The platelet-derived-growth-factor receptor, not the epidermal-growth-factor receptor, is used by lysophosphatidic acid to activate p42/44 mitogen-activated protein kinase and to induce prostaglandin G/H synthase-2 in mesangial cells

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In renal mesangial cells, activation of protein tyrosine kinase receptors may increase the activity of mitogen-activated protein (MAP) kinases and subsequently induce expression of prostaglandin G/H synthase-2 (PGHS-2, cyclo-oxygenase-2). As examples, platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) were shown to transiently enhance p42/44 MAP kinase activity, which was an essential step in the induction of PGHS-2 mRNA and protein. Inhibitors of receptor kinase activities, tyrphostins AG1296 and AG1478, specifically inhibited the effects of PDGF and EGF respectively. Activation of p42/44 and p38 MAP kinases and PGHS-2 induction were also mediated by lysophosphatidic acid (LPA), which binds to pertussis-toxin-sensitive G-protein-coupled receptors. LPA stimulation was inhibited by AG1296, but not AG1478, indicating involvement of the PDGF receptor kinase in LPA-mediated signalling. This was confirmed by pertussis-toxin-sensitive tyrosine phosphorylation of the PDGF receptor by LPA, whereas no phosphorylation of the EGF receptor was detected. For comparison, 5-hydroxytryptamine ('serotonin')-mediated signalling was only partially inhibited by AG1296, and also not affected by AG1478. A strong basal AG1296-sensitive tyrosine phosphorylation of the PDGF receptor and a set of other proteins was observed, which by itself was not sufficient to induce p42/44 MAP kinase activation, but played an essential role not only in LPA- but also in phorbol ester-mediated activation. Taken together, the PDGF receptor, but not the EGF receptor, is involved in LPA-mediated MAP kinase activation and PGHS-2 induction in primary mesangial cells, where both protein kinase receptors are present and functionally active.

Key words: 5-hydroxytryptamine, p38 mitogen-activated protein kinase, protein kinase C, tyrosine phosphorylation, tyrphostin.

INTRODUCTION

Resident renal mesangial cells are critically involved in renal inflammatory reactions and glomerular injury. Upon activation, these normally quiescent cells proliferate and secrete various types of mediators, among them prostaglandins. The key enzymes of arachidonic acid metabolism, providing prostaglandin H_2 as precursor of all types of prostanoids, are prostaglandin G/H synthases-1 and -2 (PGHS-1, -2, cyclo-oxygenases). PGHS-2 is coded for by an immediate response gene and is rapidly induced by various different types of stimuli [1,2]. Depending on the stimulus and the cell type investigated, different signalling pathways are used to induce PGHS-2 mRNA expression and protein synthesis. In mesangial cells, we and others have previously characterized induction of PGHS-2 by activation of heptahelical receptors coupled to heterotrimeric G-proteins. Thromboxane and 5-hydroxytryptamine (5-HT; 'serotonin') [3], endothelin [4] or lysophosphatidic acid (LPA) [5] rapidly induced a transient up-regulation of PGHS-2 mRNA and protein, whereas ATP, another activator of G-protein-coupled receptors, was not able to induce PGHS-2 expression [3]. Induction by LPA and 5-HT was mediated by different types of G-proteins, pertussis-toxin (PTX)-sensitive and -insensitive respectively. Further downstream, common signalling modules, such as activation of p42/44 mitogen-activated protein (MAP) kinase, were part of both pathways [5–7]. Induction of PGHS-2 in mesangial cells was not restricted to G-protein-coupled signalling. Interaction of platelet-derived growth factor (PDGF) isoforms PDGF-AB or -BB with their respective protein tyrosine kinase receptors led to an activation of similar pathways in mesangial cells [8].

Ligand-independent activation of protein tyrosine kinase receptors by G-protein-coupled receptors has been shown in several cellular systems and seems to be a general possibility of signalling networks. There is, however, considerable cell specificity concerning the type of protein tyrosine kinase receptor used for trans-activation. One of the best studied systems is activation of the epidermal-growth-factor (EGF) receptor by LPA, which has been observed in fibroblasts [9–11], keratinocytes [10], HeLa cells [11] or transfected Cos cells [10]. These results are not without controversy, as EGF receptor trans-activation in Rat-1 cells described by Daub et al. [9,12] was not detected by Kranenburg et al. [13]. Trans-activation by LPA is not restricted to the EGF receptor: in cells lacking the EGF receptor, ligand-independent activation of the PDGF receptor was observed [12]. LPA-mediated trans-activation is PTX-sensitive, indicating par-

Abbreviations used: EGF, epidermal growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPA, lysophosphatidic acid; MAP kinase, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase; PDGF, platelet-derived growth factor; PGHS, prostaglandin G/H synthase; PKC, protein kinase C; PTX, pertussis toxin; 5-HT, 5-hydroxytryptamine ('serotonin'); FCS, fetal-calf serum; DMEM, Dulbecco's modified Eagle's medium.

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ticipation of G-proteins of the G_i family, which release the $\beta\gamma$ subunit that initiates the trans-activation pathway characterized by protein tyrosine phosphorylation [14]. Less is known about ligand-independent tyrosine kinase receptor activation by receptors coupling to PTX-insensitive G-proteins. Angiotensin II was studied in rat vascular smooth-muscle cells and shown to activate the PDGF receptor in one study [15] and the EGF receptor in another [16]. EGF receptor trans-activation was PTX-insensitive, whereas toxin sensitivity was not specified in the earlier study by Linseman et al. [15].

We used cultured renal mesangial cells in our investigations, because these are primary cell cultures which express receptors for both, PDGF and EGF. Furthermore, PTX-sensitive as well as PTX-insensitive activation of MAP kinases and induction of PGHS-2 mRNA and protein offered us the possibility of investigating receptor cross-talk between these different systems without the need to overexpress one or the other receptor.

MATERIALS AND METHODS

Materials

Recombinant human PDGF-BB was kindly provided by J. Hoppe, Biozentrum Würzburg, Würzburg, Germany. Recombinant human EGF, the tyrphostins AG1296 and AG1478, SB203580, GF109203X and PD98059 were obtained from Calbiochem, Bad Soden, Germany. LPA and 5-HT were from Sigma, Deisenhofen, Germany. PTX was from Biomol, Hamburg, Germany. Cell-culture reagents were from Biochrom, Berlin, Germany; fetal-calf serum (FCS) was from Gibco, Eggenstein, Germany.

Cell culture

Rat mesangial cells were isolated as described in [17] and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 5 μg/ml insulin, 4.5 g/l glucose, 100 units/ml penicillin and 100 μg/ml streptomycin containing 10 % FCS. Mesangial cells [$(0.5–1.0) \times 10^6$ cells/10 ml] were plated in 100 mm-diameter Petri dishes in medium with 10 % FCS. At subconfluency (after 3–4 days), cells were serum-starved in DMEM containing 0.5 % FCS for 3 days.

