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**Thromboxane A<sub>2</sub> receptor mediated activation of the mitogen activated protein kinase cascades in human uterine smooth muscle cells.**

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**Key Words:** thromboxane A<sub>2</sub> receptor, TP $\alpha$ , TP $\beta$ , 8-epiPGF<sub>2 $\alpha$</sub> , smooth muscle, human, MAPK, ERK, JNK, mitogenesis.

**Abbreviations:** 8-epiPGF<sub>2 $\alpha$</sub> , 8-epi prostaglandin F<sub>2 $\alpha$</sub> ; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; FCS, foetal calf serum; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; PI3K, phosphoinositide 3-kinase; PTX, pertussis toxin; SAPK, stress-activated protein kinase; SMC, smooth muscle cell; TXA<sub>2</sub>, thromboxane A<sub>2</sub>; TP, TXA<sub>2</sub> receptor.

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### Summary

Both thromboxane (TX) A<sub>2</sub> and 8-epi prostaglandin (PG) F<sub>2α</sub> have been reported to stimulate mitogenesis of vascular smooth muscle (SM) in a number of species. However, TXA<sub>2</sub> and 8-epiPGF<sub>2α</sub> mediated mitogenic signalling have not been studied in detail in human vascular SM. Thus, using the human uterine ULTR cell line as a model, we investigated TXA<sub>2</sub> receptor (TP) mediated mitogenic signalling in cultured human vascular SM cells. Both the TP agonist U46619 and 8-epiPGF<sub>2α</sub> 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 SQ29,548 abolished U46619-mediated signalling, it only partially inhibited 8-epiPGF<sub>2α</sub> mediated ERK and JNK activation in ULTR cells. Both U46619 and 8-epiPGF<sub>2α</sub> induced ERK activations were inhibited by the protein kinase (PK) C, PKA and phosphoinositide 3-kinase inhibitors GF 109203X, H-89 and wortmannin, respectively, but were unaffected by pertussis toxin. In addition, U46619 mediated ERK activation in ULTR cells involves transactivation of the EGF receptor. In humans, TXA<sub>2</sub> 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<sub>2α</sub> 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, SQ29,548 abolished 8-epiPGF<sub>2α</sub> mediated ERK and JNK activation through both TPα and TPβ in HEK 293 cells providing further evidence that 8-epiPGF<sub>2α</sub> may signal through alternative receptors, in addition to the TPs, in human uterine ULTR cells.

## Introduction.

The prostanoid thromboxane (TX)<sub>2</sub> 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<sub>2</sub>, termed TP $\alpha$  and TP $\beta$ , 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 2 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 desensitization [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<sub>2</sub> mimetics I-BOP and U46619 activate extracellular signal regulated kinase (ERK) 1 and ERK 2 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<sub>2</sub> mediated signal cascades [16,17].

The F2-isoprostane, 8-epiPGF<sub>2 $\alpha$</sub>  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<sub>2 $\alpha$</sub>  is less potent than U46619 in inducing contraction of rat aorta. 8-epiPGF<sub>2 $\alpha$</sub>  has been reported to induce ERK activity through the TP in porcine carotid arteries [24]. It has also been suggested that 8-epiPGF<sub>2 $\alpha$</sub>  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<sub>2</sub> and 8-epiPGF<sub>2 $\alpha$</sub>  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<sub>2</sub> and 8-epiPGF<sub>2 $\alpha$</sub>  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<sub>2</sub> mimetic and in response to the F2-isoprostane 8-epiPGF<sub>2 $\alpha$</sub> . Thus, the immortalised human uterine ULTR cell line [27] was used as a model vascular SM cell line to investigate U46619 and 8-epiPGF<sub>2 $\alpha$</sub>  mediated mitogenesis and activation of mitogen activated protein kinase (MAPK) cascades (ERK and JNK). Thereafter, as TXA<sub>2</sub> signals in humans through 2 TP isoforms both of which were demonstrated to be expressed in ULTR cells, human embryonic kidney (HEK) 293 cell lines stably over-expressing either TP $\alpha$  (HEK. $\alpha$ 10 cells) or TP $\beta$  (HEK. $\beta$ 3 cells) were used to establish whether both TP $\alpha$  and TP $\beta$  individually contribute to U46619 and 8-epiPGF<sub>2 $\alpha$</sub> -mediated MAPK activation in human tissues. Both TP isoforms mediated ERK and JNK activation in response to U46619 and 8-epiPGF<sub>2 $\alpha$</sub>  in the ULTR and the HEK 293 cell lines. Whereas the selective TP antagonist SQ29,548 abolished 8-epiPGF<sub>2 $\alpha$</sub>  mediated ERK and JNK activation in HEK.TP $\alpha$ 10 and HEK.TP $\beta$ 3 cells, it resulted in only partial inhibition of 8-epiPGF<sub>2 $\alpha$</sub>  mediated MAPK activation in ULTR cells suggesting that 8-epiPGF<sub>2 $\alpha$</sub>  may signal through both TP $\alpha$  and TP $\beta$  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 SM cells in response to the TXA<sub>2</sub> mimetic U46619 and the F2-isoprostane 8-epiPGF<sub>2 $\alpha$</sub> . Additionally, this study provides the first evidence that TP $\alpha$  and TP $\beta$  individually contribute to TP mediated MAPK activation in human tissue.

