Smad3 and PKCδ mediate TGF-β1-induced collagen I expression in human mesangial cells

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Runyan, Constance E., H. William Schnaper, and Anne-Christine Poncelet. Smad3 and PKCδ mediate TGF-β1-induced collagen I expression in human mesangial cells. Am J Physiol Renal Physiol 285: F413–F422, 2003. First published May 20, 2003; 10.1152/ajprenal.00082.2003.—Transforming growth factor (TGF-β) has been associated with fibrogenesis in clinical studies and animal models. We previously showed that Smad3 promotes COL1A2 gene activation by TGF-β1, human mesangial cells. In addition to the Smad pathway, it has been suggested that TGF-β1 could also activate more classical growth factor signaling. Here, we report that protein kinase C (PKCδ) plays a role in TGF-β1-stimulated COL1A2 production. In an in vitro kinase assay, TGF-β1 treatment specifically increased mesangial cell PKCδ activity in a time-dependent manner. Translocation to the membrane was detected by immunocytochemistry and immunoblot, suggesting activation of PKCδ by TGF-β1. Inhibition of PKCδ by rottlerin decreased basal and TGF-β1-stimulated COL1A2 promoter activity, whereas blockade of conventional PKCs by Gö 6976 had little or no effect. In a Gal4-LUC assay system, promoter activity, whereas blockade of conventional PKCs by the Smad pathway, it has been suggested that TGF-β1 could also activate more classical growth factor signaling. Here, we report that protein kinase C (PKCδ) plays a role in TGF-β1-stimulated collagen I production. In an in vitro kinase assay, TGF-β1 treatment specifically increased mesangial cell PKCδ activity in a time-dependent manner. Translocation to the membrane was detected by immunocytochemistry and immunoblot, suggesting activation of PKCδ by TGF-β1. Inhibition of PKCδ by rottlerin decreased basal and TGF-β1-stimulated COL1A2 promoter activity, whereas blockade of conventional PKCs by Gö 6976 had little or no effect. In a Gal4-LUC assay system, inhibition of PKCδ abolished TGF-β1-induced transcriptional activity of Gal4-Smad3 and Gal4-Smad4 (266–552). Overexpression of Smad3 or Smad3D, in which the three COOH-terminal serine phosphoacceptor residues have been mutated, increased activity of the SBE-LUC construct, containing four DNA binding sites for Smad3 and Smad4. This induction was blocked by PKCδ inhibition, suggesting that rottlerin decreased Smad3 transcriptional activity independently of COOH-terminal serine phosphorylation. Blockade of PKCδ abolished ligand-independent and ligand-dependent stimulation of COL1A2 promoter activity by Smad3. These data indicate that PKCδ is activated by TGF-β1 in human mesangial cells. TGF-β1-stimulated PKCδ activity positively regulates Smad transcriptional activity and is required for COL1A2 gene transcription. Thus cross talk among multiple signaling pathways likely contributes to the pathogenesis of glomerular matrix accumulation.

transforming growth factor-β signal transduction; cross talk; gene regulation; extracellular matrix accumulation; glomerulosclerosis

Clinical studies have associated transforming growth factor (TGF)-β production with glomerular matrix accumulation in diabetic nephropathy, focal segmental glomerulosclerosis, lupus nephritis, and IgA nephropathy (5). Transgenic mice with increased levels of TGF-β1 develop progressive renal disease (26). In addition, TGF-β has been shown to mediate fibrogenesis in experimental models of glomerulonephritis and diabetic nephropathy (5, 45). Extracellular matrix (ECM) accumulation in experimental glomerulonephritis induced by anti-thymocyte serum is suppressed by administration of anti-TGF-β antibody (7); by the natural inhibitor of TGF-β, decorin (6); or by TGF-β antisense oligonucleotides (1). In vitro, we and others showed that TGF-β induces type I and type IV collagen and fibronectin synthesis by human mesangial cells (16, 40). Because many fibrogenic stimuli, including stretch, high glucose, platelet-activating factor, and angiotensin II, may induce TGF-β1 expression or activation (14, 24, 42, 43), TGF-β1 action could represent a common pathway mediating glomerulosclerosis. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
tivation. The conventional PKCs (cPKCs), including PKCa, PKCβI, PKCβII, and PKCγ, depend on calcium and phospholipids. The novel PKCs, PKCδ, PKCe, PKCζ, and PKCd, do not require Ca2+ but are phospholipid dependent. The atypical enzymes, PKCζ and PKCd, require neither Ca2+ nor phospholipid. PKC isoenzymes are expressed in a tissue-specific fashion and their subcellular localization varies depending on the cell type (22).

