Nephrol Dial Transplant (2003) 18: 670–676
DOI: 10.1093/ndt/gfg141

Original Article

Nephrology
Dialysis
Transplantation

Pentoxifylline modulates intracellular signalling of TGF-β in cultured human peritoneal mesothelial cells: implications for prevention of encapsulating peritoneal sclerosis

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Abstract

Background. Peritoneal matrix accumulation is a major characteristic of encapsulating peritoneal sclerosis (EPS), which is a serious complication in long-term peritoneal dialysis (PD) patients. We reported previously that TGF-β stimulates collagen gene expression in cultured HPMC, and is attenuated by pentoxifylline (PTX). The SMAD family and the mitogen-activated protein kinase (MAPK) (ERK1/2, JNK and $p38^{HOG}$) pathways have been shown to participate in TGF-β signalling. However, how PTX modulates the intracellular signalling downstream to TGF-β remains undetermined in HPMC. In this study, we explored these signalling pathways in HPMC, and investigated the molecular mechanisms involved in the inhibitory effects of PTX on TGF-β-induced collagen gene expression in HPMC.

Methods. HPMC was cultured from human omentum by an enzyme digestion method. The expression of collagen α1(I) mRNA was determined by northern blotting, while the SMAD proteins and the MAPK kinase activity were determined by western blotting.

Results. TGF-β-stimulated collagen α1(I) mRNA expression of HPMC was inhibited by PTX. The Smad2, ERK1/2 and $p38^{HOG}$ pathways were activated in response to TGF-β. However, TGF-β displayed no activation of the JNK pathway in HPMC. The addition of PD98059 and SB203580, which blocked the activation of ERK1/2 and $p38^{HOG}$, respectively, suppressed the TGF-β-induced collagen α1(I) mRNA expression. At a concentration (300 μg/ml) that inhibited the collagen gene expression, PTX suppressed the ERK1/2 and $p38^{HOG}$ activation by TGF-β. In contrast, PTX had no effect on the TGF-β-induced activation of Smad2, under the same concentration.

Conclusion. PTX inhibits the TGF-β-induced collagen gene expression in HPMC through modulating the ERK1/2 and $p38^{HOG}$ pathways. Our study of PTX may provide the therapeutic basis for clinical applications in the prevention of EPS.

Keywords: encapsulating peritoneal sclerosis; mesothelial cell; pentoxifylline; signal transduction; TGF-β

Introduction

The term 'peritoneal fibrosing syndrome' (PFS) represents a wide range of peritoneal alterations observed in long-term peritoneal dialysis (PD) patients [1]. At least two extreme patterns were frequently mentioned. One is simple peritoneal sclerosis (SS), which is of low clinical impact but of high prevalence in regular PD subjects. The other is encapsulating peritoneal sclerosis (EPS), which is relatively rare but often associated with high mortality. Both SS and EPS are probably driven by the same processes and involve collagen deposition. However, as the management of EPS is still unsatisfactory, our work focused on the therapeutic potentiality of EPS.

EPS is a serious complication that develops in long-term PD patients [2]. Fibrogenic matrix accumulation is important in the pathogenesis of EPS [3]. Transforming growth factor-β (TGF-β) has been regarded as the central mediator of the fibrosing process in clinical diseases. PD patients with persistent TGF-β in their drained effluent were found to associate with an increased risk of EPS [4]. In addition, we had reported previously that TGF-β stimulates the expression of types I and III collagen mRNA in cultured HPMC [5]. These observations imply that TGF-β may mediate the

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development of EPS in PD patients. Pharmacological agents that attenuate TGF-β-induced matrix synthesis of HPMC may have clinical implications in the prevention or retardation of EPS.

Despite the well-recognized association between TGF-β and matrix accumulation, limited information is available regarding the mechanisms of TGF-β to induce this process [6]. The SMAD family members have been identified as major intracellular mediators of TGF-β signalling [7]. TGF-β first binds to the type II receptor on the cell membrane, then recruits the type I receptor into a complex. The phosphorylated type I receptor activates Smad2 and allows it to form a heteromultimer with Smad4. This complex is then translocated to the nucleus to regulate the transcription of target genes. In addition to the SMAD proteins, the mitogen-activated protein kinase (MAPK) pathways have been recently proposed to transmit parts of the downstream signallings of TGF-β [6,8]. The MAPK pathways contain three phosphorylation cascades: the extracellular signal-regulated protein kinase (ERK), the c-Jun N-terminal kinase (JNK), and p38 ^{HOG}. In our previous report [9], we demonstrated the presence of SMAD proteins, which acted in response to TGF-β in HPMC. We also showed that ERK1/2 was activated by TGF-β, and the blockade of ERK1/2 activity resulted in the decrease of TGF-β-induced α1(I) collagen gene expression. In other cell systems, the p38^{HOG} [10] and/or the JNK [11] pathways have been demonstrated to be one of the downstream targets required for TGF-β-mediated matrix expansion. In HPMC, however, the role of the JNK and the p38^{HOG} pathways in response to TGF-β has never been investigated.

