Parathyroid hormone-potentiated connective tissue growth factor expression in human renal proximal tubular cells through activating the MAPK and NF-κB signalling pathways

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Abstract

Background. Secondary hyperparathyroidism is a universal complication of chronic renal diseases. One of the pathological consequences of hyperparathyroidism is impairment of the renal interstitium and tubules. However, the molecular mechanism of renal tubular interstitial impairment induced by parathyroid hormone (PTH) remains unclear. Enhanced and prolonged expression of connective tissue growth factor (CTGF) has been associated with fibrosis and inflammation in the kidney. The purpose of this study was to investigate the effects of PTH on CTGF expression patterns in human proximal tubular cell line—HK-2 cells.

Methods. We treated cells with various concentrations of PTH for the indicated periods of time in the presence or absence of the mitogen-activated protein kinase (MAPK) inhibitor (PD98059) or the NF-κB inhibitor (PDTC).

Results. Quantitative real-time RT–PCR analysis revealed that PTH at a concentration of 10⁻¹²–10⁻¹⁰ M increased the mRNA levels of CTGF, which was similar to the trends of CTGF protein levels detected by immunoblotting assay. Our data clearly show the ability of human proximal tubular HK-2 cells to produce CTGF after the treatment with PTH. In addition, we showed that PTH induced the phosphorylation of MAPK p42 and p44, and increased NF-κB binding activities in the PTH-treated cells. Moreover, both PD98059 and PDTC inhibited the effect of PTH on the expression of CTGF, which strongly suggests that these pathways play important roles in the PTH-induced CTGF upregulation in renal tubular cells.

Conclusions. Our results indicated for the first time that PTH may enhance the expression of CTGF in human kidney proximal tubular cells, suggesting that PTH may play an important role in the fibrotic and inflammatory process that is a hallmark for progression of chronic kidney disease.

Keywords: chronic kidney disease; NF-κB; parathyroid hormone (PTH); renal proximal tubular cells

Introduction

Hyperparathyroidism is an invariable complication of chronic renal diseases. Biochemical and/or histological evidence of hyperparathyroidism typically occurs when the glomerular filtration rate is < 70 mL/min [1,2]. Secondary hyperparathyroidism is associated with renal impairment, coronary artery disease, hypertension, higher in-hospital mortality and perioperative myocardial injury [3]. Patients with secondary hyperparathyroidism caused by chronic kidney disease are characterized by major impairment of the renal interstitium and tubules [4]. Parathyroid hormone (PTH) has proliferative effects on the kidney and induces compensatory renal growth [5,6]. The receptor for PTH (PTHR) has been detected in the convoluted and straight proximal tubules, the cortical straight ascending limbs, and the distal convoluted tubules in the kidney, which are consistent with known sites of PTH action. However, the physiologic role of PTH in renal tubulointerstitial impairment and its related molecular mechanisms remain unknown.

The PTHR/PTH-related protein (PTHrP) receptor couples to the G-protein signalling pathways, which regulate mitogen-activated protein kinases (MAPKs) in many systems [7–10]. MAPKs are serine and threonine protein kinases that play an important role in the regulation of cell growth and differentiation. The MAPK signalling pathway is tightly coupled to the regulation of cell proliferation and viability, and may be involved in cardiac and renal fibrosis [11]. Renal fibrosis is the principal process underlying the progression from chronic kidney disease to end-stage renal disease (ESRD) which is a relatively uniform response involving glomerulosclerosis, tubulointerstitial fibrosis and abnormal renal vascular changes. Furthermore, tubulointerstitial fibrosis has evolved as the most consistent predictor of irreversible loss of renal function and progression to ESRD. Two of the best characterized MAPKs, ERK1/p42mapk and ERK2/p44mapk, are implicated in the regulation
of cellular growth and differentiation by a variety of G-protein-coupled receptor agonists. The first aim of the present investigation was to identify signalling components involved in PTH-stimulated ERK activity in regulation of renal tubulointerstitial fibrosis.

