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Inhibition by pentoxifylline of extracellular signal-regulated kinase activation by platelet-derived growth factor in hepatic stellate cells

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1 It has been proposed that pentoxifylline (PTF) acts an antifibrogenic agent by reducing the synthesis of extracellular matrix components, and this possibility has been confirmed in animal models of hepatic fibrosis. In this study the effects of PTF on the proliferation of extracellular matrix producing cells induced by platelet-derived growth factor (PDGF) were evaluated. The study was performed on hepatic stellate cells, currently indicated as the major source of extracellular matrix in fibrotic liver.

2 PTF caused a dose-dependent reduction of PDGF-induced mitogenesis with an IC_{50} of 170 μM, identical to the EC_{50} for the increase in intracellular cyclic AMP levels. Preincubation with PTF did not affect either PDGF-receptor autophosphorylation or phosphotidylinositol 3-kinase activity, whereas it markedly reduced PDGF-stimulated extracellular signal-regulated kinase (ERK) activity and ERK isoform phosphorylation. PTF also reduced PDGF-induced *c-fos* mRNA expression, which is dependent on activation of the RAS/ERK pathway. In addition, the PDGF-induced increase in cytsolic-free calcium was almost completely prevented by pretreating the cells with PTF.

3 The results of the present study indicate that PTF, in addition to its effect on collagen deposition and degradation, may exert an antifibrogenic effect by reducing the PDGF-induced proliferation of extracellular matrix producing cells. This effect appears to be mediated by a reduction of PDGF-stimulated ERK activity as well as of other intracellular signalling pathways such as the PDGF-induced elevation of cytosolic-free calcium.

Keywords: Cyclic AMP; extracellular-signal regulated kinase (ERK); hepatic fibrosis; hepatic stellate cells (HSCs); intracellular calcium; mitogen-activated protein kinase (MAPK); pentoxifylline; platelet-derived growth factor (PDGF); phosphatidylinositol 3-kinase (PI 3-K)

Introduction

Several *in vitro* and *in vivo* studies have shown that hepatic stellate cells (HSCs, also known as Ito cells, fat-storing cells or lipocytes), liver-specific pericytes, play a fundamental role in the progression of liver fibrogenesis (for review see: Friedman, 1993; Pinzani, 1995). Indeed, in conditions of chronic liver tissue damage and inflammation, HSCs undergo a process of activation and phenotypical modulation leading to cell proliferation and to a remarkable increase in their synthetic rate of extracellular matrix components, particularly collagen type I and type III. Among other polypeptide growth factors potentially involved in chronic tissue inflammation, platelet-derived growth factor (PDGF), a dimer of two polypeptide chains referred to as A- and B-chain, has been shown to be the most potent mitogen for cultured HSCs isolated from rat, mouse, or human liver (Pinzani *et al.*, 1989; 1992a; 1995) and, importantly, codistribution of PDGF with cells expressing the relative receptor subunits (termed α and β) has been clearly demonstrated following both acute and chronic liver tissue damage (Pinzani *et al.*, 1994; 1996), thus confirming a functional role of this polypeptide mitogen in the tissue repair process and in the development of hepatic fibrosis.

Pentoxifylline (PTF), a trisubstituted xanthine-derived phosphodiesterase inhibitor, is currently used in the treatment of peripheral vascular disorders because of its effects on erythrocyte deformability and tissue oxygen delivery (Ward & Clissold, 1987). *In vitro* studies, performed in cultured dermal fibroblasts, have suggested that PTF may exert an antifibrogenic effect by reducing the synthesis of extracellular matrix (ECM) components, including collagen type I and type III, by increasing collagenase activity, and by decreasing cell proliferation (Berman *et al.*, 1989; Chang *et al.*, 1993; Duncan *et al.*, 1995).

Although PTF has been shown to dose-dependently reduce PDGF-induced DNA synthesis in dermal fibroblasts (Peterson, 1993), it is unknown whether this effect could be relevant in antagonizing the proliferative effect of PDGF on cells primarily involved in the progression of liver fibrogenesis, i.e. activated HSCs. This issue becomes even more relevant after the recent demonstration of a possible antifibrogenic effect of PTF in two animal models of hepatic fibrosis: the yellow phosphorus porcine model (Peterson, 1993) and the rat bile duct ligation and scission model (Boigk *et al.*, 1995).

The present study was specifically designed in order to evaluate the effect of PTF on PDGF-induced cell proliferation in HSCs isolated from normal human liver and activated in culture, and to establish at which level PTF may interact with the complex cascade of intracellular signalling events leading to cell proliferation elicited by PDGF-receptor physical association with signalling molecules (Claesson-Welsh, 1994). The phosphotyrosines on the activated receptor operate as high-affinity binding sites for several molecules involved in downstream transmission of the signal through binding to src-homology-2 (SH-2) domains or phosphotyrosine-binding domains (Cohen *et al.*, 1995; Pawson, 1995). In particular, the attention was focused on PDGF-induced activation of three major pathways recently shown to be highly relevant for transducing the mitogenic effect of this polypeptide mitogen, namely phosphatidylinositol 3-kinase (PI 3-K), extracellular signal regulated kinase (ERK), and changes in intracellular calcium concentration ($[Ca^{2+}]_i$).

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Methods

Reagents

Monoclonal, agarose-conjugated antiphosphotyrosine antibodies were purchased from Oncogene Science (Uniondale, NY, U.S.A.). Rabbit antisera against extracellular-signal regulated kinase (ERK) were kindly donated by Dr M.J. Dunn (Medical College of Wisconsin, U.S.A.). The antisera recognised both ERK-1 and ERK-2 (Wang *et al.*, 1994). Phosphatidylinositol, and myelin basic protein were purchased by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Protein A-sepharose was from Pharmacia (Uppsala, Sweden). [γ-^{32}P]-ATP (3000 Ci mmol^{-1}) and [α-^{32}P]-dCTP (3000 Ci mmol^{-1}) were from New England Nuclear (Boston, MA, U.S.A.). Human recombinant PDGF-AA, -AB and -BB were purchased from Boehringer Mannheim (Mannheim, Germany). Highly purified pentoxifylline was kindly provided by Dr G. Rinaldi (Hoechst Italia S.p.A., Milan, Italy). All other reagents were of analytical grade.

