Epidermal-growth-factor receptor and metalloproteinases mediate thromboxane A₂-dependent extracellular-signal-regulated kinase activation

Carole GALLET*¹, Stéphanie BLAIE*¹, Sylviane LÉVY-TOLEDANO* and Aïda HABIB*^{†‡2}

*INSERM U348, Institut Fédératif 6-Circulation-Paris 7, 8 rue Guy Patin, Hôpital Lariboisière, 75010 Paris Cedex, France, †Department of Biochemistry, American University of Beirut, PO Box 11-0236, Riad El-Solh, Beirut, Lebanon, and ‡Department of Internal Medicine, American University of Beirut, PO Box 11-0236, Riad El-Solh, Beirut, Lebanon

The signalling pathways that link G-protein-coupled receptors to mitogen-activated protein kinases involve receptor and non-receptor tyrosine kinases and protein kinase C (PKC). We explored the pathways that are implicated in the thromboxane (TX) A₂-dependent activation of extracellular-signal-regulated protein kinase (ERK) and the role of the two TX receptor (TP) isoforms, TP α and TP β . ERK activation by IBOP, a TX analogue, was dependent on epidermal-growth-factor receptor (EGFR) in TP α - or TP β -transfected cells and in human aortic smooth muscle cells (hASMCs), since AG1478, a selective inhibitor of tyrosine phosphorylation of the EGFR, strongly blocked ERK and EGFR phosphorylation. In addition, EGFR transactivation leading to ERK activation involved matrix metalloproteinases (MMPs), since BB2516, an inhibitor of MMP, decreased ERK and EGFR phosphorylation in TP α - or TP β -transfected

cells. Moreover, we showed that both isoforms activate ERK phosphorylation in an Src-kinase-dependent manner, whereas PKC was mainly implicated in ERK activation and EGFR phosphorylation by TP β . In hASMCs, we showed that ERK activation depended on both pertussis-sensitive and -insensitive G α -proteins. We demonstrated further that EGFRs, PKC, Src kinase and MMPs are involved in ERK activation by TX. The results of the present study highlight a role for MMPs and PKC in EGFR transactivation triggered by the TPs and demonstrate this mechanism for the first time in primary cells, i.e. hASMCs.

Key words: marimastat, protein kinase C (PKC), Src, thromboxane receptor, growth-factor-receptor transactivation, vascular cell.

INTRODUCTION

A diverse group of agonists, including peptide hormones, neurotransmitters and lipids, act through the G-protein-coupled receptors (GPCRs) to activate receptor tyrosine kinases (RTKs) and induce cell proliferation and differentiation. Extracellular-signal-regulated kinase (ERK) activation has been reported to be involved in receptor activation of multiple heterotrimeric G-proteins, including both pertussis toxin (PTX)-insensitive G α_q and -sensitive G $\alpha_{i/o}$ [1–3]. Although the signalling pathways that are involved in ERK activation can be subject to differential regulations between the different cellular systems, it is generally thought that activation of G α_q -coupled receptors involves protein kinase C (PKC), whereas G $\alpha_{i/o}$ -coupled receptors involve the β and γ subunits of G-proteins and c-Src-dependent activation of Ras [4,5]. Characterization of the activation of RTKs by GPCRs demonstrated the involvement of the activation of the epidermal-growth-factor (EGF) receptor (EGFR) [6,7] or the platelet-derived-growth-factor receptor (PDGFR) [6], independently of detected exogenous growth factor. In some cellular models, this transactivation also implicated the activation of matrix metalloproteinases (MMPs) and the shedding of heparin-binding EGF (HB-EGF), one of the ligands of the EGF receptor (EGFR) [8–11]. This transactivation of the RTK has a role in

ERK activation and subsequent DNA synthesis and expression of transcription factors [12].

Thromboxane (TX) A₂ is a product of the sequential metabolism of arachidonic acid by cyclo-oxygenases and TXA₂ synthase [13]. It exhibits a potent biological activity ranging from platelet secretion and aggregation, vaso- or broncho-constriction, and has been implicated in a number of cardiovascular and bronchial diseases. The biological effects of TXA₂ are mediated through membrane receptors that are identified as members of the GPCR superfamily, coupled mainly to G α_q [14–16]. Two variants of the TX receptor (TP), TP α and TP β [17], have been reported for the human receptor and are derived from an alternative splicing [18–20].

Mitogenic and hypertrophic effects, and activation of mitogen-activated protein kinases (MAPKs) by TXA₂ analogues have been reported mainly in porcine and rat vascular smooth muscle cells [21–23]. We have demonstrated in porcine aortic smooth muscle cells that U46619, a TXA₂ analogue, induced c-Jun N-terminal kinase (JNK) activation and cyclo-oxygenase-2 expression in a protein kinase A (PKA)-dependent manner [24]. Recently, Gao et al. [25] have demonstrated in a human endothelial cell line, ECV304, that TXA₂ ligands trigger a PKC-, PTX-, Src- and EGFR-dependent activation of ERK. Miggin and Kinsella [26] reported in a human uterine smooth muscle cell line, ULTR,

Abbreviations used: ADAM, a disintegrin and metalloproteinase-like; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular-signal-regulated kinase; FGF-2, fibroblast growth factor 2; GPCR, G-protein-coupled receptor; hASMC, human aortic smooth muscle cell; HB-EGF, heparin-binding EGF; HEK-293-TP α/β , HEK-293 cells stably overexpressing TP α or TP β ; IBOP, [1S-[1 α ,2 α (Z),3 β (1E,3S),4 α]]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; JNK, c-Jun N-terminal kinase; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinases; MEK1, MAPK/ERK kinase 1; MMP, matrix metalloproteinase; PDGFR, platelet-derived-growth-factor receptor; PKA, protein kinase A; PKC, protein kinase C; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PP3, 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine; PTX, pertussis toxin; RTK, receptor tyrosine kinase; SmGM, smooth-muscle-cell growth medium; SQ 29548, [(1S)1 α ,2 β (5Z)3 α ,4 β]-7-[3-[2-(phenylamino)carbonyl-hydrazino-methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; TP, TX receptor; TX, thromboxane.

¹ These authors contributed equally to this work.

² To whom correspondence should be addressed (e-mail ah31@aub.edu.lb).

ERK activation by TX that was dependent on PKC, PKA, phosphoinositide 3-kinase and EGFRs, but was independent of PTX.

In the present study, we compared the activation of the human TP isoforms in human embryonic kidney HEK-293 cells. We demonstrated that, in human aortic smooth muscle cells (hASMCs), TP-induced activation of ERK is dependent on activation of the EGFR and partially on MMPs. Our results support the existence of cross-talk between TPs and RTKs in normal hASMCs involving MMPs, EGFR, Src kinase and PKC leading to ERK phosphorylation.