Northern-blot analysis

Northern-blot analysis was performed as described previously [3]. After stimulation for the indicated times, total RNA was extracted according to the protocol of Chomczynski and Sacchi [18] with minor alterations. Usually, RNA yield was about 30–40 μg/10 cm-diameter Petri dish. Separation of total RNA (10 μg/lane) was achieved by use of 1.2 %-agarose gels containing 1.9 % formaldehyde with 1 × Mops (20 mM morpholinopropanesulphonic acid, pH 7.0, 5 mM sodium acetate, 1 mM EDTA) as gel running buffer. Separated RNA was transferred to nylon membranes by capillary blotting and fixed by baking at 80 °C for 2 h.

Hybridization was performed with cDNA probes labelled with [^{32}P]dCTP using the NonaPrimer kit from Appligene, Heidelberg, Germany. The specific PGHS-2 probe was a 1.156 kb *Eco*RI fragment from the 5′ end of mouse cDNA [19]. The GAPDH (glyceraldehyde 3-phosphate dehydrogenase) probe was obtained with a 500 bp reverse-transcribed fragment. DNA–RNA hybrids were detected by autoradiography using Kodak X-Omat AR film.

Western-blot Analysis

Cellular proteins were isolated using RIPA buffer [50 mM Tris/HCl (pH 7.5)/1 % (v/v) Triton X-100/0.1 % deoxycholic acid/0.1 % SDS/150 mM NaCl/1 mM PMSF/1 mM sodium vanadate/14 μg/ml aprotinin]. For Western-blot analysis, 10–30 μg of protein were separated by SDS/10 %-PAGE, transferred on to PVDF membrane (Pall Biosupport Division Dreieich, Germany) and probed with specific polyclonal antibodies. Polyclonal rabbit antibodies directed against specific peptides of p44 MAP kinase or its phosphorylated form, P-p44 MAP kinase, were obtained from New England Biolabs. Both antibodies detected the 42 kDa and 44 kDa MAP kinase isoforms. The polyclonal antibody directed against the phosphorylated form of p38 MAP kinase was from New England Biolabs. The polyclonal rabbit antibody directed against mouse PGHS-1 was kindly provided by Professor D. DeWitt, Department of Biochemistry, Michigan State University, East Lansing, MI, U.S.A. PGHS-1 expression is not subject to change during short-time incubations with the stimuli used and thus was taken as control for equal protein loading. The polyclonal antibody against PGHS-2 was obtained from Cayman, Ann Arbor, MI, U.S.A. The antibodies directed against phosphotyrosine, PDGF receptor β and the adapter protein Shc were obtained from Santa Cruz, Heidelberg, Germany. The peroxidase-conjugated anti-rabbit secondary antibody was obtained from Amersham (Braunschweig, Germany). Protein–antibody complexes were revealed using the enhanced-chemiluminescence detection system (ECL®; Amersham).

Immunoprecipitation

Lysates containing 500 μg of protein were precleared with 20 μl of G-Plus agarose (Santa Cruz, Heidelberg, Germany) in a total volume of 500 μl for 20 min at 4 °C. To precipitate the PDGF receptor, 0.5 μg of anti-(PDGF-β receptor) antibody (Santa Cruz) was added for 2 h, followed by overnight incubation with G-Plus agarose (20 μl). Immune complexes were collected by centrifugation, washed three times with RIPA buffer and resuspended in SDS/PAGE sample buffer. After heating for 10 min at 95 °C, the samples were subjected to SDS/8 %-PAGE.

Determination of MAP kinase activity

p42/44MAP kinase activity was determined in cellular lysates using the p42/44 MAP kinase assay kit of Amersham Pharmacia Biotech, Freiburg, Germany, according to the manufacturer's instructions. The reaction time was 10 min. The reaction was linear with respect to protein over the range 2–10 μg.

Statistics

Data were statistically assessed by Student's t test. The significance level was set at $P < 0.05$.

RESULTS

Involvement of p42/44 MAP kinase in PDGF-mediated PGHS-2 expression

PDGF isoforms AB and BB have been shown previously to be potent inducers of PGHS-2 mRNA and protein in mesangial cells [8]. PDGF-BB was used throughout this study. Induction of

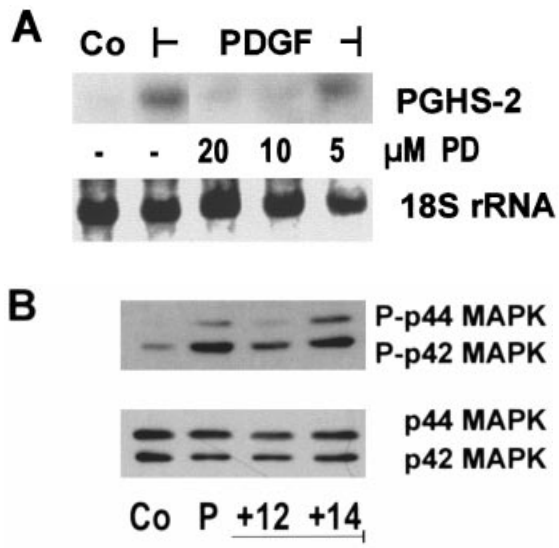

Figure 1 Activation of p42/44 MAP kinase is related to PGHS-2 mRNA induction by PDGF

(**A**) Mesangial cells were preincubated with the inhibitor of MAP kinase (MAPK) activation PD98059 (PD; 5, 10 and 20 μM) for 30 min and then stimulated with PDGF (20 ng/ml) for 2 h. PGHS-2 mRNA expression was detected by Northern-blot analysis. 18 S rRNA was stained with Methylene Blue to confirm equal loading of the gel. (**B**) Mesangial cells were preincubated with AG1296 (+12; 10 μM) or AG1478 (+14; 100 nM) for 30 min and then stimulated with PDGF (P) for 10 min. Cellular protein (25 μg) was subjected to Western-blot analysis using antibodies specific for p42/44 MAP kinase and their phosphorylated forms respectively; Co, control cells. The blot is representative of three independent experiments.

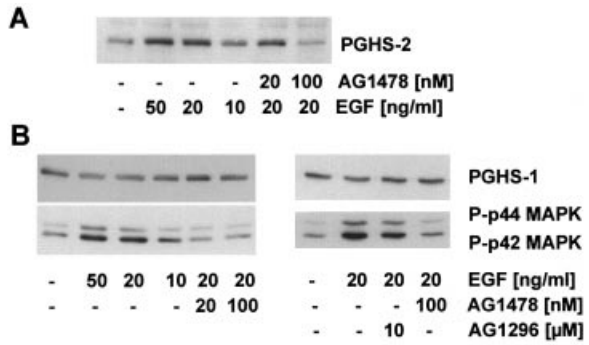

Figure 2 Activation of p42/44 MAP kinase and induction of PGHS-2 by EGF

(**A**) Mesangial cells were stimulated with EGF (10–50 ng/ml) as indicated for 2 h, with or without preincubation with the tyrphostin AG1478 (20 and 100 ng/ml) for 30 min. PGHS-2 protein expression was detected by Western-blot analysis. (**B**) Mesangial cells were stimulated with EGF (10–50 ng/ml) as indicated for 10 min, with or without preincubation with the tyrphostins AG1478 (20 and 100 ng/ml) or AG1296 (10 μM) for 30 min. Western blots were detected with an antibody specific for the phosphorylated form of p42/44 MAP kinase (MAPK). The upper part of the blot was probed with an antibody specific for PGHS-1. The expression of this enzyme is not changed by short-term incubation with the stimuli used and thus served as control for equal loading of the blots.