Isolation and culture of human HSCs

Human HSCs were isolated from wedge sections of normal human liver unsuitable for transplantation as previously reported (Pinzani *et al.*, 1992b; Casini *et al.*, 1993). Briefly, after a combined digestion with collagenase/pronase, HSCs were separated from other liver nonparenchymal cells by ultracentrifugation over gradients of stractan (Larex-LO, Larex International Co., Tacoma, WA, U.S.A.). Extensive characterization was performed as described elsewhere (Casini *et al.*, 1993). Cells were cultured on plastic culture dishes (Falcon, Becton Dickinson, Lincoln Park, NJ, U.S.A.) in Iscove's modified Dulbecco's medium supplemented with 0.6 u ml^{-1} insulin, 2.0 mmol l^{-1} glutamine, 0.1 mmol l^{-1} nonessential amino acids, 1.0 mmol l^{-1} sodium pyruvate, antibiotic antifungal solution (all provided by Gibco Laboratories, Grand Island, NY, U.S.A.) and 20% foetal bovine serum (Imperial Laboratories, Andover, U.K.). Experiments described in this study were performed on cells between first and fifth serial passages (1 : 3 split ratio) using three independent cell lines. As already reported (Casini *et al.*, 1993), in our culture conditions, human HSCs showed transmission electron microscopy features of 'transitional cell' (smooth cell surface with slender nucleus, flattened profiles of rough endoplasmic reticulum interposed among numerous lipid droplets) during the early phases of subculture (passages 1 and 2), whereas features of 'myofibroblast-like cell' were clearly predominant after the third serial subculture (passages 3–5). These phenotypical changes are currently considered analogous to those observed *in vivo* following chronic liver injury (Mak & Lieber, 1988).

DNA synthesis

DNA synthesis was measured as the amount of [methyl-^{3}H]-thymidine ([^{3}H]-TdR) incorporated into trichloroacetic acid-precipitable material. Cells were plated in 24-well dishes at a density of 2×10^4 cells/well in complete culture medium containing 20% FBS. Confluent cells (approximately 1×10^5 cells/well) were made quiescent by incubation in SFIF medium for 48 h. Unless otherwise specified, cells were incubated with or without agonists at the indicated doses and modalities for 20 h and then pulsed for an additional 4 h with 1.0 μCi ml^{-1} [^{3}H]-TdR (6.7 Ci mmol^{-1}) (New England Nuclear, Boston, MA, U.S.A.). At the end of the pulsing period, [^{3}H]-TdR incorporation into cellular DNA was measured as previously reported (Pinzani *et al.*, 1989). Cell number was determined in three separate wells from each dish and results were expressed as c.p.m./10^5 cells.

Cell growth assay

Human HSCs were plated in 12-well dishes at a density of 2×10^4 cells/well in complete culture medium. After 24 h cells were washed twice with SFIF medium and were placed in the same medium containing PDGF-BB (10 ng ml^{-1}) with or without PTF (170 μM) pretreatment (Day 0). Cell counts were performed on quadruplicate wells at Day 0 and after 2, and 4 days by trypsinizing the cells and using a Coulter counter (Coulter Electronics Inc., Hialeah, FL). Fresh SFIF medium containing PDGF with or without pretreatment with PTF at the same concentrations was added to the remaining wells at each time point. Results are expressed as absolute cell number at each day of observation.

Phosphatidylinositol 3-kinase (PI 3-K) assay

Confluent HSCs were incubated in SFIF medium for 48 h, and then treated as indicated in the Results section. They were quickly placed on ice and washed with ice-cold PBS. The cell monolayer was lysed in RIPA buffer (composition mM: Tris-HCl 20, pH 7.4, NaCl 150, EDTA 5, Nonidet P-40 1%, Na_3VO_4 1, phenyl methyl sulphonyl fluoride (PMSF) 1, [w/v] aprotinin 0.05%). Insoluble proteins were discarded by high-speed centrifugation at 4°C. Protein concentration in the supernatant was measured in triplicate using a commercially available assay (Bio Rad, Hercules, CA, U.S.A.). PI 3-K assay was performed as described elsewhere (Ghosh Choudhury *et al.*, 1991). Briefly, identical amounts of protein were immunoprecipitated with antiphosphotyrosine antibodies. After washing, the immunobeads were resuspended in 50 μl of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5 mM EGTA; 0.5 μl of phosphatidylinositol (20 mg ml^{-1}) was added, and the samples were incubated at 25°C for 10 min. One μl of 1 M $MgCl_2$ and 10 μCi of [γ-^{32}P]-ATP were added simultaneously, and the incubation was continued for an additional 10 min. The reaction was stopped by addition of 150 μl of chloroform/methanol/37% HCl 10 : 20 : 0.2. Samples were extracted with chloroform and dried. Radioactive lipids were separated by thin-layer chromatography and developed with chloroform/methanol/30% ammonium hydroxide/water 46 : 41 : 5 : 8. After drying, the plates were subjected to autoradiography. Identity of the 3-OH phosphorylated lipids after separation by thin-layer chromatography had been previously assessed by high-pressure liquid chromatography (Ghosh Choudhury *et al.*, 1991). The radioactive spots were finally scraped and counted.

