Pentoxifylline Attenuates Tubulointerstitial Fibrosis by Blocking Smad3/4-Activated Transcription and Profibrogenic Effects of Connective Tissue Growth Factor

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Pentoxifylline (PTX) is a potent inhibitor of connective tissue growth factor (CTGF), but its underlying mechanism is poorly understood. Here, it was demonstrated that PTX inhibited not only TGF-β1–induced CTGF expression but also CTGF-induced collagen I (α1) [Col I (α1)] expression in normal rat kidney fibroblasts (NRK-49F) and α-smooth muscle actin expression in normal rat kidney proximal tubular epithelial cells (NRK-52E). Furthermore, PTX attenuated tubulointerstitial fibrosis, myofibroblasts accumulation, and expression of CTGF and Col I (α1) in unilateral ureteral obstruction kidneys. The mechanism by which PTX reduced CTGF in NRK-49F and NRK-52E was investigated. Activation of Smad3/4 was essential for TGF-β1–induced CTGF transcription, but PTX did not interfere with TGF-β1 signaling to Smad2/3 activation and association with Smad4 and their nuclear translocation. However, PTX was capable of blocking activation of TGF-β1–induced Smad3/4-dependent reporter as well as CTGF promoter, suggesting that PTX affects a factor that acts cooperatively with Smad3/4 to execute transcriptional activation. It was found that PTX increased intracellular cAMP and caused cAMP response element binding protein phosphorylation. The protein kinase A antagonist H89 abolished the inhibitory effect of PTX on Smad3/4-dependent CTGF transcription, whereas dibutyryl cAMP and forskolin recapitulated the inhibitory effect. In conclusion, these results indicate that PTX inhibits CTGF expression by interfering with Smad3/4-dependent CTGF transcription through protein kinase A and blocks the profibrogenic effects of CTGF on renal cells. Because of the dual blockade, PTX potently attenuates the tubulointerstitial fibrosis in unilateral ureteral obstruction kidneys.

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Almost all forms of kidney diseases that progress to end-stage renal failure are characterized by diffuse fibrosis, in which tubulointerstitial fibrosis is considered the most important determinant of progressive renal injury (1). Tubulointerstitial fibrosis is characterized by the accumulation of myofibroblasts and extracellular matrix (ECM) (1). Increasing evidence indicates that TGF-β1 is a key mediator in renal fibrosis (2–4). Therefore, it would be of great importance to elucidate downstream mediators of TGF-β1's profibrogenic effects and to develop new antifibrotic strategies. Connective tissue growth factor (CTGF) has been known to act downstream of TGF-β1 to regulate ECM synthesis (5). CTGF expression is increased in mesangial cells, proximal tubular epithelial cells, and interstitial fibroblasts of fibrotic kidneys (6–12). Neutralization of CTGF by antisense oligodeoxynucleotide significantly attenuates tubulointerstitial fibrosis despite the sustained TGF-β1 level (11,12). These data suggest that CTGF may be a potential target to prevent progressive tubulointerstitial fibrosis.

Pentoxifylline (PTX), a nonselective phosphodiesterase inhibitor, exerts potent inhibitory effects against cell proliferation, inflammation, and ECM accumulation (13–18). There is increasing evidence that PTX markedly reduces proteinuria in patients with membranous nephropathy or diabetes (19–21). PTX can downregulate the gene expression of CTGF and collagen in remnant nephropathy or acute Thy 1 glomerulonephritis (14,15). PTX downregulates CTGF despite sustained TGF-β1 in angiotensin II–stimulated interstitial fibroblasts and mesangial cells (15). Although PTX inhibits TGF-β1–induced collagen expression in human peritoneal mesothelial cells through modulating mitogen-activated protein kinase (MAPK) (22), the detailed mechanism by which PTX downregulates CTGF in renal cells is unknown. Moreover, it remains unclear whether PTX reduces profibrogenic effects of CTGF on interstitial fibroblasts and proximal tubular epithelial cells, which act in concert in the progressive tubulointerstitial fibrosis.

In this study, we investigated the mechanism by which PTX inhibited TGF-β1–induced CTGF in interstitial fibroblasts and proximal tubular epithelial cells. Our results indicate that PTX inhibits not only CTGF expression by interfering with Smad3/4-dependent CTGF transcription through protein kinase A (PKA) but also profibrogenic effects of CTGF on renal cells.

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Because of the dual blockade, PTX potently attenuated tubulointerstitial fibrosis in kidneys of ureteral obstruction.

Materials and Methods

Antibodies and Reagents

PTX, antibodies against α-smooth muscle actin (α-SMA; 1A4 clone), and β-actin were from Sigma (St. Louis, MO). Antibodies against Ser433/435-phosphorylated Smad2/3 (p-Smad2/3), Smad2/3, Smad4, and CTGF were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Ser133-phosphorylated cAMP responsive element binding protein (p-CREB) were from Upstate Biotechnology (Lake Placid, NY). Antibody against ED-1 antigen was from Chemicon (Temecula, CA). Recombinant TGF-β1 was from R&D Systems (Minneapolis, MN). Dibutyryl cAMP (db-cAMP), forskolin (FK), 3-isobutyl-1-methylxanthine (IBMX), and *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline (H89) were from Calbiochem (San Diego, CA). G418, hygromycin B, and doxycycline were from Clontech (Palo Alto, CA).

Plasmids

Mammalian expression vectors for the wild (CTGF/wt) and antisense (AS-CTGF) forms of CTGF were from Dr. M.L. Kuo (National Taiwan University, Taiwan) (23,24). pTRE2-hygromycin-CTGF (pTRE2-CTGF) was generated by subcloning CTGF/wt into the pTRE2-hygromycin (pTRE2) and was provided with pUHD15.1 by Dr. T.S. Jou (National Taiwan University, Taiwan). C-terminally truncated forms of Smad3 (Smad3DN) and Smad4 (Smad4DN) were from Dr. R. Derynck (University of California, San Francisco) (25). Luciferase promoter constructs, pGL3-CTGF that contained a 3.8-kb fragment upstream of CTGF (from −14 to −3873 relative to the transcription start site), and pGL3-CTGF-Δ Smad that contained mutated Smad-binding element were from Dr. D.F. Higgins (University of Pennsylvania, Philadelphia) (10). $(CAGA)_9$-MLP-Luc, a Smad3/4-specific reporter, was from Dr. J.M. Gauthier (Glaxo-Wellcome, France) (26).

Cell Culture and Transfection

Normal rat kidney fibroblasts (NRK-49F) and proximal tubular epithelial cells (NRK-52E) were cultured as stated previously (15). Subconfluent cells were placed in medium with 0.5% FBS (Life Technologies BRL, Rockville, MD) for 24 h before TGF-β1 or PTX treatment. Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) was used for transfection of plasmids. G418 was used to select cell lines after transfection with CTGF/wt, AS-CTGF, and pCDNA3, and hygromycin B was used to select NRK-52E with pTRE2 or pTRE2-CTGF. NRK-52E with pTRE2-CTGF was maintained in medium that contained doxycycline (2 μg/ml) to inhibit CTGF expression.

RNA and Protein Extraction and Northern and Western Blot Analyses

Total RNA isolated for Northern blot analysis was performed as described previously (13). Human type I collagen (α1) [Col I (α1)], rat TGF-β1, CTGF, and glyceraldehyde-3-phosphate dehydrogenase RNA probes were synthesized as described previously (14,15).