PGHS-2 mRNA expression was strongly inhibited by PD98059, an inhibitor of MAP kinase activation (Figure 1A). PD98059 was effective at a concentration of 10 μM, just above the IC_{50} of 2–7 μM [20–22]. A rapid activation of p42/44 MAP kinases was observed within minutes after stimulation of the cells with PDGF as detected by Western-blot analysis using an antibody directed against the phosphorylated MAP kinase (Figure 1B) or by activity measurements in cellular homogenates (Figure 4 below). By Western-blot analysis, the p44 MAP kinase (ERK1) appeared to be at least as abundant as p42 MAP kinase (ERK2). Activation of the cells by PDGF, however, preferentially increased the phosphorylation, and thus activation, of p42 MAP kinase (ERK2). Phosphorylation of MAP kinase was inhibited by the tyrphostin AG1296, an inhibitor specific for the PDGF receptor kinase, but not by AG1478, an inhibitor specific for the EGF receptor kinase. AG1478 was used at a concentration of 100 nM (IC_{50} 3 nM; [23]), which completely suppressed EGF-mediated MAP kinase activation (Figure 2B).

Induction of PGHS-2 expression by EGF

Effects of EGF on PGHS-2 expression in mesangial cells had not been reported previously. We could then show that EGF was able to induce PGHS-2 synthesis in a concentration-dependent manner (Figure 2A). Induction was receptor-mediated, as shown by the inhibitory effect of AG1478. Similarly, as observed with PDGF, EGF induced a rapid concentration-dependent activation of MAP kinase detected by phosphorylation of the p42/44 MAP kinase within 5–12 min (Figure 2B). Phosphorylation of MAP kinase was specifically inhibited by AG1478 (20 and 100 nM), but not affected by AG1296 at concentrations where PDGF-mediated MAP kinase activation was completely inhibited (Figure 1B), thus confirming the specificity of these tyrphostins.

Involvement of the PDGF receptor in LPA-mediated PGHS-2 induction

PDGF- and LPA-mediated induction of PGHS-2 mRNA (Figure 3A) and protein (Figure 3B) were inhibited when the cells were preincubated with the inhibitor of the PDGF receptor kinase AG1296. The effect was concentration-dependent, with LPA signalling being more sensitive than PDGF signalling: the lower concentration of AG1296 (10 μM) inhibited the induction of PGHS-2 protein by PDGF by $45.7 \pm 4.2\,\%$ compared with $68.2 \pm 14.4\,\%$ when LPA was used as stimulus ($n = 3$, means $\pm$ S.D., $P < 0.05\,\%$). The higher concentration of AG1296 (25 μM) led to an almost complete cessation in PGHS-2 expression induced by both stimuli. Preincubation of the cells with AG1478, the inhibitor of EGF receptor kinase, did not affect LPA-induced PGHS-2 synthesis (Figure 3C). 5-HT has been shown previously to induce PGHS-2 via 5-HT_{2A} receptors, which couple to PTX-insensitive G-proteins [6]. Induction of PGHS-2 by 5-HT was only partially inhibited by AG1296 (Figures 3A and 3D). Under conditions where LPA- and PDGF-mediated PGHS-2 protein synthesis was decreased to baseline values, 5-HT-mediated induction was only decreased by about 60 % ($58.9 \pm 1.4\,\%$; mean $\pm$ S.D., $n = 3$). Pretreatment of the cells with AG1478 did not significantly alter induction of PGHS-2 synthesis by 5-HT (results not shown).

Requirement for the PDGF receptor kinase in MAP kinase activation by LPA and 5-HT

Induction of PGHS-2 by LPA was inhibited by the MAP kinase kinase inhibitor PD98059 [5]. The concentration-dependence was comparable with that observed when the cells were stimulated with PDGF (Figure 1A). At a concentration of 10 μM, PD98059 inhibited LPA (10 μM)-induced PGHS-2 mRNA expression by $83 \pm 5\,\%$ (mean $\pm$ S.D., $n = 3$). Incubation of mesangial cells with LPA rapidly enhanced p42/44 MAP kinase activity and phosphorylation, both of which were inhibited by treatment of

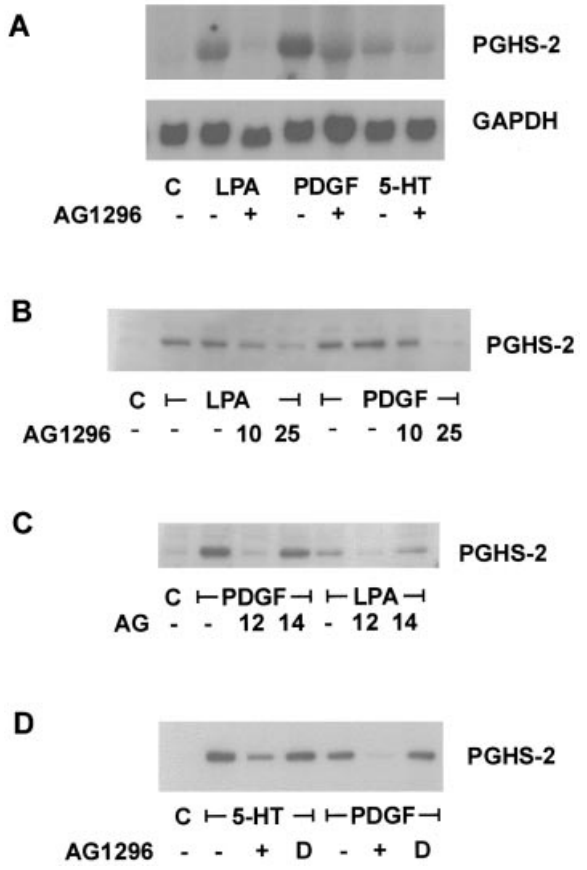

Figure 3 Involvement of the PDGF receptor in LPA-mediated PGHS-2 induction

(**A**) Mesangial cells were stimulated with LPA (25 μM), PDGF (20 ng/ml) or 5-HT (1 μM) for 2 h with or without preincubation with AG1296 (10 μM) for 30 min. Expression of PGHS-2 mRNA was detected by Northern-blot analysis. As a control for mRNA loading the blot was reprobed for GAPDH expression. (**B**) Concentration-dependent inhibition of PGHS-2 protein synthesis by AG1296 (10 and 25 μM) was detected by Western-blot analysis (stimulation for 2 h with 20 ng/ml PDGF or 10 μg/ml LPA). (**C**) Western-blot analysis of PGHS-2 protein after incubation with PDGF (20 ng/ml) or LPA (10 μg/ml) for 2 h with or without preincubation with AG1296 (10 μM) or AG 1478 (100 nM) for 30 min. (**D**) Mesangial cells were preincubated with AG1296 (+; 10 μM) or DMSO (D; 0.1%) for 30 min and then incubated with 5-HT (1 μM) or PDGF (20 ng/ml) for 3 h. PGHS-2 protein was detected by Western-blot analysis.