## **Materials & Methods.**

**Materials.** U46619, 8-epiPGF<sub>2α</sub> and SQ29,548 were purchased from Cayman Chemical Company, Ann Arbor, MI., U.S.A. PD 98059, GF 109203X, tyrphostin AG1478 and H-89 were from Calbiochem-Novabiochem, Nottingham, U.K. Anti-ACTIVE™ MAPK and Anti-ACTIVE™ JNK rabbit polyclonal antibodies and EGF were purchased from Promega Corporation, Madison, WI., U.S.A. Affinity purified rabbit polyclonal anti-JNK and anti-ERK antibodies were from Santa Cruz Laboratories, Santa Cruz, CA., U.S.A. Anisomycin, myelin basic protein (MBP), platelet derived growth factor-ββ (PDGF-ββ) and wortmannin were purchased from Sigma Chemical Company, St. Louis, MO., U.S.A.

### ***Culture of human uterine SMCs, HEK.TPα10 and HEK.TPβ cells:***

The uterine SMC line ULTR, described by Perez-Reyes *et al.*, [27] was obtained from J. K. McDougall, Dept. Pathology, University of Washington, Seattle, USA. Cells were routinely grown in Dulbecco's minimal essential medium (DMEM), 10% 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% foetal calf serum (FCS), unless otherwise indicated. The recombinant HEK.TPα10 and HEK.TPβ3 cell lines stably over-expressing TPα and TPβ, respectively, have been previously described [9, 28].

### ***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 haemocyanin 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-phenylindole (DAPI) as per the manufacturer's instructions (Sigma).

### ***Determination of DNA and Protein synthesis:***

ULTR cells were seeded at  $2.5 \times 10^5$  cells/35 mm dish in DMEM, 10% FCS. After 24 hr, cells were exposed to DMEM, 0.5% FCS to induce quiescence. After a further 24 hr, the media was supplemented with 1.5 μCi [<sup>3</sup>H] thymidine (60 Ci / mmol – Sigma) or 1.5 μCi [<sup>3</sup>H] leucine (60 Ci / mmol – American Radiochemicals) and cells were exposed to test agents (U46619, 1μM 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).

### ***Determination of ERK activity using an in vitro kinase assay:***

ULTR cells were seeded at  $4 \times 10^5$  cells/60 mm dish in DMEM, 10% FCS. After 24 hr, cells were exposed to DMEM, 0.5% FCS to induce quiescence. After a further 48 hr, 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 ERK 1/2 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).

***Determination of ERK/JNK activation by immunoblot analysis:***

ULTR cells were seeded at  $1.5 \times 10^6$  cells/10 cm dish in DMEM, 10% FCS. After 24 hr, cells were exposed to DMEM without any FCS to induce quiescence. HEK.TP $\alpha$ 10 and HEK.TP $\beta$ 3 cells were seeded at  $1.5 \times 10^6$  cells/10 cm dish in MEM, 10% FCS. After 24 hr, cells were exposed to medium without any FCS to induce quiescence. After a further 48 hr, 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 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 (pp ERK1/2) and dual phosphorylated JNK (pp JNK1/2), respectively, as recommended by the 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 visualised 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 pp ERK1 and pp ERK2 or pp JNK1 and pp JNK2 were quantified by scanning densitometry using a UVP gel documentation system and the combined pp ERK1/2 or pp JNK1/2 signals are presented as mean fold increase of basal ERK phosphorylation  $\pm$  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  $\leq 0.05$  were considered to indicate a statistically significant difference.