ERK assay

ERK was measured as the myelin basic protein kinase activity of ERK immunoprecipitates. RIPA lysates (60 μg of protein) obtained as described above were immunoprecipitated with rabbit polyclonal anti-ERK antibodies and protein A sepharose. After washing, the immunobeads were incubated in a buffer containing (mM): HEPES 10, pH 7.4, $MgCl_2$ 20, dithiotreitol 1, Na_3VO_4 1, [γ-^{32}P]-ATP 1 mCi and myelin basic protein 0.5 mg ml^{-1} for 30 min at 30°C. At the end of the incubation, 15 ml of the reaction were spotted onto phosphocellulose disks, washed four times in 1% phosphoric acid, and the radioactivity counted in a beta-counter. Another aliquot (10 ml) of the reaction mixture was run on 15% SDS–PAGE. After electrophoresis, the gel was dried and subjected to autoradiography.

Western blotting

Identical amounts of protein (15–25 μg) were separated by 7.5% or 12% SDS–PAGE, as appropriate, and electroblotted on a polyvinylidendifluoride membrane. The membranes were blocked overnight with 2% bovine serum albumin in 0.1% PBS-Tween, and sequentially incubated at room temperature with primary and then horseradish peroxidase-conjugated secondary antibodies (Amersham, Arlington Heights, IL, U.S.A.). Detection was carried out using chemiluminescence according to the manufacturer's protocol (Amersham).

Northern blot analysis

RNA was isolated according to Chomczynski & Sacchi (1987). Ten mg of total RNA were fractionated by 1% agarose-formaldehyde gel electrophoresis, and blotted on a nylon membrane. Procedures for DNA radiolabelling and filter hybridization have been described elsewhere (Marra *et al.*, 1993).

Digital video imaging of intracellular free calcium concentration in individual human HSCs

Digital video imaging of intracellular free calcium concentration ($[Ca^{2+}]_i$) in individual human HSCs was performed as described by Pinzani *et al.* (1992b) and Failli *et al.* (1995). Human HSCs were grown till subconfluence in complete culture medium on round glass cover slips (25 mm diameter, 0.2 mm thick) for 72 h, and then incubated for 48 h in SFIF medium. Cells were then loaded with 10 μM Fura-2-AM (Calbiochem Corp., San Diego, CA, U.S.A.), 15% Pluronic F-127 for 30 min at 22°C. $[Ca^{2+}]_i$ was measured in Fura-2-loaded cells in HEPES-$NaHCO_3$ buffer containing (mM): NaCl 140, KCl 3, NaH_2PO_4 0.5, $NaHCO_3$ 12, $MgCl_2$ 1.2, $CaCl_2$ 1.0, HEPES 10 and glucose 10, pH 7.4. Ratio images (340/380 nm) were collected every 3 s and calibration curves were obtained for each cell preparation as described by Pinzani *et al.* (1992b). PDGF-BB (10 ng ml^{-1}) was added directly to the perfusion chamber immediately after recording the $[Ca^{2+}]_i$ basal value. In parallel experiments, cells were preincubated with PTF (170 μM) for 15 min before the addition of PDGF-BB.

Assay of intracellular cyclic AMP levels

Confluent HSCs in 22 mm well dishes were incubated in serum-free/insulin-free (SFIF) medium for 24 h. SFIF medium was then removed and replaced with 200 μl/well of a buffer containing 0.025 M Tris-HCl, 0.25 M sucrose, 0.5% BSA, pH 7.4. Increasing doses of PTF were added to the wells and cells were incubated at 37°C for the indicated time points. The reaction was stopped by the addition of 500 μl of 100% ice-cold ethanol and dishes were placed at −20°C for at least 24 h. Samples were then centrifuged at 2500 *g* at 4°C for 20 min and supernatants were collected, lyophilized, and reconstituted with sodium acetate buffer (50 mM), pH 6.2. Intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels were measured by RIANEN cyclic AMP [^{125}I] Radioimmunoassay Kit (Du Pont Company, Billerica, MA, U.S.A.) according to the instructions provided by the manufacturer. Cell number was determined in three separate wells from each dish after trypsinization and counting.

Statistical analysis

Results, relative to the number of experiments indicated, are expressed as means ± s.d. Statistical analysis was performed by one-way ANOVA and when the *F* value was significant, by Duncan's test.

Results

Effect of PTF on the increase in DNA synthesis and cell proliferation induced by different PDGF dimeric forms in human HSCs

The effect of PTF on PDGF-induced DNA synthesis, measured as [^{3}H]-TdR incorporation into DNA, was first evaluated. In a preliminary set of experiments, PTF was able to reduce significantly the increase in DNA synthesis induced by PDGF dimeric forms only when cells were preincubated with PTF for 10–15 min before the addition of PDGF. Conversely, either the simultaneous addition of both compounds or the delayed addition of PTF (10 min after PDGF) did not cause a significant reduction of the PDGF-induced increase in DNA synthesis. These observations were confirmed with doses of PDGF-BB ranging from 1.0 to 10 ng ml^{-1}. As shown in Figure 1, preincubation of cell monolayers with PTF caused a dose-dependent reduction of the increase in DNA synthesis induced by PDGF-AA, PDGF-AB and PDGF-BB. PTF inhibited PDGF-BB-induced DNA synthesis with an IC_{50} (50% in-