Pentoxifylline (PTX) is a widely used anti-platelet agent. In addition to its anti-platelet effect, we demonstrated previously in human vascular smooth muscle cells [12] and in HPMC [5] that PTX may attenuate TGF-β-induced collagen synthesis. Nevertheless, the molecular mechanism of this inhibitory effect of PTX on TGF-β-induced collagen gene expression in HPMC remains undetermined.

In this work, we initially elucidated the intra-cellular signalling network (SMAD proteins, ERK1/2, JNK and the p38^{HOG} pathways) downstream of TGF-β in HPMC. Next, we further explored the inhibitory mechanism of PTX on TGF-β-treated HPMC by modulating these signalling pathways. Our results may provide a pharmacological basis of PTX for the treatment of EPS.

Materials and methods

Materials

Fetal calf serum (FCS) was obtained from Biochrome KG (Berlin, Germany). Culture flasks and plates were purchased from Corning (Corning, NY). Trypsin–EDTA, RPMI-1640 medium, glutamine and trypan blue were obtained from Gibco (Grand Island, NY). Aprotonin, ATP, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na_3VO_4), dibutyryl-cAMP (DBcAMP), 3-isobutyl-1-methylxanthine and other tissue culture reagents were purchased from Sigma (St Louis, MO). TGF-β1 was obtained from R&D (Minneapolis, MN). A selective cAMP-dependent protein kinase (PKA) inhibitor, H-89, was obtained from Calbiochem (La Jolla, CA). BCA reagents were from Pierce (Rockford, IL). Phosphorylated and non-phosphorylated polyclonal antibodies to ERK1/2, JNK, p38^{HOG}, Smad2 and Smad4 were all purchased from Cell Signaling (New England, MA). Human collagen α1(I) cDNA was purchased from the American Type Culture Collection (Rockville, MD). Agents used for isolating total RNA and northern blot analysis were obtained from Boehringer Mannheim (Mannheim, Germany) unless otherwise specified. PTX was generously provided by Aventis. All other chemicals used were of analytical grade.

Establishment of HPMC culture

Specimens of human omentum were obtained from omentectomy for elective gastric cancer resection, in which the omentum was grossly normal. HPMC culture was carried out as we previously reported [5]. Briefly, the surgically removed human omentum was washed thrice with PBS and then digested with trypsin EDTA (0.125%, Gibco) for 15 min. After centrifugation, the cell pellet was washed with culture medium and then seeded into a gelatin-coated (1 mg/ml) flask. Medium was changed on the third day. RPMI-1640 medium containing 20% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml) and insulin (30 µg /ml) was used. After 2–4 days, the cells became confluent and were subcultured with medium containing 10% FCS. HPMC was identified by the presence of vimentin and cytokeratin but without desmin and factor VIII-related antigen by the immunofluorescence method. All experiences were performed in passages 1–3 cells.

Northern blot analysis

To determine the effect of PTX on collagen gene expression, HPMC was grown in RPMI supplemented with 10% FCS until subconfluent. HPMC was arrested by 0.5% FCS for 24 h and then treated with TGF-β (1–2.5 ng/ml) in the absence or presence of various concentrations of PTX. After 24 h, cells were harvested for isolation of total RNA, as described previously [5,9]. The concentration of each sample was determined using spectrophotometry with the absorbance at 260 nm (A_{260}). The purity of each sample was determined based on the ratio of A_{260} to A_{280}. Ten micrograms of RNA were electrophoresed on a 1% agarose gel containing 1 M formaldehyde in MOPS buffer (0.2 M morpholinopropanesulfonic acid, 0.05M Na acetate, 0.01 M EDTA). The equivalency of sample loading and the lack of degradation were verified by ethidium bromide staining of 28S and 18S rRNA bands. The RNA was then transferred to nylon membranes by overnight capillary action and followed by fixation in a UV cross-linker.