MATERIALS AND METHODS

Materials

TP β cDNA was kindly provided by Dr J. A. Ware (Albert Einstein College of Medicine, New York, NY, U.S.A.) and HEK-293 cells stably overexpressing TP α or TP β were kindly provided by Dr Garret FitzGerald (University of Pennsylvania, Philadelphia, PA, U.S.A.). Control HEK-293 cells were obtained from ATCC (Rockville, MD, U.S.A.). Cell culture media were from Life Technologies Inc. (Paisley, U.K.). hASMCs and the corresponding culture medium (smooth-muscle-cell growth medium; SmGM) were from Clonetics (BioWhittaker Europe, Verviers, Belgium). PD98059 and lysophosphatidic acid (LPA) were from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). 4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)-pyrazolo[3,4-*d*]pyrimidine (PP1) was from Alexis (San Diego, CA, U.S.A.). Triton X-100, sodium orthovanadate, benzamidine, leupeptin, sodium fluoride, sodium pyrophosphate, PMA, ATP and myelin basic protein were from Sigma Aldrich Co. (St. Louis, MO, U.S.A.). ECL[®] (enhanced chemiluminescence) reagents and protein A-Sepharose were from Amersham Biosciences (Les Ulis, France). GF109203X, AG1478, BB2516 (also known as marimastat), PD158780, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)-pyrazolo[3,4-*d*]pyrimidine (PP2), 4-amino-7-phenylpyrazolo[3,4-*d*]pyrimidine (PP3) and PTX were from Calbiochem (San Diego, CA, U.S.A.). IBOP {[1S-[1 α ,2 α (Z),3 β (1E,3S),4 α]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid]} and SQ 29548 {[1S]-1 α ,2 β (5Z)3 α ,4 β -7-[3-[2-(phenylamino)carbonyl-hydrazino-methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid]} were from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). A polyclonal antibody raised against phosphorylated active ERK was from Promega Life Science (Madison, WI, U.S.A.). Rabbit polyclonal anti-ERK and anti-EGFR antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Monoclonal anti-phosphotyrosine (4G10) antibody was from Upstate Biotechnologies Inc. (Lake Placid, NY, U.S.A.). Peroxidase-conjugated donkey anti-rabbit and rabbit anti-mouse IgGs were from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA, U.S.A.). Recombinant human fibroblast growth factor 2 (FGF-2) was from Roche (Meylan, France) and recombinant human EGF and anti-(HB-EGF) antibody were from R & D Systems (Minneapolis, MN, U.S.A.). Orthophosphoric acid was from Carlo Erba Reagenti (Milan, Italy). [γ -³²P]ATP was from PerkinElmer Life Sciences (Great Shelford, Cambridge, U.K.). Electrophoresis reagents were from J.T. Baker (Noisy-le-Sec, France) and Invitrogen (Carlsbad, CA, U.S.A.). P-81 phosphocellulose paper was from Whatman (Maidstone, Kent, U.K.).

Cell culture

HEK-293 cells stably overexpressing TP α or TP β were developed as previously described in [27]. Two clones were used expressing

similar amounts of receptors (approx. 1.6 pmol/mg of total protein) [27]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) heat-inactivated foetal calf serum, 2 mM glutamine, 5 units/ml penicillin, under 5 % CO₂ at 37 °C containing 0.75 mg/ml geneticin. Cells were plated in 12-well plates for total inositol phosphate formation, in six-well plates for immunoblotting or MAPK assay, and in 100 mm-diameter dishes for immunoprecipitation of EGFRs. Cells were serum-deprived for 24 h before stimulation. hASMCs were cultured in SmGM according to the manufacturer's instructions. Cells were used following the eighth passage and cultured to subconfluence.

Western blotting

Subconfluent cells (10⁶ cells per well in six-well plates) were serum-deprived overnight in DMEM containing 0.5 % BSA and 20 mM Hepes, pH 7.4, for HEK-293 cells and for 36–72 hours for hASMCs in Clonetics smooth-muscle-cell basal medium (SmBM) containing 0.5 % BSA. The culture medium was changed and cells were kept for 1 h before the addition of agonist and various inhibitors. After incubation, cells were washed twice with PBS, pH 7.4, and lysed in 1 ml of ice-cold lysis buffer [50 mM Tris, pH 7.5, containing 1 % (v/v) Triton X-100, 100 mM NaCl, 40 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.2 mM sodium orthovanadate, 1 μ g/ml leupeptin, 5 mM EDTA and 1 μ M benzamidine]. Lysates were centrifuged at 10 000 *g* for 10 min at 4 °C, and the protein concentration in the resulting supernatant was determined by the micro-bicinchoninic acid ('BCA') assay (Pierce) using BSA as standard. Proteins (10 μ g), prepared in Laemmli sample buffer, were separated by SDS/PAGE (10 % gels), and transferred on to nitrocellulose membranes in 25 mM Tris/HCl, 192 mM glycine buffer, pH 8.3, containing 0.01 % (w/v) SDS and 10 % (v/v) methanol. Blots were saturated for 2 h in Tris-buffered saline [50 mM Tris, pH 7.5, 250 mM NaCl and 0.1 % (v/v) Tween 20], containing 5 % (w/v) fat-free powdered milk. Membranes were incubated overnight at 4 °C with a 1:5000 dilution of anti-(phosphorylated ERK) antibody. Membranes were washed five times for 10 min in Tris-buffered saline and incubated with a 1 in 5000 dilution of donkey anti-rabbit antibody coupled to horseradish peroxidase. Excess antibody was washed and positive bands were revealed by ECL[®], according to the manufacturer's instructions. All the membranes were stripped using the glycine/HCl buffer [0.2 M glycine, 0.1 % (w/v) SDS and 1 % (v/v) Tween 20], pH 2.2, before reprobing with anti-ERK polyclonal antibody (diluted 1 in 5000) to determine total ERK expression.

MAPK assay

MAPK assay was performed on immunoprecipitates of ERK [28,29]. Cell lysates (500 μ l at 0.5 mg/ml), prepared as described above, were incubated for 3 h at 4 °C with 1 μ l of anti-ERK polyclonal antibody pre-adsorbed to protein A-Sepharose. Immune complexes were washed three times with lysis buffer and twice with kinase assay buffer (50 mM Hepes, pH 7.4, 5 mM magnesium acetate, 2 mM dithiothreitol, 1 mM EGTA and 0.2 mM sodium orthovanadate). MAPK activity was assayed by resuspending the final pellet in a total volume of 50 μ l of kinase assay buffer containing myelin basic protein as substrate (0.5 mg/ml). Reactions were initiated by the addition of [γ -³²P]ATP (50 μ M with unlabelled ATP; 3000 c.p.m./pmol specific radioactivity). After incubation at 30 °C for 15 min, assays were terminated by spotting 50 μ l of the mix on to a 2 cm²-sized P-81 phosphocellulose paper. The papers were washed three times for

Figure 1 Time-dependent activation of ERK1/2 by IBOP in HEK-293-TP α and HEK-293-TP β cells

(A) Western blot analysis of ERK1/2 phosphorylation (upper panel). Crude cell lysates (10 μ g/lane) prepared from HEK-293-TP α and HEK-293-TP β cells stimulated with 100 nM of IBOP for 2–180 min at 37 °C were separated by SDS/PAGE (10 % gels), transferred on to a nitrocellulose membrane and blotted with an anti-phosphorylated ERK1/2 antibody as described in the Materials and methods section. Membranes were stripped and re probed with a polyclonal antibody raised against total ERK1/2. ERK1/2 signals observed in Western blots were quantified by densitometric analysis (lower panel). The ratio of phosphorylated ERK1/2 to total ERK was determined. Results are presented as fold increase in ERK1/2 phosphorylation compared with basal (B) ERK1/2 phosphorylation in untreated cells. (B) An immune-complex kinase assay was performed using myelin basic protein as a substrate as described in the Materials and methods section. Results are expressed in pmol of ATP/15 min per mg of total protein and are representative of three separate experiments.