Interstitial inflammation is one of the predominant features of chronic kidney disease that precedes fibrosis. In primary hyperparathyroidism, circulating levels of inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) are elevated [12]. In patients with glucocorticoid-induced osteoporosis, treatment with PTH caused a rapid and significant increase in the circulating levels of nuclear factor-κB (NF-κB) ligand, the receptor activator for NF-κB ligand (RANKL), IL-6 and IL-6 soluble receptor (IL-6sR) [13]. NF-κB is one of the most important regulators of pro-inflammatory gene expression [14], and the activation of NF-κB is associated with tubulointerstitial fibrosis, which correlates with the outcome of chronic renal failure [15].

Connective tissue growth factor (CTGF) is a novel inflammatory and pro-fibrotic factor that is upregulated in different human kidney diseases and contributes to renal fibrosis and tubulopelithelial transdifferentiation [16,17]. Angiotensin II-induced tubular cell epithelial–mesenchymal transition in human proximal tubular epithelial cells was attenuated by an anti-sense CTGF oligonucleotide [18,19]. Furthermore, over-expression of the CTGF gene was implicated in the progression of many chronic inflammatory/fibroliferative disorders, such as glomerulosclerosis, pulmonary fibrosis and hepatic cirrhosis [20]. These observations suggest a critical role of CTGF in the development of renal fibrosis and inflammation. In the current study, we examined the effects of PTH on CTGF expression in human renal proximal tubular epithelial cells and attempted to identify the signalling pathway that was activated by PTH to upregulate CTGF expression.

Materials and methods

Cell culture

Human kidney proximal tubular cell line HK-2 was obtained from the Institute of Basic Medical Sciences, Beijing, China and grown in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Germany)/F-12 plus 10% fetal bovine serum (FBS) supplemented with 1000 mg/L glucose, 2 mmol/L pyrrolidine dithiocarbamate (PDTC, Sigma, St. Louis, MO, USA), an inhibitor of NF-κB, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L GAG TAT AGT AGA GCC A-3’ and anti-sense 5’-CCG TCG GTA CAT CCT CCA CA-3’, yielding a PCR product of 211 bp; for β-actin (NM_001101.3), sense 5’-GCT GCA ACT CAA CAC AG-3’ and anti-sense 5’-ATA CTC CGT TGT GCT GAT CC-3’, yielding a PCR product of 299 bp. Each real-time PCR reaction contained SYBR Green PCR Master Mix (Shanghai Shine Co., Shanghai, China), and every pair of primers in a volume of 20 μL was carried out. Briefly, after 1 μL of total RNA was converted to cDNA with Superscript II reverse transcriptase, PCR was performed with CTGF or β-actin primers mixed with SYBR Green JumpStat TaqReadyMix (Sigma, St. Louis, MO, USA) using a DNA Engine OPTICON (MJ Research,Waltham, MA, USA) as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 10 s, 55°C for 15 s and 72°C for 20 sec for 40 cycles followed by further extension for 5 min at 72°C. The mRNA abundance β-actin was used as the internal reference for gene evaluation. To determine the gene expression, genes investigated in the present study were calculated as 2(ΔΔCt gene) using Ct of each gene was performed five times to obtain a mean value.

Immunoblotting assay

Cellular lysates were prepared as described [21] and resolved by SDS–PAGE. Immunoblotting procedures were carried out as previously described [22], and the following antibodies were used: anti-CTGF and anti-β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-p42/44 MAPK and phospho-p42/44 MAPK antibodies (Cell Signaling Technology, MA, USA). Bands were analysed by scanning densitometry (GeneGenius, with Genesnap version 4, Syngene).

