Thromboxane A_2 receptor mediated activation of the mitogen activated protein kinase cascades in human uterine smooth muscle cells

Sinead M. Miggin, B. Therese Kinsella *

Department of Biochemistry, Conway Institute of Biomolecular and Biomedical Research, Merville House, University College Dublin, Belfield, Dublin 4, Ireland

Abstract

Both thromboxane (TX) A_2 and 8-epi prostaglandin (PG) $F_{2\alpha}$ have been reported to stimulate mitogenesis of vascular smooth muscle (SM) in a number of species. However, TXA_2 and 8-epiPGF$_{2\alpha}$ mediated mitogenic signalling has not been studied in detail in human vascular SM. Thus, using the human uterine ULTR cell line as a model, we investigated TXA_2 receptor (TP) mediated mitogenic signalling in cultured human vascular SMCs. Both the TP agonist U46619 and 8-epiPGF$_{2\alpha}$ elicited time and concentration dependent activation of the extracellular signal regulated kinase (ERK)s and c-Jun N-terminal kinase (JNK)s in ULTR cells. Whereas the TP antagonist SQ29548 abolished U46619 mediated signalling, it only partially inhibited 8-epiPGF$_{2\alpha}$ mediated ERK and JNK activation in ULTR cells. Both U46619 and 8-epiPGF$_{2\alpha}$ induced ERK activations were inhibited by the protein kinase (PK) C, PKA and phosphoinositide 3-kinase inhibitors GF109203X, H-89 and wortmannin, respectively, but were unaffected by pertussis toxin. In addition, U46619 mediated ERK activation in ULTR cells involves transactivation of the epidermal growth factor (EGF) receptor. In humans, TXA_2 signals through two distinct TP isoforms. In investigating the involvement of the TP isoforms in mitogenic signalling, both TPα and TPβ independently directed U46619 and 8-epiPGF$_{2\alpha}$ mediated ERK and JNK activation in human embryonic kidney (HEK) 293 cells over-expressing the individual TP isoforms. However, in contrast to that which occurred in ULTR cells, SQ29548 abolished 8-epiPGF$_{2\alpha}$ mediated ERK and JNK activation through both TPα and TPβ in HEK 293 cells providing further evidence that 8-epiPGF$_{2\alpha}$ may signal through alternative receptors, in addition to the TPs, in human uterine ULTR cells. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Thromboxane A_2 receptor; TPα; TPβ; 8-EpiPGF$_{2\alpha}$; Smooth muscle; Human; MAPK; ERK; JNK; Mitogenesis

1. Introduction

The prostanoid thromboxane A_2 (TXA_2) mediates a number of responses in smooth muscle (SM) cells including constriction of aortic and uterine SM [1] and stimulation of mitogenic/hypertrophic responses in vascular SM [2]. In humans, molecular cloning has identified two receptors for TXA_2, termed TXA_2 receptor (TP)α and TPβ, which are encoded by a single TP gene but which arise by differential splicing and

which differ exclusively in their carboxyl terminal tail regions [3–5]. Whereas the physiologic significance for the existence for two TP receptors in humans, but not in other species, is currently unknown, differences in their expression profiles [6], G protein coupling specificity [7] and patterns of desensitisation [8–10] have been identified.

Of the many intracellular protein kinase (PK)s implicated as mediators of mitogenesis, the extracellular signal regulated kinases (ERKs) play an integral role in the response of cells to various growth promoting agents [11,12]. Both the TXA_2 mimetics I-BOP and U46619 activate ERK1 and ERK2 in porcine coronary artery smooth muscle cells (SMCs), in rat aortic and bovine SMCs, respectively [2,13–15]. Recently, the stress activated protein kinases (SAPKs), c-Jun N-terminal kinase (JNK) and p38, have been also implicated in TXA_2 mediated signal cascades [16,17].

The F2-isoprostane, 8-epi prostaglandin $F_{2\alpha}$ (8-epiPGF$_{2\alpha}$) is produced in large quantities in vivo in response to free radical induced mechanisms, and is a potent renal, pulmonary and arterial vasoconstrictor [18–20] with its actions being mediated, at least in part, through interaction with vascular TPs [21,22]. Zhang et al. [23] demonstrated that 8-epiPGF$_{2\alpha}$ is less potent than U46619 in inducing contraction of rat aorta. 8-EpiPGF$_{2\alpha}$ has been reported to induce ERK activity through the TP in porcine carotid arteries [24]. It has also been suggested that 8-epi-PGF$_{2\alpha}$ may exert its biological actions in SMC through activation of receptor sites related to but distinct from TPs [25]. Thus, conflicting evidence exists regarding the actions of TXA_2 and 8-epiPGF$_{2\alpha}$ in vascular SM. These discrepancies may be due to differences in the species investigated [2,13] or in tissue and culture conditions employed [2,26]. Despite these diverse studies, TXA_2 and 8-epiPGF$_{2\alpha}$ mediated mitogenesis have not been investigated in detail in human vascular SM.

Thus, in the present study, we sought to investigate TP mediated mitogenesis in human vascular SM in response to a TXA_2 mimetic and in response to the F2-isoprostane 8-epiPGF$_{2\alpha}$. Thus, the immortalised human uterine ULTR cell line [27] was used as a model vascular SMC line to investigate U46619 and 8-epiPGF$_{2\alpha}$ mediated mitogenesis and activation of

mitogen activated protein kinase (MAPK) cascades (ERK and JNK). Thereafter, as TXA_2 signals in humans through two TP isoforms both of which were demonstrated to be expressed in ULTR cells, human embryonic kidney (HEK) 293 cell lines stably overexpressing either TPα (HEK.TPα10 cells) or TPβ (HEK.TPβ3 cells) were used to establish whether both TPα and TPβ individually contribute to U46619 and 8-epiPGF$_{2\alpha}$ mediated MAPK activation in human tissues. Both TP isoforms mediated ERK and JNK activation in response to U46619 and 8-epiPGF$_{2\alpha}$ in the ULTR and the HEK 293 cell lines. Whereas the selective TP antagonist SQ29548 abolished 8-epiPGF$_{2\alpha}$ mediated ERK and JNK activation in HEK.TPα10 and HEK.TPβ3 cells, it resulted in only partial inhibition of 8-epiPGF$_{2\alpha}$ mediated MAPK activation in ULTR cells suggesting that 8-epiPGF$_{2\alpha}$ may signal through both TPα and TPβ and through other unidentified, SQ29548 insensitive receptors in ULTR cells. This study provides the first in depth analysis of the regulation of TP mediated MAPK activation in human SMCs in response to the TXA_2 mimetic U46619 and the F2-isoprostane 8-epiPGF$_{2\alpha}$. Additionally, this study provides the first evidence that TPα and TPβ individually contribute to TP mediated MAPK activation in human tissue.

2. Materials and methods

2.1. Materials

U46619, 8-epiPGF$_{2\alpha}$ and SQ29548 were purchased from Cayman Chemical Company, Ann Arbor, MI, USA. PD98059, GF109203X, tyrphostin AG1478 and H-89 were from Calbiochem-Novabiochem, Nottingham, UK. Anti-ACTIVE MAPK and Anti-ACTIVE JNK rabbit polyclonal antibodies and epidermal growth factor (EGF) were purchased from Promega Corporation, Madison, WI, USA. Affinity purified rabbit polyclonal anti-JNK and anti-ERK antibodies were from Santa Cruz Laboratories, Santa Cruz, CA, USA. Anisomycin, myelin basic protein (MBP), platelet derived growth factor-$\beta\beta$ (PDGF-$\beta\beta$) and wortmannin were purchased from Sigma Chemical Company, St. Louis, MO, USA.

2.2. Culture of human uterine SMCs, HEK.TPα10 and HEK.TPβ3 cells

The uterine SMC line ULTR, described by Perez-Reyes et al. [27] was obtained from J.K. McDougall, Department of Pathology, University of Washington, Seattle, WA, USA. Cells were routinely grown in Dulbecco's minimal essential medium (DMEM), 10% foetal calf serum (FCS). Human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection and were routinely grown in minimal essential medium (MEM), 10% FCS, unless otherwise indicated. The recombinant HEK.TPα10 and HEK.TPβ3 cell lines stably overexpressing TPα and TPβ, respectively, have been previously described [9,28].