## Results.

### Effect of U46619 on ERK activation in ULTR cells.

To elucidate the mitogenic/hypertrophic capabilities of TXA<sub>2</sub> in human uterine smooth muscle cells, 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  $1.23 \pm 0.07$  fold ( $P \leq 0.001$ ) and  $1.21 \pm 0.03$  fold ( $P \leq 0.001$ ) increase in [<sup>3</sup>H] thymidine incorporation, respectively. Whereas PDGF resulted in a  $1.51 \pm 0.17$  fold ( $P \leq 0.001$ ) increase in [<sup>3</sup>H] leucine incorporation, U46619 resulted in a  $1.89 \pm 0.11$  fold ( $P \leq 0.001$ ) increase in [<sup>3</sup>H] 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 (Figure 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 (Figure 1A,  $P \leq 0.05$ ). Exposure of the quiescent ULTR cells to PDGF-ββ also induced MBP phosphorylation.

As an alternative method to evaluate U46619 induced ERK 1 and ERK 2 activation, anti-ACTIVE™ ERK antibodies that preferentially recognize their dually phosphorylated, active forms (phosphorylated ppERK 1 / 2) were utilised. Furthermore, an affinity-purified anti-ERK antibody was routinely used to detect total ERK 1 and ERK 2 (ERK) protein present in the cellular lysate, thus ensuring equal expression and protein loading of ERK 1 and ERK 2. U46619 mediated a dose dependent phosphorylation of ERK 1 (ppERK 1) and ERK 2 (ppERK 2) in ULTR cells with maximal activation observed at 100-300 nM U46619 (Figure 1B & 1D). U46619 also mediated a time dependent activation of ERK 1 and ERK 2 with maximal activation observed at 5-10 min (Figure 1C & 1E). 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 ERK 1 and ERK 2 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 SQ29,548 (Figure 2) or with PD 98059 (Figure 2) reduced U46619 induced ERK 1/2 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 ERK 1 / 2 (Figure 2), PTX did not affect U46619 mediated activation of ERK 1 / 2 (Figure 2). The PKC inhibitor GF 109203X (Figure 2) or the PI3K inhibitor wortmannin (Figure 2) each resulted in a partial, though significant, inhibition of ERK 1 / 2 activation when compared to ULTR cells exposed exclusively to U46619. H-89, GF 109203X and wortmannin combined did not fully abolish U46619 mediated ERK activation (data not shown). These data confirm that U46619-induced activation of ERK 1 / 2 in ULTR cells is mediated through the TP(s) in a MEK 1/2-dependent manner and suggest that PKA, PKC and PI3K play a role in these events.

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

Many G-protein coupled receptor (GPCR)s 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 epidermal growth factor (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 activation of ERK 1 and ERK 2 (Figure 3A & 3C). Moreover, pretreatment of ULTR cells with AG1478 significantly inhibited both EGF and U46619 mediated ERK 1 and ERK 2 activation (Figure 3A & 3C). These findings suggest that U46619 mediated activation of the ERK signalling cascade also involves transactivation of the EGF receptor.

#### **Effect of 8-epiPGF<sub>2α</sub> on ERK activation in ULTR cells.**

Exposure of ULTR cells to 8-epiPGF<sub>2α</sub> resulted in a dose dependent activation of ERK 1 and ERK 2 with maximal activation observed when cells were exposed to 300 nM 8-epiPGF<sub>2α</sub> (Figure 4A & 4C). Additionally, 8-epiPGF<sub>2α</sub> induced a time dependent activation of ERK 1 and ERK 2 with maximal phosphorylation occurring after 10 min reaching near basal levels after 60 min (Figure 4B & 4D).