For northern blotting, a 1.5-kb *Eco*RI fragment of collagen α1(I) was subcloned into pBSII/SK (Stratagene, La Jolla, CA) and used as templates for *in vitro* transcription of antisense digoxigenin-conjugated riboprobes according to the manufacturer's instructions (Boehringer Mannheim). The blots were developed using CSPD (Boehringer Mannheim) as the substrate for alkaline phosphatase, as described by the supplier. The signal intensity recorded on X-ray film was

then quantified with computerized densitometry and was normalized against the signal of glyceraldehyde-3-phosphate dehydrogenase messages.

Cell preparations and protein extraction

HPMC was grown in 10 cm dishes until subconfluent, growth-arrested (0.5% FCS) for 24 h and then was harvested after treatment at indicated periods of time with 200 µl ice-cold lysis buffer. The lysis buffer contains: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 40 mM β-glycerophosphate, 50 mM NaF, 10 mM sodium pyrophosphate, 200 µM sodium orthovanadate, 10 µg leupeptin/ml, 200 U aprotinin/ml, 1 µM pepstatin A, 1 mM PMSF, 100 nM okadaic acid. The obtained cell lysate was centrifugated at 14 000 r.p.m. for 5 min, and the protein concentration of the supernatant measured by BCA protein assay (Pierce).

Western blot analysis

The western blotting analysis of the MAPK pathways and SMAD proteins was performed by using phospho-specific antibodies according to the manufacturer's protocol. Briefly, HPMC was low serum (0.5% FCS) starved for 24 h, then stimulated with TGF-β (2.5 ng/ml) for different time intervals. For evaluating the inhibitory effect of PTX on TGF-β-stimulated activation of MAPK (ERK1/2, JNK, $p38^{HOG}$) and/or SMAD pathways, we incubated HPMC with 300 µg/ml PTX for 30 min before stimulation with TGF-β. The cells were harvested at the indicated time period then for western blotting. In addition, lanes of cells were treated with 3 or 10 µM H89 for 30 min before PTX. Cell lysates (30 µg protein) were separated by SDS–PAGE (12%), then transferred to PVDF membrane (Millipore, Bedford, MA). For immunodetection, the membranes were probed with primary antibody followed by incubation with peroxidase-conjugated secondary antibodies. Bands were visualized by the ECL system (Amersham).

Statistical analysis

The results are expressed as mean $\pm$ SEM, unless otherwise stated. These statistical analyses were carried out using StatView IV on a personal computer. Statistical significance ($P < 0.05$) was evaluated using the Student's t-test or one way analysis of variance with modified t-test performed using the Bonferroni correction.

Results

Previously in dose–response experiments [5,9], we had demonstrated that TGF-β (1–5 ng/ml) stimulated collagen gene expression dose–responsively in cultured HPMC. PTX at concentrations of 3–300 µg/ml caused a dose-dependent inhibition of serum- and TGF-β-stimulated collagen synthesis in HPMC [5]. Via cell viability test, we [4,8,11] had further excluded significantly cytotoxic effects of TGF-β and PTX under the above dose-dependent concentration. As the theme of this study is the intracellular signalling of HPMC stimuated and inhibited by TGF-β and PTX, respectively, the dosage of 2.5 ng/ml for TGF-β and 300 µg/ml for PTX were selected in these experiments.

PTX inhibits TGF-β-induced α1(I) mRNA expression in HPMCs

The inhibitory effect of PTX on TGF-β-induced expression of collagen α1(I) mRNA level was analysed in HPMC. Similar to what we reported previously [5], TGF-β induced collagen α1(I) mRNA expression in HPMC (Figure 1). Compared with untreated cells (control), TGF-β enhanced the collagen α1(I) mRNA levels. The treatment with PTX (300 µg/ml) potently down-regulated the collagen α1(I) mRNA expression by TGF-β in HPMC. PTX had been reported to exert its biological effect through the cAMP–PKA pathway [5,12]. Accordingly, we also examined the effect of DBcAMP, a membrane-permeable analogue of cAMP, on TGF-β-induced collagen α1(I) mRNA expression and tested whether the blockade of the cAMP–PKA pathway can overcome the inhibitory effect of PTX in TGF-β-treated HPMC (Figure 1). As expected, the exposure of HPMC to DBcAMP (1 mM) inhibited the induction of collagen α1(I) mRNA expression by TGF-β. On the other hand, the combination of PTX and H-89 (10 µM), a non-specific PKA inhibitor, prevented the suppressive effect of PTX on collagen α1(I) mRNA expression by TGF-β.