CTGF enzyme-linked immunosorbent assay

CTGF was measured using a capture sandwich ELISA with biotinylated and non-biotinylated affinity-purified goat polyclonal antibodies to human CTGF. Briefly, a flat-bottom ELISA plate was coated with 50 μL of goat anti-human CTGF antibody at a concentration of 10 μg/mL in PBS/0.02% sodium azide for 1 h at 4°C. Wells were washed three times and incubated with 300 μL of blocking buffer (PBS/0.02% sodium azide/1% BSA) for 1 h at room temperature. The wells were washed three times, and 50 μL of recombinant human CTGF protein (from 0.156 to 100 ng/ml) or the cell supernatants in different groups were added and incubated at room temperature for 2 h. After washing three times, 50 μL of biotinylated goat anti-human CTGF (2 μg/mL) was added, incubated at room temperature in the dark for 1 h and then washed three times, and 50 μL of alkaline phosphatase-conjugated streptavidin (1 μg/mL, Zymed, South San Francisco, CA, USA) was added and incubated at room temperature for 1 h. The wells were washed again three times and incubated with 100 μL of alkaline phosphatase substrate solution (1 mg/mL p-nitrophenyl phosphate, Sigma Chemicals, St. Louis, MO, USA) in sodium carbonate/bicarbonate buffer. Absorbance at 405 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). CTGF levels were expressed as nanogram per milliliter for three replicate samples for each condition.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as previously depicted [23]. The NF-κB sequence in the immunoglobulin gene was used for EMSA (5’-AGT TGA GCC GAC TTT ACC AGG C-3’; the core NF-κB sequence was underlined). EMSA was performed as previously described [24]. Specifically, the NF-κB oligonucleotides used for EMSAs were kinased with [γ-32P]-ATP using T4 polynucleotide kinase (New England BioLabs, Beverly, MA, USA). The labelling efficiency of the oligonucleotides was carefully monitored by spectrometry and scintillation counting to ensure that equal amounts of radioactive oligonucleotides were included in each reaction. Nuclear extracts (3.5 μg) were incubated with 20 fmol of [γ-32P]-ATP-labelled NF-κB oligonucleotide in a binding reaction mixture containing 10 mmol/L Tris–HCl (pH 7.5), 80 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 5% glycerol and 1.0 μg of poly(dI–dC) using a first-strand cDNA synthesis kit according to the manufacturer’s protocol (Invitrogen, Inc., Carlsbad, CA, USA). PCR was performed using 1 μL of first-strand cDNA and gene-specific primers for CTGF and β-actin (as an internal control); for CTGF (NM_001901.2), sense 5’-CAT GCA ACT ATT ATG GCC A 3’ and anti-sense 5’-CGG TCG GTA CAT CCT CCA CA-3’, yielding a PCR product of 211 bp; for β-actin (NM_001101.3), sense 5’-GCT GCA ACT CAA CAC AGT GC-3’ and anti-sense 5’-ATA CTC CGT TGT GCT GAT CC-3’, yielding a PCR product of 299 bp. Each real-time PCR reaction contained SYBR Green PCR Master Mix (Shanghai Shine Co., Shanghai, China), and every pair of primers in a volume of 20 μL was carried out. Briefly, after 1 μL of total RNA was converted to cDNA with Superscript II reverse transcriptase, PCR was performed with CTGF or β-actin primers mixed with SYBR Green JumpStat TaqReadyMix (Sigma, St. Louis, MO, USA) using a DNA Engine OPTICON (MJ Research,Waltham, MA, USA) as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 10 s, 55°C for 15 s and 72°C for 20 sec for 40 cycles followed by further extension for 5 min at 72°C. The mRNA abundance β-actin was used as the internal reference for gene evaluation. To determine the gene expression, genes investigated in the present study were calculated as 2(ΔΔCt gene) using Ct of each gene was performed five times to obtain a mean value.

Drug treatments

HK-2 cells were incubated with serum-free DMEM/F-12 for 24 h before treatment with the indicated drugs. The cells were treated with various concentrations of PTH for the indicated period of time and with 5 × 10−3 M PD98059 (Promega, Madison, WI, USA), a MAPK inhibitor, or 20 μmol/L pyrrolidine dithiocarbamate (PDTC, Sigma, St. Louis, MO, USA), an inhibitor of NF-κB, where indicated. Untreated HK-2 cells were used as controls.