mesangial cells with the MAP kinase kinase/ERK kinase ('MEK') inhibitor PD98059 in a concentration-dependent manner (Figure 4A). Maximal values of MAP kinase activity were obtained after 2–5 min of stimulation with LPA (Figures 4B and 4C). No significant difference was observed between 10 and 25 μM LPA. Induction of MAPK phosphorylation by PDGF or 5-HT was slower and more prolonged: the ratio of activation by PDGF (20 ng/ml) to activation by LPA (10 or 25 μM) was 1.5 ± 0.2 ($n = 6$)-fold after 5 min and 2.3 ± 0.4 ($n = 4$)-fold after 10 min. Preincubation of the cells with AG1296 (10 or 25 μM) strongly decreased the LPA-mediated activation of p42/44 MAP kinase (Figures 4B–4E). In accordance with the data obtained with respect to PGHS-2 induction, the effect of AG1296 on 5-HT-mediated activation was less pronounced.

Interference of AG1296 with LPA-mediated MAP kinase activation was observed even after 2 min, when PDGF barely affected MAP kinase activity. This discrepancy hinted at a functional role for the basal activation of the PDGF receptor

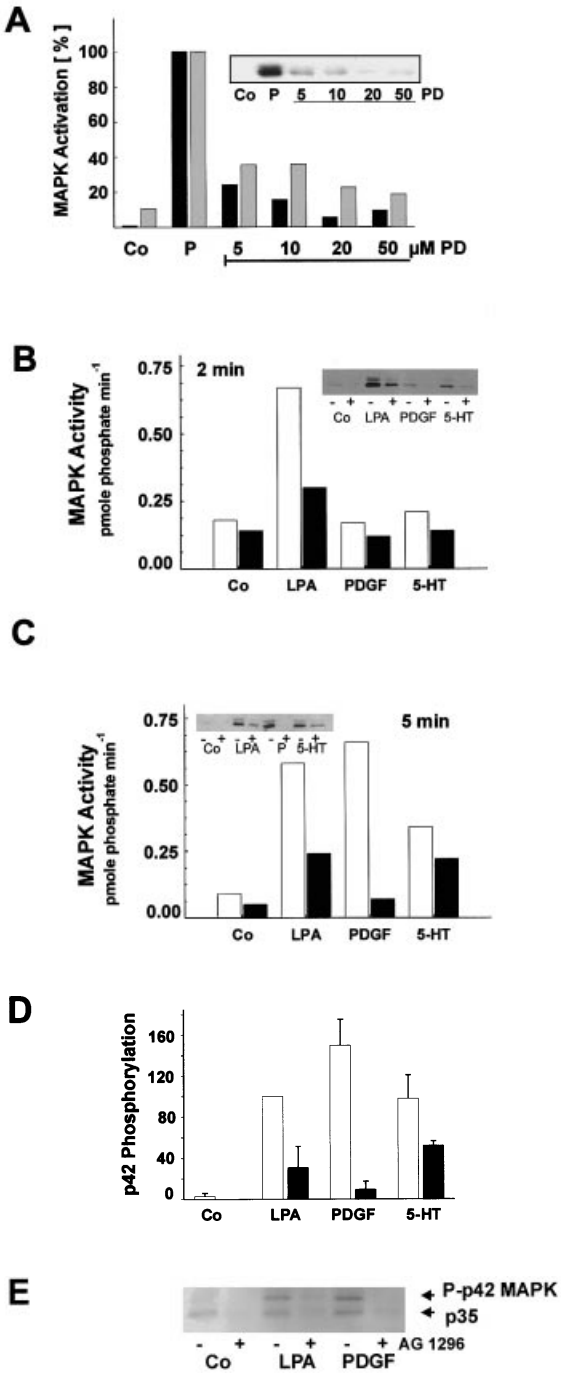

Figure 4 Sensitivity of p42/44 MAP kinase activation to tyrphostin AG1296

(**A**) p42/44 MAP kinase (MAPK) activation by PDGF (P, 20 ng/ml) was assessed by Western-blot analysis with antibodies directed against the phosphorylated forms (inset and black bars) and by determination of the specific activity in cellular homogenates as described in the Materials and methods section (grey bars, means of duplicate determinations). Before stimulation, cells were preincubated with PD98059 as indicated (5–50 μM). (**B** and **C**) Mesangial cells were preincubated with AG1296 (25 μM) for 30 min (black bars) and then stimulated for 2 and 5 min with LPA (25 μM), PDGF (20 ng/ml) or 5-HT (1 μM); open bars represent cells stimulated without AG1296. MAP kinase activity was determined in cellular homogenates; the insets show the corresponding Western blots of the phosphorylated form of p42/44 MAP kinase with or without preincubation with AG1296. (**D**) Densitometric evaluation of p42 MAP kinase phosphorylation after 5 min of stimulation. Conditions were as described in (**C**); the concentration of LPA was 10 or 25 μM. Phosphorylation induced by LPA was set to 100%. Data are means $\pm$ S.D. for five experiments. (**E**) Cells were stimulated as described in (**C**). The Western blot was probed with an antibody directed against phosphotyrosine. The blot is representative of five experiments.

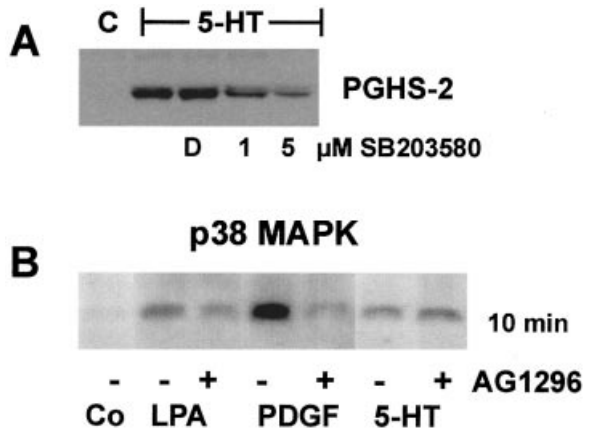

Figure 5: Role of p38 MAP kinase activation

(**A**) Mesangial cells were pretreated with 1 or 5 μM SB203580 or DMSO (D, corresponding to 5 μM SB203580) for 30 min and then stimulated with 1 μM 5-HT for 3 h. C, non-treated control cells. PGHS-2 protein expression was detected by Western-blot analysis. The blot is representative of three independent experiments. (**B**) Mesangial cells were stimulated as described in (**A**). Phosphorylation of p38 MAP kinase (MAPK) was detected using a phosphospecific antibody. The blot is representative of three independent experiments with similar results.