Total cellular proteins were extracted by RIPA buffer, and nuclear proteins were obtained using methods as described previously (27). For detection of CTGF secreted into by renal cells, media were concentrated with Centricon-10 (Millipore, Bedford, MA) as described previously (27). Equal amounts of protein were subjected to Western blot analysis (27).

Luciferase Assay

pRL-TK vectors were co-transfected to normalize the experimental firefly reporter activities. Firefly and renilla luciferase activities were quantified with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

Immunoprecipitation

Immunoprecipitation was performed as described previously (16). Briefly, 250 μg of total cellular extracts was immunoprecipitated overnight with 1 μg of anti–p-Smad2/3 followed by precipitation with 20 μl of protein A/G Plus-Agarose (Santa Cruz Biotechnology). The resulting precipitated complexes were separated by SDS-PAGE and analyzed by Western blot analyses.

Experimental Unilateral Ureteral Obstruction

Male Wistar rats that weighed 200 to 220 g were obtained from the Experimental Animal Center in our institute. The animal care and treatment were conducted in accordance with the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health. Under anesthesia, unilateral ureteral obstruction (UUO) was induced by ligation of the left ureter according to the procedure described previously (4). Catheters were placed in internal jugular veins for drug treatments. UUO rats were treated with either PBS ($n = 4$) or PTX (0.1 g/kg per d; $n = 4$) from day 0 to day 6. Sham operation (SHM) was performed in four age-matched rats. All rats were killed at day 7. UUO and SHM kidneys were divided coronally into three parts. The first part was fixed in 10% neutral-buffered formalin for pathology, the second was fixed in 4% paraformaldehyde/PBS for immunopathology, and the third was quickly frozen in liquid nitrogen and stored at −70°C for total RNA extraction.

Renal Pathology

Tubulointerstitial damage was graded in periodic acid-Schiff (PAS)-stained sections on a scale from 0 to 4 (0, no changes; 1, changes affecting <25%; 2, changes affecting 25 to 50%; 3, changes affecting 50 to 75%; 4, changes affecting 75 to 100% of the section) as described previously (15). For further analyzing the degree of interstitial collagen deposition, Masson trichrome–stained sections were graded (0, no staining; 1, <25% staining; 2, 25 to 50% staining; 3, 50 to 75% staining; 4, 75 to 100% staining of the section). These examinations evaluated the areas overlying the tubular basement membrane and interstitial space while avoiding glomeruli and large vessels. Twenty cortical tubulointerstitial fields that were randomly selected at ×400 magnification were assessed in each rat, and the average for each group then was analyzed.

Renal Immunopathology

Cryostat sectioning and immunostaining were performed as described previously (15,16). The cells that were positive for ED-1 were counted in 20 consecutive ×400 microscopic fields of the cortical interstitium using a 0.0625-mm^2 ocular grid for each rat. Data from each group were expressed as mean ± SD per 0.0625 mm^2. The extents of α-SMA immunostaining were graded on a scale from 0 to 5 under a micrometric ocular grid in 20 consecutive ×400 microscopic fields of the cortical tubulointerstitium (0, no staining; 1, 1 to 5%; 2, 5 to 25%; 3, 25 to 50%; 4, 50 to 75%; 5, 75 to 100% of the section). These examinations avoided glomeruli and large vessels. Data from each group were expressed as mean ± SD α-SMA staining score. The observer was blinded to the animal group.

Enzyme Immunoassay

cAMP and cyclic guanine monophosphate concentrations were measured using enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI) as described previously (16).

Statistical Analyses

Statistical analyses were carried out using GraphPad Prizm (GraphPad Software, San Diego, CA). The statistical significance was evaluated by one-way ANOVA using the Bonferroni correction application.

Results

PTX Inhibited Profibrogenic Gene Expression in Renal Cells Stimulated by Angiotensin II or TGF-β1

Levels of CTGF mRNA and protein in NRK-49F were increased with time and reached their highest levels approximately 8 to 24 h after TGF-β1 stimulation (Figure 1, A and B). The cellular CTGF increased accompanied with CTGF secretion (Figure 1B). In PTX-treated cells, the CTGF expression was lowered at all time points examined (Figure 1, A and B). In agreement with a previous report (28), PTX reduced the increased levels of Col I (α1) mRNA and α-SMA protein in TGF-β1–stimulated NRK-49F (Figure 1, A and C). Because the CTGF expression is mainly regulated at the level of transcription (6), we further studied the effect of PTX on CTGF transcription by introduction of luciferase reporters that carry the CTGF promoter into NRK-49F. PTX consistently suppressed the promoter activities induced by TGF-β1 (Figure 1D).

Evidence has suggested that proximal tubular epithelial cells, in addition to interstitial fibroblasts, participate in tubulointerstitial fibrosis (12,15,29,30); we therefore examined the effect of PTX on the profibrogenic gene expression in angiotensin II–stimulated NRK-52E. Consistent with our previous findings in NRK-49F (15), Northern blot analyses revealed increased levels of TGF-β1 and CTGF in angiotensin II–stimulated NRK-52E, and only the upregulation of CTGF but not TGF-β1 was significantly inhibited by PTX (Figure 2A). We further elucidated the effect of PTX on TGF-β1–stimulated NRK-52E. PTX reduced both the cellular and the soluble CTGF and Col I (α1) expression in TGF-β1–stimulated NRK-52E in a dose-dependent manner (Figure 2, B and C). Furthermore, TGF-β1 was found to increase α-SMA expression in NRK-52E, a marker of myofibroblastic phenotype, which was again inhibited by PTX (Figure 2D). PTX also suppressed the promoter activities in NRK-52E induced by TGF-β1 (Figure 2E).

These data indicate that PTX can inhibit CTGF expression in angiotensin II– or TGF-β1–stimulated renal cells, possibly through suppressing its transcription. Furthermore, TGF-β1–induced myofibroblastic differentiation in NRK-49F and NRK-52E is also blocked by PTX.

PTX Blocked CTGF-Mediated Induction of Collagen and α-SMA in Renal Cells

Next, we investigated the mechanism that leads to PTX-elicited blockage of profibrogenic gene expression in response to TGF-β1. Antisense CTGF gene transfection blocked collagen and α-SMA expression in TGF-β1–stimulated NRK-49F and NRK-52E, respectively (Figures 3A and 4A), suggesting that

