTGTCGTAGCAGGGTTCTTTC-3′; collagen IIIα1, 5′-CGAGGTAACAGAGGTGAAAGA-3′ and 5′-AACCC-AGTATTCTCCGCTCTT-3′; and β-actin, 5′-CAAAGACCTGTACGC-CAACAC-3′ and 5′-CATACTCCTGCTTGCTGATCC-3′. All cDNA samples were tested in duplicate using an ABI PRISM 7700 (Applied Biosystems, Foster City, CA). The thermal profile for all real-time PCR reactions was 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 55°C for 1 min. Fluorescence data from each sample was analyzed by the $2(-[\Delta\Delta C_t]$ method: fold induc-

tion $= 2(-[\Delta\Delta C_t]$, where $\Delta\Delta C_t$ is (C_t GI of treated sample $- C_t$ β-actin of treated sample) $- [C_t$ GI (vehicle-treated sample) $- C_t$ β-actin (vehicle-treated sample)]. GI is the gene of interest, and C_t is the cycle threshold (the cycle number where the fluorescent signal crosses an arbitrary intensity threshold).

Data Analysis and Statistics. Data are presented as the mean $\pm$ S.E.M. and in some cases as representative images of at least three separate experiments. Statistical comparisons (t tests and one-way analysis of variance) were performed, and graphics were produced using Prism 4.0 (GraphPad Software Inc., San Diego, CA).
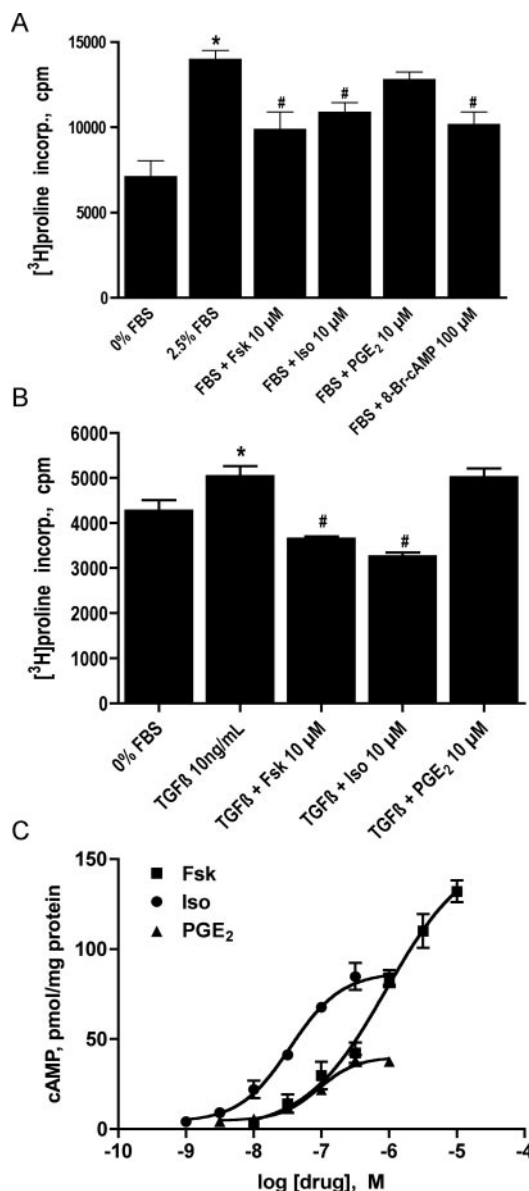

Fig. 1. cAMP-elevating agents inhibit collagen synthesis in rat cardiac fibroblasts [³H]proline incorporation stimulated by 2.5% FBS (A) or 10 ng/ml TGF-β (B) was measured in the absence or presence of maximal concentrations of Fsk, Iso, or PGE$_2$. Cells were serum-starved for 24 h and then treated the indicated drug for 20 min before treatment with 10 ng/ml TGF-β for another 48 h (see *Materials and Methods*). Each bar or point represents the mean $\pm$ S.E.M. of three to six experiments. ∗, $p <$ 0.05 compared with unstimulated condition; #, $p <$ 0.05 compared with stimulated condition by ANOVA. cAMP accumulation in cardiac fibroblasts was measured in response to various concentrations of Fsk, Iso or PGE$_2$ (C). Each point or bar represents the mean $\pm$ S.E.M. of six to eight experiments.

Results

Previous studies demonstrate that cAMP-elevating agents inhibit myofibroblast differentiation and collagen synthesis in cardiac fibroblasts (Ostrom et al., 2003; Swaney et al., 2005). We investigated the potential antifibrotic effect of cAMP-elevating agents in cardiac fibroblasts using [^{3}H]proline incorporation (an assay of collagen synthesis) in cardiac fibroblasts. Cells were serum-starved for 24 h in 0.25% FBS and then cultured for 24 h with 2.5% FBS (a maximally effective concentration of serum; data not shown) and either vehicle or various concentrations of cAMP-elevating agonists. Fsk, a direct activator of adenylyl cyclases, and Iso, a β-adrenergic receptor agonist, each inhibited FBS-stimulated [^{3}H]proline incorporation (Fig. 1A). The effect of Iso is presumed to be through the activation of β_2-adrenergic receptors because these are the only isoform expressed in cardiac fibroblasts (Gustafsson and Brunton, 2000). PGE$_2$ had no effect on FBS-stimulated [^{3}H]proline incorporation, but the cell-permeable cAMP analog 8-Br-cAMP (100 μM) significantly reduced this response. TGF-β also stimulated [^{3}H]proline incorporation, and this level of activation was also inhibited by the inclusion of Fsk or Iso but not by PGE$_2$ (Fig. 1B). Fsk and Iso each inhibited TGF-β-stimulated [^{3}H]proline incorporation to levels lower than that of unstimulated conditions yet did not alter basal incorporation in the absence of either FBS or TGF-β (data not shown).