15 min at 4 °C in orthophosphoric acid (1 %, w/v), once overnight at 4 °C and twice for 15 min at room temperature (25 °C). Papers were transferred into 6 ml plastic scintillation vials containing 4 ml of scintillation fluid and radioactivity was determined using a Beckman scintillation counter. Results were expressed in pmol of ATP/15 min per mg of protein.

Immunoprecipitation and immunoblotting of the EGFRs

Cells in 100 mm dishes were lysed in 1.5 ml of lysis buffer and centrifuged for 10 min at 14 000 *g*. Total protein samples (3 mg for IBOP- and 1 mg for EGF-activated cells) were mixed in a total volume of 1 ml with 15 μ l of polyclonal anti-EGFR antibody coupled to agarose and incubated for 3 h at 4 °C. Immunoprecipitates were washed four times with lysis buffer, solubilized in Laemmli buffer, resolved by SDS/PAGE (7 % gel) and transferred on to nitrocellulose membranes. After blocking with 5 % (w/v) non-fat powdered milk in Tris-buffered saline, membranes were incubated with the anti-phosphotyrosine antibody (1:1000 dilution) for 1 h at room temperature,

followed by a secondary antibody coupled to peroxidase (1:5000 dilution). Immunoreactive proteins were detected as described for ERK above. Membranes were stripped and re probed with the polyclonal anti-EGFR antibody to ensure appropriate immunoprecipitation of the receptors.

Data analysis

Phosphorylated and total ERK were detected in each experiment. Autoradiograms were scanned using an Agfa Arcus II scanner and densitometric analyses were performed using SigmaGel[®] (SPSS, Chicago, IL, U.S.A.). Statistical analysis of data was performed using a paired Student's *t* test.

RESULTS

Time- and dose-dependent activation of ERK1/2 by TX

The time-response and dose-response activation of ERK by each TP receptor isoform in response to IBOP, a stable analogue

Figure 2 Effect of SQ 29548, the TP antagonist, and of PD98059, the MEK1 inhibitor, on IBOP-induced activation of ERK1/2

HEK-293-TP α (left panel) or HEK-293-TP β (right panel) cells were pre-treated with or without 10 μ M SQ 29548 (A) or 30 μ M PD98059 (B) for 30 min before the addition of 10 nM IBOP. Total and phosphorylated ERK1/2 levels were determined by Western blotting. Results are representative of three experiments.

of TXA₂, was compared. HEK-293 cells stably overexpressing TP α (HEK-293-TP α) or TP β (HEK-293-TP β) were used with comparable amounts of receptors as described previously [27]. The kinetics of phosphorylation of ERK1/2 were similar for the two TP isoforms. In fact, when HEK-293-TP α or HEK-293-TP β cells were incubated with 100 nM IBOP, a rapid and transient activation of ERK1/2 MAPKs was obtained after detection with an antibody selective for the phosphorylated form of ERK1/2 (Figure 1A). HEK-293 cells transfected with the vector alone did not show any significant ERK1/2 phosphorylation in response to IBOP (results not shown). Furthermore, we checked the activity of ERK by assessing the phosphorylation of the myelin basic protein on immunoprecipitated ERK and observed results comparable with the Western blots of phosphorylated ERK (Figure 1B). In addition, IBOP induced the ERK1/2 activation in a dose-dependent manner, with an EC₅₀ value of 0.16 ± 0.03 nM and 0.6 ± 0.2 nM for TP α and TP β , respectively ($n = 3$).

Effect of SQ 29548 and PD98059 on ERK1/2 activation

SQ 29548, the highly selective antagonist of the TP receptors [30], significantly inhibited ERK 1/2 activation in both HEK-293-TP α and HEK-293-TP β cells, suggesting that this activation is mediated by the TP receptor (Figure 2A). In addition, PD98059, a specific inhibitor of MAPK/ERK kinase 1 (MEK1), the kinase responsible for the phosphorylation of ERK1/2, blocked ERK activation. PD98059, which is not a protein kinase inhibitor, but a compound that stops Raf from activating MEK1, was described by Davies et al. [31] as a compound with an impressive selectivity profile to block the MAPK cascade. A 3.7 ± 0.98 -fold increase over untreated cells was found with IBOP compared with a 1.2 ± 0.23 -fold increase with IBOP + PD98059 for TP α , and a 5 ± 1.6 -fold increase over untreated cells was found with IBOP compared with a 1.3 ± 0.2 -fold increase with IBOP + PD98059 for TP β ($n = 3$) (Figure 2B).

Role of PKC and Src in the activation of ERK1/2 by TP α and TP β

The pathways involved in the TP-dependent activation of ERK were investigated further, using 5 μ M GF109203X, a specific inhibitor of PKC. We have previously determined an optimal and selective concentration of GF109203X, i.e. 5 μ M, in the same cells that blocked PMA-dependent phosphorylation of the TP isoforms [27]. When TP α -transfected cells were pre-treated with GF109203X, TP α -dependent activation of ERK was partially decreased (a 4.6 ± 1.5 -fold increase over untreated cells was found with IBOP compared with a 3.1 ± 1.1 -fold increase with IBOP + GF109203X; $n = 4$) (Figure 3A). On the other hand, the PKC inhibitor significantly inhibited TP β -induced ERK activation. IBOP induced a 6.2 ± 1.6 -fold increase over untreated cells compared with a 2.6 ± 0.7 -fold increase with IBOP + GF109203X ($n = 4$; $P < 0.03$), suggesting a role of PKC in ERK activation by TP β rather than TP α . In these cells, as a positive control for the inhibitory effect of GF109203X, PMA was used. PMA-dependent activation of ERK activation was strongly inhibited by GF109203X (Figure 3A).

To examine the possible involvement of Src kinases in ERK activation by IBOP we used PP1, a highly selective Src kinase inhibitor, and assessed its regulatory effect on the TP-dependent activation of ERK. PP1 was first used at 3 and 10 μ M, and an inhibitory effect was found at 10 μ M. At this concentration, PP1 was able to inhibit both TP α -dependent ERK activation by 84 % (a 7.1-fold increase over untreated cells with IBOP compared with a 1.1-fold increase with IBOP + PP1) (Figure 3B). Furthermore, PP1 demonstrated an 81 % decrease in ERK phosphorylation for TP β (a 13.8-fold increase over untreated cells with IBOP compared with a 2.6-fold increase with IBOP + PP1 for TP β ; $n = 2$) (Figure 3B). Similar results were obtained in HEK-293-TP α cells with a more recently described Src inhibitor, PP2, at 10 μ M (a 4 ± 0.85 -fold increase over untreated cells with IBOP compared with 1.5 ± 0.02 with IBOP + PP2; $n = 3$; $P = 0.04$). No significant effect was observed when cells were pre-treated with 3 μ M PP2 (a 3.2 ± 1 -fold increase over untreated cells) or when cells were pre-treated with PP3, an inactive control for PP2 (a 4.2 ± 0.4 -fold increase over untreated cells) (Figure 3C), supporting the selectivity of the effect observed.