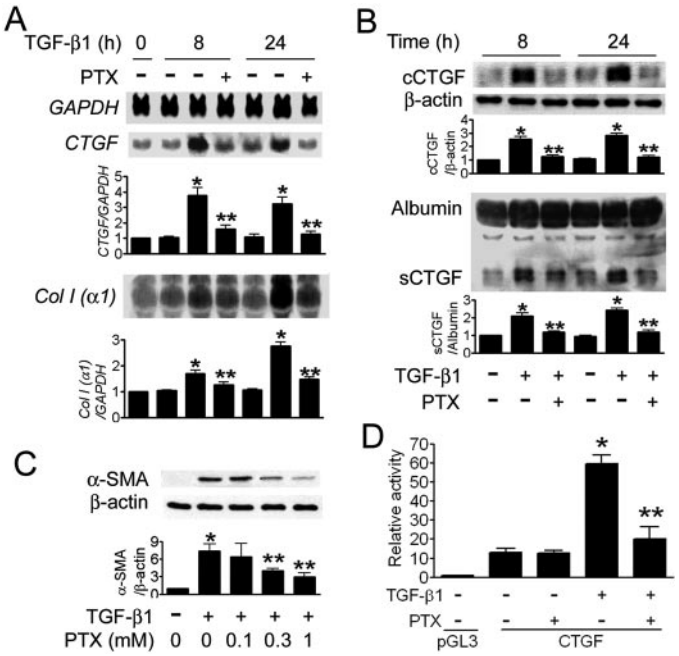

Figure 1. Pentoxifylline (PTX) inhibited profibrogenic gene expression in renal interstitial fibroblasts stimulated by TGF-β1. (A) PTX downregulated connective tissue growth factor (CTGF) and type I collagen (α1) [Col I (α1)] mRNA expression in TGF-β1–stimulated interstitial fibroblasts. Interstitial fibroblasts (NRK-49F) were stimulated with 5 ng/ml TGF-β1 in the presence or absence of 1 mM PTX for various times as indicated. The levels of CTGF, Col I (α1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were determined by Northern blot analyses. Normalized data from three independent experiments were expressed in bar charts as mean ± SD and analyzed by one-way ANOVA. $^*P < 0.05$ *versus* non–TGF-β1–stimulated cells; $^{**}P < 0.05$ *versus* TGF-β1–stimulated cells in the absence of PTX. (B) PTX reduced CTGF protein expression in TGF-β1–stimulated NRK-49F. Total cellular extracts and concentrated conditioned media were prepared and then subjected to Western blot analyses as indicated. The signals of cellular CTGF (cCTGF) and soluble CTGF (sCTGF) were normalized against those of β-actin and albumin, respectively. Normalized data from three independent experiments were expressed and analyzed as described in A. (C) PTX downregulated α-smooth muscle actin (α-SMA) expression in TGF-β1–stimulated NRK-49F. Cells that were stimulated with TGF-β1 were treated with PTX of varying concentrations for 48 h. Total cellular extracts were subjected to Western blot analyses as indicated. Normalized data were expressed and analyzed as described in A. (D) PTX suppressed CTGF transcription in TGF-β1–stimulated NRK-49F. pGL3-CTGF–transfected cells were stimulated with TGF-β1 in the presence or absence of PTX for 4 h. The results of luciferase assays were expressed as the relative activity of pGL3-CTGF over pGL3-basic and normalized to the renilla luciferase activity from co-transfected pRL-TK vectors. The raw luciferase activities for pGL3-basic were 134.8 ± 18.3 from four independent experiments. The results were analyzed as described in A.

CTGF mediates these effects of TGF-β1 in renal cells (31,32). We further demonstrated that the increased Col I (α1) mRNA in NRK-49F with CTGF overexpression was inhibited by PTX

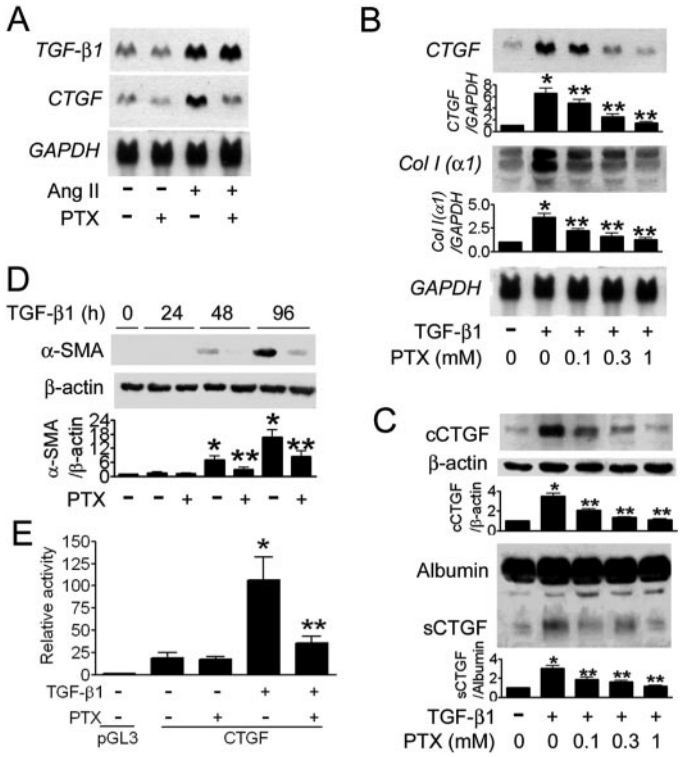

Figure 2. PTX inhibited profibrogenic gene expression in proximal tubular epithelial cells that were stimulated by angiotensin II or TGF-β1. (A) PTX downregulated the gene expression of CTGF but not TGF-β1 in angiotensin II–stimulated proximal tubular epithelial cells. Proximal tubular epithelial cells (NRK-52E) were stimulated with 1 μM angiotensin II in the presence or absence of PTX for 8 h. The levels of TGF-β1, CTGF, and GAPDH mRNA were determined by Northern blot analyses. (B) PTX downregulated CTGF and Col I (α1) in TGF-β1–stimulated NRK-52E. Cells that were stimulated with 5 ng/ml TGF-β1 were treated with PTX of varying concentrations for 24 h. The signals of CTGF and Col I (α1) form three independent experiments were normalized and analyzed as described in Figure 1A. (C) PTX reduced CTGF protein expression in TGF-β1–stimulated NRK-52E. Normalized data from Western blot analyses performed for total cellular extracts and concentrated conditioned media were expressed in bar charts and analyzed as described in Figure 1B. (D) PTX downregulated the expression of α-SMA in TGF-β1–stimulated NRK-52E. Cells were stimulated with TGF-β1 in the presence or absence of 1 mM PTX for various times. Total cellular extracts were subjected to Western blot analyses as indicated. Normalized data from three independent experiments were expressed and analyzed as described in Figure 1A. (E) PTX suppressed CTGF transcription in TGF-β1–stimulated NRK-52E. Luciferase assays were performed, and the normalized relative activity of pGL3-CTGF over pGL3-basic were analyzed as described in Figure 1D. The raw luciferase activities for pGL3-basic were 271.3 $\pm$ 37.2 from four independent experiments.

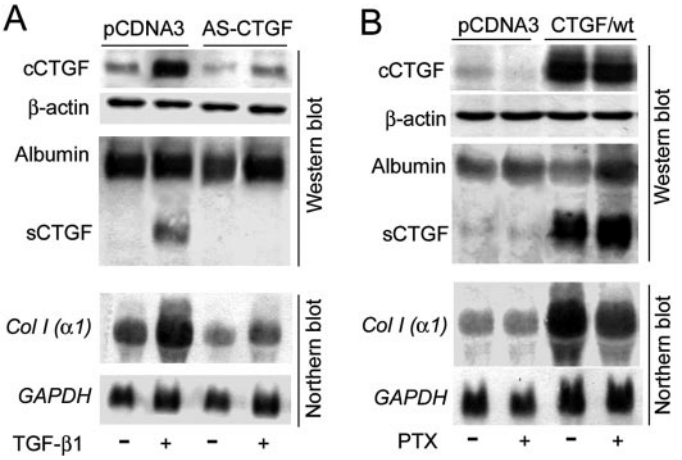

Figure 3. PTX blocked CTGF-mediated induction of collagen in renal interstitial fibroblasts. (A) Antisense CTGF gene transfection blocked the Col I (α1) expression in TGF-β1–stimulated NRK-49F. Antisense CTGF (AS-CTGF)- or pCDNA3-transfected cells were stimulated with or without 5 ng/ml TGF-β1 for 24 h. Total cellular extracts and concentrated conditioned media were subjected to Western blot analyses as indicated. Total RNA was subjected to Northern blot analyses. (B) PTX blocked CTGF-mediated induction of collagen in NRK-49F. Cells with constitutive expression of CTGF (CTGF/wt) or control vector (pCDNA3) were treated with or without 1 mM PTX for 24 h. Western and Northern blot analyses were performed as described in A. Representatives of Northern and Western blot analyses in A and B are one of three independent experiments with similar results.