TGF-β activates ERK1/2, Smad2 and the $p38^{HOG}$ pathways in HPMC

We [9] demonstrated previously in HPMC the presence of ERK1/2 and Smad2/4 proteins, which were both

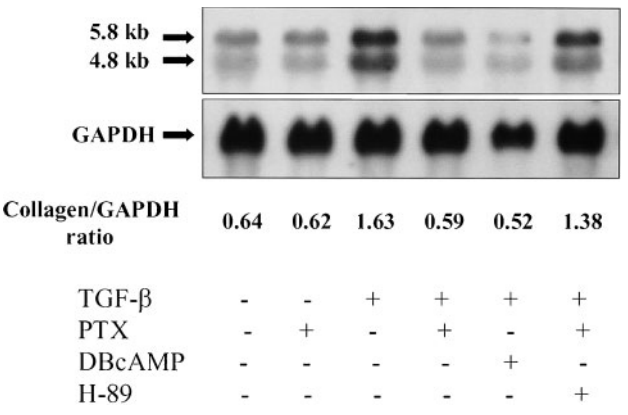

Fig. 1. PTX inhibits TGF-β-induced collagen α1(I) mRNA expression. HPMCs were grown as described in the Materials and methods section. PTX (300 µg/ml), DBcAMP (1 mM), H-89 (10 µM) or TGF-β (2.5 ng/ml) was added alone or in combinations, as indicated. After these additions, HPMC were incubated for 24 h before harvest. Untreated HPMC expressed low levels of α1(I) mRNA, and the abundance was not markedly altered by PTX alone. Representative northern blots of collagen α1(I) mRNA from four separate experiments with similar results is shown.

activated in response to TGF-β. Briefly, after adding TGF-β (2.5 ng/ml), Smad2 (58 kDa) was rapidly phosphorylated, which began within 15 min, peaked within 15–30 min, and persisted for 8 h. In contrast, the protein levels of Smad4 (62 kDa) were not changed in response to TGF-β (data not shown). In HPMC, TGF-β also activated ERK1/2, which began within 15 min after TGF-β, peak occurring in 30 min, and persisted for around 4–8 h (data not shown).

In this work, we further evaluated the effect of TGF-β on the other two MAPK pathways, the JNK and the $p38^{HOG}$ pathways, in HPMC. As shown in Figure 2A, $p38^{HOG}$ was activated within 15 min after TGF-β (2.5 ng/ml), peak occurring in 30 min, and returned to baseline by 8 h. In contrast, the JNK1/2 pathway displayed no activation in response to TGF-β (2.5 ng/ml) (Figure 2B). Similar results were observed at a higher concentration of TGF-β (5 ng/ml, data not shown). Protein levels of JNK1/2 and $p38^{HOG}$ remained constant during treatment with TGF-β. Conclusively, we found that ERK1/2 [9], Smad2 [9] and $p38^{HOG}$ (Figure 2) were activated by TGF-β, which may be responsible for the consequence of enhanced collagen α1(I) gene expression in HPMC.

PTX suppressed downstream signalling of TGF-β through modulations of ERK1/2 and $p38^{HOG}$ pathways

To explore the inhibitory mechanism of PTX on collagen α1(I) gene induction by TGF-β, we examined whether PTX modulates these intracellular signalling pathways downstream to TGF-β in HPMC. As shown in Figure 3, the activation of Smad2 by TGF-β was not significantly altered by PTX (300 μg/ml) in the presence or absence of H-90. In contrast, PTX (300 μg/ml) markedly suppressed ERK1/2 and $p38^{HOG}$ activation by TGF-β (2.5 ng/ml), which were rescued in the presence of H-89. We also tested the inhibitory effect of DBcAMP on activation of ERK1/2 or and $p38^{HOG}$ by TGF-β (Figure 4). In TGF-β-treated HPMC, DBcAMP (0.3–1 mM) suppressed the activation of ERK1/2 and $p38^{HOG}$. Pre-treatment with H-89 (10 μM) before the addition of DBcAMP preserved the activation of ERK1/2 and $P38^{HOG}$ by TGF-β. We next examined the effects of blockade of these transduction pathways (ERK1/2 and $p38^{HOG}$) on collagen α1(I) gene induction by TGF-β. Similar to what we observed in a previous report [9], PD98059 (25 μM) markedly inhibited the induction of collagen α1(I) gene expression by TGF-β (Figure 5). Similarly, the ability of TGF-β to stimulate collagen α1(I) gene expression was also reduced by SB203580 (30 μM), a selective inhibitor of the $p38^{HOG}$ pathway. These observations indicated that ERK1/2 and $p38^{HOG}$, but not the Smad2 pathway, were involved in the inhibitory effect of PTX on TGF-β-induced collagen α1(I) gene expression in HPMC. The modulations of ERK1/2 and $p38^{HOG}$ by PTX may mainly operate through the cAMP–PKA pathway.