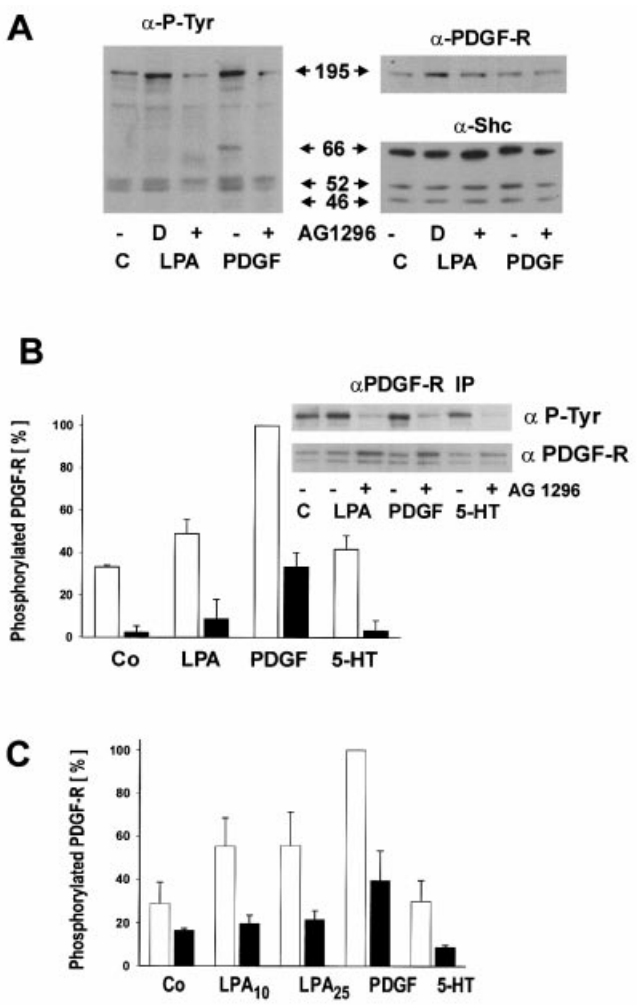

Figure 6 Phosphorylation of the PDGF receptor by LPA

(**A**) Mesangial cells were preincubated with AG1296 (+; 25 μM) or DMSO (D; 0.25%) for 30 min and then stimulated with PDGF (20 ng/ml) or LPA (25 μM) for 10 min. The Western blot was detected with antibodies directed against phosphotyrosine (α-P-Tyr), the PDGF receptor (α-PDGF-R) or Shc (α-Shc). p52 Shc co-migrated with the lower band of the tyrosine-phosphorylated doublet. The blot is representative of five independent experiments with similar results. (**B**) Cellular protein was precipitated with an anti-(PDGF receptor) antibody and probed with an antibody directed against phosphotyrosine (α P-Tyr) and against the PDGF receptor (α PDGF-R). Open bars show cells without AG1296; black bars represent protein of cells pretreated with AG1296. Phosphorylation of control cells stimulated with PDGF was set to 100%. Data are means $\pm$ S.D. for three experiments. (**C**) Densitometric quantification of the PDGF receptor phosphorylation of three to five Western-blot analyses probed with anti-phosphotyrosine antibodies. Cells were stimulated with LPA (10 and 25 μM), PDGF (20 ng/ml) or 5-HT (1 μM) for 5 min (open bars). Preincubation time with AG1296 (black bars, 25 μM) was 30 min. Data are means $\pm$ S.D.; phosphorylation after PDGF stimulation was set to 100%.

system (see below), which was suppressed during the 30 min preincubation period with AG1296. Basic tyrosine phosphorylation observed in serum-deprived cells only marginally activated p42/44 MAP kinase, because in most experiments, detection of phosphorylated p42/44 MAP kinases with the phosphospecific antibodies was at or beyond the detection limit. Consistently, tyrosine phosphorylation of a band co-migrating with the phosphorylated form of p42 MAP kinase was only detected in stimulated mesangial cells (Figure 4E). The identity of another tyrosine-phosphorylated band with a molecular mass of 35 kDa is not yet known. Phosphorylated p35 was detectable in control cells. Its phosphorylation was only slightly enhanced by LPA (25 μM LPA; 1.3 ± 0.1-fold after 5 min, $n = 3$; no increase after 10 min). Phosphorylation of p35 was more pronounced by PDGF (1.8 ± 0.4-fold after 5 min, $n = 3$; 2.7-fold after 10 min), whereas 5-HT or PMA ('TPA') had no significant effect (results not shown). Treatment of mesangial cells with AG1296 abolished tyrosine phosphorylation of this protein in control cells and stimulated cells.

Previous studies showed activation of p38 MAP kinase to be part of the LPA-mediated signal-transduction pathway leading to PGHS-2 expression [5]. Preincubation of mesangial cells with SB203580, a rather specific inhibitor of p38 MAP kinase, also decreased 5-HT-mediated PGSH-2 induction, indicating an involvement of p38 MAP kinase in 5-HT signalling (Figure 5A; $58.9 \pm 2.7\%$ inhibition by 5 μM SB203580, mean $\pm$ S.D., $n = 3$). This was confirmed by analysis of the phosphorylated, and thus activated, form of p38 MAP kinase. All three of the stimuli PDGF, LPA and 5-HT induced phosphorylation of p38 MAP kinase within 5–10 min (Figure 5B). Pretreatment of mesangial cells with AG1296 (25 μM) decreased LPA-mediated phosphorylation of p38 MAP kinase (58 and 57% in two independent experiments), whereas no significant effect was observed regarding stimulation by 5-HT. Pretreatment of the cells with 10 μM AG1296 did not affect p38 phosphorylation (results not shown).