We characterized the responsiveness of cardiac fibroblasts to Fsk, Iso, and PGE$_2$ by assaying cAMP production in response to various concentrations of each agonist over a 10-min incubation. Iso stimulated cAMP production with an EC$_{50}$ value of 46 nM and a maximum of 100 pmol of cAMP/mg of protein (Fig. 1C). PGE$_2$ stimulated cAMP production with lower potency and efficacy than Iso, exhibiting an EC$_{50}$ value of 89 nM and a max of 40 pmol of cAMP/mg of protein. Fsk was less potent than either Iso or PGE$_2$, with an EC$_{50}$ value of 0.9 μM, but it displayed a much larger maximum (156 pmol of cAMP/mg of protein). Thus, Iso and Fsk are effective stimulators of cAMP production in cardiac fibroblasts, whereas PGE$_2$ is less capable of eliciting large increases in intracellular cAMP. The inability of PGE$_2$ to inhibit [^{3}H]proline incorporation probably relates to the smaller cAMP signals it generates but may also be due to the more labile nature of this drug in long-term incubations (we did not treat repeated applications of drug over the 24 h treatment time used in the [^{3}H]proline incorporation assays).

cAMP exerts most of its transcriptional effects via activation CREB, whose activity is stimulated by PKA-promoted phosphorylation of serine 133 (Mayr and Montminy, 2001). We used a monoclonal antibody that recognizes serine 133-phosphorylated CREB (pCREB) to assay CREB activation in whole-cell lysates. Treatment of cells with either 10 μM Fsk,

1 μM Iso, or 1 μM PGE$_2$ increased pCREB immunoreactivity in cells at 5 min, the earliest time point we measured (Table 1). pCREB immunoreactivity remained elevated out to 1 h in the presence of Fsk or Iso but out to only 5 min in the presence of PGE$_2$. The levels of pCREB immunoreactivity decreased steadily out to 24 h in Fsk- and Iso-treated conditions, an effect not due to decreased cell number or viability.

To confirm that the observed effects of cAMP-elevating agents on [^{3}H]proline incorporation are specific to the synthesis of collagen, immunoblot analyses were used to assess levels of expression of collagen I, collagen III (predominant collagen isoforms in the heart), and α-smooth muscle actin (a marker of myofibroblast differentiation). Cells were starved for 24 h with 0% FBS and pretreated with 10 μM Fsk or 10 μM Iso for 20 min. After stimulation of cells with TGF-β (10 ng/ml), cells were treated with Fsk, Iso, or vehicle for another 24 h, and then cell lysates were collected for immunoblot analysis. TGF-β significantly increased immunoreactivity of collagen I, collagen III, and α-smooth muscle actin (Fig. 2). Cotreatment of cells with 10 μM Fsk or Iso significantly decreased TGF-β-stimulated collagen I, collagen III, and α-smooth muscle actin immunoreactivity. Treatment of cells with Fsk also reduced the expression of collagen mRNAs, as measured by quantitative RT-PCR (Fig. 7D). Thus, cAMP-elevating agents specifically inhibit the expression of collagen types I and III and the differentiation of cardiac fibroblasts into myofibroblasts.

Recent reports indicate that cAMP-elevating agents inhibit TGF-β-induced gene transcription via CREB-mediated sequestration of CBP1 and p300, coactivators required for full transcriptional activity of numerous transcription factors, including Smad3/4 (Schiller et al., 2003). To investigate the role of this mechanism in cardiac fibroblasts, we performed electrophoretic mobility shift assays (EMSAs) to assess the impact of TGF-β and cAMP-elevating agents on CREB and Smad3/4 DNA binding. Cells were serum-starved for 24 h and then pretreated with 10 μM Fsk or 10 μM Iso for 20 min before being activated by serum (2.5%) or TGF-β (10 ng/ml) for 1 h. Nuclear extracts were then isolated and analyzed by EMSA. Fsk and Iso stimulated CREB binding to specific DNA probes several fold over basal levels (Fig. 3A). The probe-protein complexes were confirmed by both unlabeled probes and supershift analysis using a CREB antibody (data not shown). TGF-β also caused an increase in basal levels of CREB DNA binding, consistent with reports by others (Zhang et al., 2004). However, combining TGF-β and either Fsk or Iso treatment resulted in CREB DNA binding that was significantly less than TGF-β or cAMP-elevating agents alone. Thus, cotreatment with TGF-β inhibits Iso- or Fsk-stimulated CREB binding to CRE sequences in cardiac fibroblasts, implying that TGF-β signaling, perhaps via Smad3/4

TABLE 1
CREB phosphorylation in response to cAMP-elevating agents in rat cardiac fibroblasts

Cells were treated with the indicated agent for the given amount of time before levels of pCREB were measured by immunoblot analysis (see *Materials and Methods*). Immunoreactive bands were quantified and then normalized to the levels of total CREB immunoreactivity. The data are expressed as the mean $\pm$ S.E.M. of four to five experiments.

	Basal	5 min	20 min	30 min	1 h	3 h	24 h
Fsk 10 μM	12.4 $\pm$ 0.1	17.9 $\pm$ 0.1*	15.5 $\pm$ 0.5*	18.2 $\pm$ 2.6*	17.5 $\pm$ 0.9*	11.7 $\pm$ 2.0	6.8 $\pm$ 0.8*
Iso 1 μM	9.3 $\pm$ 1.4	14.6 $\pm$ 1.1*	14.1 $\pm$ 1.0*	17.5 $\pm$ 2.2*	15.8 $\pm$ 1.3*	15.8 $\pm$ 2.2	12.8 $\pm$ 2.1
PGE$_2$ 1 μM	13.8 $\pm$ 0.6	19.2 $\pm$ 2.0*	14.0 $\pm$ 0.6	13.1 $\pm$ 0.3	13.7 $\pm$ 0.4	13.0 $\pm$ 0.7	13.0 $\pm$ 1.3

*, $p < 0.05$ compared with basal by ANOVA.

competition for transcriptional cofactors, reduces CREB interaction with DNA.

Conversely, we examined whether cAMP-elevating agents might affect the DNA-binding ability of Smad3/4 transcriptional complexes using EMSAs. Fsk or Iso was added 20 min before incubation of cells with TGF-β (10 ng/ml) for 1 h. The level of Smad3/4-DNA complex formation was increased by TGF-β but was not attenuated by cAMP-elevating agents such as Fsk and Iso (Fig. 3B). The probe-protein complexes were confirmed by both unlabeled probes and by supershift analysis using a Smad3 antibody (data not shown). Whereas these results indicate that cAMP-elevating agents do not impair Smad3/4 DNA-binding activity, the data do not rule out that Smad3/4 transcriptional activity is reduced by

cAMP-elevating agents because CBP1 and p300 coactivators act primarily by aiding the uncoiling of DNA and do not necessarily enhance transcription factor-DNA interaction (Schiller et al., 2003, 2005).