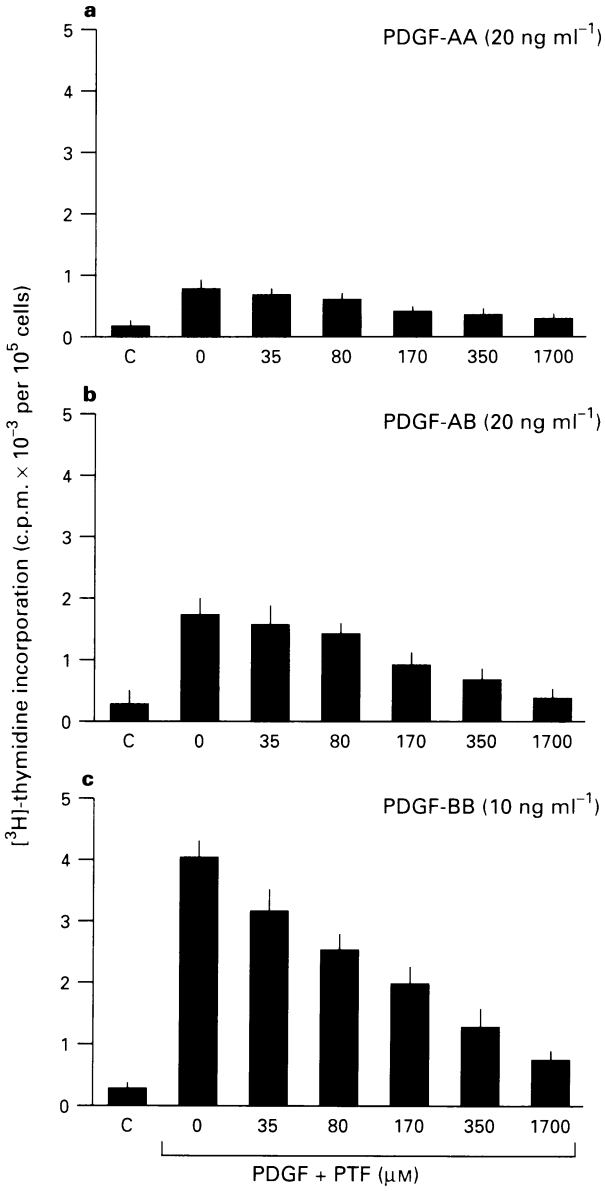

Figure 1 Effect of pentoxifylline (PTF) pretreatment on DNA synthesis induced by different PDGF dimeric forms in human hepatic stellate cells. Dose-response for the effect of increasing concentrations of PTF on DNA synthesis, evaluated as [^{3}H]-thymidine incorporation into DNA, induced by different PDGF dimeric forms. Confluent cells were made quiescent by maintaining them in serum-free/insulin-free medium for 48 h. Cells were then pretreated for 10–15 min with increasing concentrations of PTF before starting an incubation with fixed doses of (a) PDGF-AA (20 ng ml^{-1}), (b) PDGF-AB (20 ng ml^{-1}), (c) PDGF-BB (10 ng ml^{-1}), for a total of 24 h. Cells were pulsed with [^{3}H]-thymidine during the last 4 h of incubation (see Methods for details). C, in each panel, refers to control untreated wells. Data are means ± s.d. for five experiments performed in triplicate. When compared to the effect of PDGF isoforms alone (PTF concentration = 0 μM), changes were statistically significant ($P < 0.05$ or higher degree of significance) starting at PTF 350 μM for PDGF-AA, PTF 170 μM for PDGF-AB and 35 μM for PDGF-BB.

hibitory concentration) of approximately 170 μM. Because of the more relevant effect of PTF on PDGF-BB-induced DNA synthesis, the following experiments were focused on the biological effects and signalling elicited by this PDGF dimeric form. In addition, in all the following experiments a standard dose of PTF (170 μM) was consistently used.

Growth curve experiments for human HSCs in response to PDGF-BB with or without PTF were performed to demonstrate whether or not the decrease in PDGF-induced increase in [^{3}H]-TdR incorporation into DNA is associated with an actual decrease of cell growth. As shown in Figure 2, PDGF-BB at a dose of 10 ng ml^{-1}, significantly increased HSC growth after 2 and 4 days of incubation when compared with unstimulated control cells ($P<0.001$). This effect was clearly reduced by pretreating the cells with PTF before the addition of PDGF, and this reduction was already statistically significant after 2 days of culture.

Effect of PTF on PDGF-receptor autophosphorylation

Experiments were performed to elucidate at which step in PDGF mitogenic signalling the inhibitory effect of PTF occurs. Upon binding to PDGF, the receptor increases its tyrosine kinase activity and 'autophosphorylates'. As shown in Figure 3 (results of one representative experiment out of three), incubation with PDGF-BB resulted in a marked increase in tyrosine phosphorylation of the receptor (190 kDa band) (lane 3) when compared to control (lane 1). Preincubation of cells with PTF did not reduce the extent of receptor phosphorylation (lane 4). Incubation with PTF alone did not induce any effect on PDGF-R autophosphorylation (lane 2).

Effect of PTF on the increase of PI 3-K activity induced by PDGF-BB

The effect of PTF preincubation on the PDGF-induced activation of PI 3-K was then evaluated. As shown in Figure 4, (results of one representative experiment out of two) little or no PI 3-K activity was detectable in unstimulated, serum-starved HSCs (lane 1). Incubation with PDGF-BB (10 ng ml^{-1}) remarkably increased the PI 3-K activity associated with phosphotyrosine immunoprecipitates (lane 3). Pretreatment with PTF did not result in a detectable reduction of this activity (lane 4). Incubation with PTF alone (lane 2) did not affect PI 3-K activity when compared to unstimulated cells.