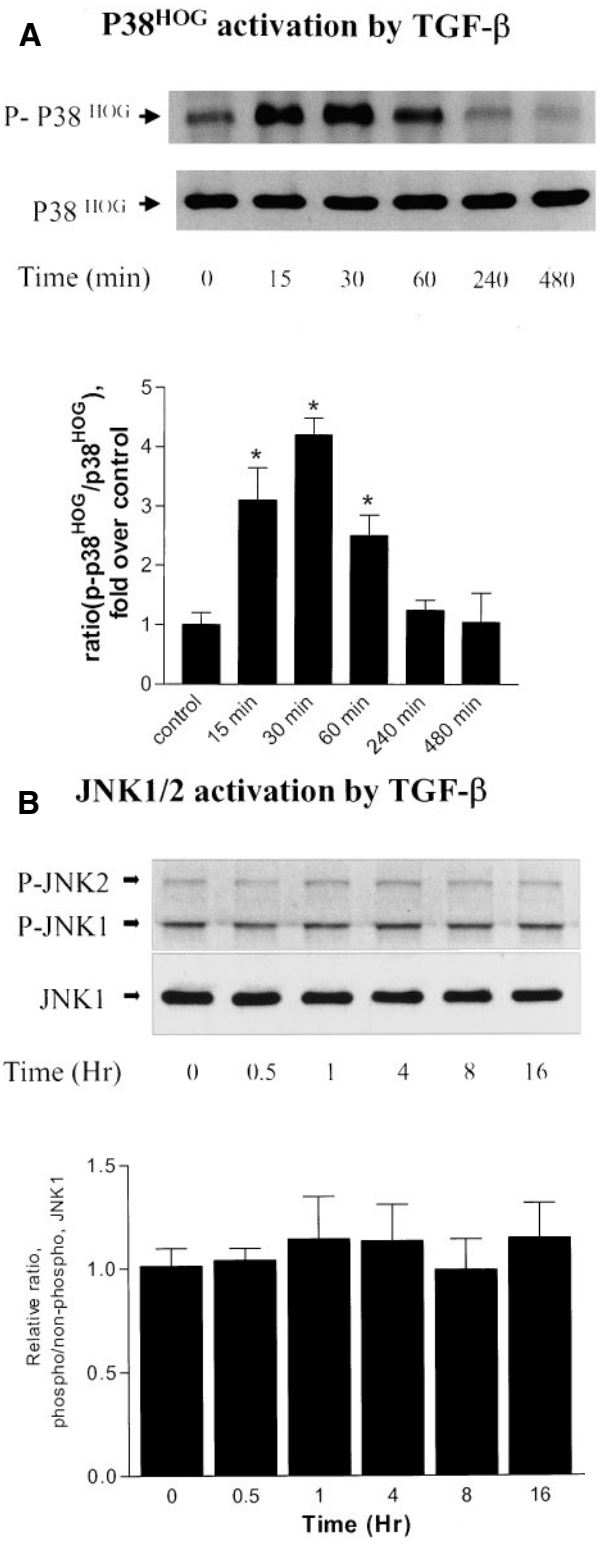

Fig. 2. Time course of (**A**) $p38^{HOG}$ and (**B**) JNK1/2 activation by TGF-β in HPMC. HPMC were treated with TGF-β (2.5 ng/ml) for the indicated time periods. Kinase activation was determined by western blot analysis using phosphospecific [p-$p38^{HOG}$ and p-JNK1/2] antibodies. As controls, the protein levels of $p38^{HOG}$ and JNK1/2 were determined using corresponding non-phosphorylated form antibodies. Results of densitometric analysis, expressed as a ratio between phospho- and non-phospho-antibody, from four separate experiments are shown.