Phosphorylation of the PDGF receptor by LPA

At higher concentrations, AG1296 has been shown to inhibit other kinases which might be involved in LPA signalling [24]. To confirm the involvement of the PDGF receptor, mesangial cells were treated with LPA, 5-HT or PDGF, and tyrosine phosphorylation was assessed by Western-blot analysis. Control cells, although kept in medium with low serum (0.5%) for 3 days before the start of the experiments, showed a variable degree of tyrosine phosphorylation of the receptor (Figures 6A, 7B and 8C), which was sensitive to treatment with AG1296. As expected,

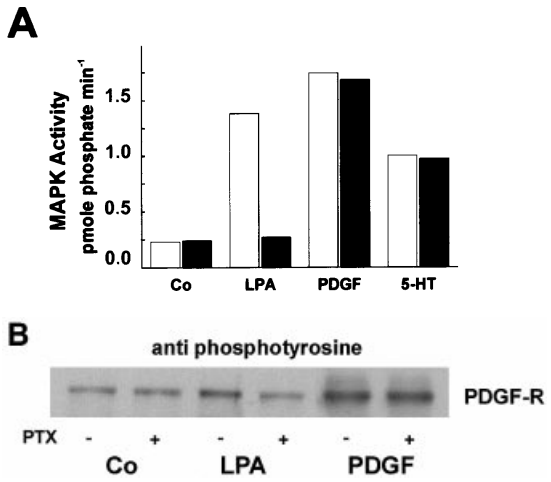

Figure 7 Involvement of a PTX-sensitive G-protein in LPA-mediated MAP kinase activation and PDGF receptor phosphorylation

Mesangial cells were preincubated with PTX (PTX, 100 ng/ml, black bars) for 18 h and then stimulated with LPA (25 μM), PDGF (20 ng/ml) or 5-HT (1 μM) for 5 min (open bars, stimulated control cells). MAP kinase (MAPK) activity (**A**) was determined in the cellular homogenates as described in the Materials and methods section; data are means of duplicate determinations. PDGF receptor phosphorylation was detected by Western-blot analysis (**B**) with an antibody directed against phosphotyrosine.

PDGF strongly phosphorylated its receptor (Figure 6A). The same band was also phosphorylated by LPA (10 and 25 μM). The identity of the receptor was confirmed by probing a parallel blot with an antibody specific for the PDGF receptor (Figure 6A) and by immunoprecipitation with an antibody directed against the PDGF receptor, and subsequent detection of the blot with antibodies directed against phosphotyrosine or the PDGF receptor (Figure 6B). To compare different experiments, the phosphorylation of the receptor by PDGF was set to 100% (Figure 6B, immunoprecipitation; Figure 6C, Western-blot analysis). No significant difference was seen with 10 and 25 μM LPA as stimuli.

Phosphorylation of the PDGF receptor by LPA was transient. After 10 min there was no significant increase in tyrosine phosphorylation above that of control cells (ratio of tyrosine phosphorylation of stimulated cells and control cells after 10 min: 1.0 ± 0.2, $n = 3$, compared with 1.8 ± 0.5, $n = 5$, after 5 min). With the sensitivity of the techniques used, activation of the cells with 5-HT did not significantly increase PDGF receptor phosphorylation above basal values. Pretreatment of mesangial cells with AG1296 for 30 min strongly decreased basal and stimulated PDGF receptor phosphorylation.

Two of the protein bands phosphorylated by PDGF co-migrated with the 66 kDa and 52 kDa bands of the adapter protein Shc (Figure 6A). Treatment of mesangial cells with AG1296 decreased phosphorylation of both bands. The phosphorylated 52 kDa band was also detectable in control cells and was slightly enhanced on treatment with LPA, whereas phosphorylation of the 66 kDa band was not observed. The nature of the tyrosine-phosphorylated band, which migrated above the 52 kDa band, was not identified.

Sensitivity of LPA-mediated PDGF receptor phosphorylation to PTX

We have shown previously that LPA-mediated PGHS-2 induction is PTX-sensitive, indicating involvement of a G-protein

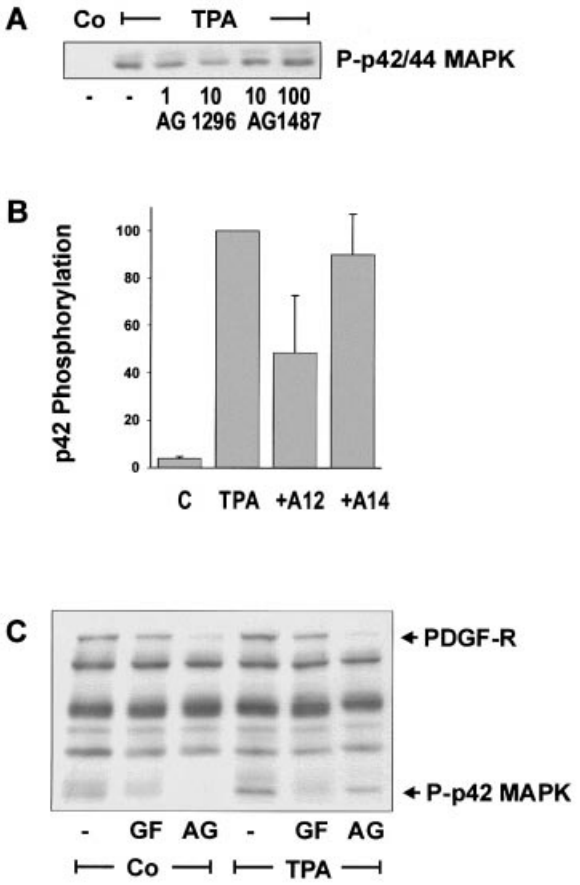

Figure 8 Effect of tyrphostin AG1296 on PMA-mediated p42/44 MAP kinase activation

(**A**) Mesangial cells were preincubated with the tyrphostins AG1296 (1 and 10 μM) or AG1478 (10 and 100 nM) for 30 min and then stimulated with PMA ('TPA'; 0.1 μM) for 5 min. p42/44 MAP kinase phosphorylation was detected by specific antibodies. (**B**) Densitometric quantification of three experiments with 10 μM AG1296 (+A12) and 100 nM AG1478 (+A14). Phosphorylation after PMA treatment was set to 100% (means $\pm$ S.D.). (**C**) Mesangial cells were treated with GF109203X (GF, 1 μM) or AG1296 (AG, 10 μM) and then stimulated with PMA ('TPA', 0.1 μM). Phosphorylated proteins were detected with an antibody directed against phosphotyrosine. The blot is representative of three experiments.

of the G_i family [5]. In line with these results, activation of p42/44 MAP kinases by LPA was abrogated by pretreatment with PTX, whereas activation by PDGF or 5-HT was not affected (Figure 7A). Trans-activation of the PDGF receptor by LPA also proved to be PTX-sensitive (Figure 7B).

Role of protein kinase C (PKC)

We have shown previously that induction of PGHS-2 by PDGF or 5-HT is strongly decreased when PKC isoforms, which are sensitive to phorbol ester activation, are down-regulated [6,8]. LPA-mediated activation of p42/44 MAP kinase was partially decreased when the cells were pretreated with a specific inhibitor of PKC, GF109203X [25], at a concentration which abrogated PMA-induced p42/44 MAP kinase activation (1 μM GF109203X, 40.2 ± 20.4% inhibition of LPA-induced p42 MAP kinase phosphorylation, mean $\pm$ S.D., $n = 3$). To test whether there was an interaction between PKC and tyrosine kinase receptors, PMA-mediated activation of p42/44 MAP kinase was

investigated in the presence of the tyrphostins AG1296 and AG1478 (Figure 8A). While AG1478, the tyrphostin specific for the EGF receptor, had no effect on p42/44 MAP kinase activation, AG1296 decreased p42/44 MAP kinase phosphorylation by about 50% (Figure 8B). Tyrosine phosphorylation of p42/44 MAP kinase was also observed when Western blots of whole cell lysates were detected with phosphotyrosine-specific antibodies, whereas there was no detectable effect of PMA on PDGF receptor phosphorylation (Figure 8C).