TP α - and TP β -dependent ERK activation involves EGFR and MMP activation

Since EGFRs may link GPCRs to MAPK activation, we explored the role of the activation of these receptors in both TP α - and TP β -dependent activation of ERK1/2. AG1478, a highly selective inhibitor of tyrosine-dependent phosphorylation of the EGFR, was used. TP α - and TP β -dependent activation of ERK was strongly inhibited by 250 nM AG1478 (Figure 4A). For TP α , IBOP induced a 3.8 ± 0.2 -fold increase in ERK activation over untreated cells compared with a 1.1 ± 0.2 -fold increase with IBOP + AG1478 ($n = 3$; $P < 0.02$). For TP β -expressing cells, a 16 ± 5 -fold increase over untreated cells was obtained with IBOP compared with a 2.6 ± 0.7 -fold increase with IBOP + AG1478 ($n = 3$; $P < 0.02$). As a positive control for the selectivity of the inhibition by AG1478, EGF was used, which showed a strong inhibition, whereas FGF-2-dependent ERK1/2 activation was not significantly modified (Figure 4A). To confirm the effect observed with AG1478, a structurally different inhibitor of the EGFR kinase activity, PD158780, was used at 250 nM, and similar results were obtained with IBOP [a 5 ± 1.02 -fold increase over untreated cells with IBOP compared with a 1.9 ± 0.25 -fold increase with IBOP + PD158780 ($n = 3$;

Figure 3 Effect of PKC inhibitor, GF109203X, and of Src kinase inhibitors, PP1 and PP2, on ERK activation

Western blots of phosphorylated ERK1/2 (p-ERK) and densitometric analysis results of HEK-293-TP α (left panel) and HEK-293-TP β (right panel) cells, treated with or without 5 μ M GF109203X (A), 10 μ M PP1 (B) or 3 and 10 μ M PP2 (C) for 30 min before activation with 10 nM IBOP for 10 min. Total and phosphorylated ERK levels were determined. As a positive control of inhibitory effects of GF109203X on PKC-dependent activation, cells were incubated with 200 nM PMA (A) under similar conditions. As a control of inhibitory effects of the two Src kinase inhibitors, cells were incubated with their inactive analogue PP3 (C). Results are means $\pm$ S.E.M. for four (A) or three (C) independent experiments. Two experiments were performed for (B). *, $P = 0.025$.

$P < 0.04$] or EGF-dependent activation of ERK (a 8.5 ± 1.8 -fold increase over untreated cells with EGF compared with a 2.8 ± 0.5 -fold increase with EGF + PD158780) and no modifications of the ERK phosphorylation by FGF-2 (Figure 4B).

Extracellular MMPs have been shown to have a role in the activation of EGFRs by some GPCRs, resulting, in some

cases, in the shedding of HB-EGF, which subsequently activated the EGFR. Two concentrations, 3 and 10 μ M, of BB2516, a specific inhibitor of the majority of these MMPs were first tested on ERK phosphorylation by IBOP in HEK-293-TP α cells, and a significant inhibition was observed at 10 μ M (results not shown). Treatment of HEK-293-TP α with 10 μ M BB2516

Figure 4 Inhibition of IBOP-induced ERK activation by the EGFR kinase inhibitors AG1478 and PD158780

(A) HEK-293-TP α (left panel) and HEK-293-TP β (right panel) cells were pre-treated with or without 250 nM AG1478 for 30 min and further stimulated with 10 ng/ml EGF or FGF-2 and 10 nM IBOP for 10 min. (B) HEK-293-TP α cells were pre-treated with or without 250 nM PD158780 for 30 min before the addition of 10 nM IBOP, or 10 ng/ml EGF or FGF-2 for 10 min. Total and phosphorylated ERK levels were determined. Western blots of phosphorylated ERK1/2 and densitometric analysis results are presented. Results are means $\pm$ S.E.M. for three independent experiments.

partially blocked ERK phosphorylation in response to IBOP. A 6.6 ± 1 -fold increase over basal levels resulted with IBOP compared with a 3 ± 0.3 -fold increase with IBOP + BB2516 for TP α ($n = 3$; $P < 0.04$) (Figure 5A). Under these conditions, BB2516 did not modify EGF-dependent activation of ERK. This confirms that the effect observed with BB2516 is selective and is not because of a direct effect on EGFR. The ratio of phosphorylated ERK to total ERK was 1.1 ± 0.14 with EGF compared with 1.1 ± 0.2 with EGF + BB2516 ($n = 6$; $P = 0.84$). Furthermore, a neutralizing antibody for the HB-EGF decreased the ERK activation by IBOP, as shown for TP α in Figure 5(A). Similarly, treatment of HEK-293-TP β with 10 μ M BB2516 partially blocked ERK phosphorylation in response to IBOP. A 6.6 ± 1.6 -fold increase over basal cells resulted with IBOP compared with a 2.1 ± 0.8 -fold increase with IBOP + BB2516 for TP β ($n = 3$; $P < 0.05$) (Figure 5B).

Mechanisms involved in the phosphorylation of the EGFR by IBOP

To assess the direct involvement of TXA₂ in EGFR phosphorylation, HEK-293-TP α and HEK-293-TP β cells were treated with IBOP and the phosphorylation of the immunoprecipitated EGFR on tyrosine residues was assayed. Figure 6(A) shows that

Figure 5 Effect of the MMP inhibitor, BB2516, on activation of ERK1/2

HEK-293-TP α (A) and HEK-293-TP β (B) cells were pre-treated with or without 10 μ M BB2516 or 20 μ g/ml anti-(HB-EGF) antibody for 30 min before incubation with 10 nM IBOP or 10 ng/ml EGF for 10 min. Total and phosphorylated ERK1/2 levels were determined. Results are representative of three independent experiments.

phosphorylation of EGFR was obtained for both TP α and TP β and was blocked by both AG1478 and SQ 29548. Incubation of cells with EGF resulted in an increase in EGFR phosphorylation, which was inhibited by AG1478 (Figure 6A). We then checked whether activation of extracellular MMP is important in this transactivation.

IBOP-dependent phosphorylation of EGFR was also blocked when both HEK-293-TP α and HEK-293-TP β cells were pre-treated with BB2516 (Figure 6B). Moreover, since PKC was implicated more in TP β - rather than TP α -dependent activation of ERK, we checked whether activation of this kinase was involved in the phosphorylation of EGFRs. GF109203X, the PKC inhibitor, blocked this phosphorylation strongly in HEK-293-TP β cells and to a lesser extent in HEK-293-TP α (Figure 6B).