To investigate whether cAMP-elevating agents are able to interfere with the recruitment of CBP1 to CREB or Smad transcriptional complexes, we used immunoprecipitations of nuclear proteins to assess the interaction between these endogenous transcriptional proteins in cardiac fibroblasts after treatment with TGF-β and/or Iso. Immunoprecipitation of endogenous CREB followed by immunoblotting for CBP1 indicates that Iso and TGF-β each induce the recruitment of CBP1 to CREB complexes (Fig. 4). Cotreatment of cells with Iso and TGF-β led to a reduction in CBP1 binding to CREB
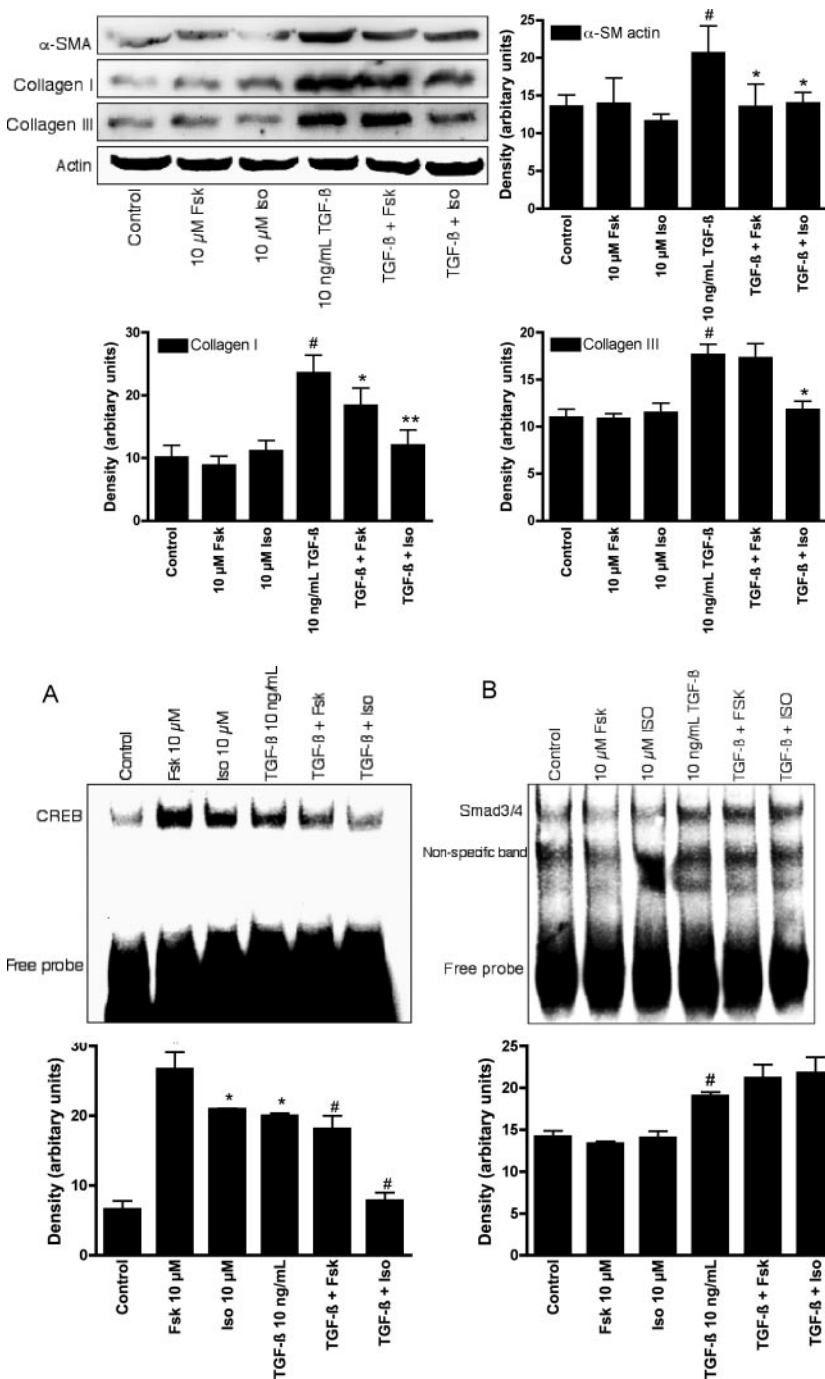

Fig. 2. cAMP-elevating agents inhibit expression of collagen I, collagen III, and α-smooth muscle actin. Cells in maintenance media were incubated with the indicated concentrations of Fsk, Iso, TGF-β, or all three (Fsk, Iso, and TGF-β) for 24 h. Whole-cell lysates were analyzed by immunoblot analysis using antibodies specific for collagen types I and III and β actin (see *Materials and Methods*). Representative images of at least three experiments are shown along with quantification of immunoreactive bands (adjusted for the density of β-actin loading controls). Data are shown as the mean $\pm$ S.E.M. of four to six experiments. #, $p < 0.05$ compared with control; $*$, $p < 0.05$ compared with TGF-β treatment alone by ANOVA.

Fig. 3. TGF-β and cAMP-elevating agents alter CREB and Smad3/4 DNA binding. Rat cardiac fibroblasts were treated with either vehicle (control), 10 μM Fsk, 10 μM Iso, 10 ng/ml TGF-β, or a combination. Nuclear extracts were then isolated and incubated with either CREB (A) or Smad3/4 (B) specific probes and separated by 6% polyacrylamide gel (see *Materials and Methods*). Representative gels images are shown (top), and protein-probe complexes were quantified, adjusted for protein loading, and are shown as the mean $\pm$ S.E.M. of three experiments (bottom). $*$, $p < 0.05$ compared with unstimulated condition; #, $p < 0.05$ compared with stimulated condition by ANOVA.

compared with Iso or TGF-β alone. Immunoblot analyses of CBP1 immunoprecipitates from untreated cardiac fibroblasts indicate that CBP1 complexes with Smad2, Smad3, and Smad4. Pretreatment of cells with TGF-β, but not with Iso, increased the coimmunoprecipitation of Smad 2 and Smad3 with CBP1. Treatment with both TGF-β and Iso resulted in a reduction of Smad2 and Smad 3 in CBP1 immunoprecipitates. Conversely, immunoblot analyses of Smad3 immunoprecipitates also indicate that Iso treatment of cells reduces the interaction between Smad3 and the transcriptional coactivator CBP1 and with Smad 4. Taken together, these results imply that cAMP-elevating agents repress TGF-β signaling by activating CREB, which recruits CBP1, effectively competing with Smad transcriptional complexes for binding to CBP1.

