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 in 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 (PKC)δ 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 collagen I production, mRNA expression, and 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.

transfoming 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 fibrinectin 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.

Memories of the TGF-β superfamily transmit their signal via heteromeric complexes of transmembrane serine/threonine kinases, the type I and type II receptors (TβRI and TβRII). The Smads are a series of proteins that function downstream from the TGF-β family receptors to transduce signal to the nucleus (2, 34, 38). The receptor-regulated or pathway-restricted Smads (R-Smads), Smad2 and Smad3, contain a SSXS phosphorylation site in their COOH-terminal end that is a direct target of TβRI. Upon ligand binding, the R-Smads are phosphorylated and associate with the common partner Smad, Smad4. The resulting heteromultimer translocates to the nucleus where it regulates expression of TGF-β target genes by direct binding to DNA and/or interaction with other transcription factors (2, 34, 38). The inhibitory Smads, Smad6 and Smad7, may participate in a negative feedback loop to control TGF-β responses by competitive interaction with TβRI (18, 21, 34, 36). R-Smad and Smad4 are composed of Mad-homology (MH)1 and MH2 domains separated by a variable linker region. Smad3 and Smad4 can bind directly to DNA through their MH-1 domain (2, 34, 38).

Although most studies of TGF-β signal transduction have focused on Smad activity, the data also suggest a role for more classical growth factor signaling, such as protein kinase C (PKC). The PKC family is composed of at least 11 serine/threonine kinases. These are grouped according to the biochemical requirement for their ac-
tivation. The conventional PKCs (cPKCs), including PKCα, PKCβI, PKCβII, and PKCγ, depend on calcium and phospholipids. The novel PKCs, PKCδ, PKCe, PKCζ, and PKCd, do not require Ca<sup>2+</sup> but are phospholipid dependent. The atypical enzymes, PKCe and PKCd, require neither Ca<sup>2+</sup> 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-β1 promoter activity in mesangial cells (51). This could be the mechanism leading to increased TGF-β1 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 p38. 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 isozyme inhibitors, we showed that PKCδ, but not PKCα 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-Smad2/3 (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, rottlerin, 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, gluta- tmine, 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-PKCδ or anti-PKCε antibody and 30 µl protein G-agarose 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 [γ<sup>32</sup>P] 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 radioactive 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 an AlexaFluor 546-conjugated secondary antibody 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.

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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-PKCβ 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 same blots were successively rehybridized with additional probes after confirmation of complete stripping. cDNAs for human α1(I) [clone Hf677 (8)] and α2(I) collagen [clone Hf1131 (4)] chains were obtained from Dr. Y. Yamada. Quantification of the bands on autoradiograms was performed using densitometric analysis. The signals obtained by hybridization with these probes were corrected for loading using the signal obtained with a bovine cDNA for 28S ribosomal RNA provided by Dr. H. Sage.

Transient transfection and luciferase assay. The day before the transfection, 6.5 to 8 × 10⁴ cells were seeded in six-well plates. Eighteen hours later, cells were switched to 1% FBS medium and transfected with the indicated constructs along with 0.5 µg of CMV-SPORT-β-galactosidase (Gibco BRL) as a control of transfection efficiency. Transfection was performed with the FuGene6 transfection reagent (Roche Applied Science, Indianapolis, IN) as previously described (39). After 3 h, 1 ng/ml TGF-β1 or control vehicle was added to the cells. In some experiments, the transfected cells were pretreated for 1 h with PKC inhibitors before addition of TGF-β1. Twenty-four hours later, the cells were harvested in 300 µl reporter lysis buffer (Promega). Luciferase and β-galactosidase activities were measured as previously described (39). Luciferase assay results were normalized for β-galactosidase activity. Experimental points were performed in triplicates in several independent experiments. One arbitrary unit was set up as the ratio between luciferase and β-galactosidase activities. The promoter-reporter construct and the empty expression vector were cotransfected with the transfection, 6.5 to 8

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 PKCβ (12); and Gö 6976, an inhibitor of Ca²⁺-dependent PKCs and PKCβ I isoforms (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 Gö 6976 did not affect TGF-β1-increased 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 PKCβ. Moreover, while PKCβ is expressed in human mesangial

Fig. 1. Inhibition of protein kinase C (PKCβ), but not conventional PKCs, blocks transforming growth factor (TGF-β1)-increased 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; Gö, Gö 6976) or DMSO as a control vehicle (−). TGF-β1 (1 ng/ml) or control vehicle (1 mg/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).

