Regulation of connective tissue growth factor expression by prostaglandin E₂

DENNIS A. RICUPERO, DAVID C. RISHIKOF, PING-PING KUANG, CHRISTINE F. POLIKS, AND RONALD H. GOLDSTEIN
Pulmonary Center and Department of Biochemistry, Boston University School of Medicine, Boston 02118-2394; and Boston Veterans Affairs Medical Center, Boston, Massachusetts 02130

Ricupero, Dennis A., David C. Rishikof, Ping-Ping Kuang, Christine F. Poliks, and Ronald H. Goldstein.

Regulation of connective tissue growth factor expression by prostaglandin E₂. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L1165–L1171, 1999.—Transforming growth factor-β (TGF-β) stimulates α₁(I) collagen mRNA synthesis in human lung fibroblasts through a mechanism that is partially sensitive to cycloheximide and that may involve synthesis of connective tissue growth factor (CTGF). Northern blot analyses indicate that TGF-β stimulates time- and dose-dependent increases in CTGF mRNA. In TGF-β-stimulated fibroblasts, maximal levels of CTGF mRNA (3.7-fold above baseline) occur at 6 h. The TGF-β-stimulated increase in CTGF mRNA was not blocked by cycloheximide. Nuclear run-on analysis indicates that TGF-β increases the CTGF transcription rate. The TGF-β-stimulated increases in CTGF transcription and steady-state levels of CTGF mRNA are attenuated in prostaglandin E₂ (PGE₂)-treated fibroblasts. PGE₂ fails to attenuate luciferase activity induced by TGF-β in fibroblasts transfected with the TGF-β-responsive luciferase reporter construct p3TP-LUX. In amino acid-deprived fibroblasts, PGE₂ and insulin regulate α₁(I) collagen mRNA levels without affecting CTGF mRNA levels. The data suggest that the regulation of α₁(I) collagen mRNA levels by TGF-β and PGE₂ may function through both CTGF-dependent and CTGF-independent mechanisms.

transforming growth factor-β; insulin; amino acid deficiency; type I collagen; human lung fibroblast

In human lung fibroblasts, transforming growth factor-β (TGF-β) stimulates an increase in α₁(I) collagen mRNA (10) that is attenuated by prostaglandin E₂ (PGE₂) and forskolin (9, 11). This rise in α₁(I) collagen mRNA is due to increased transcription and increased mRNA stability (50, 53). Investigations into the mechanism by which TGF-β stimulates collagen synthesis indicate that TGF-β induces the synthesis of an autocrine factor that is directly responsible for activating α₁(I) collagen transcription (41). This putative autocrine factor has been tentatively identified as connective tissue growth factor (CTGF) (27, 51).

CTGF, a member of the CCN (CTGF, Cyr61/Cef10, Nov) family, is a cysteine-rich, heparin-binding, 349-amino acid polypeptide (2). Other members of the CCN family include the serum-induced immediate-early genes cyr61 and Fisp12/Big2M (4, 46), a v-src-induced peptide (Cef10) (49), a putative protooncogene (nov) (30), and a potential tumor suppressor (Elm1) (23). The high degree of amino acid homology (50–90%) among CCN members is distinguished by conservation of 38 cysteine residues. In addition, all CCN proteins possess a secretory signal peptide and four distinct protein modules: an insulin-like growth factor binding domain, a von Willebrand factor type C repeat, a thrombospondin type 1 repeat, and a COOH-terminal module (1).

TGF-β but not epidermal growth factor, fibroblast growth factor, or platelet-derived growth factor stimulates CTGF transcription in NRK fibroblasts (35). In adult mammals, CTGF is expressed in high levels during wound repair and at sites of connective tissue formation in a variety of fibrotic disorders (15, 25, 26, 29, 46). Recombinant CTGF stimulates fibroblast proliferation and extracellular matrix protein synthesis (14, 35).

In these studies, we examined the effects and interactions of TGF-β and PGE₂ on the steady-state levels of CTGF and α₁(I) collagen mRNA. We report that PGE₂ inhibits transcription of the CTGF gene. TGF-β and PGE₂ appear to affect steady-state levels of α₁(I) collagen mRNA via CTGF-dependent and -independent mechanisms.

METHODS

Tissue culture. Human embryonic lung fibroblasts (IMR-90, Institute for Medical Research, Camden, NJ) were grown in Dulbecco’s modified Eagle’s medium supplemented with 0.37 g sodium bicarbonate/100 ml, 10% (vol/vol) fetal bovine serum, 100 U penicillin/ml, 10 µg streptomycin/ml, 0.1 mM pyruvate, and 0.1 mM nonessential amino acids. After confluence, the serum content of the medium was reduced to 0.4% fetal bovine serum for 24 h. Cell numbers were determined by triplicate cell counts with an electronic particle counter (Coulter Counter ZM).