One alternative interpretation of the above results is that cAMP-elevating agents directly attenuate TGF-β-induced activation of Smads. Thus, we used immunoblot analyses to measure the levels of phosphorylated Smad2 (pSmad2) after treatment of cells with TGF-β with or without cotreatment with a cAMP-elevating agent. Cardiac fibroblasts were starved for 24 h with media containing 0% FBS and then pretreated with either vehicle, Fsk or Iso (10 and 1 μM, respectively), 20 min before incubation with TGF-β (10 ng/ml) for another 30 min. Fsk and Iso had no effect on vehicle-treated (basal) levels of pSmad2, but TGF-β treatment of cells induced a 2-fold increase in pSmad2 immunoreactivity (Fig. 5). The cotreatment of TGF-β with either Fsk or Iso had no effect of the levels of pSmad2. Thus, cAMP-elevating agents do not seem to impinge upon the ability of TGF-β to activate "upstream" Smad signaling.
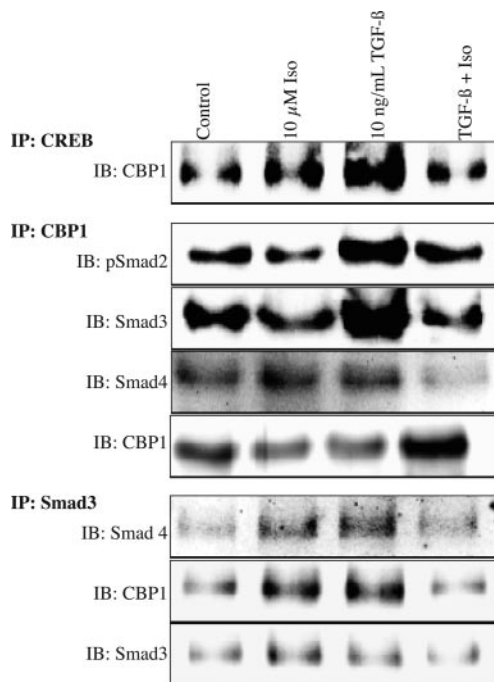
Because TGF-β is known to also signal via Smad-independent pathways, particularly through activation of the MEK/ERK, JNK, and p38 MAP kinase pathways, we assessed whether these kinases are involved in TGF-β-mediated signaling. We assessed the activation of these pathways using immunoblot analyses of phosphorylated ERK (pERK1/2), phosphorylated JNK (pJNK), or phosphorylated p38. TGF-β, Fsk, and Iso treatments alone each increased pERK1/2 levels in cardiac fibroblasts (Fig. 6A). By contrast, the combined treatment of cells with TGF-β and either Fsk or Iso resulted in a reduction in pERK1/2 levels to basal levels. Thus, cAMP-elevating agents can dually regulate ERK1/2 activation in a contextual manner: they stimulate pERK levels on their own but inhibit pERK levels in conditions in which TGF-β is present. TGF-β also increased pJNK and p38 immunoreactivity, but Fsk or Iso alone had no effect (Fig. 6, B and C). Fsk or Iso inhibited TGF-β-simulated pJNK immunoreactivity (Fig. 6B) but had no effect on TGF-β-stimulated p38 levels (Fig. 6C). Thus, the cAMP pathway seems to affect ERK1/2 and JNK activity but does not effect TGF-β signaling via p38.

To determine whether MEK/ERK signaling is required for the TGF-β-mediated activation of collagen synthesis in cardiac fibroblasts, we measured TGF-β-stimulated collagen I and III immunoreactivity with and without treatment with the MEK inhibitor PD98059. Cells were starved for 24 h with 0% FBS and pretreated with 50 μM PD98059 or vehicle for 30 min before being stimulated with TGF-β (10 ng/ml) or vehicle for another 24 h. PD98059 inhibited TGF-β-stimu-

Fig. 4. cAMP-elevating agents disrupt the interaction between Smads and the coactivator CBP1. Cells were serum-starved for 24 h and pretreated with 10 μM Iso for 20 min before the addition of TGF-β for 24 h. Immunoprecipitates of the indicated proteins were separated by SDS-PAGE and probed with antibodies specific for CBP1, Smad2, Smad3, or Smad4 were used to examine levels of the indicated proteins. IgG control antibodies were used to control for nonspecific interaction (data not shown). Representative images of three to five experiments are shown.
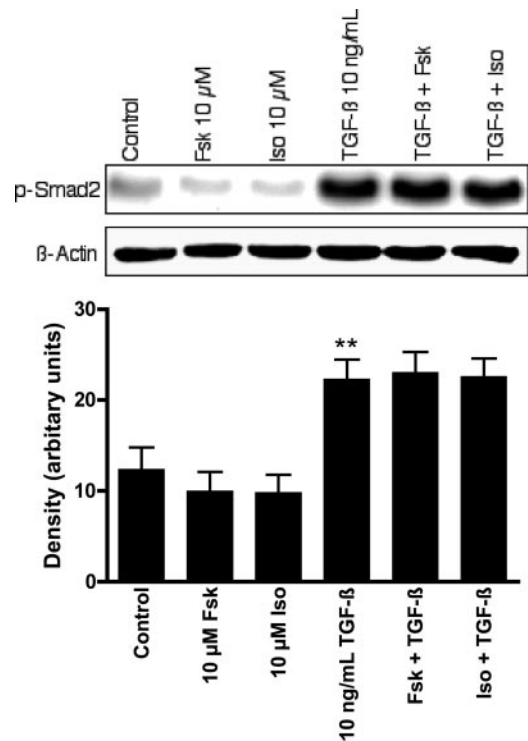

Fig. 5. cAMP-elevating agents do not inhibit TGF-β stimulated Smad2 phosphorylation. Cells were starved for 24 h and pretreated with 10 μM Fsk or 10 μM Iso for 20 min before treating with TGF-β for 1 h. Whole-cell lysates were separated by SDS-PAGE and analyzed by immunoblot analysis using a monoclonal antibody specific for phosphoserine 465/467 Smad2 (pSmad2) or β-actin. Representative images of three experiments are shown (top) along with quantification of immunoreactive bands (bottom). Quantified data are expressed as the mean ± S.E.M. of five experiments. **, p < 0.01 compared with control by ANOVA.

lated immunoreactivity of collagen isoforms I and III to levels equivalent to those in basal conditions (Fig. 7A). To address the potential nonspecificity of PD98059, we used a different MEK inhibitor, U0126 (25 μM). U0126 significantly inhibited TGF-β-stimulated collagen III immunoreactivity (Fig. 7B) and mRNA expression (as measured by quantitative RT-PCR; Fig. 7D). Thus, pharmacological inhibition of the MAP kinase pathway causes a sizable attenuation of TGF-β-stimulated collagen mRNA and protein expression, implying that ERK signaling is a critical pathway, but not the only one, through which TGF-β stimulates collagen synthesis in cardiac fibroblasts.

We used a similar approach to test for the role of JNK in TGF-β activation of collagen synthesis by measuring TGF-β-stimulated proline incorporation in cells treated with either vehicle or 20 μM SP600125, a JNK inhibitor. Cells were starved for 24 h with 0% FBS and pretreated with 20 μM SP600125 or vehicle for 30 min before being stimulated with TGF-β (10 ng/ml) or vehicle for another 24 h. SP600125 reduced both basal and TGF-β-stimulated collagen III immunoreactivity (Fig. 7C) and mRNA levels (Fig. 7D). SP600125 had similar effects on basal and TGF-β-stimulated [^{3}H]proline incorporation (20 $\pm$ 1.1% inhibition of basal, 35 $\pm$ 4.2% inhibition of TGF-β-stimulated). Inhibition of basal collagen expression may be due to this inhibitor's effects on other signaling pathways (Bennett et al., 2001) or may reflect a more complex role for JNK in regulating collagen synthesis. Thus, whereas JNK seems to play a role in collagen synthesis in cardiac fibroblasts, its precise role in mediating the effects of either TGF-β or cAMP is unclear.