2.3. Indirect immunofluorescence of TPα and TPβ

TP isoform specific antibodies directed to peptide sequences unique to TPα (amino acid residues SLSLQPQLTQRSGLQ; α peptide) and TPβ (amino acid residues LPFEPPTGKALSRKD; β peptide) intracellular C-tail sequences were raised in rabbits following conjugation to the carrier protein keyhole limpet haemacyanin according to standard procedures. Indirect immunofluorescent detection of TPα and TPβ in permeabilised (2% paraformaldehyde) or, as controls, in non-permeabilised cells, was performed essentially as previously described [29]. As additional negative controls, primary antibody was omitted or the primary antibody was pre-incubated with its cognate α or β peptide prior to exposure to cells. Indirect immunofluorescence detection of the anti-rabbit fluorescein isothiocyanate (FITC) conjugated secondary antibody was observed using an Olympus BX60 fluorescence microscope. In parallel, nuclei were co-stained with 4,6-diamidino-2-phenyl-indole (DAPI) as per the manufacturer's instructions (Sigma).

2.4. Determination of DNA and protein synthesis

ULTR cells were seeded at 2.5×10^5 cells/35 mm dish in DMEM, 10% FCS. After 24 h, cells were exposed to DMEM, 0.5% FCS to induce quiescence. After a further 24 h, the medium was supplemented with 1.5 μCi [³H]thymidine (60 Ci/mmol, Sigma) or 1.5 μCi [³H]leucine (60 Ci/mmol, American Radiochemicals) and cells were exposed to test agents (U46619, 1 μM for 0–10 min; PDGF-ββ, 20 ng/ml for 10 min) at 37°C. Protein and DNA synthesis was determined essentially as previously described [30]. Results are presented as the mean data ± S.E.M. ($n = 3$).

2.5. Determination of ERK activity using an in vitro kinase assay

ULTR cells were seeded at 4×10^5 cells/60 mm dish in DMEM, 10% FCS. After 24 h, cells were exposed to DMEM, 0.5% FCS to induce quiescence. After a further 48 h, the cells were exposed to test agents (U46619, 1 μM for 0–10 min; PDGF-ββ, 20 ng/ml for 10 min) at 37°C. ERK activity was determined by monitoring ERK mediated phosphorylation of its substrate myelin basic protein as previously described [31] with the following modification. Endogenous ERK1/2 was recovered from the cleared cellular lysates (150 μg) by the addition of a 1/2000 dilution of a non-inhibitory affinity purified anti-ERK-1/2 antibody (Santa Cruz).

2.6. Determination of ERK/JNK activation by immunoblot analysis

ULTR cells were seeded at 1.5×10^6 cells/10 cm dish in DMEM, 10% FCS. After 24 h, cells were exposed to DMEM without any FCS to induce quiescence. HEK.TPα10 and HEK.TPβ3 cells were seeded at 1.5×10^6 cells/10 cm dish in MEM, 10% FCS. After 24 h, cells were exposed to medium without any FCS to induce quiescence. After a further 48 h, cells were exposed to test compounds at 37°C, as indicated in the respective figure legends. Cellular lysates were prepared as previously described [32]. Aliquots (30 μg) of the cellular lysates were fractionated by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis on 12.5% gels followed by electroblotting onto PVDF membrane. Thereafter, membranes were screened by immunoblot analysis using a rabbit anti-ACTIVE ERK antibody (Promega) or a rabbit anti-ACTIVE JNK antibody (Promega) as appropriate, to detect dual phosphorylated ERK (ppERK1/2) and dual phosphorylated JNK (ppJNK1/2), respectively, as recommended by the
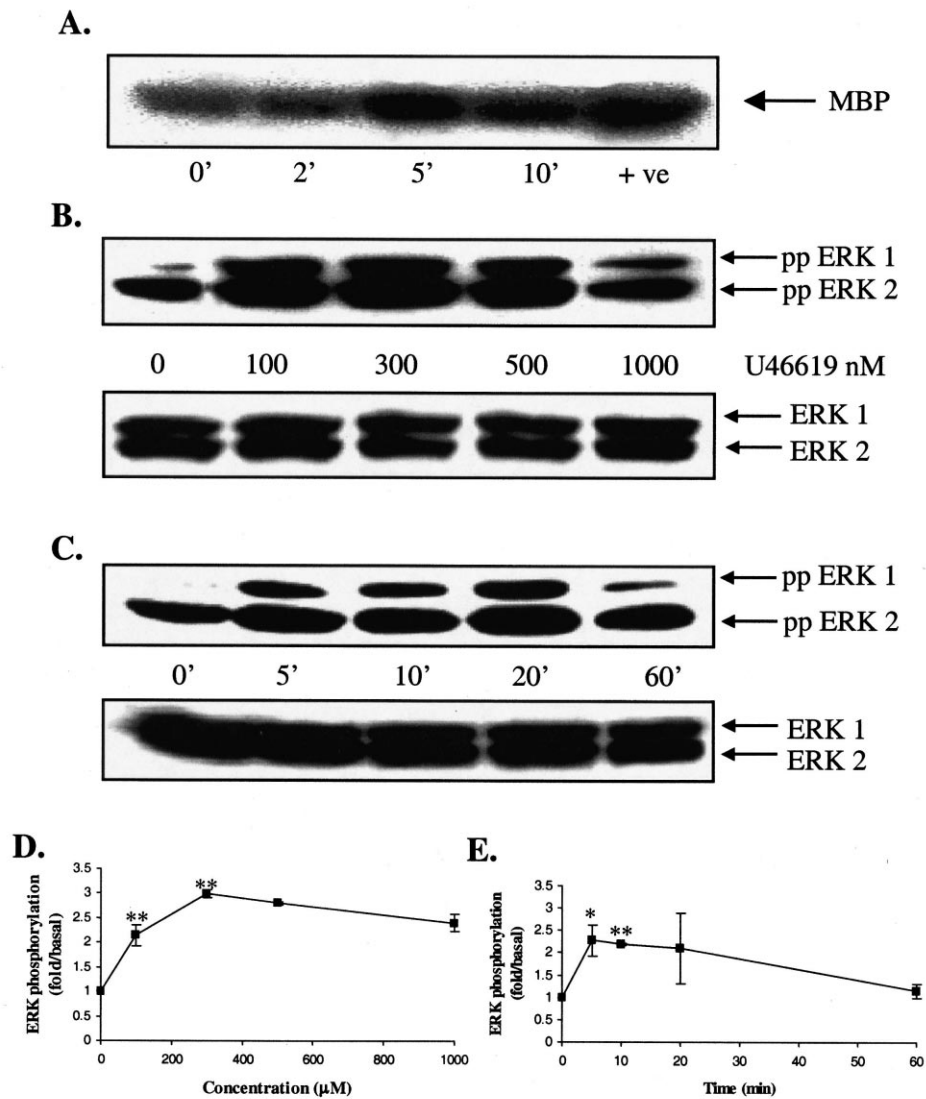
A.

B.

C.

D.

E.

Fig. 1. U46619 mediated ERK activation in ULTR cells. (A) ULTR cells were stimulated with U46619 for 0–10 min (0–10′) and U46619 mediated ERK activation was determined using an in vitro kinase assay as described in Section 2. As a positive control (+ve), cells were incubated with PDGF-ββ. Positions of the [γ^{32}P]-labelled myelin basic protein (MBP) are indicated by the arrow. (B) Concentration dependent effect of U46619 (0–1000 nM, 10 min) and (C) time dependent effect of U46619 (100 nM, 0–60 min) on ERK1 and ERK2 activation in ULTR cells. Upper panels of (B) and (C) Blots were screened with anti-ACTIVE ERK to detect the phosphorylated, active forms of ERK (ppERK1/2) whereas in the lower panels of (B) and (C) blots were screened with anti-ERK antibodies to detect ERK1/2 immunoreactive protein. Results are representatives of at least three independent experiments. Panels (D) and (E) Fold increases in ERK phosphorylation (ppERK1/2) in panels (B) and (C), respectively, are presented as mean fold increases of basal ERK phosphorylation $\pm$ S.E.M. ($n = 3$), where the levels of basal ERK phosphorylation in vehicle treated cells are assigned a value of 1.0. *$P \leq 0.05$ and **$P \leq 0.01$ indicate that the levels of U46619 mediated ERK activation (ppERK) were significantly greater relative to basal levels.

supplier. Subsequently, membranes were re-screened with either affinity purified rabbit anti-ERK antibody (Santa Cruz) or anti-JNK antibody (Santa Cruz) to detect total ERK protein and total JNK protein, respectively. Immunocomplexes were visual-

ised using the chemiluminescence detection system, as described by the supplier (Roche); in each case, immunoblots are presented as a representative blot from at least three independent experiments. Alternatively, signals from ppERK1 and ppERK2 or

ppJNK1 and ppJNK2 were quantified by scanning densitometry using a UVP gel documentation system and the combined ppERK1/2 or ppJNK1/2 signals are presented as mean fold increase of basal ERK phosphorylation ± standard error of the mean (S.E.M.), where the levels of basal ERK phosphorylation in vehicle treated cells are assigned a value of 1.0. Statistical analyses were carried out using the unpaired Student's t-test using the Statworks Analysis Package. P-values ≤ 0.05 were considered to indicate a statistically significant difference.