again (Figure 3B). To avoid constitutive expression-induced phenotype change in NRK-52E during stable cell line selection, we introduced Tet-off inducible pTRE2-CTGF or control vector into NRK-52E. After doxycycline was withdrawn, CTGF expression was increased markedly only in pTRE2-CTGF–transfected cells, which was accompanied by an induction of α-SMA expression (Figure 4B). Although PTX was incapable of blocking CTGF induced by withdrawing doxycycline, it did downregulate α-SMA (Figure 4B). In contrast with the stimulatory effect of overexpressed CTGF on Col I (α1) in NRK-49F (Figure 3B), CTGF overexpression did not significantly affect Col I (α1) expression in NRK-52E (Figure 4B). These data indicate that CTGF mediates TGF-β1–induced collagen synthesis and epithelial-myofibroblastic transdifferentiation in renal cells, and these functions of CTGF are blocked by PTX (Figure 5).

PTX Attenuated Tubulointerstitial Fibrosis in Rats with UUO

We then examined whether the dual-blockade effect of PTX on TGF-β1/CTGF signaling could be applied *in vivo* in the tubulointerstitial fibrosis system. Indeed, PTX treatment attenuated tubulointerstitial damage and interstitial collagen deposition by 55 and 49%, respectively, in comparison with vehicle-treated UUO kidneys (Figure 6).

Having demonstrated that PTX attenuated renal pathology, we next examined the effect of PTX on myofibroblasts and macrophages. In contrast with the low expression in SHM kidneys, α-SMA staining was highly increased in tubulointerstitium of UUO kidneys, whereas PTX was found to reduce the staining by 44% (Figure 7, A and B). Intriguing was that highly

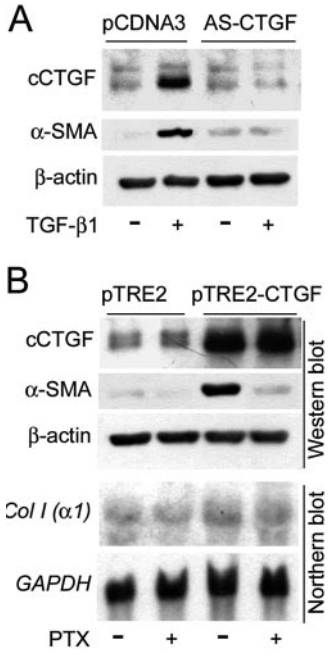

Figure 4. PTX blocked CTGF-mediated induction of α-SMA in proximal tubular epithelial cells. (A) AS-CTGF gene transfection blocked α-SMA expression in TGF-β1–stimulated NRK-52E. AS-CTGF– or pCDNA3-transfected cells were stimulated with or without 5 ng/ml TGF-β1 for 96 h. Total cellular extracts were subjected to Western blot analyses as indicated. (B) PTX blocked CTGF-mediated induction of α-SMA in NRK-52E. After doxycycline was withdrawn, cells with inducible expression of CTGF (pTRE2-CTGF) or control vector (pTRE2) were treated with or without 1 mM PTX for 96 h. Western blot analyses were performed as described in A, and Northern blot analyses were performed for Col I (α1) and GAPDH. Representatives of Northern and Western blot analyses in A and B are one of three independent experiments with similar results.

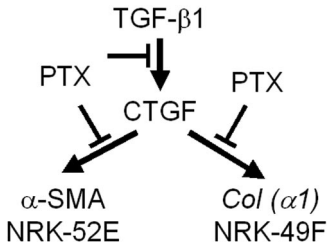

Figure 5. Diagram summarizing that PTX blocks TGF-β1/CTGF signaling pathways in both NRK-49F and NRK-52E.

accumulated ED-1(+) macrophages in interstitium of UUO kidneys were also reduced 62% by PTX (Figure 7, A and B).

Because CTGF is a soluble factor, we examined its gene expression instead of protein level in UUO kidneys. The mRNA levels of CTGF and Col I (α1) were increased in UUO kidneys; however, they were reduced by PTX consistently (Figure 7C). These data indicate that, under both *in vitro* and *in vivo* situations, downregulation of CTGF accounts for one mechanism by which PTX attenuates fibrosis.

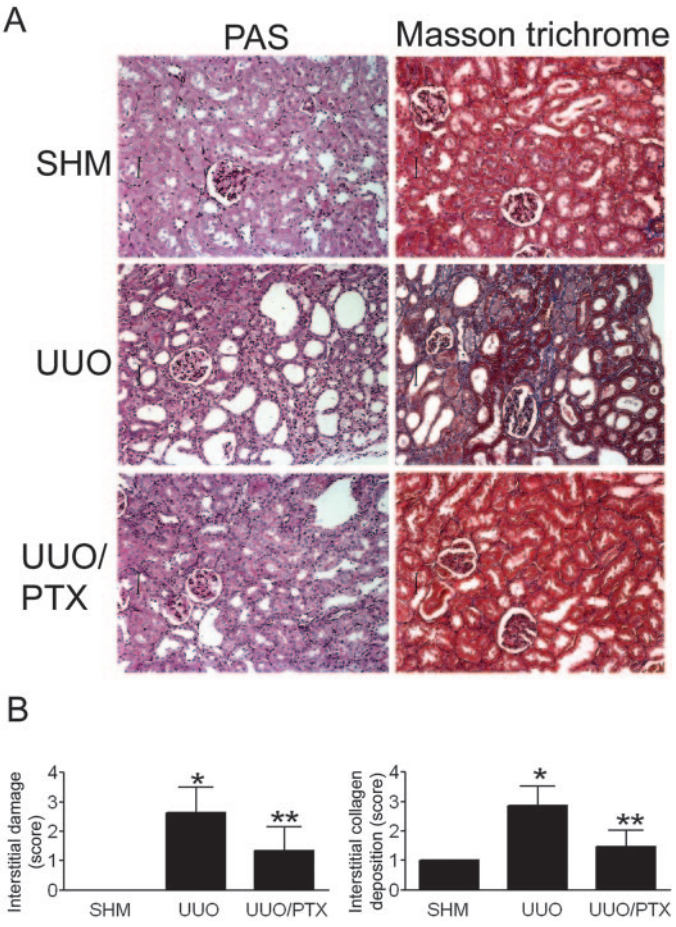

Figure 6. PTX ameliorated the renal pathology in rats with unilateral ureteral obstruction (UUO). (A) Renal cortex stained with periodic acid-Schiff (PAS) or Masson trichrome for sham rats that were given vehicle (SHM) and UUO rats that given vehicle or PTX (UUO/PTX) for 1 wk after surgery. Magnification, ×200. Bars = 12.5 μM. (B) PTX attenuated tubulointerstitial damage and interstitial collagen deposition in UUO kidneys. The tubulointerstitial damage in PAS-stained sections and the degree of interstitial collagen deposition in Masson trichrome–stained sections of the renal cortex graded semiquantitatively as described in Materials and Methods were averaged for rats of each group ($n = 4$ for each group). Data were expressed as mean ± SD for each group. *$P < 0.05$ *versus* SHM rats; **$P < 0.05$ *versus* UUO rats.