As an alternative approach to pharmacological inhibition of the MEK/ERK pathway, we expressed a constitutively active form of MEK (caMEK) using an adenoviral construct to induce maximal activation of this pathway (Sreejayan et al., 2002). Cells exposed to the caMEK adenovirus for 24 h displayed increased basal pERK immunoreactivity, indica-
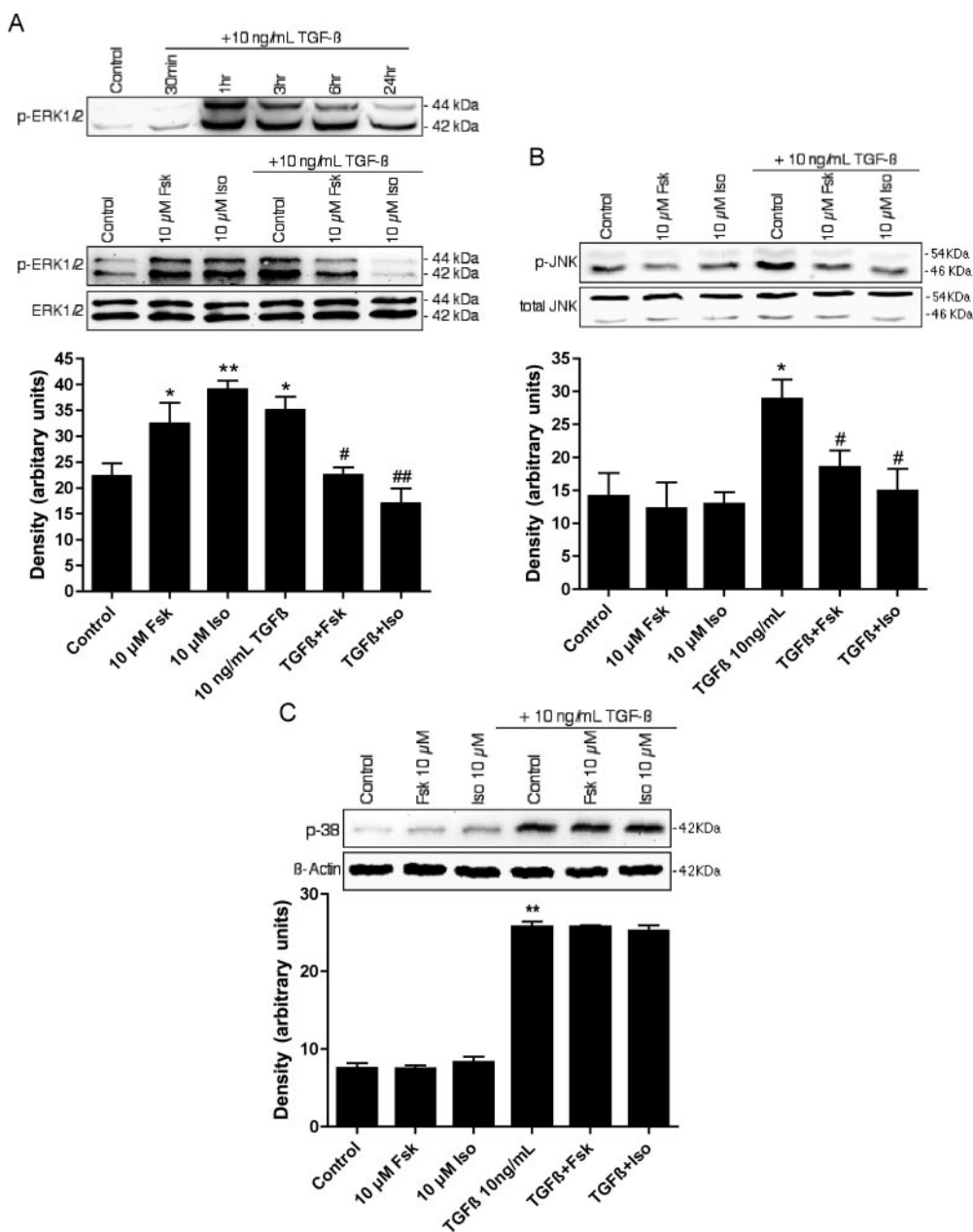
Fig. 6. cAMP-elevating agents inhibit TGF-β-stimulated ERK and JNK, but not p38, phosphorylation. Cells were serum-starved for 24 h and were pretreated with the indicated concentrations of Fsk or Iso for 20 min before treating with TGF-β for 1 h. Whole-cell lysates were separated by SDS-PAGE and analyzed by immunoblot analysis using a monoclonal antibody specific for phosphorylated-ERK1/2, -JNK, or -p38. Representative images of at least three experiments are shown (top) along with quantification of immunoreactive bands (bottom). Quantified data are expressed as the mean $\pm$ S.E.M. of three to five experiments. *, $p < 0.05$ and **, $p < 0.01$ compared with control; #, $p < 0.05$ compared with TGF-β treatment alone by ANOVA.

tive of a high level of constitutive MEK activity (Fig. 8A). The basal level of collagen III immunoreactivity in caMEK-expressing cells was similar to that control cardiac fibroblasts treated with a maximal concentration of TGF-β. However, TGF-β treatment of caMEK-expressing cells led to even greater collagen III immunoreactivity than either treatment alone. Proline incorporation assays of total collagen synthesis revealed similar results (data not shown). Taken together, these manipulations to either inhibit or activate the MEK/ERK cascade imply that TGF-β stimulates collagen synthesis via both ERK-dependent and independent pathways but that the ERK pathway is sufficient to cause up-regulation.

To gain more information regarding the mechanism by which cAMP-elevating agents inhibit ERK activation, we examined the ability of forskolin to inhibit collagen synthesis stimulated by expression of caMEK. Collagen III immunoreactivity was increased in caMEK-expressing cells, but treatment of these cells with 10 μM forskolin led to a significant inhibition of collagen III levels (Fig. 8B). Thus, cAMP can still impinge upon collagen synthesis stimulated by a constitutively activated ERK signaling pathway. These results

may indicate that cAMP exerts it effects downstream of MEK activity.