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adding TGF-β1 (Fig. 3). PKC/H9254 kinase activity correlates with changes in cellular location of PKC activity, but not PKCε, increases in a time-dependent manner following TGF-β1 treatment. Cells were treated with 1 ng/ml TGF-β1 for the indicated time periods, leading to simultaneous harvest. Cell lysates were immunoprecipitated with anti-PKCδ or anti-PKCε antibody. Immunocomplexes were used in an in vitro kinase assay with the PKCδ pseudosubstrate. Results are presented as fold induction over untreated cells. *P < 0.02 compared with untreated cells (unpaired Student’s t-test, n = 3).

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, PKCε 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 PKCε, is activated in human mesangial cells in response to TGF-β1.

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

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PKC, protein kinase C; ND, not determined; +, detected; −, not detected.
slightly increased basal mRNA expression with minimal effect on TGF-β1 fold induction. To further define a role for PKCδ in TGF-β1-induced collagen I gene expression, we performed transient transfection experiments with 376COL1A2-LUC, a construct containing the sequences from -376 to +58 of the human COL1A2 promoter in front of the luciferase reporter gene (39). The transfected cells were pretreated with PKC inhibitors for 1 h. TGF-β1 was then added for 24 h and luciferase activity was determined. Similar to the results with protein and mRNA, calphostin C and rottlerin blocked TGF-β1-induced COL1A2 promoter activity, whereas Gö6976 did not affect the response (Fig. 7). Together, these results suggest that PKCδ plays a role in basal collagen I expression and is necessary for the transcriptional response to TGF-β1.

Regulation of Smad by PKCδ. We previously showed that Smad3 is required for TGF-β1-stimulated COL1A2 gene transcription. Therefore, we investigated whether the inhibitory effect of PKCδ blockade on collagen I expression is due to modulation of Smad3 expression and/or activity. Rottlerin decreased basal expression of Smad3, although not consistently, suggesting the inhibitory effect of rottlerin on collagen I production could, at least in part, be due to decreased Smad3 expression (data not shown). 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. 4. TGF-β1 induces changes in the cellular localization of PKCδ. Cells were treated with 1 ng/ml TGF-β1 or 100 nM PMA for the indicated time periods before fixing and immunostaining with anti-PKCδ or anti-PKCε antibody. Arrows show membrane staining.](Image)

![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.](Image)
inhibits Smad3 activity. To investigate whether PKC\(\delta\) modulation of Smad3 activity is dependent on phosphorylation at the T\(\beta\)RI-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 PKC\(\delta\) 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 T\(\beta\)RI target site. Of note, Smad3D can function as a transcriptional activator in the absence of TGF-\(\beta\) 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-\(\beta\) for 24 h. Similar to the results obtained with the SBE-LUC reporter construct, inhibition of PKC\(\delta\) 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-\(\beta\)\(\beta\)-induced PKC\(\delta\) activity contributes to increased COL1A2 gene transcription by modulating Smad3 expression and transcriptional activity.

**DISCUSSION**

In the present paper, we showed that TGF-\(\beta\) stimulates PKC\(\delta\) activity in human mesangial cells and that PKC\(\delta\) mediates TGF-\(\beta\)\(\beta\)-induced collagen I expres-
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\textsubscript{H9254} is activated by TGF-\textsubscript{H9252} in a time-dependent manner, beginning at 5 min with maximal activation at 60 min. Similarly, Perillan and colleagues (37) showed that TGF-\textsubscript{H9252} causes PKC\textsubscript{H9254} translocation to the membrane in rat-reactive astrocytes. In contrast, Studer et al. (46) suggested that TGF-\textsubscript{H9252} does not stimulate PKC(s) in rat mesangial cells, whereas Uchiiyama-Tanaka et al. (48) showed a rapid and transient stimulation in murine mesangial cells. However, it has been reported that TGF-\textsubscript{H9252} 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\textsubscript{\text{8}} staining increases at the membrane as well as at the nuclear area after 60 min of treatment with TGF-\textsubscript{\text{1}}.