Quantitative real-time RT–PCR

Total RNA was subsequently extracted using the TRizol reagents. Three microgram of total RNA was reverse-transcribed in a 10-μL reaction by using 5 μL of cDNA and gene-specific primers for CTGF and β-actin (as an internal control). PCR was performed using 1 μL of first-strand cDNA and gene-specific primers for CTGF and β-actin (as an internal control): for CTGF (NM_001901.2), sense 5’-GAC CCA ACT ATT ATG GCC A 3’ and anti-sense 5’-CCG TCG GTA CAT CCT CCA CA-3’, yielding a PCR product of 211 bp; for β-actin (NM_001101.3), sense 5’-GCT GCA ACT CAA CAC AGT GC-3’ and anti-sense 5’-ATA CTC CGT TGT GCT GAT CC-3’, yielding a PCR product of 299 bp. Each real-time PCR reaction contained SYBR Green PCR Master Mix (Shanghai Shine Co., Shanghai, China), and every pair of primers in a volume of 20 μL was carried out. Briefly, after 1 μL of total RNA was converted to cDNA with Superscript II reverse transcriptase, PCR was performed with CTGF or β-actin primers mixed with SYBR Green JumpStat TaqReadyMix (Sigma, St. Louis, MO, USA) using a DNA Engine OPTICON (MJ Research,Waltham, MA, USA) as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 10 s, 55°C for 15 s and 72°C for 20 sec for 40 cycles followed by further extension for 5 min at 72°C. The mRNA abundance β-actin was used as the internal reference for gene evaluation. To determine the gene expression, genes investigated in the present study were calculated as 2(ΔΔCt gene) using Ct of each gene was performed five times to obtain a mean value.
for 1 h at 4°C. Two microgram of Hela Nuclear extract (Promega, WI, USA) was used for positive control. For competitive EMSA, unlabelled NF-κB oligonucleotide was added at a 100-fold molar excess to the binding reaction mixture, where indicated, before the addition of the radiolabelled NF-κB probe. The protein DNA complexes were resolved by electrophoresis on 4% non-denaturing polyacrylamide gels (acrylamide:bisacrylamide = 40:1) in 0.5 × Tris–borate–EDTA (TBE) buffer. The gels were dried and exposed to an autoradiography film (DuPont) using Reflection intensifying screens (DuPont).

Luciferase reporter gene assay

The human CTGF promoter spanning from −818 to +19 bp of the transcription initiation site was amplified by PCR using KOD plus DNA polymerase (TOYOBO, Osaka, Japan). The PCR profile was as follows: denaturation at 94°C for 3 min; followed by 36 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 68°C for 45 s; and a final extension at 68°C for 5 min. The core binding site of NF-κB was −GGA ATG TCC C−. An amplified DNA fragment was inserted into the Kpn I-HindIII site upstream of a luciferase reporter gene in pGL3-Basic vector (Promega, Madison, WI, USA) and sequenced to verify the identity of the amplified DNA. Amplified DNA was transformed to competent DH5α (TOYOBO) by incubation for 30 min on ice, propagated in LB medium and purified with the Qiagen plasmid kit (Qiagen, Chatsworth, CA, USA) and used for transient transfection. The following primers were used: 5'-GGG GTA CCT TGA TGG CCA CTC GTC CCT T-3' (forward) and 5'-GGA AGC TTG AGG GTG GAG TCG CAC TGG CT-3' (reverse).

HK-2 cells were seeded in a 24-well plate at a density of 1 × 10⁵ cells/well and subsequently transfected with the CTGF promoter–reporter gene construct and the pRL-SV40 control vector (Promega) using Lipofectamine™ 2000 by the manufacturer’s instructions (Invitrogen). At 48 h post-transfection, these cells were incubated with 10⁻¹²M PTH for 12 h, and the cellular lysates were subsequently prepared for measurement of firefly luciferase and renilla luciferase activities using a dual-luciferase kit in a luminometer as instructed by the manufacturer (Lumat LB9507, Germany).

Statistical analysis

Data were expressed as mean ± SE. Statistical analysis was performed by ANOVA, followed by the non-parametric Kruskal–Wallis test for multiple comparisons. Statistical significance level was defined as P < 0.05.

Fig. 1. PTH induced CTGF expression in HK-2 cells. (A) HK-2 cells were exposed to 10⁻¹², 10⁻¹¹, 10⁻¹⁰, 10⁻⁹ or 10⁻⁸ M PTH for 48 h. β-actin was used as an internal control. (B) HK-2 cells were exposed to 10⁻¹⁰ M PTH for 12, 24, 36, 48 and 72 h. (C) HK-2 cells were treated as A. Cell lysates were prepared, and CTGF expression was detected by immunoblotting assay with anti-CTGF antibody. (D) HK-2 cells were treated as B. Cell lysates were prepared, and CTGF expression was detected by immunoblotting assay with anti-CTGF antibody. β-actin was used as an internal control. Results are expressed as the mean ± SE (*P < 0.05, **P < 0.01 compared with control).
Results