RNA isolation and Northern analysis. Total cellular RNA was isolated with the single-step method employing guanidine thiocyanate-phenol-chloroform extraction as described by Chomczynski and Sacchi (6). RNA was quantified by absorbance at 260 nm. Purity was determined by absorbance at 280 and 310 nm. RNA (10 µg) was electrophoresed through a 1% agarose-6% formaldehyde gel and transferred to a nitrocellulose filter. RNA loading was assessed by ethidium bromide staining of ribosomal bands and by cohybridization with Gs, a constitutively expressed mRNA that codes for a GTP binding protein (31), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Hybridization was performed with 0.5–1.0 × 10⁶ counts·min⁻¹·lane⁻¹·labeled probe (specific activity, 4–10 × 10⁶ counts·min⁻¹·µg⁻¹), and the filter was washed according to methods previously described (13). The filter was exposed to X-ray film for autoradiography at several different times to ensure that the bands could be quantified by densitometry within the linear range. The α₁(I)
collagen probe came from a rat α1(I) collagen cDNA that specifically binds human α1(I) collagen mRNA (16). A nuclear run-on assay. Confluent, quiescent IMR-90 fibroblast cultures in 150-mm dishes were washed twice with Puck's saline and scraped into a Nonidet P-40 lysis buffer. After two low-speed spins, the pellet was reconstituted in a glycerol buffer. In vitro labeling of nascent RNA and hybridization with cDNA immobilized on nitrocellulose filters were performed according to the methods previously reported (19, 21). No hybridization occurred to filters containing plasmids without inserts.

Western blotting. PAGE was performed under reducing conditions with 7.5% polyacrylamide minigels as previously described (33). Samples (100 μg) for SDS-PAGE and Western blotting were prepared from the cell layer of quiescent confluent IMR-90 fibroblasts grown in 35-cm tissue culture dishes. The cells were stimulated with 1 ng/ml of TGF-β and harvested after 6 h. The cell layer was dissolved in radiomunoprecipitation assay buffer at 4°C and centrifuged 14,000 g for 10 min. Proteins were transferred to nitrocellulose membranes, blocked for 2 h at room temperature with 10% evaporated milk in phosphate-buffered saline with 0.1% Tween (33), and incubated with a 1:1,000 dilution of rabbit anti-CTGF (247–260) peptide antiserum overnight at 4°C. Anti-CTGF antibody was generously provided by David R. Brigstock (Wexner Institute for Pediatric Research, Columbus, OH).

Statistics. Student's t-test was used for means of unequal size. Probability values < 0.05 were considered significant.

RESULTS

In TGF-β-stimulated fibroblasts, increases in α1(I) collagen mRNA were detected after ~4–6 h, with maximal α1(I) collagen mRNA levels detected after 18 h (12). The time-course studies of TGF-β-stimulated increases in CTGF mRNA indicated that increases in CTGF mRNA preceded increases in α1(I) collagen mRNA. Increased CTGF mRNA appears after 2 h, with a peak response (3.7-fold above baseline) appearing 6 h after TGF-β stimulation (P < 0.05; n = 3 experiments; Fig. 1A). In contrast, insulin induces an increase in α1(I) collagen mRNA (18) without increasing CTGF mRNA levels (Fig. 1B). It appears that increases in α1(I) collagen mRNA may occur through CTGF-dependent and CTGF-independent pathways.

TGF-β (at concentrations of 0.5 ng/ml or greater) stimulates an increase in α1(I) collagen mRNA and protein (12). Dose-response studies indicate that comparable concentrations of TGF-β are required to stimulate increases in CTGF mRNA levels (Fig. 2). The TGF-β-stimulated increases in α1(I) collagen mRNA are composed of cycloheximide-sensitive and cycloheximide-insensitive components (37). As expected of a TGF-β-induced immediate-early gene, increases in CTGF mRNA were insensitive to cycloheximide (5 μM; Fig. 3A). At 4 h, cycloheximide alone did not affect CTGF mRNA levels or the TGF-β-induced increase in CTGF mRNA (Fig. 3B).