Smad Mediated TGF-β1's Signaling to Activation of CTGF Transcription

In certain cell types, cross-talk between Smad and other signaling pathways such as MAPK and phosphatidylinositol 3-kinase (PI3K) determine the CTGF expression (7,33,34). As shown in Figure 8A, TGF-β1 rapidly induced Smad2/3 phosphorylation in NRK-49F. Neither the MAPK nor the PI3K/Akt pathway was activated as evident by lack of phosphorylated forms of extracellular signal-regulated kinase, Jun N-terminal kinase, p38 MAPK, and Akt observed from Western blot analyses (data not shown). We next transfected NRK-49F with luciferase reporters that contained CTGF promoter with or without mutated Smad-binding element to study the role of

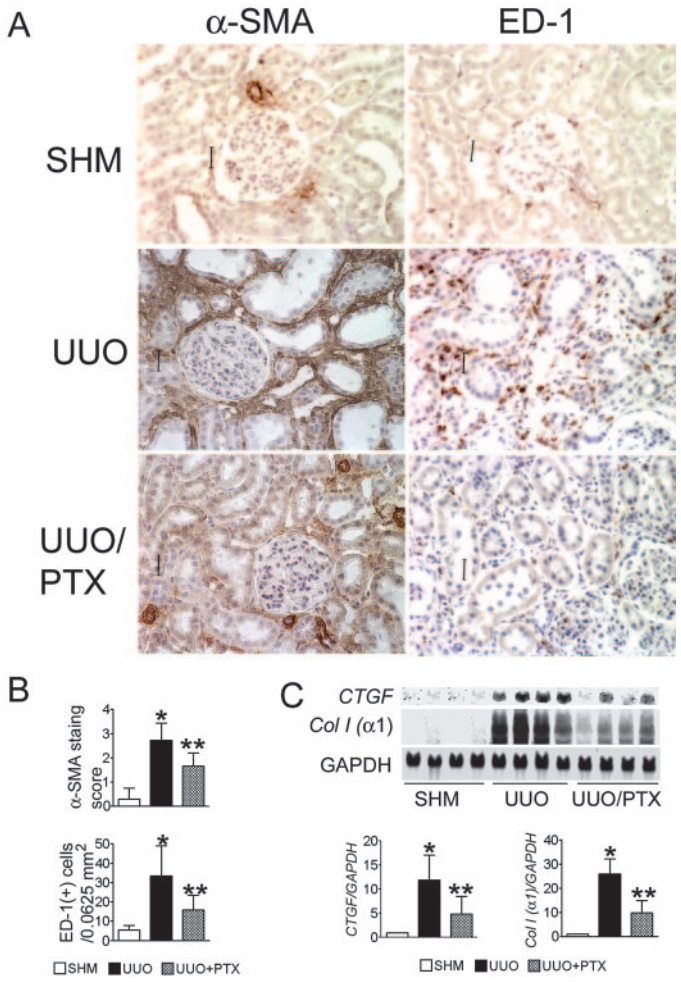

Figure 7. PTX reduced the profibrogenic gene expression and accumulation of myofibroblasts and macrophages in UUO kidneys. (A) Renal cortex immunopathology for SHM, UUO, and UUO/PTX kidneys 1 wk after surgery. Immunostaining was performed for the detection of α-SMA (+) myofibroblasts and ED-1 (+) macrophages. Magnification, ×400. Bar = 25 μM. (B) PTX reduced the accumulation of myofibroblasts and macrophages in UUO kidneys. The numbers of ED-1 (+) cells and the extents of α-SMA staining in renal cortexes assessed semiquantitatively as described in Materials and Methods were averaged for rats of each group ($n = 4$ for each group). Data were expressed as mean ± SD in bar charts and analyzed as described in Figure 1A. *$P < 0.05$ *versus* SHM rats; **$P < 0.05$ *versus* UUO rats (C) PTX downregulated the expression of CTGF and Col I (α1) in UUO kidneys. Total RNA was prepared from the whole-thickness kidneys and then subjected to Northern bolt analyses for CTGF, Col I (α1), and GAPDH. Normalized data were expressed and analyzed as described in B.

Smad in TGF-β1–induced CTGF transcription. TGF-β1 markedly increased the promoter activity in NRK-49F, whereas the stimulatory effect was absent in cells that were transfected with constructs that contained mutated Smad-binding element (Figure 8B). We further demonstrated that expression of C-terminally truncated forms of Smad3 or Smad4, which have been shown to act as specific dominant negative inhibitors (25),

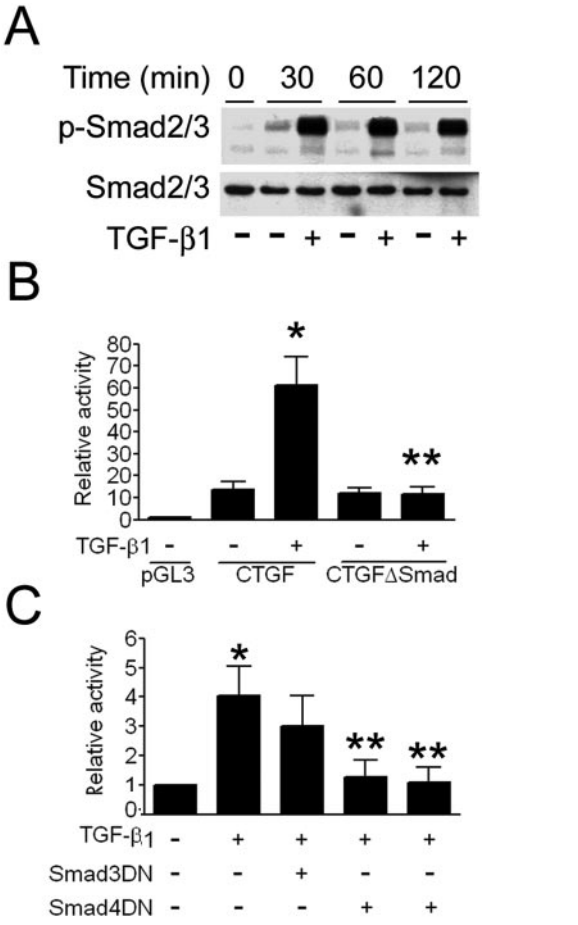

Figure 8. Smad mediated TGF-β1 signaling to the activation of CTGF transcription. (A) TGF-β1 activated Smad2/3. NRK-49F were stimulated with or without 5 ng/ml TGF-β1 for various times as indicated. Total cellular extracts were subjected to Western blot analysis with antibodies to phosphorylated Smad2/3 (p-Smad2/3) and Smad2/3. Representatives of Western blot analyses are one of three independent experiments with similar results. (B) Smad-binding element in the CTGF promoter region was essential for TGF-β1–induced gene transcription. pGL3-CTGF– or pGL3-CTGF-ΔSmad–transfected NRK-49F were stimulated with or without TGF-β1 for 4 h. Luciferase assays were performed, and the results were expressed as the relative activity of pGL3-CTGF or pGL3-CTGF-ΔSmad over pGL3-basic after normalization to the renilla luciferase activity from co-transfected pRL-TK vectors from four independent experiments. (C) Dominant negative Smads inhibited TGF-β1–induced CTGF transcription. pGL3-CTGF–transfected NRK-49F were co-transfected with or without C-terminally truncated forms of Smad3 (Smad3DN) and Smad4 (Smad4DN) as indicated. Transfected cells with or without TGF-β1 stimulation for 4 h were harvested for luciferase assay. The results were expressed as the relative activity over those of non–TGF-β1–stimulated cells after normalization to the renilla luciferase activity as described previously. Data are expressed as mean ± SD in B and C. *$P < 0.05$ *versus* non–TGF-β1–stimulated cells; **$P < 0.05$ *versus* TGF-β1–stimulated pGL3-CTGF–transfected cells in B or cells without co-transfected Smad3DN or Smad4DN in C.

caused a reduction of TGF-β1–induced CTGF promoter activity, whereas the combination of C-terminally truncated Smad3 and Smad4 completely abolished the inducibility by TGF-β1 (Figure 8C).