Whereas pre-treatment of ULTR cells with PD 98059 inhibited 8-epiPGF<sub>2α</sub> induced ERK1/2 activation (Figure 4B,  $P \leq 0.05$ ), SQ29,548 reduced, but did not lead to a statistically significant inhibition of, ERK 1 and ERK 2 activation (Figure 5A & 5C,  $P \geq 0.05$ ) in response to 8-epiPGF<sub>2α</sub>. These findings suggest that 8-epiPGF<sub>2α</sub> induced activation of ERK 1 / 2 in ULTR cells is mediated only partially through TP(s) and that 8-epiPGF<sub>2α</sub> is less potent than U46619 in mediating ERK 1/2 activation.

Pre-treatment of ULTR cells with wortmannin, H-89 or GF 109203X each significantly inhibited 8-epiPGF<sub>2α</sub> mediated activation of ERK 1 / 2 compared to cells exposed to 8-epiPGF<sub>2α</sub> alone (Figure 5A & 5C). PTX did not affect 8-epiPGF<sub>2α</sub> mediated activation of ERK 1 / 2 (Figure 5A & 5C). These findings suggest that PI3K, PKA and PKC play a role in 8-epiPGF<sub>2α</sub> induced ERK activation. Moreover, pretreatment of ULTR cells with AG1478 significantly inhibited 8-epiPGF<sub>2α</sub> mediated ERK 1 and ERK 2 activation suggesting 8-epiPGF<sub>2α</sub> induced transactivation of the EGF receptor (data not shown).

#### **Effect of U46619 and 8-epiPGF<sub>2α</sub> on JNK 1 and JNK 2 activation in ULTR cells.**

To investigate the role of TP on the stress-activated protein kinase (SAPK) cascades, quiescent ULTR cells were exposed to U46619 or to 8-epiPGF<sub>2α</sub> for 10-60 min, or as a positive control, to the protein synthesis inhibitor anisomycin for 60 min. Whereas anisomycin induced activation of both JNK 1 and JNK 2 (Figure 6A & 6B), both U46619 and 8-epiPGF<sub>2α</sub> each induced a weak, time dependent activation of JNK 1 and JNK 2 (Figure 6A – 6C). Immunoblot analysis using an affinity purified anti-JNK antibody revealed that both JNK 1 and JNK 2 are abundantly expressed in ULTR cells (Figure 6A). The TP antagonist SQ29,548 blocked U46619 mediated JNK activation (Figure 6A;  $P \leq 0.05$ ) but resulted in only a marginal, statistically insignificant, inhibition of 8-epiPGF<sub>2α</sub> mediated JNK activation (Figure 6B;  $P \geq 0.05$ ).

#### **Immunodetection of TP $\alpha$ and TP $\beta$ in a human uterine SM cell line.**

Indirect immunofluorescent staining of ULTR cells with the TP $\alpha$  and TP $\beta$  specific antisera confirmed expression of both TP $\alpha$  and TP $\beta$  in membranes of permeabilised (Figure 7A & 7B) 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  $\alpha$  peptide blocked immunodetection by anti-TP $\alpha$  antisera (Figure 7C) but not by anti-TP $\beta$  antisera (data not shown) and vice versa (Figure 7D and data not shown). These data corroborate RT-PCR analyses (6) and confirm that both TP $\alpha$  and TP $\beta$  mRNA and protein are expressed in the human ULTR cell line under study.