Fine and Goldstein (8) have previously shown that activation of protein kinase A (PKA) by PGE2 attenuates the TGF-β-stimulated increase in α1(I) collagen mRNA and protein. To demonstrate that PGE2 blocks the TGF-β-stimulated increase in CTGF mRNA levels,

![Fig. 1](http://ajplung.physiology.org/) A: time course for induction of connective tissue growth factor (CTGF) mRNA by transforming growth factor (TGF)-β. Quiescent, confluent fibroblasts were stimulated with 1 ng/ml of TGF-β. Total RNA was isolated at indicated times and resolved, transferred, and probed for CTGF and Gs, a constitutively expressed mRNA that codes for a GTP binding protein, as described in METHODS. B: CTGF mRNA is refractory to insulin stimulation. Quiescent, confluent fibroblasts were stimulated with 1 ng/ml of TGF-β or 2 U/ml of insulin. Total RNA was isolated at 6 h and analyzed by Northern blot with indicated probes. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

we incubated TGF-β-stimulated fibroblasts with varying concentrations of PGE2 (Fig. 4A). The TGF-β-stimulated increase in CTGF mRNA was reduced in fibroblasts treated with 100 nM PGE2 (73% reduction; P < 0.05; n = 3 experiments). PGE2 alone did not alter CTGF mRNA levels.

CTGF is found associated with the cell layer as well as released to the medium (52). We assayed the fibroblast cell layer for CTGF by Western blotting (Fig. 4B). With the use of an antibody directed against amino acids 247–260 of CTGF, a single protein with an apparent molecular mass of 38 kDa was detected in the cell layer from TGF-β-stimulated fibroblasts. A low-

![Fig. 2](http://ajplung.physiology.org/) Induction of CTGF mRNA by various concentrations of TGF-β. Quiescent, confluent fibroblasts were stimulated with indicated concentrations of TGF-β. Total RNA was isolated after 6 h. Expression of CTGF and GAPDH mRNA levels was determined by Northern blot analysis.
intensity band was detected in control fibroblasts and fibroblasts stimulated with both PGE2 and TGF-β.

PGE2 inhibits TGF-β-stimulated increases in α1(I) collagen mRNA through increased adenylate cyclase activity (8). In fibroblasts pretreated with the adenylate cyclase activator forskolin or PGE2, TGF-β failed to increase CTGF mRNA levels (Fig. 5). Furthermore, PGE2 and forskolin did not inhibit the TGF-β-stimulated increase in endogenous TGF-β type I receptor mRNA.

Recent advances in the signal transduction mechanism of TGF-β establish that phosphorylation of Smad2 and Smad3 precedes translocation to the nucleus and activation of gene transcription (24, 39, 43, 44, 55). We used the luciferase reporter construct p3TP-LUX to further assess the repression of PGE2 on TGF-β signaling. p3TP-LUX was engineered for maximal TGF-β responsiveness with three 31-nucleotide activator protein-1 sites concatenated to a region (−636 to −740 bp) of the plasminogen activator inhibitor promoter (54). In fibroblasts transfected with p3TP-LUX, TGF-β stimulated a 17.8-fold increase in luciferase activity above that in control fibroblasts (P < 0.05; n = 3 experiments; Fig. 6A). Preincubation with PGE2 did not affect the TGF-β-induced increase in luciferase activity. When adenylate cyclase was activated directly by forskolin, the results were comparable. In forskolin-treated fibroblasts, the TGF-β-stimulated luciferase activity was not significantly different from the luciferase activity observed in TGF-β-stimulated control fibroblasts (Fig. 6B).

**Fig. 3.** Effect of cycloheximide (CHX) on TGF-β-induced CTGF mRNA. Quiescent, confluent fibroblasts were stimulated with 1 ng/ml of TGF-β or were preincubated with 5 μM CHX for 10 min and then stimulated with 1 ng/ml of TGF-β (CHX + TGF-β). A: total RNA was isolated after 4 h. RNA (10 μg) was resolved electrophoretically and hybridized with probes for CTGF and GAPDH as described in METHODS. Results are representative of 3 independent experiments. B: densitometry analysis. Values are means ± SD of 3 independent experiments. Significant difference (P < 0.05) compared with: *control; **control and CHX. ns, Not significant (P > 0.05) compared with control.

**Fig. 4.** Effect of prostaglandin E2 (PGE2) on TGF-β-stimulated increase in CTGF mRNA and protein levels. A: quiescent, confluent fibroblasts were preincubated with indicated concentrations of PGE2 for 10 min and then stimulated with 1 ng/ml of TGF-β. Total RNA was isolated after 6 h. CTGF and GAPDH mRNA levels were determined by Northern analysis. B: Western blot for CTGF. Quiescent, confluent fibroblasts were maintained in low-serum medium (control), stimulated with 1 ng/ml of TGF-β, or incubated with 100 nM PGE2 and then stimulated with 1 ng/ml of TGF-β (TGF-β + PGE2). After 6 h, cell layer was harvested, and 100 μg of total protein were resolved by PAGE. CTGF was detected with anti-CTGF antibody as described in METHODS.