Similar to the results observed in NRK-49F, we found that Smad mediated TGF-β1 signaling to the activation of CTGF transcription in NRK-52E (data not shown). These results indicate that Smad mediates TGF-β1's profibrogenic effects in renal cells and therefore is a potential target for the blockade effect of PTX.

PTX Did Not Interfere with TGF-β1–Induced Activation and Nuclear Translocation of Smad Proteins

We next examined the role of PTX in TGF-β1–induced Smad signaling. As shown in Figure 9A, TGF-β1 rapidly induced the phosphorylation of Smad2/3 in NRK-49F, whereas this induction was not affected by PTX. We further examined whether PTX affects Smad2/3 association with Smad4. Immunoprecipitation and Western blot analysis revealed that the phosphorylated Smad2/3 was physically associated with Smad4 after TGF-β1 stimulation, whereas PTX did not affect such an association (Figure 9B). Similarly, PTX did not affect nuclear accumulation of Smad2/3 and Smad4 induced by TGF-β1 (Figure 9C). To verify these findings further, we used immunostaining to visualize the phosphorylation and subcellular localization of Smad2/3. Upon TGF-β1 stimulation, the signal of phosphorylated Smad2/3 was markedly increased and concentrated in nuclei of NRK-49F and NRK-52E, and this TGF-β1–induced effect was not affected by PTX (Figure 9D).

These data indicate that PTX does not affect TGF-β1 signaling to Smad. However, PTX inhibits TGF-β1–induced transcriptional activation of a Smad-dependent promoter, which raises a possibility that PTX regulates a factor that acts in concert with Smad to activate the transcription of CTGF.

PTX Inhibited TGF-β1–Induced CTGF by Blocking Smad3/4-Dependent Transcription through PKA

To elucidate how PTX affects transcriptional activation of Smad-dependent promoter, we used a reporter $(CAGA)_9$-MLP-Luc, an artificial reporter construct that consists of the Smad3/4-specific recognition sequence CAGA in many Smad3/4-targeted genes, including CTGF (26). TGF-β1 efficiently increased the activity of this reporter in NRK-49F, which was blocked by PTX (Figure 10A). Furthermore, PTX treatment of NRK-49F increased intracellular cAMP levels but not guanine monophosphate (data not shown) in a dose-dependent manner (Figure 10B), and the increased cAMP levels were accompanied by CREB phosphorylation, similar to the effect of PKA activators db-cAMP and FK/IBMX (Figure 10C). Furthermore, the CREB phosphorylation induced by PTX could be completely prevented by PKA inhibitor H89 (Figure 10C). We next examined whether the inhibitory effects of PTX on TGF-β1/Smad/CTGF transcription is PKA dependent. Indeed, we found that H89 prevented PTX-triggered inhibition of CTGF mRNA expression, CTGF promoter activity, and Smad3/4-dependent reporter activity in TGF-β1–stimulated NRK-49F, whereas H89 alone did not display any effect (Figure 10, C through E). To

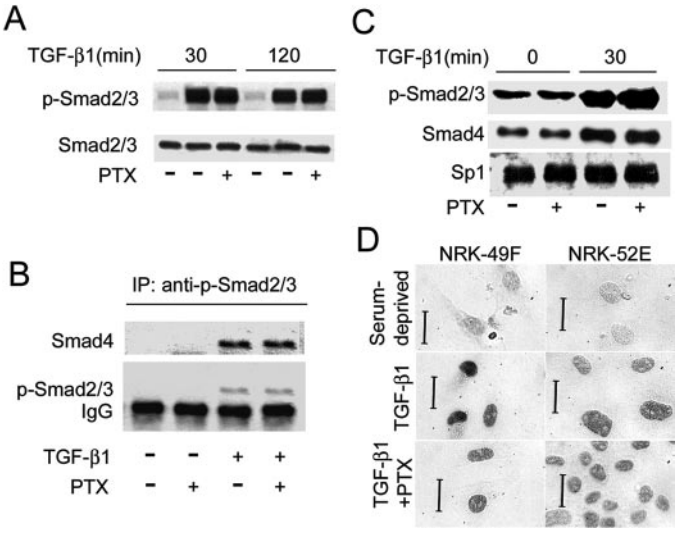

Figure 9. PTX did not interfere with activation and nuclear translocation of Smad proteins induced by TGF-β1. (A) PTX did not suppress the activation of Smad2/3 in TGF-β1–stimulated NRK-49F. Cells were stimulated with 5 ng/ml TGF-β1 in the presence or absence of 1 mM PTX for various times as indicated. Total cellular extracts were subjected to Western blot analyses with antibodies against p-Smad2/3 and Smad2/3. (B) PTX did not affect the association of activated Smad2/3 with Smad4. NRK-49F were treated with or without TGF-β1 and PTX for 30 min and then harvested for total cellular extract preparation. Total cellular extracts were immunoprecipitated with antibodies against p-Smad2/3. The precipitated complexes were subjected to Western blot analyses with antibodies against p-Smad2/3 and Smad4. (C) PTX did not block the nuclear translocation of activated Smad in TGF-β1–stimulated NRK-49F. Cells were treated as described in B. Nuclear proteins were prepared and subjected to Western blot analyses with antibodies against p-Smad2/3, Smad4, and Sp1. Representatives of Western blot analyses in A through C are one of three independent experiments with similar results. (D) PTX did not inhibit the activation and nuclear accumulation of Smad2/3 induced by TGF-β1. NRK-49F and NRK-52E were stimulated with TGF-β1 for 30 min in the presence or absence of PTX. Cells were fixed by 4% paraformaldehyde and then immunostained with antibodies against p-Smad2/3. The reactions on sections were detected with diaminobenzidine. Shown are the representatives of three independent experiments.

demonstrate further that PTX acts through PKA, we tested whether other PKA activators could mimic effects of PTX. We found that db-cAMP or FK/IBMX could efficiently recapitulate these inhibitory effects to an extent similar to that of PTX (Figure 10, C through E). Similar results were reproduced in NRK-52E (Figure 11). In summary, these studies not only indicate that the effect of PTX on TGF-β1/Smad/CTGF signal transduction is mediated by PKA but also reveal a novel cross-talk between PKA and Smad/CTGF transcription in renal cells (Figure 12).