Effect of PTF on PDGF-stimulated extracellular signal-regulated kinase (ERK) activity in human HSCs

Exposure of serum-deprived HSCs to PDGF-BB resulted in ERK activation after 5 min of incubation with a peak at 10–15 min (data not shown). As illustrated in Figure 5a (results of one representative experiment out of three), incubation for 10 min with PDGF-BB (10 ng ml^{-1}) induced a remarkable increase in ERK activity as indicated by MBP kinase activity of ERK immunoprecipitates (lane 3). Preincubation with PTF for 10–15 min before the addition of PDGF resulted in complete prevention of PDGF-stimulated ERK activity (lane 4). PTF alone (lane 2) did not affect ERK activity of unstimulated HSCs (lane 1). As shown in Figure 5b (one representative experiment out of three), exposure of HSCs to PDGF-BB (10 ng ml^{-1}) resulted in a slower electrophoretic mobility of the two ERK isoforms, i.e. ERK-1 and ERK-2, indicating phosphorylation of tyrosine and/or threonine residues (lane 2). Preincubation with PTF for 10–15 min before stimulating the cells with PDGF (lane 4) resulted in a marked reduction of the amount of protein shifted upward, indicating reduced phosphorylation of both ERK isoforms. Importantly, the effects of PTF on both ERK activity and ERK isoform phosphorylation were not observed when cells were preincubated with PTF for time periods shorter than 10 min (data not shown).

Effect of PTF on PDGF-induced c-fos *mRNA expression in human HSCs*

The effects of PTF on PDGF-induced increase in *c-fos* mRNA expression, as a downstream target of ERK activation, were then evaluated. As shown in Figure 6 (results of one representative experiment out of three), *c-fos* mRNA expression, not detectable in baseline conditions as well as 15 min after the addition of PDGF, was clearly evident after 30 min. Pre-

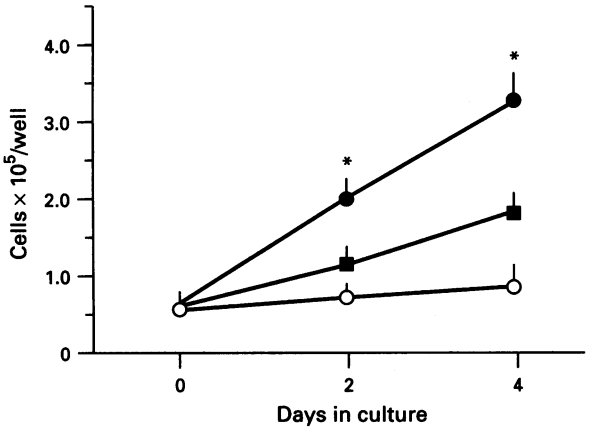

Figure 2 Effect of pentoxifylline (PTF) pretreatment on PDGF-BB-induced cell proliferation in human hepatic stellate cells. Human HSCs were plated in 12 well-dishes at the density of 0.4×10^5 cells per well in complete culture medium. After 24 h (Day 0) cells were washed twice with serum-free/insulin-free medium and then incubated in fresh serum-free/insulin-free medium alone (control, ○) or containing either PDGF-BB (●) or PTF plus PDGF-BB (■). At each time point (Day 2 and Day 4), cells were trypsinized and counted with a Coulter cell counter. Fresh medium containing the same test conditions was added to the remaining wells at each time point. PTF (170 μM) was always added 10–15 min before the addition of PDGF-BB (10 ng ml^{-1}). Data (mean ± s.d.), expressed as the absolute cell number, are from two experiments done in quadruplicate. *$P<0.005$ or higher degree of significance when compared to cells preincubated with PTF.

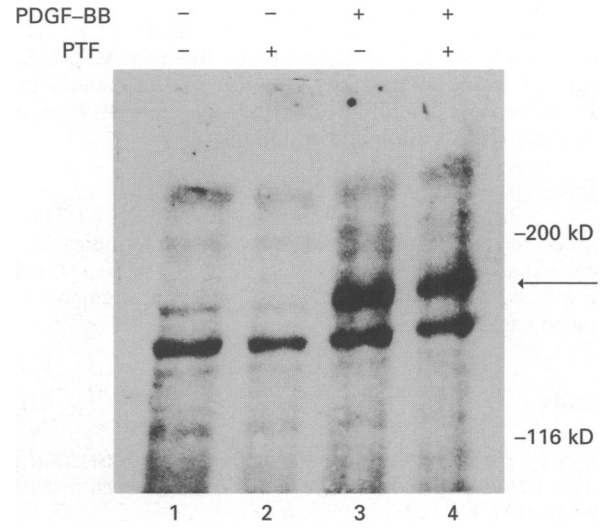

Figure 3 Effect of pentoxifylline (PTF) on PDGF receptor autophosphorylation in human hepatic stellate cells. Confluent human HSCs were maintained for 48 h in serum-free/insulin-free medium and then pretreated with PTF (170 μM; lanes 2 and 4) for 15 min before a 10 min incubation with PDGF-BB (10 ng ml^{-1}; lanes 3 and 4); 20 μg of cell lysate was separated by 7.5% SDS–PAGE and blotted with anti-phosphotyrosine antibodies. Representative experiment out of three.

treatment of cells with PTF resulted in an almost complete abrogration of *c-fos* expression, suggesting inhibition of the nuclear phase of the Ras/ERK pathway. In agreement with what had been observed previously, the inhibitory effect of PTF on PDGF-induced *c-fos* expression was not detectable when cells were preincubated with PTF for time periods less than 10 min.

Effect of PTF on PDGF-induced increase in $[Ca^{2+}]_i$

As shown in Figure 7, in individual Fura-2-loaded human HSCs, PDGF addition (open circles, average of 25 independent determinations in individual cells) caused the occurrence of an earlier cell synchronous Ca^{2+} spike (mean $[Ca^{2+}]_i$ at the peak 660 nM, $n=25$) over an average basal $[Ca^{2+}]_i$ of 70 nM. The peak phase was then followed by a long-lasting plateau (mean $[Ca^{2+}]_i$ 304 nM). Preincubation with PTF (170 μM) for 15 min before the addition of PDGF (solid squares, average of 25 independent determinations in individual cells), caused a marked reduction of both the peak and the plateau phase of $[Ca^{2+}]_i$ increase. For each single point, the standard deviation did not exceed 15% of the mean value.