Several studies have implicated TGF-β1 and PKC as mediators of ECM accumulation in diabetic animal models and in mesangial cells cultured in high glucose. In streptozotocin-induced diabetic rats, a model for type 1 diabetes, administration of LY333531, a PKCβ inhibitor, prevented increased expression of mRNA for TGF-β1, fibronectin, and type IV collagen (28). In db/db mice, a model for type 2 diabetes, the same inhibitor prevented ECM expansion (27). PKC is involved in hyperglycemia-stimulated TGF-β, promoter activity in mesangial cells (51). This could be the mechanism leading to increased TGF-β mRNA expression and protein synthesis that have been observed in murine mesangial cells cultured in high glucose. Thus high glucose could mediate its effect through PKC-induced TGF-β1 activation leading to increased ECM production (56).

Conversely, even without high concentration of glucose, TGF-β1 could exert its effect on expression of some of the ECM components through PKC activation. Halstead et al. (13) showed that treatment of a human carcinoma cell line with the PKC inhibitor calphostin C blocked TGF-β1-induced increases in plasminogen activator inhibitor-1 (PAI-1) and fibronectin mRNA expression. More recently, it has been suggested that stabilization of elastin mRNA in lung fibroblasts by TGF-β requires Smads, PKCδ, and the MAP kinase β38. These data suggest potential synergy between classical TGF-β and PKC signaling cascades. In support of this notion, Yakymovych et al. (53) recently showed that Smad2 and Smad3, the targets of TGF-β receptors, can be phosphorylated in their MH1 domain by PKC.

We previously showed that TGF-β1-induced collagen I gene expression is Smad3 dependent in human mesangial cells. Here, we investigated whether PKC might be involved in collagen I accumulation in response to TGF-β1. With the use of specific PKC isotype inhibitors, we showed that PKCδ, but not PKCa or PKCβI, mediates collagen I production by TGF-β1 in human mesangial cells. We demonstrated that TGF-β1 activates PKCδ in these cells and that this activation plays a role in TGF-β1-stimulated Smad transcriptional activity and collagen I gene transcription.

MATERIALS AND METHODS

Materials. Reagents were purchased from the following vendors: active human recombinant TGF-β1 from R&D Systems (Minneapolis, MN); rabbit anti-type I collagen from Biodesign (Saco, ME); rabbit anti-PKCδ, rabbit anti-PKCε, mouse monoclonal anti-Smad4 IgG (B-8), mouse monoclonal anti-Smad1/2/3 (H-2), and anti-mouse IgG-horseradish peroxidase (HRP) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-rabbit IgG-HRP, luciferase, and β-galactosidase assay systems from Promega (Madison, WI); PMA from Sigma (St. Louis, MO); and calphostin C, rotterlin, and Gö 6976 from Calbiochem (San Diego, CA). Stock solutions were made as follows: TGF-β1 in 4 mM HCl containing 1 mg/ml BSA; PKC inhibitors in DMSO.

Cell culture. Human mesangial cells were isolated from glomeruli by differential sieving of minced normal human renal cortex obtained from anonymous surgery or autopsy specimens. The cells were grown in DMEM/Ham’s F-12 medium, supplemented with 20% heat-inactivated FBS, glutamine, penicillin/streptomycin, sodium pyruvate, HEPES buffer, and 8 μg/ml insulin (Invitrogen Life Technologies, Carlsbad, CA) as previously described (44), and were used between passages 5 and 8.