Recent studies indicate that TGF-β/Smad signaling can be influenced by MAP kinase signaling cascades and that the direction of this regulation can be cell type-specific (Leask and Abraham, 2004). Thus, whereas the ERK1/2 cascade might directly regulate collagen gene transcription, it may also enhance Smad-dependent signaling in cardiac fibroblasts such that inhibition of ERK signaling also attenuates Smad signaling. To determine whether ERK1/2 activation alters more proximal steps in the pathway, such as TGF-β receptor activation of Smads, we pretreated cells with 50 μM PD98059 before incubating them with TGF-β and then assessed pSmad2 levels using immunoblot analyses. The phosphorylation of Smad2 is an early step in the activation of the Smad pathway by TGF-β (Derynck and Zhang, 2003). As with experiments in Fig. 5, TGF-β treatment of cells increased pSmad2 immunoreactivity (Fig. 9). PD98059 treatment alone had no effect on the basal levels of pSmad2 and did not inhibit TGF-β-stimulated pSmad2 immunoreactivity. Thus, ERK1/2 does not alter activation of Smad2 by TGF-β.
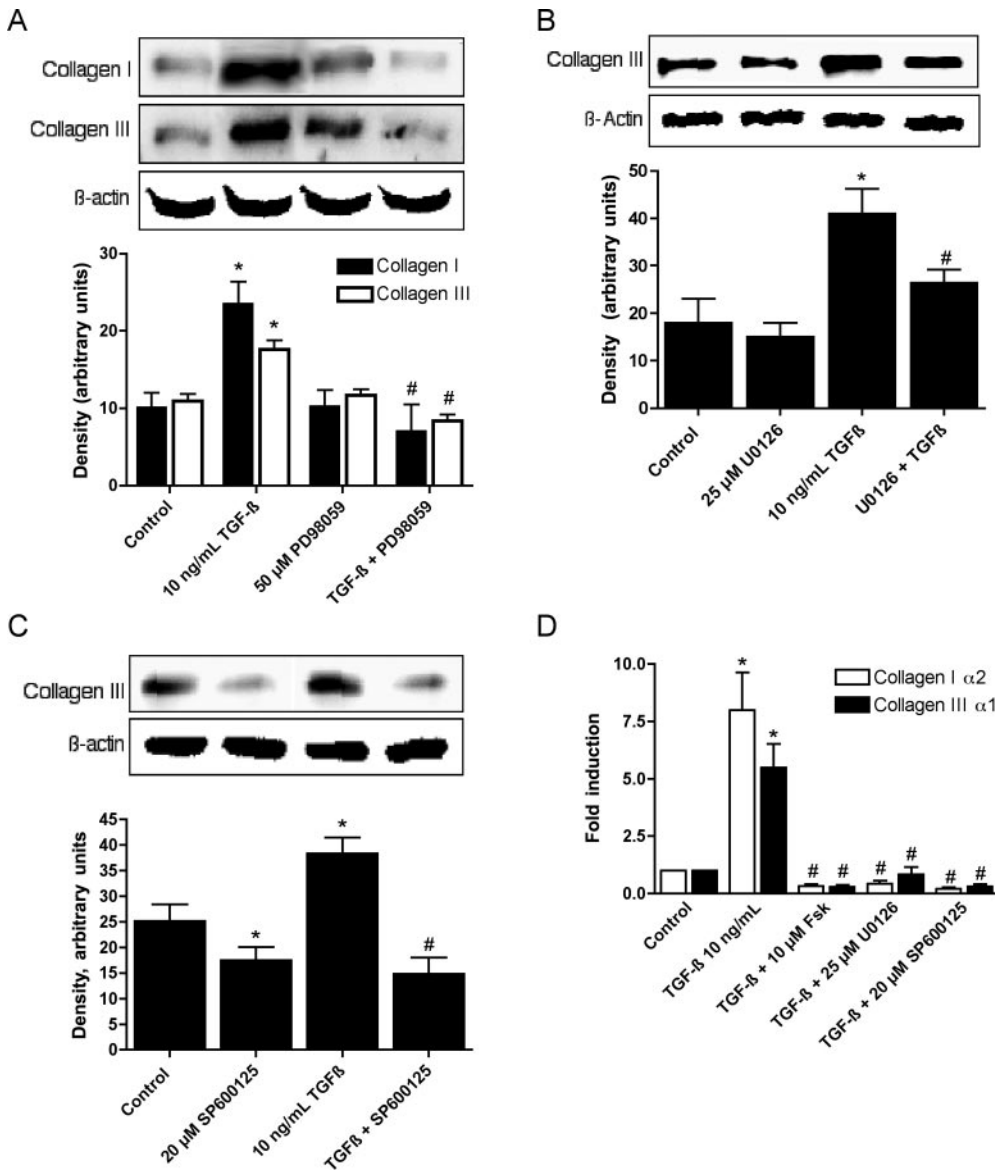

Fig. 7. MEK and JNK activities are required for the TGF-β-mediated activation of collagen synthesis in rat cardiac fibroblasts. Cells were starved for 24 h and pretreated with 50 μM PD98059 (A) or 25 μM U0126 (B), MEK inhibitors, or 20 μM SP600125 (C), a JNK inhibitor, for 20 min before treating with 10 ng/ml TGF-β for 24 h. Whole-cell lysates were separated by SDS-PAGE and analyzed by immunoblot analysis using a monoclonal antibody specific for collagen I, collagen III, or β-actin (A–C). Representative images of three to six experiments are shown (top) along with quantification of immunoreactive bands (bottom). Quantitative real-time PCR analysis of effects of TGF-β on the mRNA expression of collagen Iα2 and collagen IIIα1 (D). Rat cardiac fibroblasts were pretreated with 25 μM U0125, 20 μM SP600125, or 10 μM Fsk for 30 min followed by 24 h treatment of 10 ng/ml TGF-β before quantitative RT-PCR analysis was performed as described under *Materials and Methods*. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with TGF-β alone by ANOVA.

These studies do not rule out the possibility that ERK1/2 is involved in regulating other steps in Smad signaling (Leask and Abraham, 2004).