Effect of PTF on intracellular cyclic AMP levels in human HSCs

The following experiments were performed in order to verify whether or not: (a) PTF was able to induce changes in cyclic AMP levels in HSCs, and (b) these changes could help to explain the 10–15 min preincubation period required to observe the effects on PDGF-induced mitogenesis and signalling. In a preliminary set of experiments, PTF increased intracellular cyclic AMP levels with an EC_{50} of approximately 170 μM (dose range 35–1700 μM) within a 10–20 min time-range. Subsequently, time-course experiments, performed with PTF 170 μM, indicated a peak effect at 10 min with significantly increased levels persisting for at least 30 min after the addition of the drug (Figure 8).

No apparent differences were seen in the effects of PTF on PDGF-induced cell proliferation and intracellular signalling between different cell lines and different stages of activation in culture (passages 1–5).

Discussion

In addition to its proposed actions on defective regional microcirculation, PTF has recently been indicated as a potential antiinflammatory/immunomodulator and antifibrogenic agent. Concerning this latter effect, the bulk of the available experimental evidence is so far limited to sound studies performed on dermal fibroblasts (Berman *et al.*, 1989; Chang *et al.*, 1993; Duncan *et al.*, 1995), in which, however, very little attention has been paid to an important aspect of fibrogenesis, namely

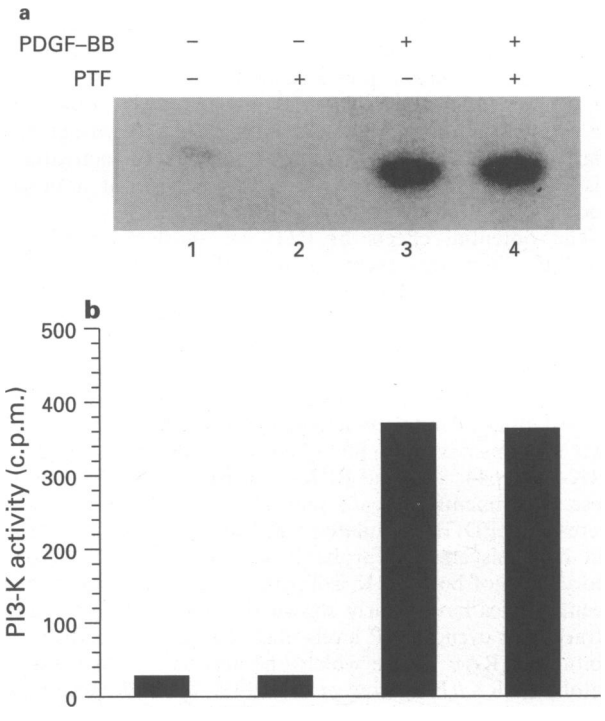

Figure 4 Effect of pentoxifylline (PTF) on PDGF-induced phosphatidylinositol 3-kinase (PI 3-K) in human stellate cells. Confluent human HSCs were maintained for 48 h in serum-free/insulin-free medium and then pretreated with PTF (170 μM; lanes 2 and 4) for 15 min before a 10 min incubation with PDGF-BB (10 ng ml^{-1}; lanes 3 and 4). Cell lysates were immunoprecipitated with antiphosphotyrosine antibodies and PI 3-K assay was performed as described in Methods. (a) Shows autoradiography of [^{32}P]-phosphatidylinositol-3-phosphate. The radioactive spots were scraped and counted in a beta-counter, as shown in (b). Representative experiment of two.

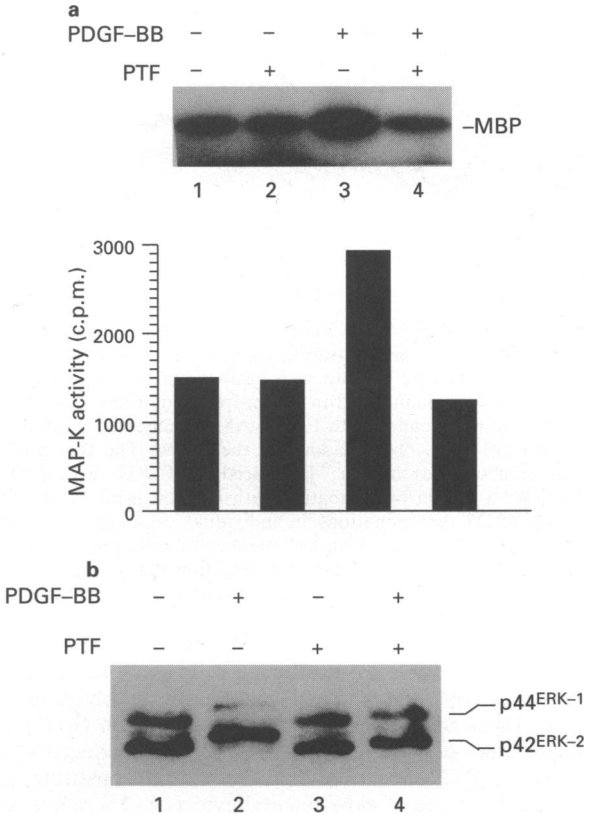

Figure 5 Effect of pentoxifylline on PDGF-induced extracellular signal-regulated kinase (ERK) activity and phosphorylation in human stellate cells. Confluent HSCs, maintained for 48 h in serum-free/insulin-free medium, were incubated as detailed in Figure 4. (a) Cell lysates (60 μg of protein) were immunoprecipitated with polyclonal anti-ERK antibodies, and the immunobeads were assayed for myelin basic protein (MBP) kinase activity as indicated in Methods. The upper part of the figure shows phosphorylated MBP after running on 15% SDS–PAGE, whereas the lowest part shows counts after spotting the reaction mix on phosphocellulose discs and extensive washings. Representative experiment out of three. (b) Twenty μg of cell lysate were separated by 12% SDS–PAGE and blotted with polyclonal anti-ERK antibodies. As shown in lane 2, addition of PDGF-BB caused a reduced electrophoretic mobility of both ERK-1 and ERK-2. Preincubation with PTF before the addition of PDGF induced a complete abrogation of this effect (lane 4). Representative experiment out of three.