Protein kinase assay. Cells were switched to medium containing 1% FBS and then treated with 1 ng/ml TGF-β1 for various time periods leading up to simultaneous harvest in RIPA buffer (50 mM Tris·HCl, pH 7.5; 150 mM NaCl; 1% Nonidet P-40; 0.5% deoxycholate; 0.1% SDS) containing protease inhibitors (1 mM PMSF, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin). After clarification by centrifugation, the protein content was determined by Bradford protein assay (BioRad, Hercules, CA). Immunoprecipitation was performed with 2 μg anti-PKCε or anti-PKCδ antibody and 30 μl protein G- sepharose for 1 h at 4°C. Immunocomplexes were incubated for 10 min at room temperature with a PKCε substrate peptide (which can be phosphorylated by both PKCε and PKCδ) (Upstate, Waltham, MA) and [γ32P] ATP in 10 mM HEPES, pH 7.0; 10 mM DTT; and 10 mM MgCl2. The reactions were then spotted onto P81 phosphocellulose paper and washed four times with 1% phosphoric acid and once with acetone. The amount of incorporated radioactivity into the substrate was determined by scintillation counting.

Immunocytochemistry. Mesangial cells were grown to 60% confluence on eight-well culture slides coated with 1 mg/ml gelatin. The cells were switched to medium containing 1% FBS and then treated with 1 ng/ml TGF-β1 for different durations before simultaneous formalin fixation and permeabilization with Triton X-100. The cells were then stained with 1 μg/ml anti-PKC antibody according to the manufacturer’s instructions. The staining was detected with a rhodamine Green 514-conjugated secondary antibodies from Molecular Probes (Eugene, OR) and evaluated under a fluorescent microscope.

Preparation of cell lysates and Western blot analysis. Cells were switched to medium containing 1% FBS and pretreated for 1 h with calphostin C (100 nM), rottlerin (5 μM), Gö 6976 (10 nM), or DMSO as vehicle control. The cells were then incubated for 24 h with 1 ng/ml TGF-β1, followed by lysis at 4°C in RIPA or lysis buffer (10 mM Tris·HCl, pH 8.0; 150 mM NaCl; 1% Nonidet P-40) containing protease and phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM sodium fluoride, 40 mM β-glycerophosphate). Lysates were clarified by centrifugation at 18,000 g for 10 min. Proteins were separated by SDS-PAGE (6 or 10% acrylamide gels), transferred onto a PVDF membrane (Millipore, Bedford, MA), and immunoblotted with anti-type I collagen, anti-Smad1/2/3, or anti-Smad4 antibody (0.2 μg/ml). The blots were developed with chemiluminescence reagents according to the manufacturer’s protocol (Santa Cruz Biotechnology). Autoradiograms were scanned with an Arcus II Scanner (AGFA) in transparency mode and densitometric analysis was performed using the National Institutes of Health Image 1.61 program for Macintosh.
**Cell fractionation.** Cells were scraped into a detergent-free buffer (20 mM Tris·HCl, pH 7.5; 0.5 mM EDTA; 0.5 mM EGTA; 10 mM β-mercaptoethanol) containing protease and phosphatase inhibitors. The cells were then disrupted by 15 strokes of a Dounce homogenizer. After centrifugation at 100,000 g, the supernatant, representing the cytosolic fraction, was saved; the pellet, representing the particulate fraction, was resuspended in buffer; and Triton X-100 was added to be 0.5% final concentration. After 30-min incubation on ice, the pellet was centrifuged at 18,000 g for 10 min to remove insoluble material. The supernatant was saved as the soluble membrane fraction. After determination of the protein content, each fraction was analyzed by immunoblotting with anti-PKCα antibody (0.2 μg/ml) as described above.

**RNA isolation and Northern blot.** Cells were plated in 100-mm culture dishes. Three days later, the cells were switched to medium containing 1% FBS. They were preincubated with PKC inhibitors for 1 h before addition of 1 ng/ml TGF-β1 or control vehicle for 24 h. Total RNA was harvested using Trizol (Invitrogen Life Technologies) and analyzed by Northern blot as described previously (40). The Southern blots were successively rehybridized with additional probes after confirmation of complete stripping. cDNAs for human Smad3 variants (32) were kindly provided by Drs. H. F. Lodish and X. Liu. The Gal4-Smad constructs (10) were kindly provided by Dr. B. Vogelstein. The vectors expressing the indicated Smad3 variants were used with a bovine cDNA for 28S ribosomal RNA provided by Dr. H. Sage. The SBE-LUC (54) reporter construct was kindly provided by Dr. Y. Yamada. Quantiﬁcation of the bands on autoradiograms was performed using densitometric analysis. The signals obtained after hybridization with these probes were corrected for loading using the signal obtained with a control probe for 28S ribosomal RNA.