Discussion

Considerable evidence indicates that CTGF plays a pivotal role in TGF-β1–dependent tubulointerstitial fibrosis (1,2,4,6–

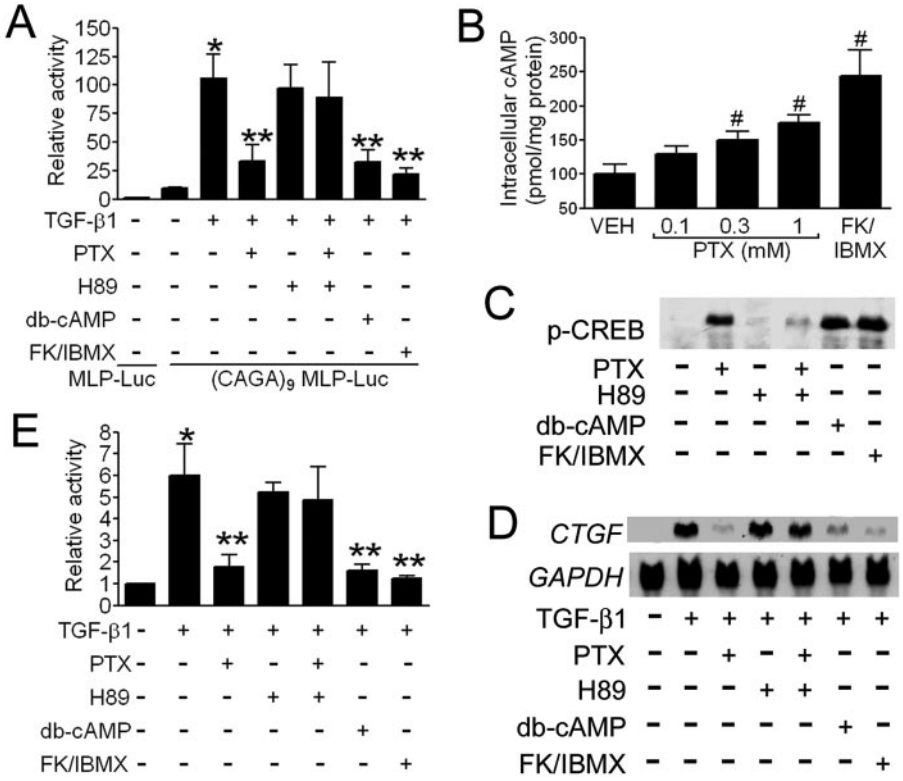

Figure 10. PTX inhibits TGF-β1–induced CTGF expression in renal interstitial fibroblasts by blocking Smad3/4-dependent transcription through activating protein kinase A (PKA). (A) PTX blocked TGF-β1–induced activation of Smad3/4-dependent reporter through PKA in NRK-49F. $(CAGA)_9$-MLP-Luc–transfected cells were stimulated with 5 ng/ml TGF-β1 for 4 h in the presence of various agents, including PTX (1 mM), *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline (H89; 5 μM), dibutyryl cAMP (db-cAMP; 2 mM), or forskolin/3-isobutyl-1-methylxanthine (FK/IBMX; 20 μM/200 μM). Results of luciferase assays were expressed as the relative activity of $(CAGA)_9$-MLP-Luc over MLP-Luc and normalized to the renilla luciferase activity from co-transfected pRL-TK vectors. The raw luciferase activities for MLP-Luc were 71.5 ± 25.1 from four independent experiments. Data are expressed as mean ± SD. $^*P < 0.05$ *versus* non–TGF-β1–stimulated cells; $^{**}P < 0.05$ *versus* TGF-β1–stimulated cells in the absence of various agents. (B) PTX caused a dose-dependent increase in intracellular cAMP levels of NRK-49F. Cells were treated with vehicle (VEH), PTX, or FK/IBMX. cAMP concentrations per milligram of cellular protein were measured using enzyme immunoassay kits and expressed as mean ± SD from three independent experiments performed in quadruplicate. $^{\#}P < 0.05$ *versus* VEH treated. (C) H89 blocked the phosphorylation of CREB induced by PTX in NRK-49F. Cells were treated with 1 mM PTX for 15 min in the presence or absence of 5 μM H89. Parallel study was performed with the treatment of db-cAMP or FK/IBMX. Nuclear extracts were prepared for Western blot analysis with antibody against phosphorylated cAMP response element-binding protein (p-CREB). Shown blot is the representative of three independent experiments with similar results. (D) PTX reduced CTGF mRNA levels in TGF-β1–stimulated NRK-49F through PKA. Cells were stimulated with TGF-β1 in the presence of various agents as indicated. Total RNA was subjected to Northern blot analyses for CTGF and GAPDH. (E) PTX blocked TGF-β1–induced CTGF transcription through PKA in NRK-49F. pGL3-CTGF–transfected cells were stimulated with TGF-β1 for 4 h in the presence of various agents as indicated. The results of luciferase assays were expressed as described in Figure 8C. Data are expressed as mean ± SD. $^*P < 0.05$ *versus* non–TGF-β1–stimulated cells; $^{**}P < 0.05$ *versus* TGF-β1–stimulated cells in the absence of various agents.

12). In this study, we present the first demonstration that PTX attenuated tubulointerstitial fibrosis possibly by blocking TGF-β1–induced CTGF and CTGF-induced profibrogenic effect on renal cells.

Although CTGF is considered to be the downstream mediator of TGF-β1 in profibrogenesis, it is found to have distinct fibrogenic effects on different renal cells. CTGF mediates the upregulatory effect of TGF-β1 on collagen I in mesangial cells and fibroblasts; however, it mainly transduces epithelial-myofibroblastic transdifferentiation signal to tubular epithelial cells (13,15,31,32,35). In fact, remnant kidney and co-culture systems have shown that CTGF secreted by tubular epithelial cells has a paracrine effect on ECM synthesis in fibroblasts (9,12). Because antisense oligodeoxynucleotide therapy to reduce CTGF attenuates kidney disease (11,12), agents that target CTGF should be promising to ameliorate tubulointerstitial fibrosis. In agreement with previous findings on mesangial cells and NRK-49F (15), we demonstrated that PTX significantly inhibited TGF-β1–induced CTGF and Col I (α1) in NRK-52E; in addition, PTX blocked TGF-β1–induced α-SMA. Thus, the inhibition of PTX on CTGF expression is a common effect in many renal cell types. Furthermore, CTGF overexpression was capable of recapitulating TGF-β1's effect in NRK-49F and NRK-52E. However, overexpression of CTGF could not overcome the downregula-