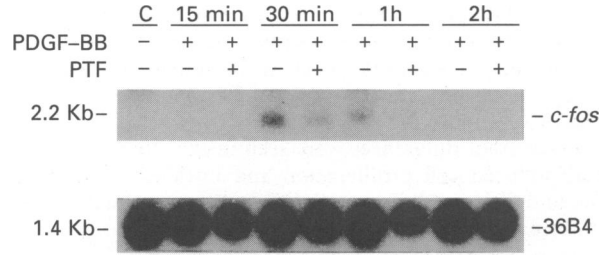

Figure 6 Effect of pentoxifylline (PTF) on PDGF-induced expression of *c-fos* mRNA. Confluent human HSCs were maintained for 48 h in serum-free/insulin-free medium and then incubated with PDGF-BB (10 ng ml^{-1}), with or without a 15 min preincubation with PTF (170 μM), for the indicated time points. RNA purification and hybridization were carried out as detailed in Methods. Control (C) refers to RNA isolated from cells incubated for 48 h in serum-free/insulin-free medium alone. *c-fos* cDNA probe was then removed by boiling, and the same blot hybridized to a cDNA encoding for the ribosomal protein 36B4 (control gene). Representative experiment of two.

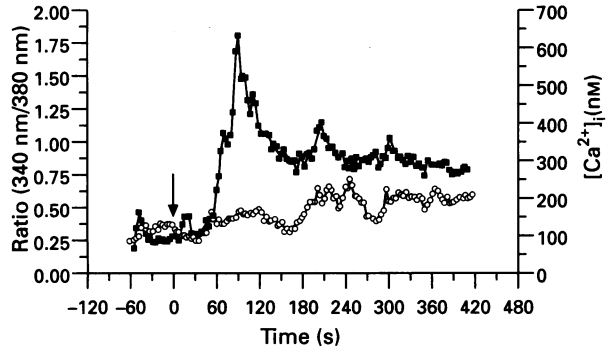

Figure 7 Effect of pentoxifylline (PTF) on PDGF-induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in individual Fura-2-loaded human hepatic stellate cells. Subconfluent (70–80% confluent) HSCs were maintained in serum-free/insulin-free medium for 48 h and were then loaded with Fura-2-AM as indicated in Methods. PDGF-BB (10 ng ml^{-1}) was added at the arrow. The two tracings illustrate the changes in $[Ca^{2+}]_i$ induced by PDGF with (○) or without (■) a 15 min preincubation with PTF 50 μg ml^{-1}. Data are the mean of 25 determinations in individual cells stimulated with PDGF compared with an identical number of cells pretreated with PTF. For each single point, standard deviation did not exceed 15% of the mean value. Measurements were obtained at 3 s time intervals.

the proliferation of ECM-producing cells and their relationship with polypeptide mitogens potentially involved in this process. Along these lines, the report by Peterson (1993), describing a dose-dependent reduction of PDGF-induced DNA synthesis by PTF in dermal fibroblasts, still constitutes the only available piece of experimental evidence. Therefore, studies specifically addressing this issue in other cell types involved in fibrogenic disorders and dissecting the molecular mechanisms of the antiproliferative effects of PTF are especially required.

The results of the present study indicate that PTF dose-dependently reduces the mitogenic effect of PDGF isoforms employed at saturating concentrations (Pinzani *et al.*, 1995). Although an inhibitory effect was observed for all PDGF dimeric forms, it was remarkably more relevant for PDGF-BB due to its well-established higher mitogenic potential for HSCs (Pinzani *et al.*, 1991; 1995), a cell type primarily involved in the progression of hepatic fibrosis. As demonstrated by cell proliferation experiments, the inhibitory effect of PTF does not appear to be limited to PDGF-induced increase in DNA synthesis, but it is also directed to an effective inhibition of cell proliferation. Subsequently, the potential effects of PTF on the

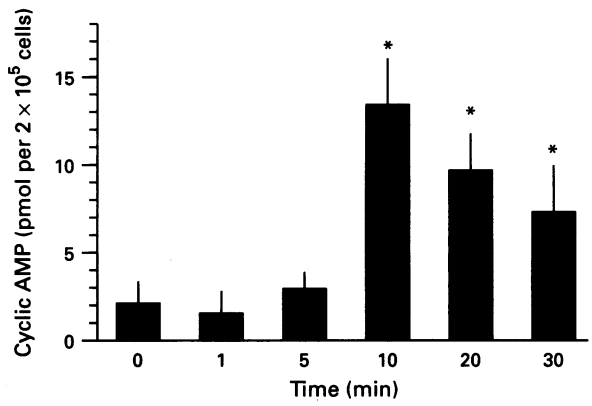

Figure 8 Effect of pentoxifylline on intracellular cyclic AMP levels in human hepatic stellate cells. Time-course for the effect of pentoxifylline (170 μM) on intracellular cyclic AMP levels. Confluent cells were incubated in serum-free/insulin-free medium for 48 h and then stimulated with pentoxifylline for the indicated time points. Intracellular cyclic AMP levels were measured as detailed in Methods. Data are means ± s.d. for three experiments performed in quadruplicate. *$P<0.005$ or higher degree of significance when compared to unstimulated cells (time 0).

main signalling pathways elicited by the autophosphorylation of the PDGF receptor and leading to mitogenesis were evaluated.