**Plasmid constructs.** The 376COL1A2-LUC construct containing the sequence 376 bp of the α1(I) collagen (COL1A2) promoter and 58 bp of the transcribed sequence fused to the luciferase (LUC) reporter gene was previously described (39). Luciferase and α-smooth muscle actin (α-SM-actin) expression were normalized for loading using the signals obtained with anti-type I collagen (Col I) or anti-Smad4 antibodies. Calphostin C and rottlerin decreased TGF-β1-induced collagen I expression, without affecting Smad4 or Sp1 expression levels (Fig. 1 and data not shown). The inhibitory effect of rottlerin on TGF-β1 induction was dose dependent (Fig. 2). In contrast, specific inhibition of cPKCs with Go6976 did not affect TGF-β1-induced collagen I production. Rottlerin has been reported to inhibit PKCθ activity in vitro (50); however, a concentration over 30 μM was necessary to achieve 50% inhibition, six times higher than the concentration used to inhibit PKCθ. Moreover, while PKCθ is expressed in human mesangial cells, TGF-β1-stimulated collagen expression was not affected by Go6976.

**Statistical analysis.** Statistical differences between experimental groups were determined by analysis of variance using StatView 4.02 software program for Macintosh. Values of P < 0.05 by Fisher’s protected least significant difference (PLSD) were considered significant. Difference between two comparative groups was further analyzed by unpaired Student’s t-test.

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**RESULTS**

**Role of PKC in collagen I expression.** To determine whether PKCs play a role in TGF-β1-stimulated collagen I expression, mesangial cells were pretreated for 1 h with PKC inhibitors before addition of 1 ng/ml TGF-β1 for 24 h. The PKC inhibitors examined were calphostin C, a general inhibitor of PKCs that competes at the binding site for diacylglycerol and phorbol esters (47); rottlerin, an inhibitor of PKCθ (12); and Go6976, an inhibitor of Ca2+-dependent PKCs and PKCβ1 isozymes (33). Cell lysates were harvested and examined by immunoblotting with anti-type I collagen and anti-Smad4 antibodies. Calphostin C and rottlerin decreased TGF-β1-induced collagen I expression, without affecting Smad4 or Sp1 expression levels (Fig. 1 and data not shown). The inhibitory effect of rottlerin on TGF-β1 induction was dose dependent (Fig. 2). In contrast, specific inhibition of cPKCs with Go6976 did not affect TGF-β1-induced collagen I production. Rottlerin has been reported to inhibit PKCθ activity in vitro (50); however, a concentration over 30 μM was necessary to achieve 50% inhibition, six times higher than the concentration used to inhibit PKCθ. Moreover, while PKCθ is expressed in human mesangial cells, TGF-β1-stimulated collagen expression was not affected by Go6976.

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**Fig. 1.** Inhibition of protein kinase C (PKCθ), but not conventional PKCs, blocks transforming growth factor (TGF)-β1-stimulated collagen I production. Human mesangial cells in 1% FBS-containing medium were pretreated for 1 h with the indicated PKC inhibitors (calphc, calphostin C; rottl, rottlerin; G0, Go6976) or DMSO as a control vehicle (−). TGF-β1 (1 ng/ml) or control vehicle (1 ng/ml BSA in 4 mM HCl) was then added for 24 h. Cell lysates were harvested and analyzed by Western blotting with anti-collagen I (Col I) or anti-Smad4 antibody. A: representative blot. B: densitometric analysis of several independent experiments. *P < 0.02 compared with TGF-β1-treated cells in the absence of PKC inhibitor (unpaired Student’s t-test, n ≥ 3).
cells, we were not able to detect PKCα in these cells by immunoblot (Table 1). Thus together, these data support a role for PKCδ in TGF-β1-stimulated collagen I production.