3. Results

3.1. Effect of U46619 on ERK activation in ULTR cells

To elucidate the mitogenic/hypertrophic capabilities of TXA_2 in human uterine SMCs, U46619 mediated changes in DNA and protein synthesis and activation of the ERK signalling cascade were investigated in ULTR cells. U46619 and PDGF resulted in a 1.23 ± 0.07-fold ($P ≤ 0.001$) and 1.21 ± 0.03-fold ($P ≤ 0.001$) increase in [³H]thymidine incorporation, respectively. Whereas PDGF resulted in a 1.51 ± 0.17-fold ($P ≤ 0.001$) increase in [³H]leucine incorporation, U46619 resulted in a 1.89 ± 0.11-fold ($P ≤ 0.001$) increase in [³H]leucine incorporation. Thereafter, an in vitro kinase assay was used to evaluate the ability of activated ERK to phosphorylate its substrate myelin basic protein (MBP; [33]) in response to U46619 stimulation. Whereas detectable levels of non-specific MBP phosphorylation were observed for non-treated cells (Fig. 1A; time zero) or vehicle only treated cells (data not shown), exposure of quiescent ULTR cells to U46619 for 2–10 min resulted in a time dependent increase in phosphorylation of MBP, with maximal phosphorylation observed after 5 min (Fig. 1A, $P ≤ 0.05$). Exposure of the quiescent ULTR cells to PDGF-ββ also induced MBP phosphorylation.

As an alternative method to evaluate U46619 induced ERK1 and ERK2 activation, anti-ACTIVE ERK antibodies that preferentially recognise their dually phosphorylated, active forms (phosphorylated ppERK1/2) were utilised. Furthermore, an affinity purified anti-ERK antibody was routinely used to
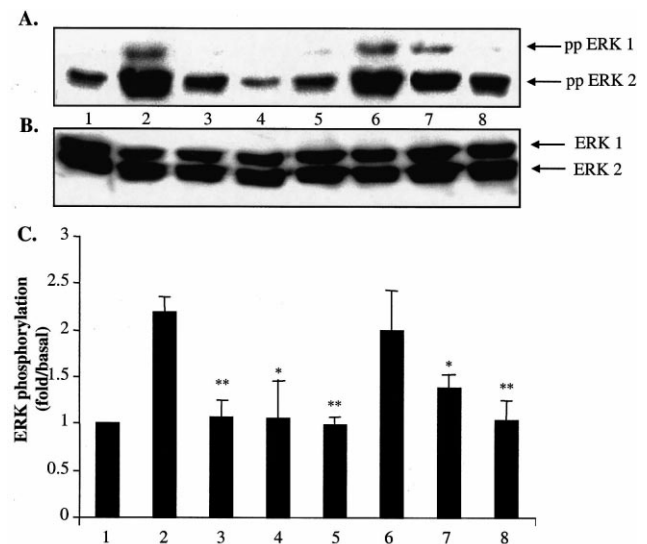

Fig. 2. Modulation of U46619 mediated ERK activation. ULTR cells were pre-incubated with either SQ29548 (1 μM, 1 min; lane 3), PD98059 (10 μM, 30 min; lane 4), H-89 (10 μM, 5 min; lane 5), PTX (50 ng/ml, 16 h; lane 6), GF109203X (500 nM, 30 min; lane 7) or wortmannin (400 nM, 30 min; lane 8). Subsequently, U46619 (100 nM) was added for 10 min (lanes 3–8), with cells exposed exclusively to U46619 (100 nM for 10 min; lane 2) or with vehicle alone (lane 1) serving as references. (A) Immunoblots were screened with anti-ACTIVE ERK to detect the phosphorylated, active forms of ERK (ppERK1/2) whereas in (B) blots were screened with anti-ERK antibodies to detect ERK1/2 immunoreactive protein. Results are representatives of at least three independent experiments. (C) Fold increases in ERK phosphorylation (ppERK1/2) in (A) are presented as mean fold increases of basal ERK phosphorylation ± S.E.M. ($n = 3$–5), where the levels of basal ERK phosphorylation in vehicle treated cells are assigned a value of 1.0. *$P ≤ 0.05$ and **$P ≤ 0.01$ indicate that the levels of U46619 mediated ppERK activation (lane 2) were significantly reduced in the presence of the respective pharmacological inhibitors (lanes 3–8).

detect total ERK1 and ERK2 (ERK) protein present in the cellular lysate, thus ensuring equal expression and protein loading of ERK1 and ERK2. U46619 mediated a dose dependent phosphorylation of ERK1 (ppERK1) and ERK2 (ppERK2) in ULTR cells with maximal activation observed at 100–300 nM U46619 (Fig. 1B,D). U46619 also mediated a time dependent activation of ERK1 and ERK2 with maximal activation observed at 5–10 min (Fig. 1C,E). It was noteworthy that in contrast to that observed with the previous in vitro kinase assays, routinely only low levels of non-specific ERK activation were observed in vehicle only treated cells using

the anti-ACTIVE ERK antibody. Furthermore, as MBP may be a substrate for phosphorylation by protein kinases other than ERK, coupled to the fact that the in vitro kinase procedure does not differentiate between ERK1 and ERK2 activation, makes the former protocol a less specific and informative method for measurement of ERK activation. For these reasons, the latter approach using anti-ACTIVE ERK antibodies was used in preference to the in vitro kinase assay for further characterisation of the ERK pathways in human SMCs.

Pre-treatment of ULTR cells with SQ29548 (Fig. 2) or with PD98059 (Fig. 2) reduced U46619 induced ERK1/2 activation to levels that were not significantly different from those observed in non-stimulated ULTR cells. Whereas pre-treatment of ULTR cells with the PKA inhibitor H-89 resulted in a near complete inhibition of U46619 mediated activation of ERK1/2 (Fig. 2), pertussis toxin (PTX) did not affect U46619 mediated activation of ERK1/2 (Fig. 2). The PKC inhibitor GF109203X (Fig. 2) or the phosphoinositide 3-kinase (PI3K) inhibitor wortmannin (Fig. 2) each resulted in a partial, though significant, inhibition of ERK1/2 activation when compared to ULTR cells exposed exclusively to U46619. H-89, GF109203X and wortmannin combined did not fully abolish U46619 mediated ERK activation (data not shown). These data confirm that U46619 induced activation of ERK1/2 in ULTR cells is mediated through the TP(s) in a MEK1/2 dependent manner and suggest that PKA, PKC and PI3K play a role in these events.

3.2. Effect of tyrphostin AG1478 on U46619 mediated ERK activation in ULTR cells

Many G protein coupled receptors (GPCRs) induce the activation of mitogenic signalling by stimulating tyrosine kinase signalling cascades [34–37]. For example, GPCRs stimulate tyrosine phosphorylation by inducing the transactivation of a receptor tyrosine kinase (RTK) such as the EGF receptor, platelet derived growth factor receptor and insulin like growth factor-1 [34,35]. The ability of tyrphostin AG1478, a selective EGF receptor inhibitor, to block U46619 mediated ERK activation in ULTR cells was examined. Exposure of ULTR cells to U46619 (100 nM) and EGF (10 ng/ml) for 10 min resulted in the
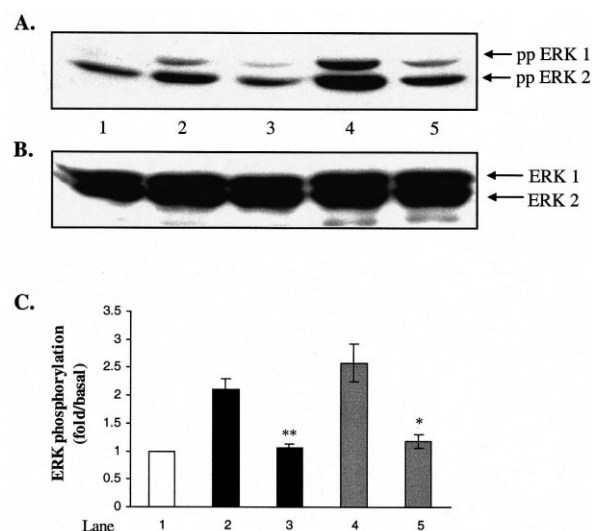