Role of kinases, MMPs and G α_i in ERK phosphorylation by IBOP in hASMCs

The involvement of EGFR activation in the TP-dependent activation of ERK was tested in cultured hASMCs. As shown in Figure 7(A), the TP antagonist SQ 29548 blocked IBOP-dependent ERK activation. When cells were pre-treated with AG1478, the ERK activation was significantly suppressed, supporting the involvement of EGFR transactivation in these cells. In parallel experiments, we assessed the role of Src kinases, MMPs and PKC in ERK activation by IBOP. ERK activation in response to IBOP was partially, but significantly, decreased by BB2516 [a 1.9 ± 0.2 -fold increase over untreated cells with IBOP compared with a 1.3 ± 0.2 -fold increase with IBOP + BB2516 ($n = 4$; $P < 0.001$)] (Figure 7B). When cells were pre-treated for 30 min with 10 μ M PP1 or 5 μ M GF109203X, selective inhibitors of Src kinases and PKC respectively, ERK activation was considerably blocked [a 2 ± 0.17 -fold increase over untreated cells with IBOP compared with a 0.73 ± 0.07 -fold increase with IBOP + PP1 and a 1.14 ± 0.04 -fold increase with IBOP + GF109203X ($n = 4, 5$; $P < 0.02$)] (Figure 7C). In these experiments, we checked whether EGF- or PMA-dependent

Figure 6 Specific tyrosine phosphorylation of EGFR by IBOP

HEK-293-TP α or HEK-293-TP β cells in 100-mm-diameter dishes were incubated with different inhibitors before the addition of 10 nM IBOP or 10 ng/ml EGF. After cell lysis, immunoprecipitation (IP) was performed with anti-EGFR antibody as described in the Materials and methods section. Note that 3 mg of total protein was immunoprecipitated except for EGF and EGF + AG1478, where only 1 mg was used. Immunoprecipitates were analysed by immunoblotting with anti-phosphotyrosine (PY) monoclonal antibody. After detection by ECL[®], nitrocellulose membranes were stripped and reprobed with anti-EGFR antibody to control appropriate immunoprecipitation. (A) Cells were pre-treated with 10 μ M SQ 29548 or 250 nM AG1478. (B) Cells were pre-treated with 10 μ M BB2516 or 5 μ M GF109203X. Results are representative of three separate experiments.

Figure 7 Involvement of EGFR and MMP activation and role of Src kinases, PKC and PTX in IBOP-induced ERK activation in hASMCs

hASMCs were serum-deprived for 36 h before stimulation. (A) Cells were pre-treated with 10 μ M SQ 29548, the TP antagonist, or 250 nM AG1478, the EGFR kinase inhibitor, for 30 min before incubation with 10 nM IBOP or 10 ng/ml EGF for 10 min. (B) Cells were pre-treated with 10 μ M BB2516, the MMP inhibitor, for 30 min and then stimulated with 10 nM IBOP for 10 min. (C) Cells were pre-treated with or without 10 μ M PP1, the Src kinase inhibitor, or 5 μ M GF109203X, the PKC inhibitor, for 30 min before incubation with 10 nM IBOP for 10 min. (D) Cells were pre-treated overnight with 100 ng/ml PTX before stimulation with 10 nM IBOP or 10 μ M LPA for 5 min. Total and phosphorylated ERK levels were determined. Western blots are representative of three separate experiments.

ERK activation was altered by these inhibitors. PP1, BB2516 and AG1478 had no effect on the activation of ERK by PMA (results not shown). Similarly, we verified that PP1, BB2516 and GF109203X did not modify EGF-dependent activation of ERK (results not shown).

Finally, the effect of PTX, a selective blocker of G α_i activation, on ERK activation was tested. When cells were pre-treated overnight with 100 ng/ml PTX, a decrease in ERK phosphorylation was observed in IBOP-treated cells [a 2.7 ± 0.5 -fold increase over untreated cells with IBOP compared with a 1.4 ± 0.2 -fold increase with IBOP + PTX ($n = 4$; $P < 0.02$)] (Figure 7D). As a positive control, we used LPA, described as coupling mainly G α_i in HEK-293 cells [32], which showed a strong inhibition of ERK phosphorylation [a 4.8 ± 0.5 -fold increase with LPA compared with a 0.8 ± 0.1 -fold increase with LPA + PTX ($n = 6$; $P < 0.001$)] (Figure 7D).

DISCUSSION

The major finding of this study is that TX-stimulated ERK is partially involved in activation of MMPs and EGFRs. In addition, using selective inhibitors, we demonstrated a role for PKC and Src kinases. In cells that overexpress either TP α or TP β , we showed a major role of PKC in TP β -dependent activation of ERK and EGFR phosphorylation, whereas PKC was less implicated in TP α signalling. A study that mentions the specificity and mechanism of action of some commonly used protein kinase inhibitors [31] asks us to pay attention to their use. Taking into account that most of our conclusions are drawn from the use of inhibitors of signalling, we have validated our results by using (i) structurally different inhibitors where possible, (ii) appropriate concentrations after having tested different concentrations and (iii) the inactive control for one inhibitor.

Activation of MAPKs is an important step in cell growth and differentiation for many growth factors and GPCRs. Activation of RTKs, such as EGFRs or PDGFRs, by GPCR ligands has been described in MAPK activation [33]. This transactivation of growth factor receptors leads to their association with adaptor proteins to form multicomplexes that lead to activation of the ERK pathway [4,7,34–36]. In some cell types, transactivation of EGFRs by GPCRs involved an increase in MMP activity with further cleavage of HB-EGF, which would then interact with the EGFR leading to intracellular signalling and activation of ERK [8–10].

At the present time, only humans are known to have two TP isoforms, and the mechanisms involved in the regulation by each of these isoforms are not fully explored. In cell lines of animal origin, TX have been shown to activate ERK, p38 and JNK MAPKs, leading to hypertrophy, cell growth or gene expression [21,37]. We carried out experiments to characterize the ERK activation by each TP isoform and evaluated the role of RTKs, non-RTKs, PKC and MMPs in ERK activation by a TX analogue. We showed that ERK activation in both HEK-293-TP α and HEK-293-TP β , and in vascular smooth muscle cells is dependent on EGFR activation. This was demonstrated by using the highly selective inhibitor of EGFR phosphorylation, AG1478, and demonstrating the direct phosphorylation of the receptor on tyrosine residues in response to TX. To validate these results, we used a structurally different inhibitor, PD158780, and found the same effect. Recently, two reports have described in a human endothelial cell line ECV304 [25] and an endometrial cell line UTRL-1 [26], that TP activation of ERK is dependent on EGFRs. However, these two studies were performed only in immortalized

Figure 8 Proposed model for TP α - and TP β -mediated ERK activation

TXA₂ stimulation of TP α and TP β leads to phosphorylation of ERK (ERK-P). This would involve transactivation of AG1478-sensitive EGFR. Both isoforms activate ERK phosphorylation in a Src-kinase-dependent manner, whereas PKC was mainly implicated in ERK activation and EGFR phosphorylation by TP β . Moreover, transactivation of the EGFR is, in part, dependent on the extracellular MMP responsible for the cleavage of pro-HB-EGF, thereby allowing the binding of HB-EGF to its receptor.

cell lines, where the amount of RTKs and signalling may differ considerably from primary cells.