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Inhibition of PKC\( \delta \) by rottlerin blocked TGF-\( \beta_1 \)-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-\( \beta_1 \), but that are involved in TGF-\( \beta_1 \)-induced collagen I expression (41)] are not affected by the PKC\( \delta \) inhibitor. The inhibitory effect of rottlerin on collagen I production is due, at least in part, to inhibition of \( \text{COL1A1} \) and \( \text{COL1A2} \) gene transcription because rottlerin also inhibited mRNA expression and promoter activity. Taken together with the data showing changes of PKC\( \delta \), but not PKC\( \varepsilon \), translocation and kinase activity, our findings suggest that PKC\( \delta \) stimulates collagen I expression in response to TGF-\( \beta_1 \) in human mesangial cells. Recently, Rosenbloom and colleagues (29, 30) suggested a role for PKC\( \delta \) in mediating increased fibronectin transcription and elastin mRNA stabilization by TGF-\( \beta \) in human lung fibroblasts. Thus TGF-\( \beta \) could stimulate ECM accumulation in several cell types, in part, by modulating PKC\( \delta \) activity.

In contrast to rottlerin and low concentration of the general PKC inhibitor calphostin C, Go 6976, an inhibitor of cPKCs, did not affect collagen I production or \( \text{COL1A2} \) promoter activity in response to TGF-\( \beta_1 \). However, in some of our experiments, Go 6976 increased basal collagen I protein and \( \alpha_2(1) \) mRNA expression. This result suggests that cPKC isoenzymes might inhibit collagen I expression in unstimulated cells.

In our experiments, rottlerin not only blocked TGF-\( \beta_1 \) stimulation of \( \text{COL1A2} \) transcription but also decreased promoter activity in unstimulated cells. Similarly, a recent report showed blockade of \( \text{COL1A1} \) gene transcription by inhibition of PKC\( \delta \) in scleroderma fibroblasts (23). These findings suggest that PKC\( \delta \) is involved in maintaining basal \( \text{COL1A1} \) and \( \text{COL1A2} \) gene expression as well as playing a role in gene activation by TGF-\( \beta_1 \). Because mesangial cells have been shown to produce TGF-\( \beta_1 \) (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-\( \beta_1 \).

To determine the mechanism by which a TGF-\( \beta_1 \)-induced PKC pathway modulates collagen I expression, we investigated whether PKC\( \delta \) modulates Smad expression and/or activity. Both depletion of PKC by chronic PMA treatment and inhibition of PKC\( \delta \) by rottlerin slightly decreased Smad3 protein levels. These data suggest that PKC\( \delta \) could play a role in maintaining basal Smad3 expression. Downregulation of endogenous Smad3 by rottlerin or chronic PMA correlates with decreased \( \text{COL1A2} \) promoter activity and inhibition of the TGF-\( \beta_1 \) response. Because the inhibition following PKC depletion by PMA is overcome by ectopic expression of Smad3, this observation suggests that blockade of PKC\( \delta \) might inhibit TGF-\( \beta_1 \)-induced collagen I gene transcription, at least in part, by decreasing Smad3 expression.

Although the effect of rottlerin on \( \text{COL1A2} \) 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 PKC\( \delta \) also inhibits Smad3 transcriptional activity. Thus TGF-\( \beta_1 \)-induced PKC\( \delta \) activity could stimulate Smad activity, leading to increased \( \text{COL1A2} \) gene expression. Because blockade of PKC\( \delta \) similarly decreased transcriptional activity of Smad3 and Smad3D, PKC\( \delta \) likely modulates Smad3 activity independently of phosphorylation of the specific T\( \beta \)RI 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-\( \beta_1 \) signals. In contrast, our data indicate that blocking the PKC\( \delta \) pathway decreases TGF-\( \beta_1 \)-induced collagen I production and promoter activity as well as Smad activity, suggesting a positive effect of PKC\( \delta \) on the TGF-\( \beta_1 \) 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-\( \beta_1 \). Previously, we demonstrated that inhibition of TGF-\( \beta_1 \)-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-\( \beta_1 \) in rat lung fibroblasts. Our
laboratory showed that TGF-β1 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 of PKCδ by TGF-β1 might itself require activation of other signaling pathways. It has been shown that the phoshatidylinositol 3-kinase (PI3K) associates with PKCδ in the erythroleukemia 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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