Fig. 3. Effect of tyrphostin AG1478 on U46619 mediated ERK activation. ULTR cells were pre-incubated with tyrphostin AG1478 (125 nM, 30 min; lanes 3 and 5). Subsequently, U46619 (100 nM; lane 3) or EGF (10 ng/ml; lane 5) was added for 10 min, with cells exposed to U46619 alone (100 nM for 10 min; lane 2), EGF alone (10 ng/ml for 10 min; lane 4) or with vehicle alone (lane 1) serving as references. (A) Immunoblots were screened with anti-ACTIVE ERK to detect the phosphorylated, active forms of ERK (ppERK1/2) whereas in (B) blots were screened with anti-ERK antibodies to detect ERK1/2 immunoreactive protein. Results are representatives of four independent experiments. (C) Fold increases in ERK phosphorylation (ppERK1/2) in (A) are presented as mean fold increases of basal ERK phosphorylation $\pm$ S.E.M. ($n = 4$), where the levels of basal ERK phosphorylation in vehicle treated cells are assigned a value of 1.0. *$P \leq 0.05$ and **$P \leq 0.01$ indicate that the levels of U46619 or EGF mediated ppERK activation (lanes 2 and 4, respectively) were significantly reduced in the presence of AG1478 (lanes 3 and 5, respectively).

activation of ERK1 and ERK2 (Fig. 3A,C). Moreover, pre-treatment of ULTR cells with AG1478 significantly inhibited both EGF and U46619 mediated ERK1 and ERK2 activation (Fig. 3A,C). These findings suggest that U46619 mediated activation of the ERK signalling cascade also involves transactivation of the EGF receptor.

3.3. Effect of 8-epiPGF$_{2\alpha}$ on ERK activation in ULTR cells

Exposure of ULTR cells to 8-epiPGF$_{2\alpha}$ resulted in a dose dependent activation of ERK1 and ERK2 with maximal activation observed when cells were exposed to 300 nM 8-epiPGF$_{2\alpha}$ (Fig. 4A,C). Addi-
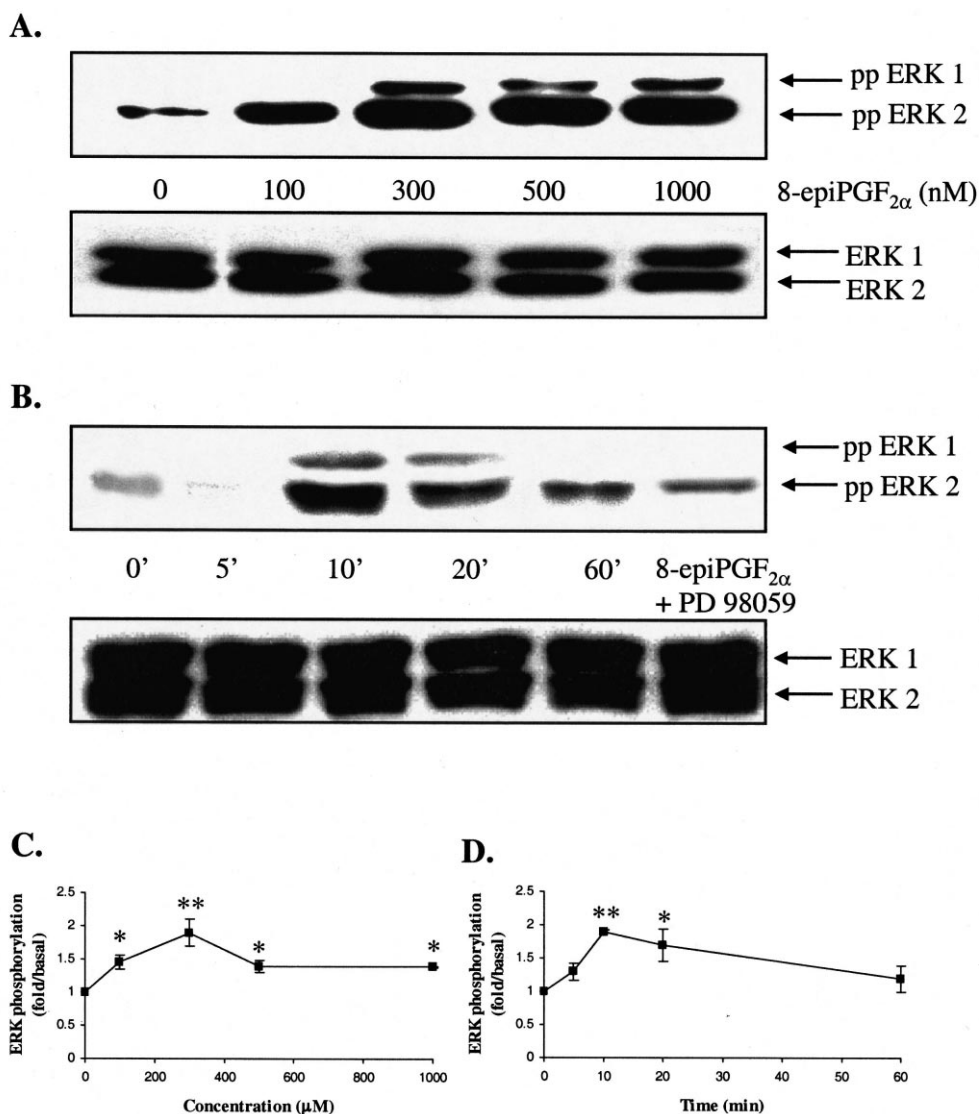
Fig. 4. 8-EpiPGF$_{2\alpha}$ mediated ERK activation in ULTR cells. (A) Dose dependent (0–1000 nM, 10 min) and (B) time dependent (300 nM, 0–60 min) activation of ERK1/2 in response to 8-epiPGF$_{2\alpha}$. Alternatively, in (B) (8-epiPGF$_{2\alpha}$ and PD98059), cells were pre-incubated with PD98059 (10 μM, 30 min) prior to stimulation of cells with 8-epiPGF$_{2\alpha}$ (300 nM, 10 min). Upper panels, blots were screened with anti-ACTIVE ERK to detect the phosphorylated, active forms of ERK (ppERK1/2) whereas in the lower panels, blots were screened with anti-ERK antibodies to detect ERK1/2 immunoreactive protein. Results are representatives of at least three independent experiments. (C) and (D) Fold increases in ERK phosphorylation (ppERK1/2) in (A) and (B), respectively, are presented as mean fold increases of basal ERK phosphorylation ± S.E.M. ($n = 3$–7), where the levels of basal ERK phosphorylation in vehicle treated cells are assigned a value of 1.0. *$P \leq 0.05$ and **$P \leq 0.01$ indicate that the levels of 8-epiPGF$_{2\alpha}$ mediated ERK activation (ppERK) were significantly greater relative to basal levels.

tionally, 8-epiPGF$_{2\alpha}$ induced a time dependent activation of ERK1 and ERK2 with maximal phosphorylation occurring after 10 min reaching near basal levels after 60 min (Fig. 4B,D).

Whereas pre-treatment of ULTR cells with PD98059 inhibited 8-epiPGF$_{2\alpha}$ induced ERK1/2 activation (Fig. 4B, $P \leq 0.05$), SQ29548 reduced, but

did not lead to a statistically significant inhibition of, ERK1 and ERK2 activation (Fig. 5A,C, $P \geq 0.05$) in response to 8-epiPGF$_{2\alpha}$. These findings suggest that 8-epiPGF$_{2\alpha}$ induced activation of ERK1/2 in ULTR cells is mediated only partially through TP(s) and that 8-epiPGF$_{2\alpha}$ is less potent than U46619 in mediating ERK1/2 activation.