One novel aspect of this report is the role of MMPs in ERK activation by TX. We showed that a synthetic MMP inhibitor, BB2516, blocked ERK activation by TX. This inhibitor attenuated EGFR phosphorylation in response to the two TP isoforms both in overexpressing systems and in hASMCs. We also demonstrated that anti-(HB-EGF) neutralizing antibody was able to decrease ERK activation in HEK-293-TP α . Our results suggest that MMP-dependent processing of pro-HB-EGF could constitute one of the pathways linking TP to EGFR and ERK activation. The identity and activation mechanism of the MMP involved in this transactivation are unidentified. GPCRs activate a pathway that stimulates MMPs, most probably of the ADAM (a disintegrin and metalloproteinase-like) family that cleaves the pro-HB-EGF. It has been demonstrated that ADAM-9 or ADAM-12 could have a role in the shedding of the HB-EGF and the further activation of the EGFR [38–40]. In our effort to compare the intracellular pathways in response to TX activation, we showed that TP β -dependent activation of ERK involves PKC, whereas for TP α , phosphorylation of ERK was less modified by PKC inhibitor. Our results suggest a difference in the regulation of intracellular signalling leading to ERK activation between the two isoforms, where PKC appeared to be more involved in EGFR activation by TP β , but less by TP α . The analysis of EGFR phosphorylation of tyrosine residues and the phosphorylation of ERK showed similar results between both isoforms. This suggests that, for TP β , PKCs are upstream of the EGFR subsequently leading to EGFR and ERK phosphorylation. For TP α , the mechanism seems different as other pathways might be involved. Miggin and Kinsella [41] have recently described a partial role of PKC in ERK activation by both TP isoforms, where PKC activates c-raf without involving the activation of EGFR.

TP α and TP β differ in their C-terminal end. Although each of the HEK-293 cell expressing the TP isoforms expresses equal amounts of receptors, it is probable that the pathways triggered

by these isoforms are different, due to the cytoplasmic C-terminal domain. At the present time, the major role described for these splice variants is in their difference in intracellular trafficking. It has been demonstrated that TP β internalizes upon treatment with TX analogues whereas TP α does not [42]. Internalization might be responsible in part for triggering different intracellular pathways [43]. It can have a role in the activation of intracellular signalling, probably allowing contact between different proteins of the transduction cascade. One other possibility is the difference in the potency of TP α to activate ERK phosphorylation, since the EC₅₀ for activating ERK is higher than for TP β .

In hASMCs, we demonstrated that TX implicates PKC in ERK activation. hASMCs have been shown by reverse-transcriptase PCR to express both TP α and TP β [44]. Whether or not ERK activation in these cells occurs in response to one of the two isoforms is not presently known. PKC-dependent, partially dependent or -independent activation of ERK in response to different G α -coupled receptors has been described: PKC involvement has been demonstrated for bradykinin [45], the purinoreceptor P2Y₂ [46] and M1 muscarinic receptors [47], but not for angiotensin AT1 receptors [48]. PKC-dependent intracellular signalling has been described as divergent between the receptors and either regulates or not the transactivation between GPCRs and RTKs. For example, for bradykinin receptors [45], PKC is not involved in EGFR transactivation and constitutes an independent pathway, whereas PKC activation is important in EGFR transactivation by the M1 muscarinic and P2Y₂ receptors [46,47]. Another type of regulation has been described where the PKC activation masks the transactivation of the EGFR by AT1 receptors in rat liver epithelial cell lines [49].

Activation of tyrosine kinases, including Src family tyrosine kinases, has been implicated in the GPCR-dependent activation of MAPKs. Our results demonstrate that Src kinases and MMPs are critical in both TP α - and TP β -transfected cells and in hASMC-dependent activation of ERK1/2. PKC and Src kinases can contribute to MMP activation by other GPCRs, as demonstrated

previously [39,50]. This indeed depends on the receptor and cell type. However, a direct role of PKC on raf-1, which leads to ERK activation, cannot be ruled out. The regulation by Src kinases and the characterization of the PKC-dependent or -independent MMPs involved in EGFR transactivation in response to TX must await further investigation.

It is known from pharmacological and biochemical evidence that the TP α couple mainly G α_q [51,52] and G $\alpha_{12/13}$ [53]; Hirata et al. [20] have indicated that TP β might couple G α_i proteins. Recently, Gao et al. [25] have described a new mechanism in the ECV 304 cell line for the transactivation of EGFR by TX, which required mainly G α_i , which in turn required PKC. In the present study, we showed that PTX-sensitive and -insensitive coupling are needed to activate ERK by TP in hASMCs.

TXA $_2$ has been shown to cause proliferation of vascular smooth muscle cells [54,55], endothelial cells, astrocytes and mesangial cells *in vitro*. Synergy has also been described between TP α and the PDGFR $_{\beta}$ in rat vascular smooth muscle cells [54,56]. Similarly, serotonin and U46619 react synergistically for the proliferation of aortic smooth muscle cells [57]. Other immediate early genes such as c-fos are expressed in response to GPCR ligands, such as thrombin or endothelin, and involve at least partial transactivation of the EGFR [12,58–61].

In summary, we show that activation of ERK by TX involves EGFR transactivation and is partially dependent on metalloproteinase activation in cells that overexpress each of the TP isoforms and in hASMCs. The isoforms of TP α differ in ERK activation where TP β -induced signalling has a greater dependency on PKC activation than TP α . A general model summarizing our results is proposed (Figure 8). Early events associated with mitogenesis could contribute to the cell proliferation induced by other factors, such as growth factors or other GPCR agonists. The role of activation of MMPs and EGFRs in cellular functions in response to TX awaits further investigation.

This work was supported by grants from the Association pour la Recherche sur le Cancer (ARC, Grant # 5884 to A.H.), the Fondation de France (Subvention no. 2000 005985 to A.H.), and graduate fellowships from the ARC and the Fondation pour la Recherche Médicale (C.G.) and Naturalia and Biologia (S.B.). We are grateful for the technical assistance of Nadège Poligne.