Activation of PKCδ by TGF-β1. Inhibition of TGF-β1-stimulated collagen I expression by blockade of PKCδ suggested that TGF-β1 could activate PKCδ. Thus we examined the timing of PKCδ activation by TGF-β1 in human mesangial cells. Cells were treated with 1 ng/ml TGF-β1 for different time periods leading up to simultaneous harvest. Lysates were immunoprecipitated with an anti-PKCδ or -PKCe antibody. Immunocomplexes were used for an in vitro kinase assay. TGF-β1 stimulates PKCδ in a time-dependent manner (Fig. 3). PKCδ activity began to increase 5 min after adding TGF-β1, although not significantly. Maximal activity was detected at 60 min and increased activity was sustained for up to 24 h. In contrast, PKCe activity was not affected by TGF-β1 treatment. Incubation for 15 min with 100 nM PMA was used as a positive control for PKC activation (not shown).

Next, we examined whether increased in vitro PKCδ kinase activity correlates with changes in cellular localization. PKCδ translocates to the plasma membrane with TGF-β1 treatment in a time-dependent manner (Fig. 4). At 5 min, staining at the cell periphery slightly increased, whereas staining decreased in the nucleus.

Table 1. Analysis of human mesangial cells for PKC isozyme expression

<table>
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<th>α</th>
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PKC, protein kinase C; ND, not determined; +, detected; -, not detected.

Staining at the plasma membrane was more apparent at 60 min, when PKCδ activity was more robust (see Fig. 3). Membrane localization remained elevated for up to 24 h of treatment. Nuclear staining began increasing at 15 min and remained elevated for up to 24 h. Translocation to the cell periphery in response to TGF-β1 was also demonstrated by immunocytochemistry studies on mesangial cells transfected with a green fluorescent protein-PKCδ construct (data not shown). In contrast to PKCδ, PKCe did not translocate to the plasma membrane in response to TGF-β1, whereas translocation of both isozymes was detected following 15 min of treatment with PMA (Fig. 4). Because increased PKCδ activity and membrane association between 5 and 30 min were subtle, we sought to further analyze early PKCδ activation. We performed Western blot analysis of cells separated into membrane and cytosolic fractions. An increase in membrane-associated PKCδ was detectable as early as 5 min after adding TGF-β1 (Fig. 5), corresponding to the low levels of increased activity shown in Fig. 3. Together, these data suggest that PKCδ, but not PKCe, is activated in human mesangial cells in response to TGF-β1.

TGF-β1-induced PKCδ activity modulates collagen I gene expression. Because PKCδ is activated by TGF-β1, and inhibition of PKCδ blocked TGF-β1-stimulated collagen I production, we investigated whether PKCδ modulates collagen I gene expression. Cells were pretreated for 1 h with PKC inhibitors before addition of 1 ng/ml TGF-β1 for 24 h. Steady-state mRNA levels for α1(I) collagen (COL1A1) and α2(I) collagen (COL1A2) were measured by Northern blot. As shown in Fig. 6, both calphostin C and rottlerin inhibited TGF-β1-induced COL1A1 and COL1A2 mRNA expression. Rottlerin also decreased basal collagen I mRNA levels in agreement with its effect on basal protein synthesis (see Fig. 1). In contrast, the cPKC inhibitor Gö 6976
were pretreated with collagen I expression by decreasing Smad3 levels, cells were cotransfected with SBE-LUC and an empty vector (pEXL) or a construct expressing wild-type Smad3 (Flag-N-Smad3) (32) or an empty vector (pEXL). To determine whether PKCδ inhibition impaired TGF-β1-induced collagen I expression by decreasing Smad3 levels, cells were pretreated with 1 μM PMA for 24 h to deplete cellular PKCs. The cells were then transfected with 376COL1A-LUC and a construct expressing wild-type Smad3 (Flag-N-Smad3) (32) or an empty vector (pEXL) before treatment with TGF-β1. Similar to the effect of incubation with rottlerin, prolonged exposure to PMA not only decreased basal COL1A2 promoter activity but also completely blocked the promoter induction by TGF-β1 (Fig. 8A). Overexpression of Smad3 restored the response to TGF-β1. Figure 8B shows a Western blot analysis indicating that chronic PMA treatment led to PKCδ downregulation and decreased Smad3 protein levels. Together, these data suggest that the inhibitory effect of PKCδ blockade or depletion on COL1A2 transcription could be partially due to the downregulation of Smad3 expression.