Pre-treatment of ULTR cells with wortmannin, H-89 or GF109203X each significantly inhibited 8-epiPGF$_{2\alpha}$ mediated activation of ERK1/2 compared to cells exposed to 8-epiPGF$_{2\alpha}$ alone (Fig. 5A,C). PTX did not affect 8-epiPGF$_{2\alpha}$ mediated activation of ERK1/2 (Fig. 5A,C). These findings suggest that PI3K, PKA and PKC play a role in 8-epiPGF$_{2\alpha}$ induced ERK activation. Moreover, pre-treatment of ULTR cells with AG1478 significantly inhibited 8-epiPGF$_{2\alpha}$ mediated ERK1 and ERK2 activation suggesting 8-epiPGF$_{2\alpha}$ induced transactivation of the EGF receptor (data not shown).
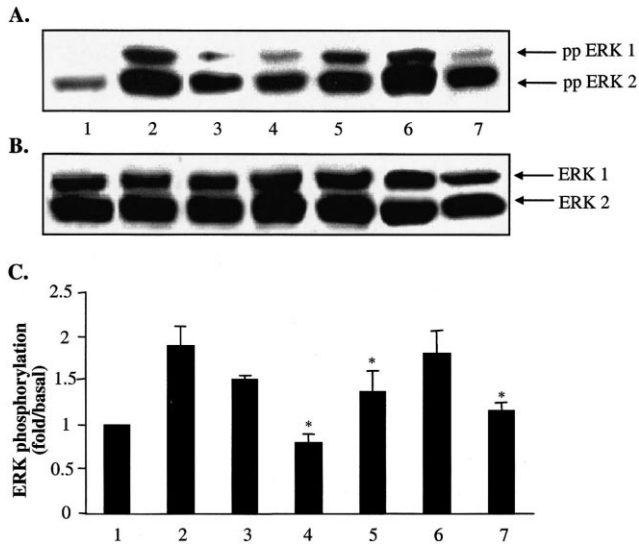

Fig. 5. Modulation of 8-epiPGF$_{2\alpha}$ mediated ERK activation. ULTR cells were pre-incubated with either SQ29548 (1 μM, 1 min; lane 3), wortmannin (400 nM, 30 min; lane 4), H-89 (10 μM, 5 min; lane 5), PTX (50 ng/ml, 16 h; lane 6) or GF109203X (500 nM, 30 min; lane 7). Subsequently, 8-epiPGF$_{2\alpha}$ (300 nM) was added for 10 min (lanes 3–7), with cells exposed to 8-epiPGF$_{2\alpha}$ alone (300 nM for 10 min; lane 2) or with vehicle alone (lane 1) serving as references. (A) Immunoblots were screened with anti-ACTIVE ERK to detect the phosphorylated, active forms of ERK (ppERK1/2). (B) Immunoblots were screened with anti-ERK antibodies to detect ERK1/2 immunoreactive protein. (C) Fold increases in ERK phosphorylation (ppERK1/2) in (A) are presented as mean fold increases of basal ERK phosphorylation ± S.E.M. ($n = 3$–4), where the levels of basal ERK phosphorylation in vehicle treated cells are assigned a value of 1.0. *$P \leq 0.05$ and **$P \leq 0.01$ indicate that the levels of 8-epiPGF$_{2\alpha}$ mediated ppERK activation (lane 2) were significantly reduced in the presence of the respective pharmacological inhibitors (lanes 3–7).
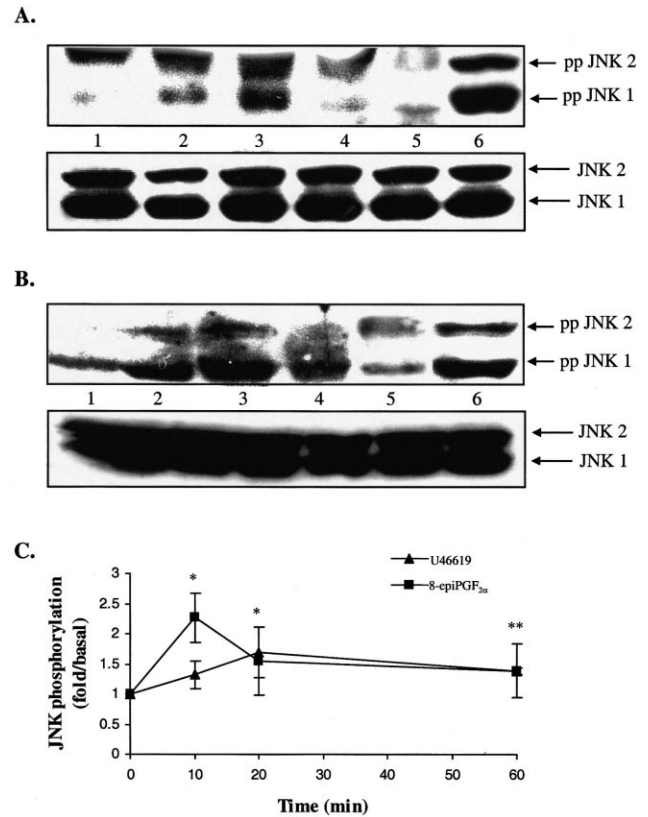

Fig. 6. Effect of U46619 and 8-epiPGF$_{2\alpha}$ on JNK activation in ULTR cells. ULTR cells were stimulated with either U46619 (100 nM; (A) lanes 2–4) or 8-epiPGF$_{2\alpha}$ (300 nM; (B) lanes 2–4) for 10, 20 and 60 min, respectively, or with vehicle alone (control; (A) and (B), lane 1) or were pre-incubated with SQ29548 (1 μM, 1 min) prior to stimulation with 100 nM U46619 (A, lane 5) or 300 nM 8-epiPGF$_{2\alpha}$ (B, lane 5) for 10 min. As positive control, cells were incubated with anisomycin (10 μg/ml) for 60 min (A and B, lane 6). Upper panels, blots were screened with anti-ACTIVE JNK to detect the phosphorylation, active forms of JNK (ppJNK1/2) whereas in the lower panels, blots were screened with anti-JNK antibodies to detect JNK1/2 immunoreactive protein. Results are representatives of three independent experiments. (C) Fold increases in U46619 and 8-epiPGF$_{2\alpha}$ mediated ppJNK1/2 in (A) and (B), respectively, are presented as mean fold increases of basal JNK phosphorylation ± S.E.M. ($n = 3$), where the levels of basal JNK phosphorylation in vehicle treated cells are assigned a value of 1.0. *$P \leq 0.05$ and **$P \leq 0.01$ indicate that the levels of U46619 or 8-epiPGF$_{2\alpha}$ mediated JNK activation (ppJNK) were significantly greater relative to basal levels.

3.4. Effect of U46619 and 8-epiPGF$_{2\alpha}$ on JNK1 and JNK2 activation in ULTR cells

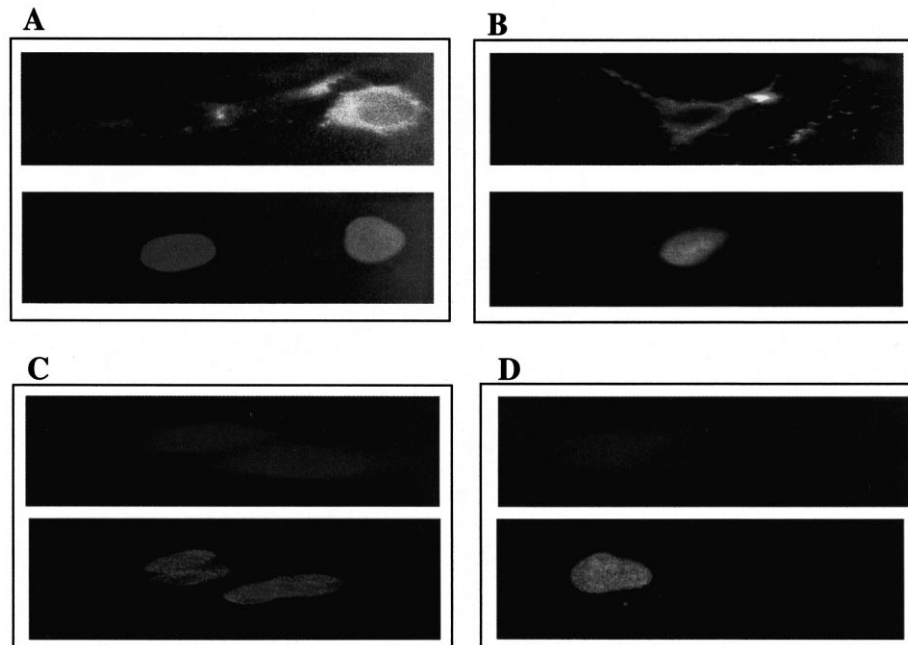To investigate the role of TP on the SAPK cascades, quiescent ULTR cells were exposed to U46619

Fig. 7. Expression of TPα and TPβ in ULTR cells. Indirect immunofluorescent staining of TPα and TPβ in permeabilised ULTR cells was performed as described in Section 2. Within each panel, the upper photograph represents the FITC immunofluorescence and the lower photograph illustrates the localisation of nuclei following DAPI staining. (A) Anti-TPα antiserum; (B) anti-TPβ antiserum; (C) anti-TPα antiserum pre-incubated with a TPα specific peptide; (D) anti-TPβ antiserum pre-incubated with a TPβ specific peptide. Results presented are representatives of at least three independent experiments.

or to 8-epiPGF$_{2\alpha}$ for 10–60 min, or as a positive control, to the protein synthesis inhibitor anisomycin for 60 min. Whereas anisomycin induced activation of both JNK1 and JNK2 (Fig. 6A,B), both U46619 and 8-epiPGF$_{2\alpha}$ each induced a weak, time dependent activation of JNK1 and JNK2 (Fig. 6A–C). Immunoblot analysis using an affinity purified anti-JNK antibody revealed that both JNK1 and JNK2 are abundantly expressed in ULTR cells (Fig. 6A). The TP antagonist SQ29548 blocked U46619 mediated JNK activation (Fig. 6A; $P \leq 0.05$) but resulted in only a marginal, statistically insignificant, inhibition of 8-epiPGF$_{2\alpha}$ mediated JNK activation (Fig. 6B; $P \geq 0.05$).