**Effect of U46619 and 8-epiPGF<sub>2α</sub> on MAPK activation in HEK.TPα10 and HEK.TPβ3 cells.**

To establish whether U46619 induced activation of ERK 1 and ERK 2 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 ERK 2 when compared to cells treated with vehicle only (Figure 8A & 8B), 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 ERK 1 and ERK 2 when compared to vehicle treated cells (Figure 8A & 8B). U46618 mediated ERK activation in HEK.TPα10 and HEK.TPβ3 cells was completely blocked by SQ29,548 and by PD 98059 (data not shown). Additionally, exposure of HEK.TPα10 and HEK.TPβ3 cells to U46619 induced a TP isoform specific activation of JNK 1 and JNK 2 when compared to the vehicle-treated control cells (Figure 8C & 8D). 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 (Figure 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-epiPGF<sub>2α</sub> elicits ERK 1 and ERK 2 activation through both TPα and TPβ, HEK.TPα10 and HEK.TPβ3 cells were exposed to 8-epiPGF<sub>2α</sub> for 0 – 60 min. Exposure of HEK.TPα10 and HEK.TPβ3 cells to 8-epiPGF<sub>2α</sub> elicited a similar time dependent activation of ERK 1 and ERK 2, with maximal activation detected after 10-20 min exposure (Figure 9A – 9C). Furthermore, pretreatment with either SQ29,548 or PD 98059 completely abolished ERK 1/2 activation when compared to cells exposed exclusively to 8-epiPGF<sub>2α</sub> (Figure 9A & 9B). Exposure of HEK.TPα10 and HEK.TPβ3 cells to 8-epiPGF<sub>2α</sub> led to time dependent JNK 1 and JNK 2 activation with maximal activation detected following 20 min incubation (Figure 10A – 10C). Pretreatment of cells with SQ29,548 resulted in a near complete inhibition of 8-epiPGF<sub>2α</sub> mediated JNK activation in both HEK.TPα10 ( $P \leq 0.001$ ) and HEK.TPβ3 ( $P \leq 0.001$ ) cells (Figure 10A & 10B).

**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<sub>2</sub> in VSM hypertrophy / mitogenesis, the ability of the TXA<sub>2</sub> mimetic U46619 and the F2-isoprostane, 8-epiPGF<sub>2α</sub> 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<sub>2</sub> signals through two related TP $\alpha$  and TP $\beta$  receptor isoforms in humans, but not in other species thus far investigated, and it was unknown whether both TP isoforms contribute to TP mediated mitogenic signalling in human tissue. Expression of both TP $\alpha$  and TP $\beta$  in the ULTR smooth muscle cell 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 MEK 1/2 inhibitor PD 98059 and by the selective TP antagonist SQ29,548. 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<sub>q</sub> family members, to mediate activation of phospholipase (PL) C and more recently it has been demonstrated that the TPs may also couple to G<sub>12</sub> members, though the effectors remain to be fully defined [38, 41-43]. In the current study, we demonstrate that TXA<sub>2</sub> exerts its effects, in part, through PKC, suggesting a role for G<sub>q</sub> members and raf-1 in TXA<sub>2</sub> 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 [<sup>3</sup>H] thymidine uptake by porcine coronary artery SMCs in response to the TXA<sub>2</sub> 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 ERK 1 / 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<sub>2</sub> 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 isoforms, TP $\alpha$  and TP $\beta$  oppositely regulate adenylyl cyclase with TP $\alpha$  activating it, through G $\alpha_s$  and TP $\beta$  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 TP $\beta$  may not activate the ERK signalling cascade in this cell type. Alternatively, stimulation of TP $\beta$  may lead to G $\alpha_i$ -derived  $\beta\gamma$ -subunits which mediate ERK activation; however, the inhibitory effects of PTX may be masked by a strong TP $\alpha$ -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<sub>2</sub> mediated mitogenic signalling in rat aortic SMCs. However, in contrast to these findings [47] and studies herein, it was recently reported that TXA<sub>2</sub> 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

basis 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 [49,11,12]. Specifically, recent studies in rat A7r5 SMC cultures transiently transfected with the human TP $\alpha$  isoform demonstrated that the TXA<sub>2</sub> mimetic I-BOP led to increased tyrosine phosphorylation of the 85 kDa adapter subunit of PI3K but also of the TP $\alpha$  receptor itself [50]. In this study, we show that PI3K plays a role in TXA<sub>2</sub> mediated ERK activation in uterine SMC, since prior exposure of ULTR cells to wortmannin partially inhibited U46619 induced ERK 1 / 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),  $\alpha_{2A}$  and  $\beta_2$  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<sub>2</sub> 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 remains to be fully explored.