After observing that PTF does not affect the extent of PDGF receptor autophosphorylation, its possible effects on PI 3-K activity were studied. PI 3-K is a lipid and protein kinase which associates with the PDGF-receptor and other tyrosine kinases (Parker & Waterfield, 1992). The signals generated by PI 3-K are only partially known, but this pathway has been shown to be sufficient to transduce a PDGF-dependent mitogenic signal (Valius & Kaslauskas, 1993), and accordingly, it has been recently demonstrated that PI 3-K activation constitutes an important pathway involved in PDGF-induced mitogenesis in human HSCs (Marra *et al.*, 1995a,b). However, the results of the experiments described in the present paper clearly indicated that PTF is unable to affect the activation of this PDGF-stimulated pathway, thus ruling out a possible modulatory effect at this level.

The potential effects of PTF on another series of intracellular signalling events, namely the Ras/ERK pathway, the activation of which has been shown to be an absolute requirement for triggering growth factor-mediated proliferative responses (Pagès *et al.*, 1993), were subsequently studies. Activated Ras triggers a kinase cascade, with sequential activation of Raf-1, MEK and ERK (Marshall, 1995). ERK belongs to the family of mitogen-activated protein kinases (MAP-K). Two isoforms have been identified so far and are referred to as ERK-1 (or $p44^{MAPK}$) and ERK-2 (or $p42^{MAPK}$). The results of these experiments indicate that PTF induces a remarkable decrease of PDGF-stimulated ERK activity in human HSCs, and that this effect is probably related to a reduced phosphorylation of both ERK isoforms. Along these lines, several recent studies have clearly shown that agents able to elevate intracellular cyclic AMP levels may reduce cell growth via inhibition of RAF kinase which operates as an upstream regulator of ERK (Burgering *et al.*, 1993; Cook & McCormick, 1993). This action has been suggested to occur through cyclic AMP-activated protein kinase A (PKA) phosphorylation of Raf-1 (Wu *et al.*, 1993; Graves *et al.*, 1993). It is therefore conceivable that, also in the cellular model employed in the present study, the dose-dependent increase in intracellular cyclic AMP levels induced by PTF, due to inhibition of cyclic nucleotide phosphodiesterases (Stefanovich *et al.*, 1974), and the subsequent activation of PKA are responsible for the inactivation of the Ras/ERK pathway elicited by PDGF. Indirect evidence supporting this hypothesis derives from the

lack of detectable effects on the PDGF-induced phosphorylation of ERK subunits and ERK activity in experiments where incubation with PTF was not performed for the minimum time period of 15 min necessary to elicit a significant increase in intracellular cyclic AMP levels, and from the coincidence observed between the EC_{50} for PTF-induced increase in intracellular cyclic AMP levels and the IC_{50} for the inhibitory effect of PTF on PDGF-BB-induced mitogenesis.

In mammalian cells, the Ras/ERK pathway directs signals to the serum response element of *c-fos* and other co-regulated genes. The results of the experiments showing an inhibition of PDGF-induced *c-fos* expression by PTF further supports an effect of this drug on the activated Ras/ERK pathway, including downstream nuclear events leading to cell proliferation. However, although these results are consistent with a model in which PTF-induced increase in cyclic AMP levels inhibits PDGF-stimulated growth by inhibiting Raf activation, the possibility that the increase in cyclic AMP may affect cell growth through Raf-independent mechanisms cannot be excluded. Indeed it is well-established that the expression of cyclic AMP-responsive genes, including *c-fos*, can be directly regulated through phosphorylation of transcription factors by catalytic subunits of cyclic AMP-activated PKA (Lalli & Sassone-Corsi, 1994; Roesler *et al.*, 1995).

Studies from this laboratory have indicated that stimulation of confluent HSCs with PDGF-BB is associated with an increase in $[Ca^{2+}]_i$ (Pinzani *et al.*, 1991; Failli *et al.*, 1995). This effect is characterized by two main components: (1) a consistent and transient increase (peak increase), probably related to Ca^{2+} release from internal stores, and (2) a lower but longer lasting increase (plateau phase) due to an influx from the external medium. The results of the present study indicate that preincubation of quiescent human HSCs with PTF for a sufficient period of time causes an almost complete abrogation of both the peak and the plateau phase of the $[Ca^{2+}]_i$ increase induced by PDGF. Analogous to what was concluded for the action of PTF on the Ras/ERK pathway, this effect could be due to the increase in intracellular cyclic AMP levels induced by PTF. Accordingly, an increase in intracellular cyclic AMP levels has been shown to reduce $[Ca^{2+}]_i$ by inhibiting Ca^{2+} influx or by activating Ca^{2+} sequestration (Furukawa *et al.*, 1988; Cooper *et al.*, 1995). Importantly, induction of replicative competence by PDGF in this cell type has been shown to be greatly influenced by the maintenance of the plateau phase of $[Ca^{2+}]_i$ increase (Failli *et al.*, 1995), although it is presently unclear at which level, in the cascade of intracellular signalling events elicited by PDGF and leading to cell proliferation, this phenomenon could play a role. This aspect, that certainly deserves further attention, may involve a direct or indirect effect of $[Ca^{2+}]_i$ on ERK stimulation as suggested by studies highlighting the existence of at least two independent pathways for the activation of this system: one that is dependent on intracellular calcium mobilisation, and one that is mediated by receptor tyrosine kinase and is calcium-independent (Chao *et al.*, 1992).

In conclusion, the observations reported here indicate that PTF, in addition to its effects on collagen deposition and degradation, may exert an antifibrogenic effect by reducing the PDGF-induced proliferation of extracellular matrix producing cells. This effect appears mediated by a reduction of PDGF-stimulated ERK activity as well as of other intracellular signalling pathways such as the PDGF-induced elevation of cytosolic-free calcium. Although the inhibitory effects of PTF were clearly detected only at high concentrations (above 10^{-4} M), these observations provide additional cellular and molecular basis for the development of phosphodiesterase inhibitors with higher bio-availability and selectivity to be employed in the treatment of organ-specific as well as systemic fibrogenic disorders.

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