3.5. Immunodetection of TPα and TPβ in a human uterine SMC line

Indirect immunofluorescent staining of ULTR cells with the TPα and TPβ specific antisera confirmed expression of both TPα and TPβ in membranes of permeabilised (Fig. 7A,B) but not in non-permeabilised ULTR cells (data not shown). The specificity of the TP isoform selective antibodies was further confirmed in peptide competition studies whereby the cognate α peptide blocked immunodetection by anti-TPα antisera (Fig. 7C) but not by anti-TPβ antisera (data not shown) and vice versa (Fig. 7D and data not shown). These data corroborate RT-PCR analyses [6] and confirm that both TPα and TPβ mRNA and protein are expressed in the human ULTR cell line under study.

3.6. Effect of U46619 and 8-epiPGF$_{2\alpha}$ on MAPK activation in HEK.TPα10 and HEK.TPβ3 cells

To establish whether U46619 induced activation of ERK1 and ERK2 was mediated through one or both TP isoforms, non-transfected HEK 293 cells, HEK.TPα10 and HEK.TPβ3 cells were exposed to U46619, with vehicle treated cells serving as a control. Exposure of HEK 293 cells to U46619 resulted in a marginal, statistically insignificant activation of ERK1 and ERK2 when compared to cells treated with vehicle only (Fig. 8A,B), probably due to low levels of endogenous TP(s) present in this cell type
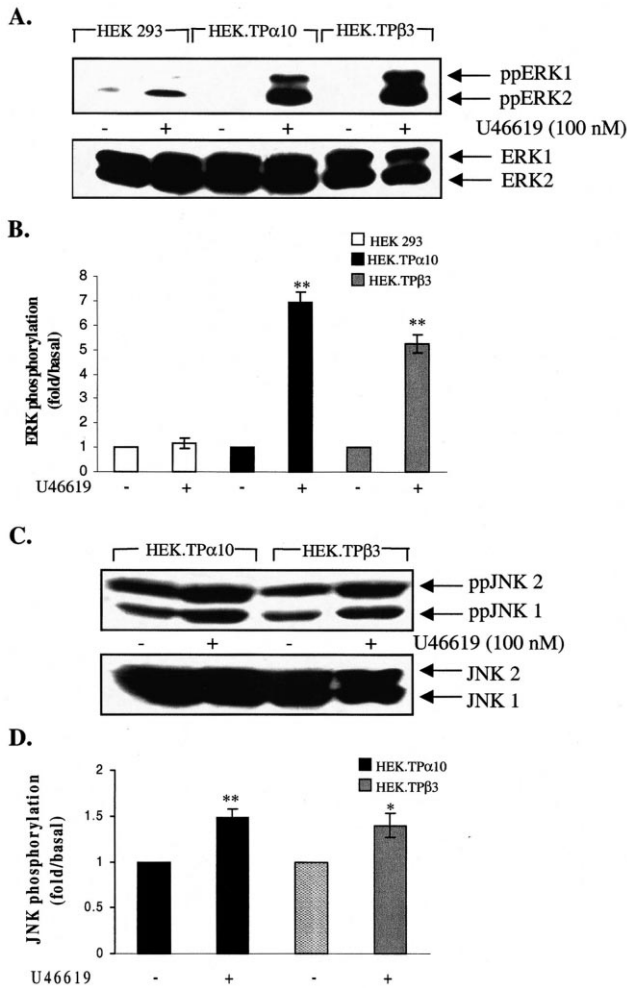
[38]. Exposure of HEK.TPα10 and HEK.TPβ3 cells to U46619 induced a TP isoform specific activation of ERK1 and ERK2 when compared to vehicle treated cells (Fig. 8A,B). U46619 mediated ERK activation in HEK.TPα10 and HEK.TPβ3 cells was completely blocked by SQ29548 and by PD98059 (data not shown). Additionally, exposure of HEK.TPα10 and HEK.TPβ3 cells to U46619 in-
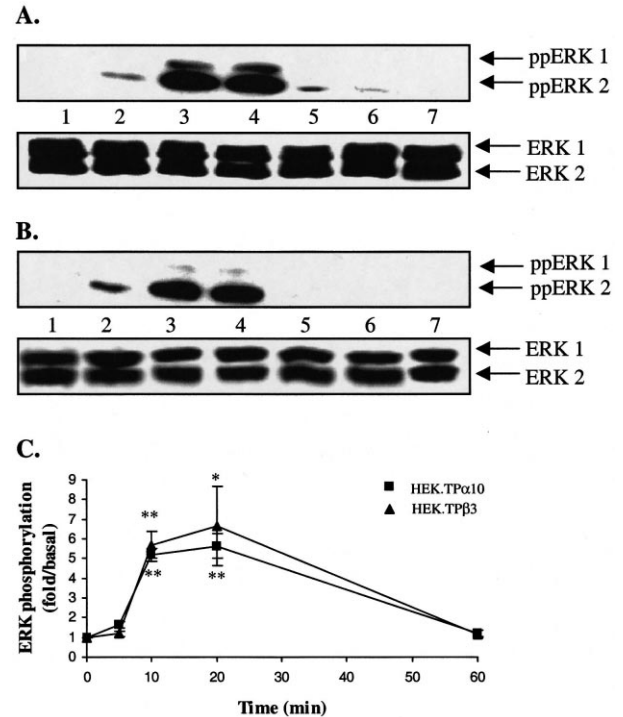

Fig. 8. Effect of U49919 on ERK1/2 and JNK1/2 activation in HEK.TPα10 and HEK.TPβ3 cells. (A) and (C) Effect of U46619 (100 nM, 10 min) on ERK and JNK activation, respectively, in HEK 293 (ERK only), HEK.TPα10 and HEK.TPβ3 cells. Upper panels of (A) and (C): Blots were screened with anti-ACTIVE ERK (A) or anti-ACTIVE JNK (C) to detect the phosphorylated, active forms of ERK (ppERK1/2), and JNK (ppJNK1/2), respectively. In the lower panels of (A) and (C), blots were screened with anti-ERK and anti-JNK antibodies to detect ERK1/2 and JNK1/2 immunoreactive protein, respectively. Results are representatives of three independent experiments. (B) and (D) Fold increases in ERK (ppERK1/2) or JNK (ppJNK) phosphorylation in (A) and (C), respectively, are presented as mean fold increases of basal phosphorylation ± S.E.M. ($n = 3$), where the levels of basal phosphorylation in vehicle treated cells are assigned a value of 1.0. *$P \leq 0.05$ and **$P \leq 0.01$ indicate that the levels of U46619 mediated ERK/JNK activation (ppERK or ppJNK) were significantly greater relative to basal levels.

Fig. 9. 8-EpiPGF$_{2\alpha}$ mediated ERK activation in HEK.TPα10 and HEK.TPβ3 cells. Time dependent effect of 8-epiPGF$_{2\alpha}$ (300 nM; 0, 5, 10, 20 and 60 min) on ERK1 and ERK2 activation in HEK.TPα10 cells (A, lanes 1–5) and in HEK.TPβ3 cells (B, lanes 1–5). Additionally, in (A) (HEK.TPα10 cells) and (B) (HEK.TPβ3 cells), cells were pre-incubated with SQ29548 (1 μM, 1 min; lane 6) or PD98059 (10 μM, 30 min; lane 7) prior to stimulation of cells with 8-epiPGF$_{2\alpha}$ (300 nM, 10 min). Upper panels of (A) and (B): Blots were screened with anti-ACTIVE ERK to detect the phosphorylated, active forms of ERK (ppERK1/2) whereas in the lower panels of (A) and (B), blots were screened with anti-ERK antibodies to detect ERK1/2 immunoreactive protein. Results are representatives of three independent experiments. (C) Fold increases in ERK (ppERK1/2) phosphorylation in (A) (HEK.TPα10 cells) and (B) (HEK.TPβ3 cells), respectively, are presented as mean fold increases of basal ERK phosphorylation ± S.E.M. ($n = 3$), where the levels of basal ERK phosphorylation in vehicle treated cells are assigned a value of 1.0. *$P \leq 0.05$ and **$P \leq 0.01$ indicate that the levels of 8-epiPGF$_{2\alpha}$ mediated ERK activation (ppERK) were significantly greater relative to basal levels.