The F2-isoprostane 8-epiPGF<sub>2 $\alpha$</sub>  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<sub>2</sub> antagonist, SQ29,548 [51,24,38]. Here, we show that 8-epiPGF<sub>2 $\alpha$</sub>  mediates a time and concentration dependent activation of ERK 1 and ERK 2 in ULTR cells. Furthermore, consistent with its partial agonist effect on TPs [51], 8-epiPGF<sub>2 $\alpha$</sub>  is slower and is less potent than U46619 in eliciting ERK activation in ULTR cells. Whereas the TXA<sub>2</sub> antagonist, SQ29,548 abolished U46619 induced activation of ERK, it only partially inhibited 8-epiPGF<sub>2 $\alpha$</sub>  mediated ERK activation. These findings suggest that 8-epiPGF<sub>2 $\alpha$</sub>  exerts its effects, at least in part, through SQ29,548-sensitive TPs but also suggest that 8-epiPGF<sub>2 $\alpha$</sub>  may activate alternative receptor(s) other than SQ29,548-sensitive TPs in human uterine SMCs. Correlating with U46619 induced activation of ERK 1 and ERK 2, 8-epiPGF<sub>2 $\alpha$</sub>  induced activation of ERK 1 and ERK 2 is partially activated by PI3K and exhibited a partial dependence on PKA and PKC. Whereas as PTX had no effect, the EGF receptor inhibitor AG1478 inhibited 8-epiPGF<sub>2 $\alpha$</sub>  mediated ERK activation in ULTR cells (data not shown) indicating that 8-epiPGF<sub>2 $\alpha$</sub>  also mediated transactivation of the EGF receptor in ULTR cells

Recently, Karim *et al.*, [17] reported that in porcine SMC, whereas U46619 weakly activated ERK 1/ 2, the stress-activated protein kinase, JNK 1 was strongly activated. To investigate if TP(s) expressed in the human VSM (ULTR) cells mediated JNK 1 and JNK 2 activation, U46619 and 8-epiPGF<sub>2 $\alpha$</sub>  mediated JNK activation was investigated. Whereas anisomycin elicited strong activation of JNK 1 and JNK 2, both U46619 and 8-epiPGF<sub>2 $\alpha$</sub>  elicited only weak activation of JNK 1 / 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<sub>2 $\alpha$</sub>  in ULTR cells. However, this does not rule out the possibility that TXA<sub>2</sub> and/or 8-epiPGF<sub>2 $\alpha$</sub>  may act as a stress factor in certain pathophysiological states.

As previously stated, TXA<sub>2</sub> signals through 2 TP receptor isoforms, termed TP $\alpha$  and TP $\beta$ . Consistent with previous findings that the mRNAs for both TP $\alpha$  and TP $\beta$  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 TP $\alpha$  (HEK.TP $\alpha$ 10 cells) or TP $\beta$  (HEK.TP $\beta$ 3 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.TP $\alpha$ 10 and HEK.TP $\beta$ 3 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 TP $\alpha$  and TP $\beta$  independently mediate ERK and JNK activation in human tissues.