Discussion

TGF-β is a major activator of fibroblast differentiation and activation, and thus is a key player in the fibrotic response. Blockade of TGF-β signaling is predicted to blunt fibrosis, and thus, much attention has been paid to TGF-β-mediated signaling. Very recently, cAMP has been identified as a negative regulator of fibroblast activation and differentiation (Liu et al., 2004; Swaney et al., 2005). The present work sheds the first light on the intracellular signaling mechanisms by which cAMP might be directly acting to reduce TGF-β-mediated activation of fibrotic responses, particularly the activation of collagen gene transcription, but also the expression of α-smooth muscle actin, a marker of myofibroblast differentiation. TGF-β activates TGF-β receptors, which signal through the activation of Smads. The receptor induces the phosphorylation of Smad2, which in turn phosphorylates Smad3 and induces recruitment of Smad4 (Nakao et al., 1997). The active Smad3/4 complex then translocates into the nucleus where it interacts with Smad binding elements within DNA promoter regions to activate transcription (Attisano and Wrana, 2002). Smads require other proteins for transcrip-
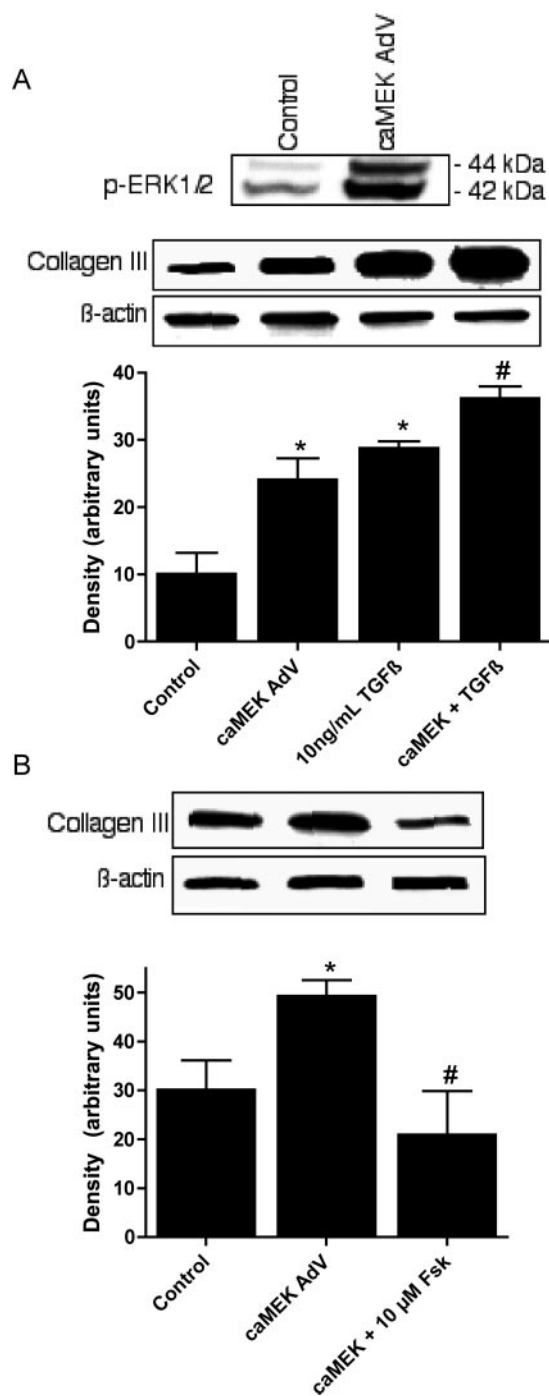

Fig. 8. caMEK stimulates collagen synthesis—a response increased by TGF-β and inhibited by a cAMP-elevating agent. Cells were starved for 24 h while simultaneously being exposed to an adenovirus expressing caMEK or control virus (A and B). In some conditions, cells were treated with 10 ng/ml TGF-β with or without 10 μM Fsk for 24 h (B). Whole-cell lysates were separated by SDS-PAGE and analyzed by immunoblot analysis using a monoclonal antibody specific for pERK (A, top) to assess the level of ERK activation or collagen III as a measure of collagen synthesis. Representative images of three experiments are shown along with quantification of immunoreactive bands. Quantified data are expressed as the mean $\pm$ S.E.M. of three experiments. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with TGF-β or caMEK alone by ANOVA.
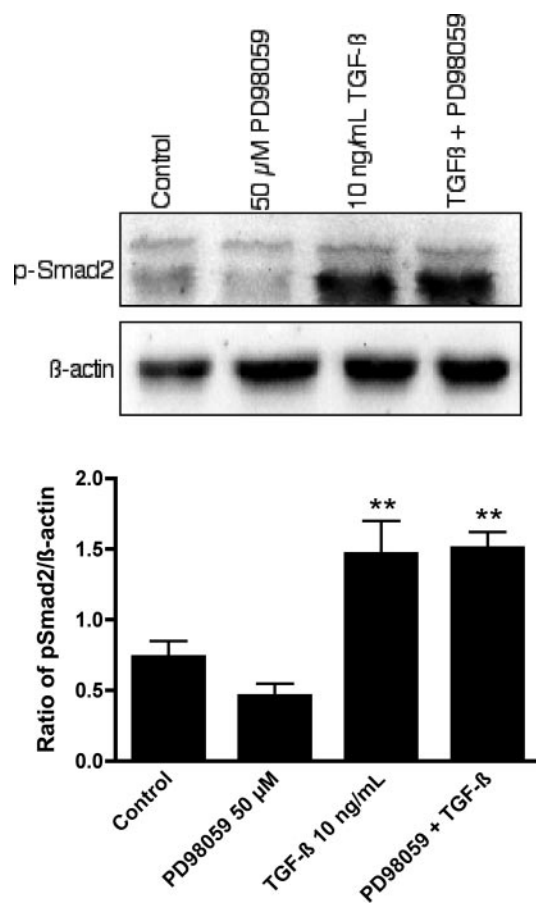

Fig. 9. The MEK/ERK pathway does not directly alter Smad2 phosphorylation. Cells were starved for 24 h and pretreated with 50 μM PD98059, a MEK inhibitor, for 20 min before treating with 10 ng/ml TGF-β for 24 h. Whole-cell lysates were separated by SDS-PAGE and analyzed by immunoblot analysis using a monoclonal antibody specific for serine 465/467 phosphorylated-Smad2. Representative images of three experiments are shown (top) along with quantification of immunoreactive bands (bottom). Quantified data are expressed as the mean $\pm$ S.E.M. of six experiments. *, $p < 0.05$; **, $p < 0.01$ compared with control by ANOVA.

tional activity, including the coactivators CBP1 and p300 (Ghosh et al., 2000). Thus, the activation of Smads, their translocation to the nucleus, and subsequent effects on gene transcription are the critical steps in modulation of signaling by this pathway. The antifibrotic actions of angiotensin receptor antagonists and angiotensin converting enzyme inhibitors in the heart seems related to the permissive role of angiotensin II on TGF-β signaling in cardiac fibroblasts (Hao et al., 2000).

cAMP is a second messenger that has been implicated as a modulator of cell proliferation and cell growth in several fibroblastic cell types (Takahashi et al., 2004). More recently, it was demonstrated that increased cAMP levels inhibit cardiac myofibroblast formation and collagen synthesis (Swaney et al., 2005). In addition, β-adrenergic receptor agonists, which stimulate production of cAMP, attenuate fibroblast-mediated contraction of collagen gels (Mio et al., 1996). Nonetheless, the precise mechanisms by which cAMP-elevating agents inhibit collagen formation remain undefined. The present work demonstrates that β-adrenergic receptor activation (Iso) or direct stimulation of adenylyl cyclase (Fsk) inhibits the ability of TGF-β to stimulate collagen synthesis in rat cardiac fibroblasts by attenuating TGF-β-stimulated ERK1/2 MAP kinase activity and via CREB-mediated competition for the transcriptional coactivator CBP1 to Smad transcriptional complexes. cAMP also inhibited TGF-β-stimulated pJNK levels, but the role of this effect in cAMP's inhibition of collagen synthesis is less clear. Whereas β-adrenergic receptors can certainly activate signaling pathways independent of cAMP, we conclude that the cAMP pathway is primarily responsible for the inhibition of collagen synthesis because the responses we measured were also elicited by Fsk and because others have recently reported that overexpression of adenylyl cyclase enhances these effects (Swaney et al., 2005).