A.

B.

C.
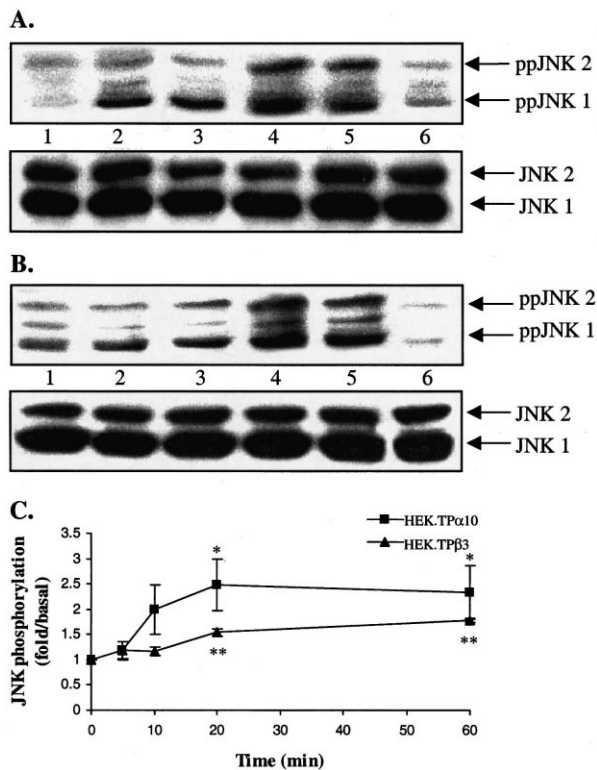
Fig. 10. 8-EpiPGF$_{2\alpha}$ mediated JNK activation in HEK.TPα10 and HEK.TPβ3 cells. Time dependent effect of 8-epiPGF$_{2\alpha}$ (300 nM; 0, 5, 10, 20 and 60 min) on JNK1 and JNK2 activation in HEK.TPα10 cells (A, lanes 1–5) and in HEK.TPβ3 cells (B, lanes 1–5). Additionally, HEK.TPα10 cells (A, lane 6) and HEK.TPβ3 cells (B, lane 6) were pre-incubated with SQ29548 (1 μM, 1 min) prior to stimulation with 8-epiPGF$_{2\alpha}$ (300 nM, 10 min). Upper panels of (A) and (B): Blots were screened with anti-ACTIVE JNK to detect the phosphorylated, active forms of JNK (ppJNK1/2) whereas in the lower panels of (A) and (B), blots were screened with anti-JNK antibodies to detect JNK1/2 immunoreactive protein. Results are representatives of three independent experiments. (C) Fold increases in JNK (ppJNK1/2) phosphorylation in (A) (HEK.TPα10 cells) and (B) (HEK.TPβ3 cells), respectively, are presented as mean fold increases of basal JNK phosphorylation $\pm$ S.E.M. ($n = 3$), where the levels of basal JNK phosphorylation in vehicle treated cells are assigned a value of 1.0. $*P \leq 0.05$ and $**P \leq 0.01$ indicate that the levels of 8-epiPGF$_{2\alpha}$ mediated JNK activation (ppJNK) were significantly greater relative to basal levels.

duced a TP isoform specific activation of JNK1 and JNK2 when compared to the vehicle treated control cells (Fig. 8C,D). However, the level of TP mediated JNK activation was considerably lower than the level of ERK activation despite the presence of high levels and JNK1/2 in these cells (Fig. 8C). These findings

suggest that both TPα and TPβ independently direct U46619 mediated ERK1/2 and JNK1/2 activation.

To establish whether the F2-isoprostane, 8-epi-PGF$_{2\alpha}$ elicits ERK1 and ERK2 activation through both TPα and TPβ, HEK.TPα10 and HEK.TPβ3 cells were exposed to 8-epiPGF$_{2\alpha}$ for 0–60 min. Exposure of HEK.TPα10 and HEK.TPβ3 cells to 8-epiPGF$_{2\alpha}$ elicited a similar time dependent activation of ERK1 and ERK2, with maximal activation detected after 10–20 min exposure (Fig. 9A–C). Furthermore, pre-treatment with either SQ29548 or PD98059 completely abolished ERK1/2 activation when compared to cells exposed exclusively to 8-epi-PGF$_{2\alpha}$ (Fig. 9A,B). Exposure of HEK.TPα10 and HEK.TPβ3 cells to 8-epiPGF$_{2\alpha}$ led to time dependent JNK1 and JNK2 activation with maximal activation detected following 20 min incubation (Fig. 10A–C). Pre-treatment of cells with SQ29548 resulted in a near complete inhibition of 8-epiPGF$_{2\alpha}$ mediated JNK activation in both HEK.TPα10 ($P \leq 0.001$) and HEK.TPβ3 ($P \leq 0.001$) cells (Fig. 10A,B).

4. Discussion

Activation of the ERK signalling cascade is an obligatory step for growth factor induced protein synthesis in aortic SMC [39]. Previous studies have demonstrated TP induced ERK activation in rat, bovine and porcine VSM [2,13,14]. However, TP mediated ERK activation has not been demonstrated in human SMC. Thus, in the present study, to ascertain the role of TXA$_2$ in VSM hypertrophy/mitogenesis, the ability of the TXA$_2$ mimetic U46619 and the F2-isoprostane, 8-epiPGF$_{2\alpha}$ to activate the MAPK signalling cascades was investigated using the previously described human uterine ULTR cells [27] as a model cell line for cultured human VSM cells. In addition, as TXA$_2$ signals through two related TPα and TPβ receptor isoforms in humans, but not in other species thus far investigated, it was unknown whether both TP isoforms contribute to TP mediated mitogenic signalling in human tissue. Expression of both TPα and TPβ in the ULTR SMC line [27] under study was confirmed by indirect immunofluorescence using TP isoform specific antibodies corroborating previous RT-PCR analyses [6].

Thus, we initially examined the effect of U46619 on activation of ERK signalling in ULTR cells. U46619 elicited both a weak hypertrophic and hyperplastic effect and also mediated a time and concentration dependent activation of ERK1/2 in ULTR cells which was inhibited by MEK1/2 inhibitor PD98059 and by the selective TP antagonist SQ29548. Thereafter, we sought to define the factors that regulate TP mediated ERK activation. The second messenger kinases, such as PKA, PKC and PI3K, play a central role in modulating the ERK signalling cascade in response to a variety of extracellular stimuli [11,12,40]. TP receptors functionally couple to G_q family members, to mediate activation of phospholipase (PL) C and more recently it has been demonstrated that the TPs may also couple to G_{12} members, though the effectors remain to be fully defined [38,41–43]. In the current study, we demonstrate that TXA_2 exerts its effects, in part, through PKC, suggesting a role for G_q members and raf-1 in TXA_2 mediated ERK activation in human SMC [11,12]. These data in human ULTR cells are in agreement with previous studies that established that the PKC inhibitor staurosporine inhibited [^{3}H]thymidine uptake by porcine coronary artery SMCs in response to the TXA_2 mimetic I-BOP [2].

A number of studies have shown that increases in cAMP and resulting activation of PKA decreases ERK activation [44], possibly by inhibiting raf-1 activation or by inhibiting certain PKC isoforms [40]. However, cAMP may also elicit a positive effect on ERK activation [45]. Here, we demonstrate that inhibition of PKA with H-89 in ULTR cells inhibited U46619 mediated ERK1/2 activation suggesting that activation of ERK through TP is partially PKA dependent. These data suggest that PKA functions as a positive regulatory element for TXA_2 induced ERK activation in human SMC by mechanisms currently unknown but which may include inhibition of PKA sensitive phosphatases or by PKA mediated activation of a MEK kinase distinct from raf-1 [40] such as A-raf or B-raf [46].