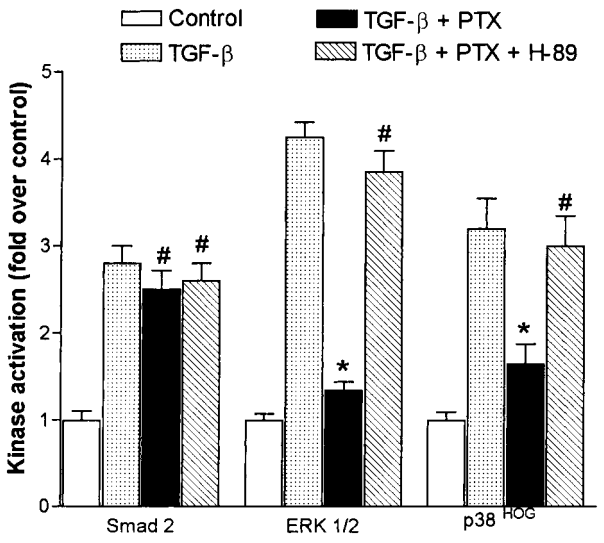

Fig. 3. Evaluation of inhibitory PTX on intracellular signalling pathways (ERK1/2, Smad2 and $p38^{HOG}$) activated by TGF-β (2.5 ng/ml) in HPMC. Cell lysates were immunoblotted as described in Figure 2. PTX (300 µg/ml) suppressed ERK1/2 and $p38^{HOG}$ MAPK activation by TGF-β, which was rescued in the presence of H-89 (10 µM). Activation of Smad2 by TGF-β was not altered by PTX. Shown are results of kinase activation obtained after densitometric analysis, expressed as a ratio between phospho- and non-phospho- levels, from four separate experiments. Values in the graph are shown as fold increase over corresponding controls.

Interaction of the ERK1/2 pathway and $p38^{HOG}$ in HPMC

Although the ERK1/2 and the $p38^{HOG}$ have been recognized as two independent transduction pathways for intracellular signalling, the nature of their possible interaction in HPMC is not clear. We performed experiments to evaluate the potential interaction between these two pathways. HPMC pretreated with PD98059 did not influence the activation of $P38^{HOG}$ upon TGF-β treatment (data not shown). Similarly, the activation of ERK1/2 by TGF-β was not altered in the presence of SB203580. These observations suggest that in HPMC, TGF-β induced collagen α1(I) gene expression through two independent pathways: the ERK1/2 and $p38^{HOG}$, which were both inhibited by PTX.

Discussion

We [5] demonstrated previously the possibility of PTX having the therapeutic potential for EPS by suppressing TGF-β-induced collagen gene expression in HPMC. As the SS and EPS both may be driven by the same processes driven by TGF-β and the other fibrogenic factors that increased collagen deposition, our observations may also be applicable to the spectrum of PFS. In this work, we explored the molecular mechanism of PTX with a special focus on the ability of PTX to modulate downstream signalling factors, SMAD proteins and the MAPK family (ERK1/2, JNK and $p38^{HOG}$ pathway) of TGF-β. In

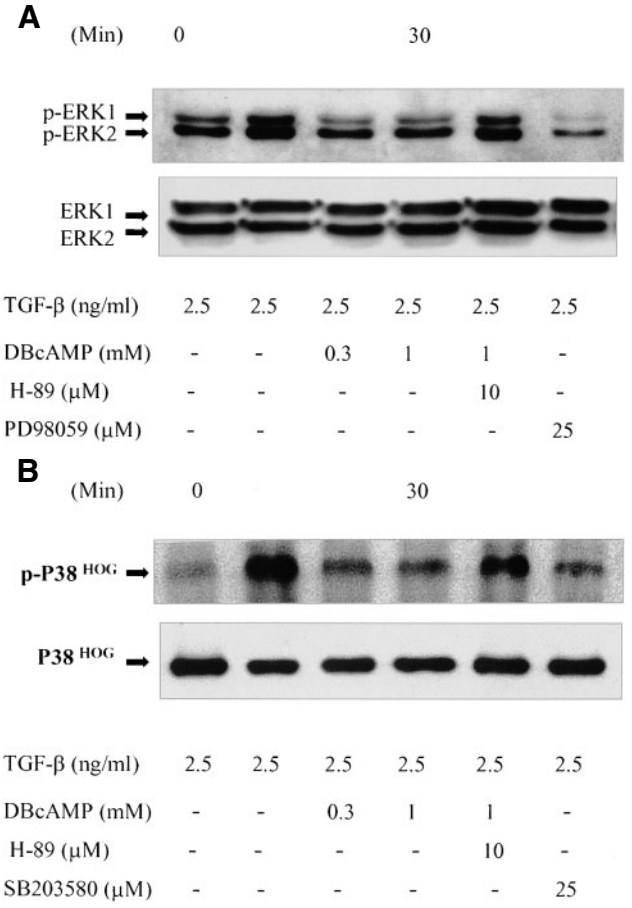

Fig. 4. Effect of DBcAMP and H-89 on TGF-β-induced (**A**) ERK1/2 and (**B**) $p38^{HOG}$ activation in HPMC. Cells were treated with DBcAMP (0.3–1 mM) in the presence or absence of H-89 (10 µM) for 1 h, then incubated with TGF-β (2.5 ng/ml) for another 30 min. Western blotting was performed as described in Figure 2. Selective kinase inhibitors, PD98059 (25 µM) and SB203580 (30 µM), were used as control for blockade of ERK1/2 and $p38^{HOG}$ kinase activity, respectively. Shown is a representative blot from four separate experiments with similar results.