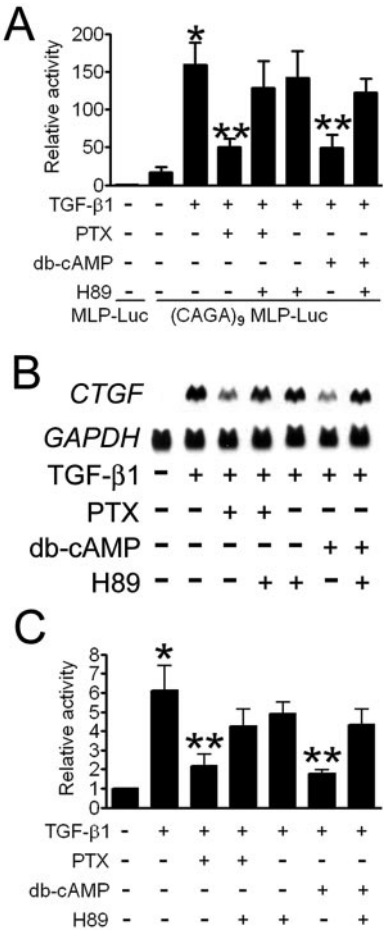

Figure 11. PTX inhibits TGF-β1–induced CTGF expression in proximal tubular epithelial cells by blocking Smad3/4-dependent transcription through activating PKA. (A) PTX blocked TGF-β1–induced activation of Smad3/4-dependent reporter through PKA in NRK-52E. $(CAGA)_9$-MLP-Luc–transfected cells were stimulated with 5 ng/ml TGF-β1 for 4 h in the presence of various agents as indicated. The results of luciferase assays were expressed and analyzed as described in Figure 10A. The raw luciferase activities for MLP-Luc were 83.7 $\pm$ 30.6 from four independent experiments. (B) PTX reduced CTGF mRNA levels in TGF-β1–stimulated NRK-52E through PKA. Cells were stimulated with TGF-β1 in the presence of various agents as indicated. Total RNA was subjected to Northern blot analyses for CTGF and GAPDH. (C) PTX blocked TGF-β1–induced CTGF transcription through PKA in NRK-52E. pGL3-CTGF–transfected cells were stimulated with TGF-β1 for 4 h in the presence of various agents as indicated. Luciferase assay and data analysis were performed as described in Figure 10E.

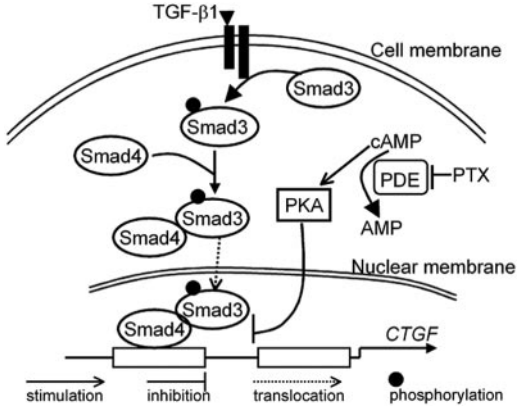

Figure 12. A model illustrating the mechanism by which PTX inhibits TGF-β1–induced CTGF transcription through PKA in renal cells. PDE, phosphodiesterase.

tory effect of PTX on Col I (α1) in NRK-49F and rescue the downregulation of α-SMA in NRK-52E, either. These findings indicate that PTX can block profibrogenic effects of TGF-β1 through inhibiting both CTGF expression and profibrogenic effects of CTGF on renal cells (Figure 5). Because of the dual blockade, we found that the inhibitory effects of PTX on CTGF could be translated into the attenuation of the *in vivo* tubulointerstitial fibrosis system.

In this study, we demonstrated that PTX downregulated TGF-β1–induced CTGF through blocking Smad3/4-dependent transcription *via* PKA. Agents that elevate intracellular cAMP have been reported to inhibit CTGF expression and cell proliferation in TGF-β1–stimulated renal fibroblasts and mesangial cells (7,15,31,33). No evidence has shown that increased cAMP in tubular epithelial cells can recapitulate such an inhibition, although it is demonstrated in ovarian granulose cells (36). Although increased cAMP increases CTGF protein by inhibiting its breakdown without affecting mRNA level in bovine endothelial cells (37), it has been reported to downregulate the expression of several additional TGF-β1–inducible genes in most sensitive cells (38–41). Thus, sensitivity to cAMP seems to be a common feature for many TGF-β1–regulated genes, and one common TGF-β1–induced signaling pathway may be the target of cAMP-activated molecules. Smad proteins transduce signals from the TGF-β1 receptors to regulate the target genes, including collagen and α-SMA (42,43). Cross-talk with other signaling pathways may contribute to the regulation of TGF-β1's effects. In this study, we demonstrated that Smad, not MAPK and PI3K/Akt pathways, mediated TGF-β1's signaling into CTGF transcription in NRK-49F and NRK-52E. We further demonstrated that PTX inhibited TGF-β1–induced Smad3/4-dependent CTGF transcription without affecting Smad activation and nuclear translocation (Figure 12), which is in agreement with findings in other cell types (44). Schiller *et al.* (44) demonstrated that transcription co-activators CREB-binding protein (CBP) and p300 are essential for Smad3/4-dependent gene transcription. In the absence of effect on nuclear translocation and DNA binding of Smad3/4 complexes, cAMP-elevating agents can interfere with Smad3/4-dependent gene transcription in nucleus *via* reduction of Smad3/4-CBP/p300 interactions and possible sequestration of CBP and p300 by phosphorylated CREB in a PKA-dependent manner.

Smad mediates TGF-β1 signaling to the activation of α-SMA transcription in mouse AKR-2B fibroblasts and normal skin fibroblasts (43,45). However, the activation of Smad is not enough to induce α-SMA expression in NRK-52E because such expression was blocked by antisense CTGF gene transfection, a finding in agreement with that in human proximal tubular

epithelial cells (32). Interference with TGF-β1–induced Smad signaling by peroxisome proliferator-activated receptor γ activation or hepatocyte growth factor treatment has been shown to downregulate α-SMA expression in normal skin fibroblasts and human kidney epithelial cells (34,45). Thus, we speculate that PTX inhibits TGF-β1–induced expression of α-SMA in NRK-52E by inhibiting CTGF expression through blocking Smad-dependent gene transcription.

The nature of signaling pathways distal to the CTGF stimulation that are regulated by PTX remains to be defined. Recent evidence shows that CTGF can activate the Smad pathway in human proximal tubule cells and cortical fibroblasts (46), a potential target of PTX in the disruption of CTGF signaling. In addition to the Smad pathway, small G protein Rho has been shown to be indispensable for α-SMA activation in porcine proximal tubular epithelial cells (47). It is interesting that PKA can phosphorylate Rho and regulate its activity negatively (48). We now are undergoing studies to examine the signaling pathways distal to recombinant CTGF stimulation in NRK-49F and NRK-52E that will be crucial for Col I (α1) and α-SMA expression and the possible functional position of PTX.

Angiotensin II participates in fibrogenesis and inflammation in UUO kidneys (29,30). Although PTX could not affect angiotensin II–induced TGF-β1, it more specifically blocked its downstream CTGF. This finding supports the hypothesis that combination of PTX with an angiotensin II blockade will further improve tubulointerstitial fibrosis. In fact, we have proved the hypothesis in remnant nephropathy (15), and human studies have also proved its efficacy in reducing proteinuria further in patients who have diabetes or membranous nephropathy and receive angiotensin II blockade (19,21). Notably, PTX further reduces *N*-acetyl-β-glucosaminidase excretion in these patients, suggesting its beneficial effects on tubulointerstitial damage in chronic kidney disease (21). In addition to its potent antifibrotic effect, we demonstrated that PTX significantly reduced macrophages in UUO kidneys, which also was noticed previously (14,15,17). Because of no apparent adverse effect in clinical application, PTX should be a safe and potent drug for kidney disease treatment in view of its potent inhibitory effects against cell proliferation, inflammation, and fibrosis (18–21).

In conclusion, our results demonstrated that PTX not only inhibited CTGF expression by interfering with Smad3/4-dependent CTGF transcription through PKA but also blocked profibrogenic effects of CTGF on renal cells. Because of the dual blockade, PTX potently attenuated tubulointerstitial fibrosis in UUO kidneys.

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