REFERENCES

- Hawes, B. E., van Biesen, T., Koch, W. J., Luttrell, L. M. and Lefkowitz, R. J. (1995) Distinct pathways of G β_1 - and G α_q -mediated mitogen-activated protein kinase activation. *J. Biol. Chem.* **270**, 17148–17153
- Sugden, P. H. and Clerk, A. (1997) Regulation of the ERK subgroup of MAP kinase cascades through G protein-coupled receptors. *Cell. Signal.* **9**, 337–351
- van Biesen, T., Luttrell, L. M., Hawes, B. E. and Lefkowitz, R. J. (1996) Mitogenic signaling via G protein-coupled receptors. *Endocr. Rev.* **17**, 698–714
- Luttrell, L. M., Daaka, Y., Della Rocca, G. J. and Lefkowitz, R. J. (1997) G protein-coupled receptors mediate two functionally distinct pathways of tyrosine phosphorylation in rat 1a fibroblasts. Shc phosphorylation and receptor endocytosis correlate with activation of Erk kinases. *J. Biol. Chem.* **272**, 31648–31656
- Luttrell, L. M., Della Rocca, G. J., van Biesen, T., Luttrell, D. K. and Lefkowitz, R. J. (1997) G $\beta\gamma$ subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G protein-coupled receptor-mediated Ras activation. *J. Biol. Chem.* **272**, 4637–4644
- Heeneman, S., Haendeler, J., Saito, Y., Ishida, M. and Berk, B. C. (2000) Angiotensin II induces transactivation of two different populations of the platelet-derived growth factor β receptor. *J. Biol. Chem.* **275**, 15926–15932
- 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) Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *J. Biol. Chem.* **273**, 8890–8896
- Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C. and Ullrich, A. (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature (London)* **402**, 884–888
- Kalmes, A., Vesti, B. R., Daum, G., Abraham, J. A. and Clowes, A. W. (2000) Heparin blockade of thrombin-induced smooth muscle cell migration involves inhibition of epidermal growth factor (EGF) receptor transactivation by heparin-binding EGF-like growth factor. *Circ. Res.* **87**, 92–98
- Eguchi, S., Dempsey, P. J., Frank, G. D., Motley, E. D. and Inagami, T. (2001) Activation of MAPKs by angiotensin II in vascular smooth muscle cells. Metalloprotease-dependent EGF receptor activation is required for activation of ERK and p38 MAPK but not for JNK. *J. Biol. Chem.* **276**, 7957–7962
- Blobel, C. P. (2000) Remarkable roles of proteolysis on and beyond the cell surface. *Curr. Opin. Cell Biol.* **12**, 606–612
- Eguchi, S., Iwasaki, H., Hirata, Y., Frank, G. D., Motley, E. D., Yamakawa, T., Numaguchi, K. and Inagami, T. (1999) Epidermal growth factor receptor is indispensable for c-Fos expression and protein synthesis by angiotensin II. *Eur. J. Pharmacol.* **376**, 203–206
- Smith, W. L. (1992) Prostanoid biosynthesis and mechanisms of action. *Am. J. Physiol.* **263**, F181–F191
- Halushka, P. V., Allan, C. J. and Davis-Bruno, K. L. (1995) Thromboxane A $_2$ receptors. *J. Lipid Mediators Cell Signalling* **12**, 361–378
- Armstrong, R. A. and Wilson, N. H. (1995) Aspects of the thromboxane receptor system. *Gen. Pharmacol.* **26**, 463–472
- Narumiya, S., Sugimoto, Y. and Ushikubi, F. (1999) Prostanoid receptors: structures, properties, and functions. *Physiol. Rev.* **79**, 1193–1226
- Coleman, R. A., Smith, W. L. and Narumiya, S. (1994) International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol. Rev.* **46**, 205–229
- Hirata, M., Hayashi, Y., Ushikubi, F., Yokota, Y., Kageyama, R., Nakanishi, S. and Narumiya, S. (1991) Cloning and expression of cDNA for a human thromboxane A $_2$ receptor. *Nature (London)* **349**, 617–620
- Raychowdhury, M. K., Yukawa, M., Collins, L. J., McGrail, S. H., Kent, K. C. and Ware, J. A. (1994) Alternative splicing produces a divergent cytoplasmic tail in the human endothelial thromboxane A $_2$ receptor. *J. Biol. Chem.* **269**, 19256–19261
- Hirata, T., Ushikubi, F., Kakizuka, A., Okuma, M. and Narumiya, S. (1996) Two thromboxane A $_2$ receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg 60 to Leu mutation. *J. Clin. Invest.* **97**, 949–956
- Morinelli, T. A., Zhang, L. M., Newman, W. H. and Meier, K. E. (1994) Thromboxane A $_2$ /prostaglandin H $_2$ -stimulated mitogenesis of coronary artery smooth muscle cells involves activation of mitogen-activated protein kinase and S6 kinase. *J. Biol. Chem.* **269**, 5693–5698
- Dorn, 2nd, G. W. (1997) Role of thromboxane A $_2$ in mitogenesis of vascular smooth muscle cells. *Agents Actions Suppl.* **48**, 42–62
- Schorr, K. and Weber, A. A. (1997) Roles of vasodilatory prostaglandins in mitogenesis of vascular smooth muscle cells. *Agents Actions Suppl.* **48**, 63–91
- Karim, S., Berrou, E., Lévy-Toledano, S., Bryckaert, M. and MacLouf, J. (1997) Regulatory role of prostaglandin E $_2$ in induction of cyclo-oxygenase-2 by a thromboxane A $_2$ analogue (U46619) and basic fibroblast growth factor in porcine aortic smooth-muscle cells. *Biochem. J.* **326**, 593–599
- Gao, Y., Tang, S., Zhou, S. and Ware, J. A. (2001) The thromboxane A $_2$ receptor activates mitogen-activated protein kinase via protein kinase C-dependent G α_i coupling and Src-dependent phosphorylation of the epidermal growth factor receptor. *J. Pharmacol. Exp. Ther.* **296**, 426–433
- Miggin, S. M. and Kinsella, B. T. (2001) Thromboxane A $_2$ receptor mediated activation of the mitogen activated protein kinase cascades in human uterine smooth muscle cells. *Biochim. Biophys. Acta* **1539**, 147–162
- Habib, A., Vezza, R., Créminon, C., MacLouf, J. and FitzGerald, G. A. (1997) Rapid, agonist-dependent phosphorylation of human thromboxane receptor isoforms: *in vivo* minimal involvement of protein kinase C. *J. Biol. Chem.* **272**, 7191–7200
- Wang, Y., Simonson, M. S., Pouyssegur, J. and Dunn, M. J. (1992) Endothelin rapidly stimulates mitogen-activated protein kinase activity in rat mesangial cells. *Biochem. J.* **287**, 589–594
- Pages, G., Lenormand, P., L'Allemand, G., Chambard, J. C., Meloche, S. and Pouyssegur, J. (1993) Mitogen-activated protein kinases p42 mapk and p44 mapk are required for fibroblast proliferation. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8319–8323
- Ogletree, M. L., Harris, D. N., Greenberg, R., Haslanger, M. F. and Nakane, M. (1985) Pharmacological actions of SQ 29,548, a novel selective thromboxane antagonist. *J. Pharmacol. Exp. Ther.* **234**, 435–441
- Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95–105