Because it has been shown that PMA-activated PKC modulates Smad3 DNA binding activity (53), we investigated whether rottlerin decreases Smad protein transcriptional activity as well as decreasing Smad3 levels. Cells were cotransfected with a reporter construct containing five Gal4 binding sites in front of the luciferase gene and a construct expressing the Gal4 DNA binding domain fused to either full-length Smad3 (Gal4-Smad3) or Smad4 (266–552) [Gal4-Smad4(ΔN)]. These constructs enable us to study the transcriptional activity of Smads independently of their DNA binding activity. We previously showed that both constructs are responsive to TGF-β1 in mesangial cells (41). The cells were pretreated for 1 h with the PKCδ inhibitor and then incubated with TGF-β1 for 24 h. Rottlerin blocked TGF-β1-stimulated Smad3 transcriptional activity (Fig. 9). Smad4 transcriptional activity was also decreased by rottlerin pretreatment. These data suggest that Smad activity is modulated by PKCδ signaling in response to TGF-β1. To further support these data, we evaluated the effect of PKCδ inhibition on the activity of the SBE-LUC reporter construct containing four copies of the CTCTAGAC sequence that has been shown to bind recombinant Smad3 and Smad4 (54). Cells were cotransfected with SBE-LUC and an empty vector (pEXL) or a construct expressing wild-type Smad3. The cells were pretreated with rottlerin for 1 h and then incubated with TGF-β1 for 24 h. As expected, TGF-β1 stimulated the activity of the SBE-LUC reporter indicating activation of Smad proteins (Fig. 10, pEXL histograms). TGF-β1-induced activity of endogenous Smads was decreased by rottlerin pretreatment. Moreover, blockade of PKCδ almost completely inhibited transcriptional activity of overexpressed Smad3. These results further confirm that blockade of PKCδ

Fig. 5. Timing of increased PKCδ activity correlates with membrane translocation. Cells treated with TGF-β1 for different time periods were fractionated into cytosolic and membrane fractions before analysis by Western blot using anti-PKCδ antibody. A representative blot from 3 separate experiments is shown.
inhibits Smad3 activity. To investigate whether PKCα modulation of Smad3 activity is dependent on phosphorylation at the TβRI-specific target site of Smad3, mesangial cells were cotransfected with a mutated Smad3 construct (Smad3D) in which the three COOH-terminal serine residues are replaced by three aspartic acid residues (32). Inhibition of PKCα drastically decreased luciferase activity induced by Smad3D. These data suggest that the inhibitory effect of rottlerin on Smad3 transcriptional activity is not due to blocking of phosphorylation at the COOH-terminal TβRI target site. Of note, Smad3D can function as a transcriptional activator in the absence of TGF-β1 as previously demonstrated (32).

Finally, we investigated whether the inhibitory effect of rottlerin on Smad activity modulates COL1A2 promoter activity. Mesangial cells were cotransfected with the 376COL1A2-LUC and an expression vector for Smad3, Smad3D, or the corresponding empty vector. One hour after adding rottlerin or control vehicle, the cells were incubated with TGF-β1 for 24 h. Similar to the results obtained with the SBE-LUC reporter construct, inhibition of PKCα partially blocked ligand-independent and ligand-dependent, Smad3-mediated COL1A2 promoter activity (Fig. 11). This inhibition did not require phosphorylation at the COOH-terminal SSXS phosphorylation site of Smad3. Taken together, these results suggest that TGF-β1-induced PKCα activity contributes to increased COL1A2 gene transcription by modulating Smad3 expression and transcriptional activity.

DISCUSSION

In the present paper, we showed that TGF-β1 stimulates PKCα activity in human mesangial cells and that PKCα mediates TGF-β1-induced collagen I expression.
sion, probably by modulating Smad transcriptional activity.