**Fig. 5.** Effect of PGE2 and forskolin on TGF-β-stimulated increase in steady-state levels of CTGF and TGF-β type I receptor mRNAs. Quiescent, confluent fibroblasts were preincubated with 100 nM PGE2 or 10 μM forskolin for 10 min and then stimulated with 1 ng/ml of TGF-β as indicated. Total RNA was isolated after 6 h. Steady-state levels of CTGF, TGF-β type I receptor (TGF-β R I), and GAPDH mRNAs were determined by Northern analysis.
Nuclear transcriptional run-on assays were performed to determine the mechanism resulting in increased CTGF mRNA. Nuclei were isolated 5 h after TGF-β stimulation in the presence and absence of PGE2. The rates of transcription for the CTGF, α1(I) collagen, TGF-β type I receptor, and GAPDH genes were determined (Fig. 7). We found that TGF-β stimulates transcription of the CTGF gene and that this increase was blocked by PGE2. At this early time point, we did not detect a change in the rate of transcription of α1(I) collagen, TGF-β type I receptor, or GAPDH genes with either TGF-β or PGE2.

Finally, to demonstrate that the PGE2-mediated reduction of α1(I) collagen mRNA is mediated by mechanisms other than those involving CTGF, we used fibroblasts cultured in amino acid-deficient medium. In amino acid-deprived fibroblasts, steady-state levels of α1(I) collagen mRNA were decreased compared with those in fibroblasts maintained in complete medium (38). Furthermore, these fibroblasts responded to insulin, resulting in increased levels of α1(I) collagen mRNA. We found that PGE2 further decreased α1(I) collagen mRNA in fibroblasts incubated in amino acid-deficient medium (Fig. 8). CTGF mRNA was not affected by PGE2 or insulin in amino acid-deprived fibroblasts.

**DISCUSSION**

TGF-β induces the transcription of several immediate-early genes including fibronectin (28), J unB (5, 22, 32, 42), and CTGF (20, 35). In addition, TGF-β treatment of fibroblasts induces an increase in extracellular matrix components, especially type I collagen (28). Depending on the cell type, TGF-β increases α1(I) collagen mRNA levels by increasing the transcription rate or stability of the transcript (50, 53). Our findings suggest that increases in CTGF mRNA precede increases in α1(I) collagen mRNA and that comparable doses of TGF-β stimulate synthesis of CTGF and α1(I) collagen. This is consistent with the hypothesis that CTGF mediates the TGF-β-stimulated increase in α1(I) collagen mRNA.

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**Fig. 6.** Effect of PGE2 and forskolin on TGF-β-stimulated increase in luciferase activity. Fibroblasts were transiently transfected with p3TP-LUX reporter construct with calcium phosphate method. Fibroblasts were incubated with 100 nM PGE2 (A) or 10 µM forskolin (B) for 10 min and then stimulated with 1 ng/ml of TGF-β. Luciferase activity was determined after 18 h as described in METHODS. CPM, counts/min. Values are means ± SD of triplicate values, representative of 3 independent experiments.

**Fig. 7.** Rate of transcription of CTGF gene. Quiescent, confluent fibroblasts were maintained in low-serum medium (control), stimulated with 1 ng/ml of TGF-β, and incubated with 100 nM PGE2 for 10 min or incubated with 100 nM PGE2 and stimulated with 1 ng/ml of TGF-β. After 5 h, nuclei were harvested, and levels of transcription for CTGF, α1(I) collagen, TGF-β R I, and GAPDH genes were assayed.
The regulation of \( \alpha_1(1) \) collagen expression by TGF-\( \beta \) and other effectors is complex (37). Our data indicate that a portion of the TGF-\( \beta \)-stimulated increase in \( \alpha_1(1) \) collagen mRNA expression is not dependent on protein synthesis (independent of CTGF production) (17). In addition, other effectors can increase \( \alpha_1(1) \) collagen mRNA levels through CTGF-independent mechanisms. For example, insulin increases \( \alpha_1(1) \) collagen mRNA levels without affecting CTGF expression in fibroblasts incubated in amino acid-deficient medium.

CTGF is present in abundance during wound healing in many tissues including the lung (15, 25, 29, 40, 46). Recombinant CTGF increases steady-state levels of \( \alpha_1(1) \) collagen mRNA (14). Anti-CTGF antibodies or antisense oligonucleotides directed against CTGF mRNA block TGF-\( \beta \)-mediated anchorage-independent growth (35). In addition, CTGF is subject to proteolytic cleavage to biologically active fragments (3, 52). Full-length CTGF binds to the extracellular matrix and may act as a matrix signaling protein (14, 52). CTGF may function in a similar manner to Cyr61, another member of the CCN family (34, 45). Its function may involve cellular activation as well as interaction with other matrix-associated proteins.