- 32 Della Rocca, G. J., Maudsley, S., Daaka, Y., Lefkowitz, R. J. and Luttrell, L. M. (1999) Pleiotropic coupling of G protein-coupled receptors to the mitogen-activated protein kinase cascade. Role of focal adhesions and receptor tyrosine kinases. *J. Biol. Chem.* **274**, 13978–13984
- 33 Zwick, E., Hackel, P. O., Prenzel, N. and Ullrich, A. (1999) The EGF receptor as central transducer of heterologous signalling systems. *Trends Pharmacol. Sci.* **20**, 408–412
- 34 Daub, H., Wallasch, C., Lankenau, A., Herrlich, A. and Ullrich, A. (1997) Signal characteristics of G protein-transactivated EGF receptor. *EMBO J.* **16**, 7032–7044
- 35 Suzuki, Y., Ozawa, Y., Murakami, K. and Miyazaki, H. (1997) Lysophosphatidic acid inhibits epidermal-growth-factor-induced Stat1 signaling in human epidermoid carcinoma A431 cells. *Biochem. Biophys. Res. Commun.* **240**, 856–861
- 36 Maudsley, S., Pierce, K. L., Zamah, A. M., Miller, W. E., Ahn, S., Daaka, Y., Lefkowitz, R. J. and Luttrell, L. M. (2000) The β_2 -adrenergic receptor mediates extracellular signal-regulated kinase activation via assembly of a multi-receptor complex with the epidermal growth factor receptor. *J. Biol. Chem.* **275**, 9572–9580
- 37 Ali, S., Davis, M. G., Becker, M. W. and Dorn, G. W. (1993) Thromboxane A_2 stimulates vascular smooth muscle hypertrophy by up-regulating the synthesis and release of endogenous basic fibroblast growth factor. *J. Biol. Chem.* **268**, 17397–17403
- 38 Blobel, C. P. (1997) Metalloprotease-disintegrins: links to cell adhesion and cleavage of TNF α and Notch. *Cell* **90**, 589–592
- 39 Asakura, M., Kitakaze, M., Takashima, S., Liao, Y., Ishikura, F., Yoshinaka, T., Ohmoto, H., Node, K., Yoshino, K., Ishiguro, H. et al. (2002) Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. *Nat. Med.* **8**, 35–40
- 40 Schlondorff, J. and Blobel, C. P. (1999) Metalloprotease-disintegrins: modular proteins capable of promoting cell–cell interactions and triggering signals by protein-ectodomain shedding. *J. Cell. Sci.* **112**, 3603–3617
- 41 Miggin, S. M. and Kinsella, B. T. (2002) Regulation of extracellular signal-regulated kinase cascades by α - and β -isoforms of the human thromboxane A_2 receptor. *Mol. Pharmacol.* **61**, 817–831
- 42 Parent, J. L., Labrecque, P., Orsini, M. J. and Benovic, J. L. (1999) Internalization of the TXA $_2$ receptor α and β isoforms. Role of the differentially spliced COOH terminus in agonist-promoted receptor internalization. *J. Biol. Chem.* **274**, 8941–8948
- 43 Luttrell, L. M., Roudabush, F. L., Choy, E. W., Miller, W. E., Field, M. E., Pierce, K. L. and Lefkowitz, R. J. (2001) Activation and targeting of extracellular signal-regulated kinases by β -arrestin scaffolds. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 2449–2454
- 44 Miggin, S. M. and Kinsella, B. T. (1998) Expression and tissue distribution of the mRNAs encoding the human thromboxane A_2 receptor (TP) α and β isoforms. *Biochim. Biophys. Acta* **1425**, 543–559
- 45 Adomeit, A., Graness, A., Gross, S., Seedorf, K., Wetzker, R. and Liebmann, C. (1999) Bradykinin B_2 receptor-mediated mitogen-activated protein kinase activation in COS-7 cells requires dual signaling via both protein kinase C pathway and epidermal growth factor receptor transactivation. *Mol. Cell. Biol.* **19**, 5289–5297
- 46 Soltoff, S. P. (1998) Related adhesion focal tyrosine kinase and the epidermal growth factor receptor mediate the stimulation of mitogen-activated protein kinase by the G-protein-coupled P2Y $_2$ receptor. Phorbol ester or $[Ca^{2+}]_i$ elevation can substitute for receptor activation. *J. Biol. Chem.* **273**, 23110–23117
- 47 Tsai, W., Morielli, A. D. and Peralta, E. G. (1997) The m1 muscarinic acetylcholine receptor transactivates the EGF receptor to modulate ion channel activity. *EMBO J.* **16**, 4597–4605
- 48 Eguchi, S., Matsumoto, T., Motley, E. D., Utsunomiya, H. and Inagami, T. (1996) Identification of an essential signaling cascade for mitogen-activated protein kinase activation by angiotensin II in cultured rat vascular smooth muscle cells. Possible requirement of G $_q$ -mediated p21^{ras} activation coupled to a Ca $^{2+}$ /calmodulin-sensitive tyrosine kinase. *J. Biol. Chem.* **271**, 14169–14175
- 49 Li, X., Lee, J. W., Graves, L. M. and Earp, H. S. (1998) Angiotensin II stimulates ERK via two pathways in epithelial cells: protein kinase C suppresses a G-protein coupled receptor-EGF receptor transactivation pathway. *EMBO J.* **17**, 2574–2583
- 50 Pai, R., Soreghan, B., Szabo, I. L., Pavelka, M., Baatar, D. and Tarnawski, A. S. (2002) Prostaglandin E_2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nat. Med.* **8**, 289–293
- 51 Knezevic, I., Borg, C. and Le Berton, G. C. (1993) Identification of G $_q$ as one of the G-proteins which copurify with human platelet thromboxane A_2 /prostaglandin H_2 receptors. *J. Biol. Chem.* **268**, 26011–26017
- 52 Shenker, A., Goldsmith, P., Unson, C. G. and Spiegel, A. M. (1991) The G protein coupled to the thromboxane A_2 receptor in human platelets is a member of the novel G $_q$ family. *J. Biol. Chem.* **266**, 9309–9313
- 53 Offermans, S., Laugwitz, K.-L., Spicher, K. and Schultz, G. (1994) G proteins of the G12 family are activated via thromboxane A_2 and thrombin receptors in human platelets. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 504–508
- 54 Grosser, T., Zucker, T. P., Weber, A. A., Schulte, K., Sachinidis, A., Vetter, H. and Schror, K. (1997) Thromboxane A_2 induces cell signaling but requires platelet-derived growth factor to act as a mitogen. *Eur. J. Pharmacol.* **319**, 327–332
- 55 Zucker, T. P., Bonisch, D., Muck, S., Weber, A. A., Bretschneider, E., Glusa, E. and Schror, K. (1998) Thrombin-induced mitogenesis in coronary artery smooth muscle cells is potentiated by thromboxane A_2 and involves upregulation of thromboxane receptor mRNA. *Circulation* **97**, 589–595
- 56 Zucker, T. P., Grosser, T., Morinelli, T., Halushka, P. V., Sachinidis, A., Vetter, H. and Schror, K. (1995) Potentiation of PDGF-induced growth responses in coronary artery smooth muscle cells by thromboxane. *Agents Actions Suppl.* **45**, 53–58
- 57 Pakala, R., Willerson, J. T. and Benedict, C. R. (1997) Effect of serotonin, thromboxane A_2 , and specific receptor antagonists on vascular smooth muscle cell proliferation. *Circulation* **96**, 2280–2286
- 58 Moriguchi, Y., Matsubara, H., Mori, Y., Murasawa, S., Masaki, H., Maruyama, K., Tsutsumi, Y., Shibasaki, Y., Tanaka, Y., Nakajima, T. et al. (1999) Angiotensin II-induced transactivation of epidermal growth factor receptor regulates fibronectin and transforming growth factor- β synthesis via transcriptional and posttranscriptional mechanisms. *Circ. Res.* **84**, 1073–1084
- 59 Miranti, C. K., Ohno, S. and Brugge, J. S. (1999) Protein kinase C regulates integrin-induced activation of the extracellular regulated kinase pathway upstream of Shc. *J. Biol. Chem.* **274**, 10571–10581
- 60 Ohnaka, K., Numaguchi, K., Yamakawa, T. and Inagami, T. (2000) Induction of cyclooxygenase-2 by angiotensin II in cultured rat vascular smooth muscle cells. *Hypertension* **35**, 68–75
- 61 Yamakawa, T., Eguchi, S., Yamakawa, Y., Motley, E. D., Numaguchi, K., Utsunomiya, H. and Inagami, T. (1998) Lysophosphatidylcholine stimulates MAP kinase activity in rat vascular smooth muscle cells. *Hypertension* **31**, 248–253

Received 2 July 2002/17 January 2003; accepted 21 January 2003

Published as BJ Immediate Publication 21 January 2003, DOI 10.1042/BJ20021030