DISCUSSION

In the present study, ligand-independent trans-activation between G-protein-coupled receptors and protein tyrosine kinase receptors was investigated in mesangial cells, primary cells which express functional receptors for EGF, PDGF, LPA and 5-HT. Only the PDGF receptor, not the EGF receptor, was involved in LPA-mediated activation of MAP kinase and subsequent induction of PGHS-2, whereas there was less trans-activation observed after activation of 5-HT_{2A} receptors.

Different types of G-proteins have been shown to couple to LPA receptors, belonging to the G_i, G_q and $G_{12/13}$ families [26]. In mesangial cells, MAP kinase activation and PGHS-2 induction were PTX-sensitive, indicating participation of a G_i protein, as also observed in other cell types. In contrast with many other cells [26], however, release of intracellular calcium mediated by phosphatidylinositol-specific phospholipase C is also sensitive to PTX in mesangial cells [5]. These data are in accordance with the PTX-sensitive calcium mobilization by transfected LPA receptors, especially the Edg2 receptor, recently shown by An et al. [27]. Furthermore, activation of PKC by LPA was attributed to G-proteins of the G_q family (discussed in [13]). In mesangial cells, however, LPA-mediated activation of p42/44 MAP kinase was decreased by inhibition of PKC by GF109203X, suggesting PKC to be located downstream of a PTX-sensitive G-protein. In smooth-muscle cells, PDGF-mediated activation of p42/44 MAP kinase was recently described as being decreased by PTX [28], a finding that was not observed in mesangial cells. Signalling pathways in mesangial cells thus diverge from those described in other cells, primarily fibroblasts.

Although both protein tyrosine kinase receptors PDGF and EGF were functional in the cells investigated, only the PDGF receptor tyrosine kinase was used in ligand-independent trans-activation by LPA. In many other cell types, the EGF receptor has been shown to be a target of LPA-mediated trans-activation ([10] and citations therein). The reason for the different preference is not yet clear. Activation of the LPA receptor has been related to cytoskeletal reorganization and phosphorylation of focal-adhesion kinase [29,30], suggesting spatial organization of LPA-receptor distribution.

Microheterogeneity of the plasma membrane might play a role in differential trans-activation in mesangial cells, which are adherent cells and show a highly structured actin cytoskeleton. Other possibilities, such as the availability of linking enzymes and adapter molecules, also remain speculative, because the target molecules of the $\beta\gamma$ subunit have not yet been well defined.

Recruitment and activation of the adapter protein Shc has been described in some, but not all, trans-activation pathways (e.g. [31]). Tyrosine phosphorylation of p52 and p66 Shc was observed in PDGF-stimulated mesangial cells, whereas only p52 was phosphorylated after activation by LPA. Phosphorylation of p52 was also observed in non-stimulated cells, similar to what has been described in L cells [12]. Shc is only one of the proposed signalling molecules involved in G-protein and protein tyrosine kinase receptor cross-talk. Receptor-independent tyrosine kinases of the Src family were described in neuronal PC12 cells as a link between LPA receptor and MAP kinase activation [32]. In a previous study we investigated mesangial cells overexpressing the oncogene c-*src* and did not detect an enhanced, but rather a decreased, inducibility of PGHS-2 [33], rendering the possibility of the oncoprotein c-Src acting as an important mediator in this signal-transduction pathway rather unlikely. Activation of PDGF receptor-associated phosphatidylinositol 3-kinase (PI 3-kinase) has been shown to be very sensitive to treatment with AG1296 [24], whereas PI 3-kinase γ has been implicated in $G_{\beta\gamma}$-mediated activation of the p42/44 MAP kinase cascade [13]. PI 3-kinase may well play a role in LPA-mediated activation of mesangial cells, because inhibition of the enzyme by LY294002 decreased p42/44 MAP kinase activation and PGHS-2 induction (results not shown). Further studies are underway to define the role of the different PI 3-kinase isoforms in PDGF receptor trans-activation and MAP kinase activation. Analysis of receptor cross-talk in mesangial cells was complicated by the high level of AG1296-sensitive phosphorylation in non-stimulated cells. Basal receptor phosphorylation was not sufficient to induce p42/44 MAP kinase activation, but obviously provided an input which was relevant for the induction of p42/44 MAP kinase. Three observations illustrate the important role of basal activation of the PDGF receptor system: (i) stimulation with 5-HT or activation of PKC by PMA did not change the phosphorylation of the PDGF receptor, whereas MAP kinase activation was still sensitive to receptor inhibition; (ii) the rapid activation of p42/44 MAP kinase by LPA was inhibited by preincubation with AG1296 at a time point when PDGF-mediated receptor activation was not yet effective; in COS-7 cells the same kinetic differences were observed in MAP kinase activation by LPA and EGF [10]; (iii) the rather modest increase in PDGF receptor phosphorylation by LPA was not in accordance with the strong effects of AG1296 on MAP kinase activation. Thus, in addition to cross-talk via receptor activation, convergence of signalling pathways seems to play a role in LPA-, 5-HT- and PMA-mediated cellular activation. An interesting candidate for such an interaction is the p35 protein, the phosphorylation of which was enhanced by PDGF, barely changed by LPA, 5-HT or PMA and abrogated by treatment of the cells with AG1296. Increased phosphorylation of the p35 protein was also observed in mesangial cells under conditions of high pressure [34], suggesting that the protein may play a functional role in mesangial cells under various conditions, regarding the physiological function of mesangial cells as structural components of the glomerulus and their pathophysiological role as proliferating cells in glomerular injury.