Thereafter, the ability of TP $\alpha$  and TP $\beta$  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 TP $\alpha$  and TP $\beta$  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 SQ29,548 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.TP $\alpha$ 10 and HEK.TP $\beta$ 3 cells were pretreated with SQ29,548. Thus, it appears that 8-epiPGF $_{2\alpha}$  exerts its effects exclusively through SQ29,548-targetted TPs in HEK.TP $\alpha$ 10 and HEK.TP $\beta$ 3 cells and, thus, may reflect tissue specific variations in the expression of the hypothesized vascular smooth muscle specific 8-epiPGF $_{2\alpha}$  receptor [25]. This may account for the different levels of SQ29,548 antagonism observed for 8-epiPGF $_{2\alpha}$  induced ERK and JNK activation in HEK.TP $\alpha$ 10 cells, HEK.TP $\beta$ 3 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 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 TP $\alpha$  and TP $\beta$  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 SQ29,548 on 8-epiPGF $_{2\alpha}$  mediated ERK and JNK activation in ULTR cells compared to HEK.TP $\alpha$ 10/ $\beta$ 3 cells, our data also indicates that 8-epiPGF $_{2\alpha}$  signals through another receptor, other than SQ29,548 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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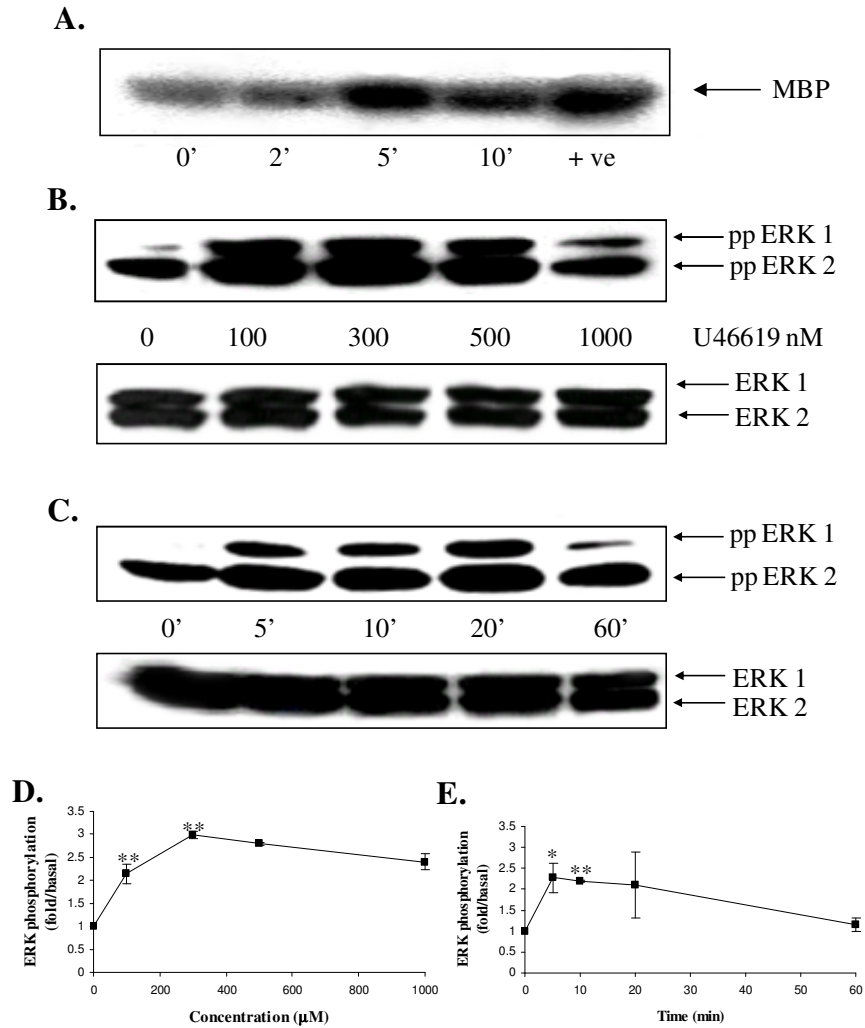
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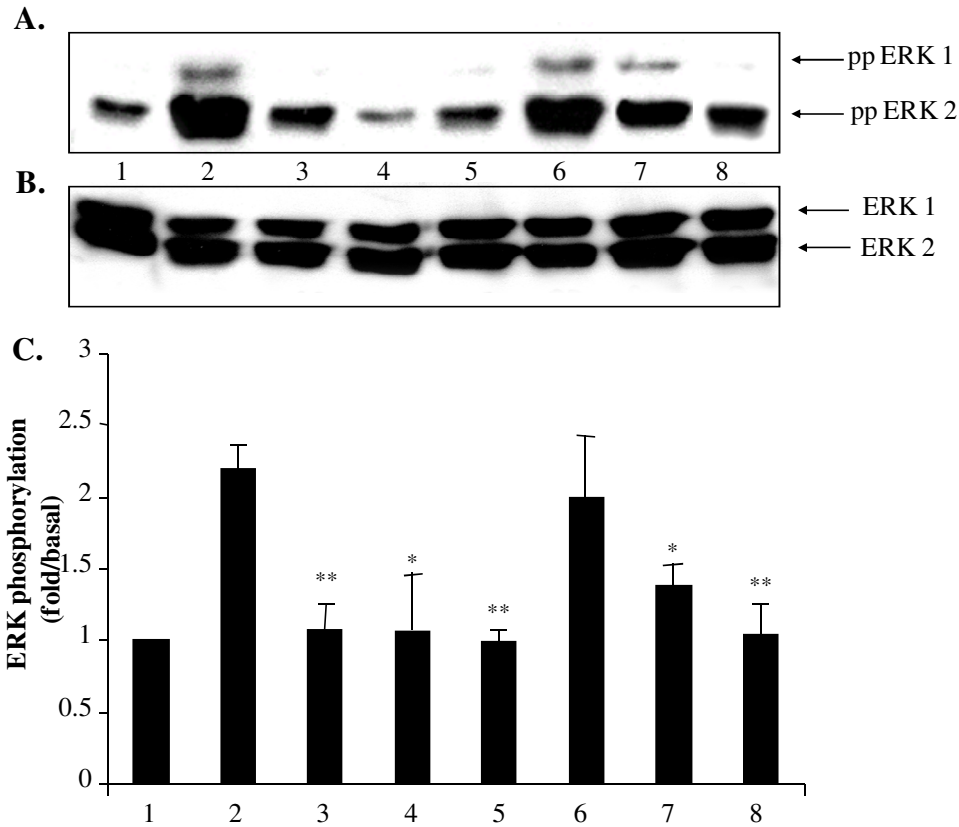
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**FIGURES.**