**PTH induced CTGF expression in HK-2 cells**

The effect of PTH to regulate CTGF expression in human proximal tubular HK-2 cells was examined by treating these cells with various concentrations of PTH. The results of quantitative real-time RT-PCR indicated that PTH at a concentration of $10^{-12}$–$10^{-10}$M induced a significant dose-dependent increase (all $P < 0.05$) in CTGF mRNA levels at 48 h post-treatment, and the further increase of PTH doses not only resulted in an attenuation enhancement in CTGF mRNA levels but also had differences with statistical significance compared with control cells (all $P < 0.05$, Figure 1A). In addition, HK-2 cells treated with $10^{-10}$M PTH for various periods of time and the results demonstrated that PTH induced a significant increase in the level of CTGF mRNA levels from 12 to 72 h post-treatment (all $<0.05$ compared with controls) (Figure 1B). The results of immunoblotting assay also demonstrated that $10^{-12}$ to $10^{-10}$M PTH induced a significant increase in the level of CTGF in a dose-dependent manner (all $P < 0.05$ compared with controls), and PTH could not induce the CTGF upregulation when its concentration was higher than $10^{-10}$M (Figure 1C). Moreover, PTH at a dose of $10^{-10}$M induced an $\sim$4-fold increase with statistical significance in the level of CTGF over the controls (3.82 $\pm$ 0.80-fold, $P = 0.03$). Furthermore, PTH ($10^{-10}$M) induced a time-dependent increase in CTGF levels (Figure 1D), with an initial increase of 1.75 $\pm$ 0.15-fold in the level of CTGF over the controls at 12 h post-treatment and an increase of 4.50 $\pm$ 0.15-fold in the level of CTGF over the controls ($P = 0.0004$) at 72 h post-treatment.

It has been demonstrated that CTGF is an autocrine downstream mediator of TGF-β1 activity in fibroblasts [25]. To determine if human proximal tubular HK-2 cells are capable of producing CTGF, the cell supernatant collected from different groups was analysed by CTGF ELISA. With the similar results of quantitative real-time RT-PCR analysis and immunoblotting assay, over a 60-h period (from 12 to 72 h post-PTH treatment), increasing levels of CTGF were released from the cells (Figure 2) confirming the ability of HK-2 cells to produce CTGF.

**MEK/ERK pathway is involved in PTH-induced CTGF expression in HK-2 cells**

We also investigated the effect of PTH on MAPK activation in HK-2 cells. As the results of immunoblotting assay, PTH ($10^{-10}$M) induced marked MAPK phosphorylation (Figure 3A) with a $\sim$1.9-fold increase over controls ($P < 0.05$ compared with control) at 30 min post-PTH treatment. In addition, it was demonstrated that PTH at a dose range of $10^{-11}$–$10^{-10}$M could increase MAPK phosphorylation in a dose-dependent manner (all $P < 0.05$ compared with control, Figure 3B). Higher doses of PTH caused an attenuated increase in MAPK phosphorylation. RT-PCR analysis showed that PTH caused a $\sim$3-fold increase in CTGF mRNA levels (Figure 3C). Moreover, pre-incubation of HK-2 cells with $5 \times 10^{-5}$M PD98059, a MAPK inhibitor, significantly attenuated PTH-induced increase in CTGF mRNA levels (all $P < 0.05$ compared with PTH-treated cells), suggesting that the MEK/ERK pathway is involved in PTH-induced CTGF expression in HK-2 cells, which was further confirmed by immunoblotting assay (Figure 3D).

**PTH activated NF-κB signalling pathway and CTGF promoter**

We further examined whether PTH induced the activation of NF-κB in HK-2 cells. It was shown that the NF-κB binding activity had risen substantially in the PTH ($10^{-10}$M)-stimulated HK-2 cells but not in the unstimulated cells (Figure 4A). However, the increase in the NF-κB binding activity induced by PTH treatment was markedly attenuated by PDTC (20 μM), a NF-κB inhibitor. In addition, PD98059, a MAPK inhibitor, also reduced the NF-κB-
binding activities induced by PTH (Figure 4B). Moreover, we found that PTH induced a ~7-fold increase in the levels of CTGF mRNA compared with those in controls (P < 0.05, Figure 4C). However, the addition of 20 μM PDTC, a NF-κB inhibitor, reduced the levels of CTGF mRNA in PTH-treated HK-2 cells. Similarly, the immunoblotting analysis also found that PDTC could attenuate the increase in the level of CTGF caused by PTH treatment (Figure 4D). Furthermore, the results from luciferase reporter assays using constructs with the luciferase gene under control of the CTGF promoter showed that increased luciferase activities were induced in HK-2 cells, suggesting the activation of CTGF promoter by PTH (P < 0.05, Figure 4E). The addition of PDTC, however, abolished the increases in luciferase activities induced by PTH, indicating that PTH triggered NF-κB signalling pathway to activate the CTGF promoter.