With the use of an in vitro kinase assay, immunocytochemistry, and immunoblotting on cytosol/membrane fractions, we demonstrated that, in human mesangial cells, PKC\(_{\text{H9254}}\) is activated by TGF-\(\beta_1\) in a time-dependent manner, beginning at 5 min with maximal activation at 60 min. Similarly, Perillan and colleagues (37) showed that TGF-\(\beta_1\) causes PKC\(_{\text{H9254}}\) translocation to the membrane in rat-reactive astrocytes. In contrast, Studer et al. (46) suggested that TGF-\(\beta\) does not stimulate PKC\(_{\text{H9254}}\) in rat mesangial cells, whereas Uchiyama-Tanaka et al. (48) showed a rapid and transient stimulation in murine mesangial cells. However, it has been reported that TGF-\(\beta\) variably affects vascular smooth muscle cell PKC translocation depending on the embryonic lineage (52). Thus discrepancies among these studies could be due to cell- or species-specific responses as well as to culture conditions.

PKC translocation to specific intracellular compartments is variable depending on the isoform, stimulus, and/or cell type (22, 35). In our immunocytochemistry experiments, PKC\(_{\text{H9254}}\) staining increases at the membrane as well as at the nuclear area after 60 min of treatment with TGF-\(\beta_1\).

Fig. 8. Inhibitory effect of PKC downregulation on TGF-\(\beta_1\)-induced COL1A2 promoter activity can be overcome by overexpressing Smad3. To deplete from PKCs, mesangial cells were treated for 18 to 24 h with 1 \(\mu\)M PMA or ethanol as control vehicle. A: cells were then cotransfected with 0.5 \(\mu\)g of 376COL1A2-LUC construct and either 0.5 \(\mu\)g of the vector encoding wild-type Smad3 or the empty expression vector (pEXL), along with 0.5 \(\mu\)g of CMV-\(\beta\)-galactosidase vector. After 3 h, the transfected cells were treated with 1 ng/ml TGF-\(\beta_1\) for 24 h. Luciferase activity was normalized to \(\beta\)-galactosidase activity. Experimental points were performed in triplicate in 2 independent experiments. Values are means \(\pm\ SE\) of triplicate wells from a representative experiment. *\(P < 0.05\). B: cells were treated with TGF-\(\beta_1\) for 30 min and lysates were harvested for immunoblotting. Chronic treatment with PMA decreases Smad3 levels. Blotting with anti-PKC\(_{\text{H9254}}\) antibody confirms downregulation of PKC\(_{\text{H9254}}\) by PMA.
Inhibition of PKCβ by rottlerin blocked TGF-β1-stimulated collagen I production. This is not due to a nonspecific effect of rottlerin on cell activity because levels of Sp1 and Smad4 [2 proteins whose expression is not modulated by TGF-β1, but that are involved in TGF-β1-induced collagen I expression (41)] are not affected by the PKCβ inhibitor. The inhibitory effect of rottlerin on collagen I production is due, at least in part, to inhibition of COL1A1 and COL1A2 gene transcription because rottlerin also inhibited mRNA expression and promoter activity. Taken together with the data showing changes of PKCβ, but not PKCε, translocation and kinase activity, our findings suggest that PKCβ stimulates collagen I expression in response to TGF-β1 in human mesangial cells. Recently, Rosenbloom and colleagues (29, 30) suggested a role for PKCβ in mediating increased fibronectin transcription and elastin mRNA stabilization by TGF-β in human lung fibroblasts. Thus TGF-β could stimulate ECM accumulation in several cell types, in part, by modulating PKCβ activity.

In contrast to rottlerin and low concentration of the general PKC inhibitor calphostin C, Gö 6976, an inhibitor of cPKCs, did not affect collagen I production or COL1A2 promoter activity in response to TGF-β1. However, in some of our experiments, Gö 6976 increased basal collagen I protein and α2(I) mRNA expression. This result suggests that cPKC isoenzymes might inhibit collagen I expression in unstimulated cells.