In the early 1990s, it was shown that the cell-permeable cAMP analogs 8-chloro-cAMP or dibutyryl cAMP blocked growth factor-stimulated ERK activation in Rat1 and NIH3T3 fibroblasts, establishing the existence of cross-talk between these pathways (Burgering et al., 1993; Wu et al., 1993). Other researchers also showed that cAMP attenuates activation of ERK in other cells (Sevetson et al., 1993). Our data show that in cardiac fibroblasts, cAMP-elevating agents alone stimulate ERK1/2 activation but in the presence of coincidental stimulatory signals (e.g., from TGF-β) converts to an inhibitory mode. The different effect of cAMP on ERK1/2 activation in other cells has been related to the expression or expression levels of either C-Raf or B-Raf and perhaps adaptor proteins that target PKA to Raf (Dumaz and Marais, 2005). However, the relative expression and function of Raf isoforms and the various proteins that regulate them is not fully defined in cardiac fibroblasts. Furthermore, our studies expressing a constitutively active form of MEK imply that cAMP-mediated inhibition of the ERK pathway occurs downstream of MEK and thus is not dependent on Raf.

Our data indicate that inhibition of the MEK/ERK pathway largely blocks TGF-β-stimulated collagen synthesis in cardiac fibroblasts. These results seemingly indicate that Smad or JNK signaling is less central to cAMP's effects. However, TGF-β/Smad signaling is tightly controlled by MAP kinase signaling cascades, particularly ERK1/2, in

many cell types (Yue and Mulder, 2000; Leask and Abraham, 2004). de Caestecker et al. (1998) showed that hepatic growth factor and epidermal growth factor, which activate the MEK/ERK pathway through their receptor tyrosine kinases, regulate Smad-mediated pathways. In addition, TGF-β-induction of CTGF and collagen expression in mesangial cells requires Smads and the MEK/ERK pathway (Stratton et al., 2002). Pharmacological inhibition of the ERK pathway did not inhibit Smad2 phosphorylation in our studies, indicating that the ERK pathway does not regulate the proximal steps of Smad activation. However, possible cross-talk between the ERK and Smad pathways could occur by other means at more distal points: 1) association of ERK-induced transcription factors with Smads and other coactivators that form transcriptional regulatory complexes; 2) separate binding of transcription factors activated by ERKs and Smads to sites on the promoter of target genes; 3) synergy between the ERK and Smad pathways before their interaction with the target gene; or 4) ERK activation enhancement of nuclear translocation of activated Smad complexes, thereby enhancing Smad-mediated transcription (Hayashida et al., 1999).

Similarly complex is the potential role of JNK in regulating collagen gene expression. Our studies show that JNK inhibition leads to decreased basal and TGF-β-stimulated collagen synthesis, implying that JNK is involved in more than just TGF-β-initiated signaling. Furthermore, inhibition of JNK by SP600125 yields different effects than inhibition of JNK by cAMP, which does not alter basal collagen synthesis. Whereas this difference may relate to nonspecific effects of the pharmacological inhibitor, the role of cAMP-mediated inhibition of JNK inhibition in the cAMP response we observe is certainly not clear from these studies. Last, JNK signaling, much like with ERK1/2, has been shown to feed back on to Smad signaling (Pessah et al., 2002), making the separation of the role of these pathways difficult.

Our data show that cAMP-elevating agents activate CREB and reduce the association between Smads and the transcriptional coactivator CBP1. This mechanism is probably one way that cAMP levels lead to a reduction of collagen synthesis stimulated by TGF-β. However, the effect of TGF-β and cAMP on the complex of proteins associated with promoters containing either a cAMP-response element or a Smad binding element is potentially complex. cAMP/PKA activation of CREB may reduce Smad-mediated transcription by recruiting CBP1 and p300, making them unavailable for recruitment to Smad transcriptional complexes (Schiller et al., 2003). At the same time, the TGF-β signaling pathway can directly activate PKA and CREB in some cells (Zhang et al., 2004). Our data indicate that such an activation occurs in cardiac fibroblasts (Fig. 3), and this would be predicted to be an autoinhibitory loop on TGF-β signaling (de Caestecker et al., 1998). However, our data also show that the combinatorial treatment with TGF-β and a cAMP-elevating agent leads to an inhibition of CREB-DNA interaction. Thus, TGF-β can stimulate or inhibit CREB transcription, dependent on coincidental cAMP signals. It is not clear whether CREB or Smad-mediated transcription alters the expression of other components that might effect transcription by these or other transcription factors. For example, cAMP/PKA/CREB may reduce the expression of

proteins involved in TGF-β signaling or Smad transcription or induce the expression of proteins that repress Smad transcription. Thus, more work is needed to define the interplay between Smad and CREB transcriptional complexes.

Our findings are consistent with previous observations that the stable prostacyclin analog, Iloprost, can alleviate symptoms of fibrosis in patients with scleroderma, including CTGF and collagen levels (Duncan et al., 1999; Stratton et al., 2001). Furthermore, iloprost reduced TGF-β induction of these proteins in vitro and in vivo, and these effects were dependent on the Iloprost-mediated elevation of cellular cAMP levels that resulted in antagonism of the MEK/ERK signaling cascade, a necessary pathway for induction of the profibrotic protein CTGF (Chen et al., 2002). In addition, other data indicate that the inhibitory effect of cAMP on TGF-β-induced transcription in HaCaT keratinocytes occurs via diminished association of Smad3 with transcription coactivators CBP1 and p300 (Schiller et al., 2003).

In summary, our results demonstrate that cAMP-elevating agents inhibit the profibrotic effects of TGF-β in cardiac fibroblasts largely by inhibiting ERK1/2 activation but also by reducing Smad-mediated recruitment of transcriptional coactivators through the activation of CREB. cAMP may further oppose fibrotic responses via inhibition of JNK. Because of the effects on multiple signaling pathways that regulate collagen gene expression and probably also myofibroblast differentiation, augmenting cAMP generation may be useful for attenuating the development or progression of fibrosis in the heart and perhaps other organs.

Acknowledgments

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Address correspondence to: Dr. Rennolds S. Ostrom, Department of Pharmacology, University of Tennessee Health Science Center, 874 Union Ave., Crowe 115, Memphis, TN 38163. E-mail: rostrom@utmem.edu