In contrast with LPA, 5-HT-mediated induction of p42/44 MAP kinase activation and PGHS-2 induction were independent of PTX. Activation of the MAP kinase pathway by 5-HT was only partially inhibited by the tyrphostin AG1296 under conditions where PDGF- as well as LPA-mediated signalling were decreased to near-background levels. Similarly, inhibition of EGF receptor tyrosine kinase activity was without effect, indicating that 5-HT-mediated signalling is largely independent of EGF or PDGF receptor tyrosine kinase activation. Trans-activation by G_q-coupled receptors has been described for angiotensin II in vascular smooth-muscle cells [16] and for bombesin in Cos-7 cells [10]. Other protein tyrosine kinase receptors present in mesangial cells cannot be excluded as trans-activation targets. Furthermore, direct interactions between $\beta\gamma$ subunits and the Raf-1 serine/threonine protein kinase, which is positioned upstream of p42/44 MAP kinase, have also been described and may well account for 5-HT-mediated MAP kinase activation [35]. Although p42/44 MAP kinase activation is an

essential step in PGHS-2 induction, it is by no means the only one. p38 MAP kinase and Rho proteins, for instance, are also part of the signalling modules involved in PGHS-2 induction by LPA in mesangial cells [5]. p38 MAP kinase was originally thought to be linked to cell activation by inflammatory cytokines such as interleukin-1 or tumour necrosis factor-α and by cellular stress, but seems to have a broader functional role, because we were then able to show that p38 MAP kinase was also activated by PDGF and 5-HT. A role for p38 MAP kinase in PGHS-2 induction was described previously by us and by others [5,36,37]. In a recent paper, Dean et al. showed regulation of PGHS-2 mRNA stability and transcription by p38 MAP kinase [38]. It is not yet clear whether this dual regulation also applies to PGHS-2 mRNA levels in mesangial cells.

Mesangial cells thus provide a model system to further study LPA-mediated trans-activation of the PDGF receptor to define links between different signalling cascades. An interesting potential study could involve including proteins of the Rho family in this network, which have been shown to play a role in LPA-mediated induction of PGHS-2 in mesangial cells [5] and, in addition, are important factors to determine cell structure and adherence.

This work was supported by the Deutsche Forschungsgemeinschaft (GO413/8-2). The technical assistance of Mrs. M. Rehm is gratefully acknowledged.

REFERENCES

1 Goppelt-Struebe, M. (1995) Prostaglandins Leukotrienes Essen. Fatty Acids **52**, 213–222
2 Herschman, H. R. (1996) Biochim. Biophys. Acta **1299**, 125–140
3 Stroebel, M. and Goppelt-Struebe, M. (1994) J. Biol. Chem. **269**, 22952–22957
4 Kester, M., Coroneos, E., Thomas, P. J. and Dunn, M. J. (1994) J. Biol. Chem. **269**, 22574–22580
5 Reiser, C. O. A., Lanz, T., Hofmann, F., Hofer, G., Rupprecht, H. D. and Goppelt-Struebe, M. (1998) Biochem. J. **330**, 1107–1114
6 Goppelt-Struebe, M. and Stroebel, M. (1998) J. Cell. Physiol. **175**, 341–347
7 Goppelt-Struebe, M., Hahn, A., Stroebel, M. and Reiser, C. O. A. (1999) Biochem. J. **339**, 329–334
8 Goppelt-Struebe, M., Stroebel, M. and Hoppe, J. (1996) Kidney Int. **50**, 71–78
9 Daub, H., Weiss, F. U., Wallasch, C. and Ullrich, A. (1996) Nature (London) **379**, 557–560
10 Daub, H., Wallasch, C., Lankenau, A., Herrlich, A. and Ullrich, A. (1997) EMBO J. **16**, 7032–7044
11 Cunnick, J. M., Dorsey, J. F., Standley, T., Turkson, J., Kraker, A. J., Fry, D. W., Jove, R. and Wu, J. (1998) J. Biol. Chem. **273**, 14468–14475
12 Herrlich, A., Daub, H., Knebel, A., Herrlich, P., Ullrich, A., Schultz, G. and Gudermann, T. (1998) Proc. Natl. Acad. Sci. U.S.A. **95**, 8985–8990
13 Kranenburg, O., Verlaan, I., Hordijk, P. L. and Moolenaar, W. H. (1997) EMBO J. **16**, 3097–3105
14 Inglese, J., Koch, W. J., Touhara, K. and Lefkowitz, R. J. (1995) Trends Biochem. Sci. **20**, 151–156
15 Linseman, D. A., Benjamin, C. W. and Jones, D. A. (1995) J. Biol. Chem. **270**, 12563–12568
16 Eguchi, S., Numaguchi, K., Iwasaki, H., Matsumoto, T., Yamakawa, T., Utsunomiya, H., Motley, E. D., Kawakatsu, H., Owada, K. M., Hirata, Y. et al. (1998) J. Biol. Chem. **273**, 8890–8896
17 Lovett, D. H., Ryan, J. L. and Sterzel, R. B. (1983) J. Immunol. **131**, 2830–2836
18 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
19 DeWitt, D. L. and Meade, E. A. (1993) Arch. Biochem. Biophys. **306**, 94–102
20 Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995) J. Biol. Chem. **270**, 27489–27494
21 Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J. and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 7686–7689
22 Watts, A. W. (1996) J. Pharmacol. Exp. Ther. **279**, 1541–1550
23 Levitzki, A. and Gazit, A. (1995) Science **267**, 1782–1788
24 Kovalenko, M., Ronnstrand, L., Heldin, C. H., Loubtchenkov, M., Gazit, A., Levitzki, A. and Bohmer, F. D. (1997) Biochemistry **36**, 6260–6269
25 Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E. and Loriolle, F. (1991) J. Biol. Chem. **266**, 15771–15781
26 Moolenaar, W. H. (1995) J. Biol. Chem. **270**, 12949–12952
27 An, S., Bleu, T., Zheng, Y. and Goetzl, E. J. (1998) Mol. Pharmacol. **54**, 881–888
28 Conway, A.-M., Rakhit, S., Pyne, S. and Pyne, N. J. (1999) Biochem. J. **337**, 171–177
29 Ridley, A. J. and Hall, A. (1994) EMBO J. **13**, 2600–2610
30 Hawes, B. E., van Biesen, T., Koch, W. J., Luttrell, L. M. and Lefkowitz, R. J. (1995) J. Biol. Chem. **270**, 17148–17153
31 van Biesen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Touhara, K., Porfiri, E., Sakaue, M., Luttrell, L. M. and Lefkowitz, R. J. (1995) Nature (London) **376**, 781–784
32 Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A. and Schlessinger, J. (1996) Nature (London) **383**, 547–550
33 Reiser, C. O. A., Marx, M., Hoppe, J. and Goppelt-Struebe, M. (1996) Exp. Cell Res. **222**, 304–311
34 Kawata, Y., Mizukamik, Y., Fujii, Z., Sakumura, T., Yoshida, K. and Matsuzaki, M. (1998) J. Biol. Chem. **273**, 16905–16912
35 Pumiglia, K. M., LeVine, H., Haske, T., Habib, T., Jove, R. and Decker, S. J. (1995) J. Biol. Chem. **270**, 14251–14254
36 Guan, Z., Buckman, A. Y., Miller, B. W., Springer, L. D. and Morrison, A. R. (1998) J. Biol. Chem. **273**, 28670–28676
37 Pouliot, M., Baillargeon, J., Lee, J. C., Cleland, L. G. and James, M. J. (1997) J. Immunol. **158**, 4930–4937
38 Dean, J. L. E., Brook, M., Clark, A. R. and Saklatvala, J. (1999) J. Biol. Chem. **274**, 264–269

Received 27 August 1999; accepted 28 October 1999