In our experiments, rottlerin not only blocked TGF-β1 stimulation of COL1A2 transcription but also decreased promoter activity in unstimulated cells. Similarly, a recent report showed blockade of COL1A1 gene transcription by inhibition of PKCβ in sclero-derma fibroblasts (23). These findings suggest that PKCβ is involved in maintaining basal COL1A1 and COL1A2 gene expression as well as playing a role in gene activation by TGF-β1. Because mesangial cells have been shown to produce TGF-β1 (25), it is also possible that the inhibitory effect of rottlerin on basal collagen I expression might be due to blockade of the autocrine/paracrine stimulation by TGF-β1.

To determine the mechanism by which a TGF-β1-induced PKC pathway modulates collagen I expression, we investigated whether PKCβ modulates Smad expression and/or activity. Both depletion of PKC by chronic PMA treatment and inhibition of PKCβ by rottlerin slightly decreased Smad3 protein levels. These data suggest that PKCβ could play a role in maintaining basal Smad3 expression. Downregulation of endogenous Smad3 by rottlerin or chronic PMA correlates with decreased COL1A2 promoter activity and inhibition of the TGF-β1 response. Because the inhibition following PKC depletion by PMA is overcome by ectopic expression of Smad3, this observation suggests that blockade of PKCβ might inhibit TGF-β1-induced collagen I gene transcription, at least in part by decreasing Smad3 expression.

Although the effect of rottlerin on COL1A2 could be partly due to the downregulation of Smad3 expression, our data with the Gal4 assay system and the transfection experiments with overexpressed Smad3 demonstrate that blockade of PKCβ also inhibits Smad3 transcriptional activity. Thus TGF-β1-induced PKCβ activity could stimulate Smad activity, leading to increased COL1A2 gene expression. Because blockade of PKCβ similarly decreased transcriptional activity of Smad3 and Smad3D, PKCβ likely modulates Smad3 activity independently of phosphorylation of the specific TBRI COOH-terminal target site.

With the use of different cell lines (Mv1Lu and NIH-3T3 cells) stably expressing tagged Smad proteins, Yakymovych et al. (53) showed that cell treatment with the PKC activator PMA resulted in phosphorylation of Smad2 and Smad3. The phosphorylation did not affect nuclear translocation but it abrogated direct DNA binding by Smad3. This phosphorylation-dependent mechanism involving PKC could selectively downregulate certain TGF-β signals. In contrast, our data indicate that blocking the PKCβ pathway decreases TGF-β1-induced collagen I production and promoter activity as well as Smad activity, suggesting a positive effect of PKCβ on the TGF-β1 signal leading to collagen I accumulation. Thus the same signaling pathway could inhibit or activate the Smad pathway depending on the cell type and/or stimuli.

In this paper, we showed modulation of the Smad pathway by PKC in response to TGF-β1. Previously, we demonstrated that inhibition of TGF-β1-activated ERK1/2 decreased Smad transcriptional activity (19). In several cell lines, PKC has been linked to ERK activation in response to some stimuli (3, 9, 15, 17, 49, 55). For example, Axmann et al. (3) demonstrated that calcium-dependent PKCs are required for ERK1/2 phosphorylation by TGF-β1 in rat lung fibroblasts. Our
laboratory showed that TGF-β, stimulation of Smad3 phosphorylation outside the COOH-terminus serines is dependent on ERK activation (20). However, this observation may not explain the role of PKC in our system because the requirement of PKC for ERK activation is dependent on the cell types and stimuli. For example, in neuronal cells, PKCδ mediates activation of ERK by fibroblast-derived growth factor and nerve growth factor but not by epidermal growth factor (9). Thus TGF-β1-induced PKCδ in human mesangial cells could modulate Smad activity directly and/or be dependent on ERK activation. On the other hand, activation could modulate Smad activity directly and/or be dependent on other signaling pathways. It has been shown that the phosphatidylinositol 3-kinase (PI3K) associates with PKCδ in the erythroblastic leukemia cell line TF-1 stimulated with cytokines (11) and that phosphorylation of PKCδ in response to serum in the human embryonic kidney 293 cells was PI3K dependent (31).

In summary, we showed that, in human mesangial cells, TGF-β1 stimulates PKCδ translocation and activity. PKCδ positively regulates Smad3 and Smad4 transcriptional activity and is required for increased collagen I production, suggesting that activation and interaction of multiple signaling pathways contribute to the pathogenesis of glomerular matrix accumulation.

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DISCLOSURES

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