**Discussion**

Secondary hyperparathyroidism is a universal complication of chronic renal failure, and elevated serum intact PTH levels are common among patients with moderate chronic kidney disease [26]. Markedly elevated PTH levels in uraemia represent a 'uraemic toxin' responsible for many abnormalities of the uraemic state. The most significant consequence of increased PTH levels in uraemia is the development of bone disease characterized by osteitis fibrosa. In addition, PTH has been implicated in the development of
Fig. 4. PTH activated the NF-κB signalling pathway. (A) HK-2 cells were exposed to 10^{-10} M to PTH for 30 min (lane 5) in the absence (lane 6) or presence of 20 μmol/L NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) (lane 7). Nuclear extracts were prepared and incubated with radiolabelled NF-κB oligonucleotides. The DNA protein complexes were resolved by non-denaturing polyacrylamide gel electrophoresis. Unlabelled NF-κB oligonucleotide was added at a 100-fold molar excess as indicated. Lane 1, negative control without protein extract; lane 2, Hela Nuclear extract was used for positive control; lane 3, competitive inhibition with excessive unlabelled NF-κB oligonucleotide; lane 4, nuclear extracts were untreated. (B) HK-2 cells were treated with 10^{-10} M PTH for 1.5 h (lane 5) in the absence (lane 6) or presence of MEK1/2 inhibitor PD98059 (5 × 10^{-5} M) (lane 7). EMSA was performed as in A. Lane 1, negative control without protein extract; lane 2, Hela Nuclear extract was used for positive control; lane 3, competitive inhibition with excessive unlabelled NF-κB oligonucleotide; lane 4, nuclear extracts were untreated. (C) HK-2 cells were treated with PDTC (20 μmol/L) 12 h before and during 48 h incubation with PTH (10^{-10} M). CTGF expression was determined by quantitative real-time RT-PCR. β-actin was used as an internal control. Results are expressed as the mean ± SE (*P < 0.05 compared with PTH). (D) HK-2 cells were treated with PDTC (20 μmol/L) for 12 h before and during 48 h incubation with PTH (10^{-10} M). CTGF expression was detected by immunoblotting analysis. β-actin was used as an internal control. (E) HK-2 cells were transfected with CTGF promoter–reporter gene construct and the phRL-SV40 control vector. The cells were pre-incubated with an indicated amount of PDTC for 12 h and then treated with 10^{-10} M PTH for 12 h. Luciferase activities in the cells were determined by the dual-luciferase assay kit. Results are expressed as the mean ± SE (#P < 0.05 versus untreated control and *P < 0.05 versus PTH).
PTH-induced CTGF upregulation by activating MAPK and NF-κB pathways

Peripheral neuropathy, carbohydrate intolerance, hyperlipidaemia and other abnormalities in uraemia. However, there have been few studies on whether PTH exerts any direct effect on the kidney. Here, we investigated for the first time the involvement of PTH in the regulation of CTGF expression in human kidney proximal tubular cells.

The severity of renal interstitial fibrosis is closely related to renal function impairment and has been considered as one of the most important indicators for the progression of chronic kidney diseases [27]. Renal tubular epithelial cells produce various cytokines, including TGF-β1 and CTGF, which promote interstitial fibrosis. TGF-β1 is a potent pro-fibrotic chemokine that can stimulate proximal tubule epithelial cells to undergo apoptosis and epithelial−mesenchymal transdifferentiation, and to secrete various extracellular matrix (ECM) proteins, which contribute to the development of interstitial fibrosis. CTGF has been recognized as one of the important downstream factors that mediate the fibrotic activity of TGF-β1 [28−33]. CTGF induced renal interstitial fibrogenesis in the remnant kidney [34], while downregulation of CTGF expression using anti-sense oligonucleotides or small interfering RNA against CTGF attenuated the production of fibronectin or collagens in response to TGF-β1 [35,36]. In the present study, we demonstrated that PTH induced the expression of CTGF at both mRNA and protein levels. Our data clearly show the ability of human proximal tubular HK-2 cells to produce CTGF after the treatment with PTH. We speculate that PTH-induced CTGF upregulation in human kidney tubular cells may contribute to tubulointerstitial fibrosis in vivo.