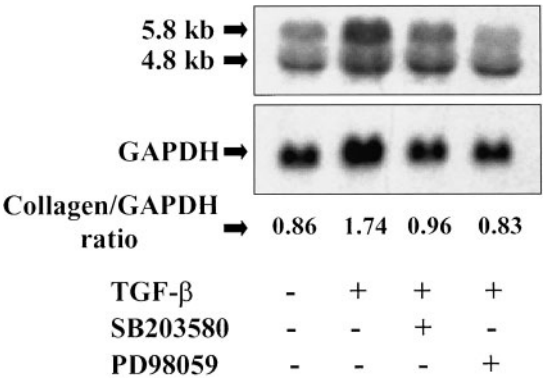

Fig. 5. Effect of PD98059 and SB203580 on TGF-β induced collagen α1(I) mRNA expression. HPMC were pre-treated with PD98059 (25 µM) or SB203580 (30 µM) for 1 h, then incubated with TGF-β (2.5 ng/ml) for 24 h. α1(I) mRNA expression was assessed as described in Figure 1. A representative northern blots from four isolated experiments with similar results is shown.

our previous study [9], we demonstrated that ERK1/2 and Smad2 were both activated by TGF-β in HPMC. After adding TGF-β to HPMC, $p38^{HOG}$ was rapidly activated (phosphorylated); in contrast, JNK1/2 displayed no activation (Figure 2). Taking these observations together, we found that in TGF-β-treated HPMC, ERK1/2 [9], Smad2 [9] and $p38^{HOG}$ were activated. These activated downstream pathways may all (or in part) contribute to the enhanced collagen α1(I) mRNA expression by TGF-β in HPMC.

As PTX inhibited collagen α1(I) gene induction by TGF-β, we next examined the effect of PTX tomodulate these intracellular signalling pathways (ERK1/2, Smad2 and $p38^{HOG}$) downstream to TGF-β in HPMC. As shown in Figure 3, PTX (300 μg/ml) did not alter the activation of Smad2 by TGF-β. In contrast, PTX (300 μg/ml) markedly suppressed ERK1/2 and $p38^{HOG}$ activation by TGF-β. Furthermore, the blockade of ERK1/2 and $p38^{HOG}$ by PD98059 (25 μM) and SB203580 (30 μM), respectively, abrogated the induction of collagen α1(I) gene expression by TGF-β (Figure 5). These observations indicated that the inhibitory mechanism of PTX on TGF-β-induced collagen α1(I) gene expression in HPMC were operated mainly through the ERK1/2 and $p38^{HOG}$ pathways.

PTX had been reported to exert its biological effect through the cAMP–PKA pathway [5,10]. Accordingly, the role of the cAMP–PKA pathway mediating the effect of PTX in TGF-β-treated HPMC was also determined. In this study, we found that the suppressive effect of PTX on ERK1/2 and $p38^{HOG}$ activation by TGF-β was reversed in the presence of H-89 (Figure 3). We also tested the inhibitory effects of DBcAMP and PTX on the induction of collagen α1(I) mRNA expression by TGF-β (Figure 1). PTX (300 μg/ml) as well as DBcAMP (1 mM) inhibited the enhancement of collagen α1(I) mRNA expression by TGF-β, which were reversed by pre-treatment of HPMC with H-89 (10 μM) before the addition of DBcAMP or PTX. The proposed possible mechanism of PTX on collagen α1(I) mRNA expression by TGF-β was described in Figure 6.