TGF-\( \beta \) increases the steady-state level of CTGF mRNA by activating transcription as assessed by nuclear run-on analysis at 5 h. In contrast, the steady-state level of \( \alpha_1(1) \) collagen mRNA is unaffected by TGF-\( \beta \) at that time. In addition, Northern blot analysis of TGF-\( \beta \)-stimulated fibroblasts demonstrates that CTGF mRNA levels are not affected by protein synthesis inhibition with cycloheximide, whereas \( \alpha_1(1) \) collagen mRNA levels are decreased (17). These results indicate that a portion of the TGF-\( \beta \)-induced increases in \( \alpha_1(1) \) collagen mRNA may be mediated by CTGF by posttranscriptional mechanisms.

PGE\(_2\) inhibits the TGF-\( \beta \)-induced increases in \( \alpha_1(1) \) collagen mRNA levels (8). PGE\(_2\) also inhibits the TGF-\( \beta \)-stimulated increase in CTGF transcription as assessed by nuclear run-on analysis. In contrast, the upregulation of TGF-\( \beta \) type I receptor mRNA by TGF-\( \beta \) is not affected by treatment with PGE\(_2\). Furthermore, PGE\(_2\) fails to inhibit TGF-\( \beta \)-induced stimulation of the reporter construct p3TP-LUX. Our data indicate that PGE\(_2\) inhibits CTGF transcription, perhaps by increasing adenylate cyclase activity. Kothapalli et al. (36) showed that forskolin and cholera toxin blocked increases in CTGF mRNA presumably by increasing adenylate cyclase activity. It is unlikely that the PGE\(_2\)-induced decrease in the CTGF mRNA level is due to phosphorylation of the TGF-\( \beta \) receptor or Smad proteins because PGE\(_2\) did not interfere with TGF-\( \beta \)-stimulated transcription of the TGF-\( \beta \) type I receptor and activity of the p3TP-LUX reporter. Alternatively, high levels of cAMP activate PKA or PKA-activated kinases that may phosphorylate transcription factors to inhibit CTGF transcription. Transcriptional coactivators CBP/p300 interact with activator protein-1 sites through a complex with Smad proteins (Smad3 and/or Smad2 bound to Smad4) (7, 47, 56). CTGF transcription may be inhibited by a mechanism that involves PKA phosphorylation of transcriptional coactivators.

Fibroblasts incubated in amino acid-deficient medium downregulate \( \alpha_1(1) \) collagen mRNA levels (38). PGE\(_2\) further downregulates \( \alpha_1(1) \) collagen mRNA under these culture conditions without affecting the steady-state levels of CTGF mRNA. As noted, insulin increases \( \alpha_1(1) \) collagen mRNA but has no effect on CTGF mRNA in amino acid-deprived fibroblasts. Our data with amino acid-deficient medium suggest that the PGE\(_2\)-induced downregulation and insulin-induced upregulation of \( \alpha_1(1) \) collagen is independent of CTGF mRNA expression.

In summary, \( \alpha_1(1) \) collagen mRNA levels are regulated by multiple mechanisms in TGF-\( \beta \)-stimulated fibroblasts. PGE\(_2\) inhibits the TGF-\( \beta \)-stimulated increases in both CTGF and \( \alpha_1(1) \) collagen mRNA levels. In contrast, PGE\(_2\) decreases \( \alpha_1(1) \) collagen mRNA levels in amino acid-deprived fibroblasts in the presence of sustained CTGF mRNA levels. CTGF expression does not appear to account for the TGF-\( \beta \)-stimulated, cycloheximide-sensitive increase in \( \alpha_1(1) \) collagen mRNA levels. In addition, \( \alpha_1(1) \) collagen mRNA levels may be regulated posttranscriptionally through CTGF-independent mechanisms. Our results suggest that the regulation of \( \alpha_1(1) \) collagen mRNA levels by TGF-\( \beta \) and PGE\(_2\) may function through both CTGF-dependent and CTGF-independent mechanisms and that the antifibrotic effects of PGE\(_2\) involve inhibition of CTGF transcription.

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Address for reprint requests and other correspondence: D. A. Ricupero, The Pulmonary Center, Boston Univ. School of Medicine, 715 Albany St., Boston, MA 02118-2394 (E-mail: ricupero@bu.edu).

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