Among the functional differences ascribed to the human TP isoform, TPα and TPβ oppositely regulate adenylyl cyclase with TPα activating it, through $G\alpha_s$ and TPβ inhibiting it, through $G\alpha_i$ [7]. Here we show that PTX did not alter U46619 induced activation in ULTR cells possibly suggesting that the PTX sensitive $G\alpha_i$ coupled TPβ may not activate the ERK signalling cascade in this cell type. Alternatively, stimulation of TPβ may lead to $G\alpha_i$ derived βγ subunits which mediate ERK activation; however, the inhibitory effects of PTX may be masked by a strong TPα mediated ERK activation through $G\alpha_s/G\alpha_q$ coupling. Our data demonstrating that PTX did not affect TP mediated ERK signalling in human ULTR cells are in agreement with previous studies by Sachinidis et al. [47] who reported that PTX did not affect TXA_2 mediated mitogenic signalling in rat aortic SMCs. However, in contrast to these findings [47] and studies therein, it was recently reported that TXA_2 mediated MAPK signalling in endothelial ECV304 cells was inhibited by PTX implicating a role for Gi/Go members in TP mediated MAPK signalling [48]. The molecular bases of these reported differences are currently unknown but may possibly be accounted for by widely reported tissue/cell dependent differences in MAPK signal transduction cascades in response to a given ligand.

PI3K has been implicated as having a central role in cell proliferation [11,12,49]. Specifically, recent studies in rat A7r5 SMC cultures transiently transfected with the human TPα isoform demonstrated that the TXA_2 mimetic I-BOP led to increased tyrosine phosphorylation of the 85 kDa adapter subunit of PI3K but also of the TPα receptor itself [50]. In this study, we show that PI3K plays a role in TXA_2 mediated ERK activation in uterine SMC, since prior exposure of ULTR cells to wortmannin partially inhibited U46619 induced ERK1/2 activation. Further studies are required to underpin the precise mechanism of U46619 mediated PI3K signalling and ERK activation.

Transactivation of receptor tyrosine kinases (RTKs) such as the EGF receptor, the PDGF receptor or the insulin like growth factor receptor, following GPCR stimulation has been implicated in GPCR mediated activation of ERK [34,37]. Specifically, transactivation of RTKs has been demonstrated for many GPCRs including the lysophosphatidic acid (LPA), α_{2A} and β_2 adrenergic and thrombin receptors [34,36,37]. In this study, we have shown that TP mediated activation of ERK in response to U46619 in ULTR cells is dependent on transactivation of the EGF receptor as AG1478, a specific inhibitor of the EGF receptor, inhibited both EGF and U46619

mediated ERK activation. Consistent with our data, Gao et al. [48] recently established that the TXA_2 mimetic I-BOP led to transactivation of the EGF receptor in ECV304 cells in a PTX dependent mechanism. The precise mechanism of TP mediated EGF receptor transactivation and whether TP signals through transactivation of other RTKs in ULTR cells remain to be fully explored.

The F2-isoprostane 8-epiPGF$_{2\alpha}$ has potent biological activity, including reversible platelet aggregation and vascular SMC contraction, the effects of which were found to be inhibited, either wholly or in part, by the TXA_2 antagonist, SQ29548 [51,24,38]. Here, we show that 8-epiPGF$_{2\alpha}$ mediates a time and concentration dependent activation of ERK1 and ERK2 in ULTR cells. Furthermore, consistent with its partial agonist effect on TPs [51], 8-epiPGF$_{2\alpha}$ is slower and is less potent than U46619 in eliciting ERK activation in ULTR cells. Whereas the TXA_2 antagonist SQ29548 abolished U46619 induced activation of ERK, it only partially inhibited 8-epiPGF$_{2\alpha}$ mediated ERK activation. These findings suggest that 8-epiPGF$_{2\alpha}$ exerts its effects, at least in part, through SQ29548 sensitive TPs but also suggest that 8-epiPGF$_{2\alpha}$ may activate alternative receptor(s) other than SQ29548 sensitive TPs in human uterine SMCs. Correlating with U46619 induced activation of ERK1 and ERK2, 8-epiPGF$_{2\alpha}$ induced activation of ERK1 and ERK2 is partially activated by PI3K and exhibited a partial dependence on PKA and PKC. Whereas PTX had no effect, the EGF receptor inhibitor AG1478 inhibited 8-epiPGF$_{2\alpha}$ mediated ERK activation in ULTR cells (data not shown) indicating that 8-epiPGF$_{2\alpha}$ also mediated transactivation of the EGF receptor in ULTR cells.

Recently, Karim et al. [17] reported that in porcine SMC, whereas U46619 weakly activated ERK1/2, the SAPK, JNK1 was strongly activated. To investigate if TP(s) expressed in the human VSM (ULTR) cells mediated JNK1 and JNK2 activation, U46619 and 8-epiPGF$_{2\alpha}$ mediated JNK activation was investigated. Whereas anisomycin elicited strong activation of JNK1 and JNK2, both U46619 and 8-epiPGF$_{2\alpha}$ elicited only weak activation of JNK1/2 in ULTR cells. This suggests that in human SMC, TP agonists act primarily as mitogenic/hypertrophic agents and may possibly explain the poor activation of the JNK cascade when compared to the ERK cascade in response to U46619 and 8-epiPGF$_{2\alpha}$ in ULTR cells. However, this does not rule out the possibility that TXA_2 and/or 8-epiPGF$_{2\alpha}$ may act as a stress factor in certain pathophysiological states.

As previously stated, TXA_2 signals through two TP receptor isoforms, termed TPα and TPβ. Consistent with previous findings that the mRNAs for both TPα and TPβ are expressed in ULTR cells [6], isoform specific antibodies permitted the immunochemical detection of both TP receptors thereby confirming their expression at the protein level in ULTR cells. Thus, to establish whether both TP isoforms activate the MAPK signalling cascades, the previously described HEK 293 cell lines which stably over-express either TPα (HEK.TPα10 cells) or TPβ (HEK.TPβ3 cells) were utilised [9,28]. Exposure of non-transfected HEK 293 cells to U46619 elicited weak activation of ERK and JNK (data not shown). However, for the stable HEK.TPα10 and HEK.TPβ3 cell lines, both TP isoforms were demonstrated to activate the ERK and JNK signalling cascades in response to U46619. These data provide the first evidence that both TPα and TPβ independently mediate ERK and JNK activation in human tissues.

Thereafter, the ability of TPα and TPβ to independently mediate 8-epiPGF$_{2\alpha}$ induced activation of ERK and JNK was investigated. It was found that 8-epiPGF$_{2\alpha}$ induced a similar time dependent activation of ERK and JNK through both TPα and TPβ isoforms. As previously stated, in ULTR cells a similar time profile of ERK activation was observed, with 10 min required for maximal ERK activation in response to 8-epiPGF$_{2\alpha}$. Interestingly, whereas SQ29548 led to only partial antagonism of 8-epiPGF$_{2\alpha}$ induced activation of ERK and JNK in ULTRs, complete inhibition of ERK and JNK activation was observed when HEK.TPα10 and HEK.TPβ3 cells were pre-treated with SQ29548. Thus, it appears that 8-epiPGF$_{2\alpha}$ exerts its effects exclusively through SQ29548 targeted TPs in HEK.TPα10 and HEK.TPβ3 cells and, thus, may reflect tissue specific variations in the expression of the hypothesised vascular smooth muscle specific 8-epiPGF$_{2\alpha}$ receptor [25]. This may account for the different levels of SQ29548 antagonism observed for 8-epiPGF$_{2\alpha}$ induced ERK and JNK activation in HEK.TPα10 cells, HEK.TPβ3 cells compared with human ULTR cells.

In conclusion, U46619 and 8-epiPGF$_{2\alpha}$ elicit strong activation of the ERK cascade in human SMC. TP mediated ERK activation in human uterine SMCs may also involve transactivation of the EGF receptor. The signalling intermediate PI3K appears to play an integral role in TXA$_2$ induced activation of the ERK cascade though the precise mechanism of PI3K signalling in response to TP activation remains to be defined. Both PKA and PKC function as positive regulatory elements in TXA$_2$ induced activation of ERK. U46619 and 8-epiPGF$_{2\alpha}$ led to weak activation of the SAPK/JNK cascade when compared with their activation of the ERK cascade. This may reflect the inherent nature of the TXA$_2$ induced signalling cascades in human SMCs, in that the ERK pathway primarily controls cellular proliferation (for example, in response to vascular injury), whereas the SAPK pathway is generally activated in response to environmental stress. In addition, both TPα and TPβ independently mediate ERK and JNK activation in response to U46619 and in response to 8-epiPGF$_{2\alpha}$. However, in view of the differential antagonistic effects of SQ29548 on 8-epiPGF$_{2\alpha}$ mediated ERK and JNK activation in ULTR cells compared to HEK.TPα10/β3 cells, our data also indicate that 8-epiPGF$_{2\alpha}$ signals through another receptor, other than SQ29548 sensitive TPs, in ULTR cells possibly through a related prostanoid/eicosanoid receptor or through a specific 8-epiPGF$_{2\alpha}$ receptor expressed in the human SMCs, as previously alluded to by others [25].

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