PTH activates multiple signalling pathways following binding to the PTH1 receptor, including the MAPK signalling pathway. PTH activated MAPK in parietal yolk sac carcinoma cells or in CHO-R15 cells [37]. MEK/ERK1/2 signalling has been implicated in fibrosis [38,39]. The results from the current study revealed that low concentrations of PTH (10−12−10−10M) stimulated the phosphorylation of p42/p44. Furthermore, PTH-induced increases in the levels of CTGF mRNA and protein were inhibited by the p42/p44 MAPK inhibitor, PD98059. Thus, the present study provides the first evidence that PTH induces CTGF via the ERK1/2 MAPK pathway. On the other hand, our studies showed that higher doses of PTH (10−8−10−7M) caused a less vigorous activation of ERK1/2. High concentrations of PTH were found to inhibit ERK1/2 activation in UMR 106 cells [40,41]. A high dose of PTH was also indicated to transiently activate ERK1/2 in opossum kidney cells and CHO cells followed by quick ERK1/2 dephosphorylation [37].

PTH is a major activator of the RANK/RANKL complex that is part of the NF-κB pathway [42], and it was found to cause dose- and time-dependent increases in NF-κB DNA binding activities in human osteoblasts [42]. The activation of NF-κB and expression of CTGF were associated with chronic inflammatory and fibrotic nephropathy. In dialysis patients, a significant correlation was found between PTH levels and inflammatory markers [43]. The interaction between MAPK and other signalling pathways activated by PTH is likely to be important in determining the diverse biological effects of PTH. We have found that PTH-induced CTGF upregulation involves the MAPK signalling pathway and NF-κB signalling cascade. Cross-talk between the NF-κB signalling pathway and Ras/MEK/MAPK pathway has been previously documented. The MAPK pathway may play a dual role in renal inflammation, and MEK/ERK pathways are involved in regulating NF-κB/IκB-dependent production of inflammatory cytokines [44]. In the present study, we demonstrated that the inhibition of NF-κB by PDTC played a critical role in the suppression of CTGF gene expression in activated HK-2 cells in vitro. Two potential NF-κB binding sites were located within 545 to 535 and 94 to 83 in the promoter of the gene encoding CTGF [45]. Additional experiments will be conducted using promoter deletion assays and site-directed mutagenesis in our lab to clarify the necessity of these two regions containing the two NF-κB binding sites in response to PDTC. Furthermore, our results also demonstrate that MEK/ERK1/2 signalling is necessary for the upregulation of both CTGF and NF-κB DNA binding by PTH in human HK-2 cells. Several reports also showed that HK-2 cells respond to PTH by an increase in cAMP−PKA [46,47]. We investigated the role of PKA and PKC signalling in MAPK phosphorylation by PTH. Inhibitions of PKA signalling with H89 and PKC signalling with calphostin C markedly suppressed PTH-mediated MAPK phosphorylation, suggesting that MAPK activation by PTH is dependent on PKA and PKC signalling in human PTECs (data not shown here). Depending on the cell type and stimulus used, other signalling pathways involving Rho, Smad and AP1 have been shown to be required for induction of CCN2. Inhibition of Rho kinase reduced the level of CTGF, leading to the reduced expression of type I collagen gene in intestinal smooth muscle cells [48]. DN-Smad3, SiRNA-Smad3, smad7 and DN-AP1 suppressed TGF-β3-mediated activation of the CTGF promoter in cells of the nucleus pulposus [49]. However, our study has limitations, and additional experiments are required.

In conclusion, we present here the first report of the signalling pathways involved in the induction of CTGF gene promoter activity and upregulation of CTGF by PTH in human HK-2 cells. PTH-induced CTGF expression is, at least in part, dependent on NF-κB activation. MAPK is also activated by PTH and is involved in NF-κB-dependent CTGF transcription. Our results indicate that PTH may be involved in the fibrotic and inflammatory process which is important in the progression of chronic renal disease.

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