In this work, we did not exclude the possible involvement of the Smad pathway as the downstream signalling factor of TGF-β in HPMC. There are four points that need to be clarified further. First, the highest concentration of PTX used in this work is 300 μg/ml, since a higher concentration would result in cytotoxicity of HPMC [5]. At this concentration (300 μg/ml), PTX attenuated the TGF-β-induced collagen gene expression (Figure 1) and ERK1/2 as well as $p38^{HOG}$ activation (Figure 3). However, the activation of Smad2 by TGF-β was not affected in the presence of PTX (300 μg/ml) (Figure 3). The inhibitory effect of PTX, at higher concentrations, on TGF-β-induced activation of SMAD proteins thus remains undetermined. Secondly, from literature survey, the Smad-binding element on promoter area of collagen α1(I) gene has never been identified. However, a functional Smad-binding element of collagen α2(I)

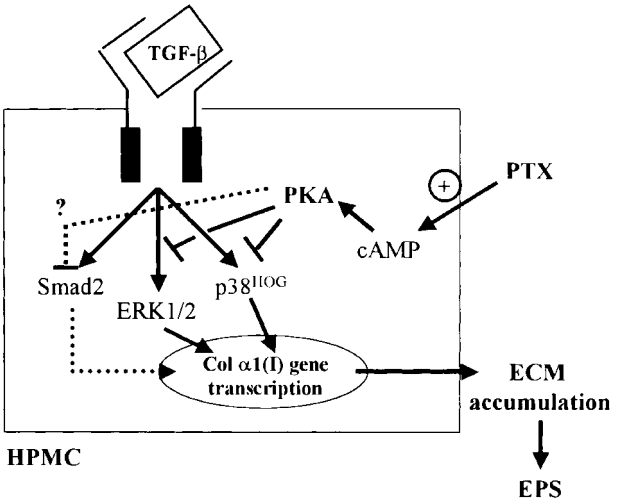

Fig. 6. Proposed possible mechanism of PTX on TGF-β-induced collagen α1(I) gene expression. TGF-β activates ERK1/2 and $p38^{HOG}$ MAPK pathways in HPMC. The role of Smad2 mediating TGF-β signalling, as well as the effect of PTX on Smad2, remains undetermined. PTX, through increased cAMP–PKA pathway, in turn results in blockade of ERK1/2 and $p38^{HOG}$ MAPK cascades.

gene promoter that is necessary for stimulation by TGF-β in human skin fibroblast has been recently identified [13]. The involvement of the Smad family in TGF-β-induced collagen gene expression of HPMC is, therefore, expected. This issue deserves further study. Thirdly, as there is still no widely available method to block the Smad2 activity, we therefore did not investigate the effect of Smad2 blockade on TGF-β induced collagen α1(I) mRNA expression. Transfection of dominant negative Smad2 in cultured HPMC may provide further information [14]. Finally, a recent study [15] in NRK fibroblast demonstrated that one protein, called connective tissue growth factor (CTGF), acts as a downstream mediator of TGF-β-induced collagen synthesis and can be down-regulated by elevated intracellular cAMP. The identification of CTGF in TGF-β-treated HPMC and their possible modulations by PTX deserves further study.

Another important issue we investigated in this work is the roles of the $p38^{HOG}$ and JNK pathways in TGF-β-treated HPMC. Our data demonstrated the involvement of $p38^{HOG}$, but not the JNK pathway, in TGF-β-induced collagen α1(I) mRNA expression of HPMC (Figures 3 and 5). During the preparation of our work, a similar observation in cultured myoblast was newly reported by Rodriguez-Barbero *et al.* [16]. They noticed that TGF-β-induced collagen α2(I) mRNA expression through the activated $p38^{HOG}$ and ERK1/2 pathways, but independent of JNK phosphorylation. PTX (Figure 3) and DBcAMP (Figure 4B) both reduced the TGF-β-induced phosphorylation of $p38^{HOG}$, which were blunted by H-89. We believe that PTX modulated the $p38^{HOG}$ pathway through the cAMP–PKA pathway. Nevertheless, the debate on possible cross-talk between the cAMP–PKA and the MAPK family continues. The elevation of intracellular

cAMP in cardiomyocyte [17] or T-cell [18] increased $p38^{HOG}$ activity. In contrast, activated cAMP–PKA activity that abolished $p38^{HOG}$ phosphorylation was also noticed in ET-1 treated osteoblast [19]. Different cells under various stimulatory conditions may exhibit diverse intracellular responses. The possible cross-talk between PKA and MAPK families in HPMC deserves further study.

In summary, we explored in HPMC the downstream signalling pathways of TGF-β. PTX, through modulations of the ERK1/2 and $p38^{HOG}$ pathways, inhibited TGF-β-induced collagen α1(I) gene expression. Our observations may provide a therapeutic basis for PTX in the prevention or retardation of EPS.

Acknowledgements. This article was supported by grants from the National Science Council (NSC 89-2314-B-002-508), Ta-Tung Kidney Foundation and Mrs Hsiu-Chin Lee Kidney Research Fund, Taipei.

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Received for publication: 20.5.02
Accepted in revised form: 17.10.02