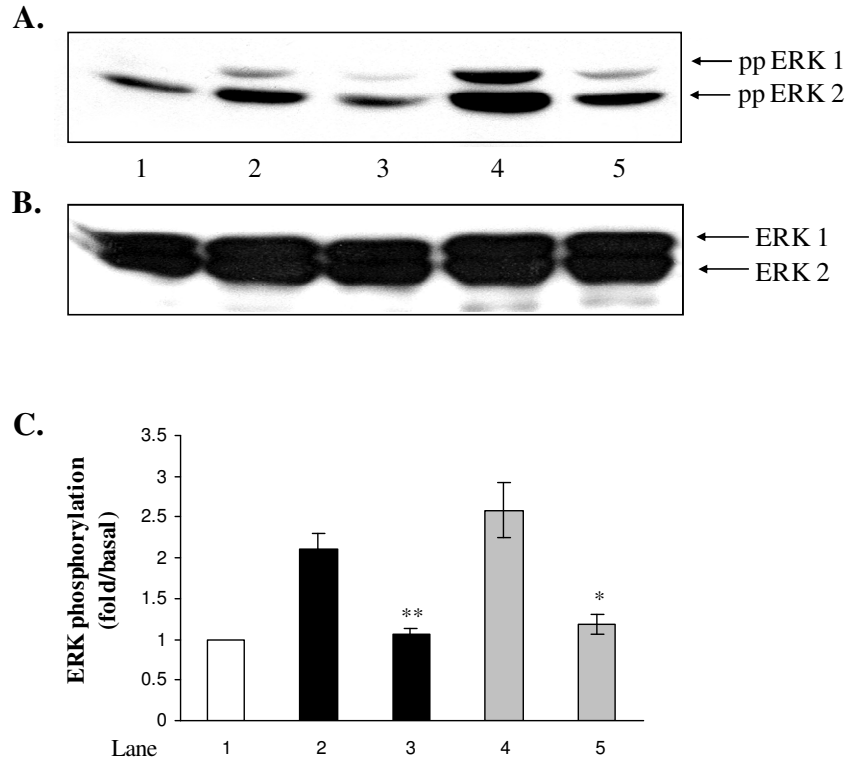
**Figure 1. U46619 mediated ERK activation in ULTR cells.**

Panel A: ULTR cells were stimulated with U66619 for 0 – 10 min (0 – 10' ) and U66619 mediated ERK activation was determined using an *in vitro* kinase assay as described under Materials and Methods. As a positive control (+ve), cells were incubated with PDGF- $\beta$ . Positions of the [ $\gamma$ - $^{32}$ P]-labelled myelin basic protein (MBP) are indicated by the *arrow*. Panel B: Concentration dependent effect of U46619 (0 – 1000 nM, 10 min) and Panel C, time dependent effect of U46619 (100 nM, 0 – 60 min) on ERK 1 and ERK 2 activation in ULTR cells. Upper panels B & C, blots were screened with anti ACTIVE<sup>TM</sup> ERK to detect the phosphorylated, active forms of ERK (pp ERK1/2) whereas in the lower panels B & C, blots were screened with anti-ERK antibodies to detect ERK1/2 immunoreactive protein. Results are representative of at least three independent experiments. Panels D and E: Fold increases in ERK phosphorylation (pp ERK1/2) 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 (pp ERK) were significantly greater relative to basal levels.



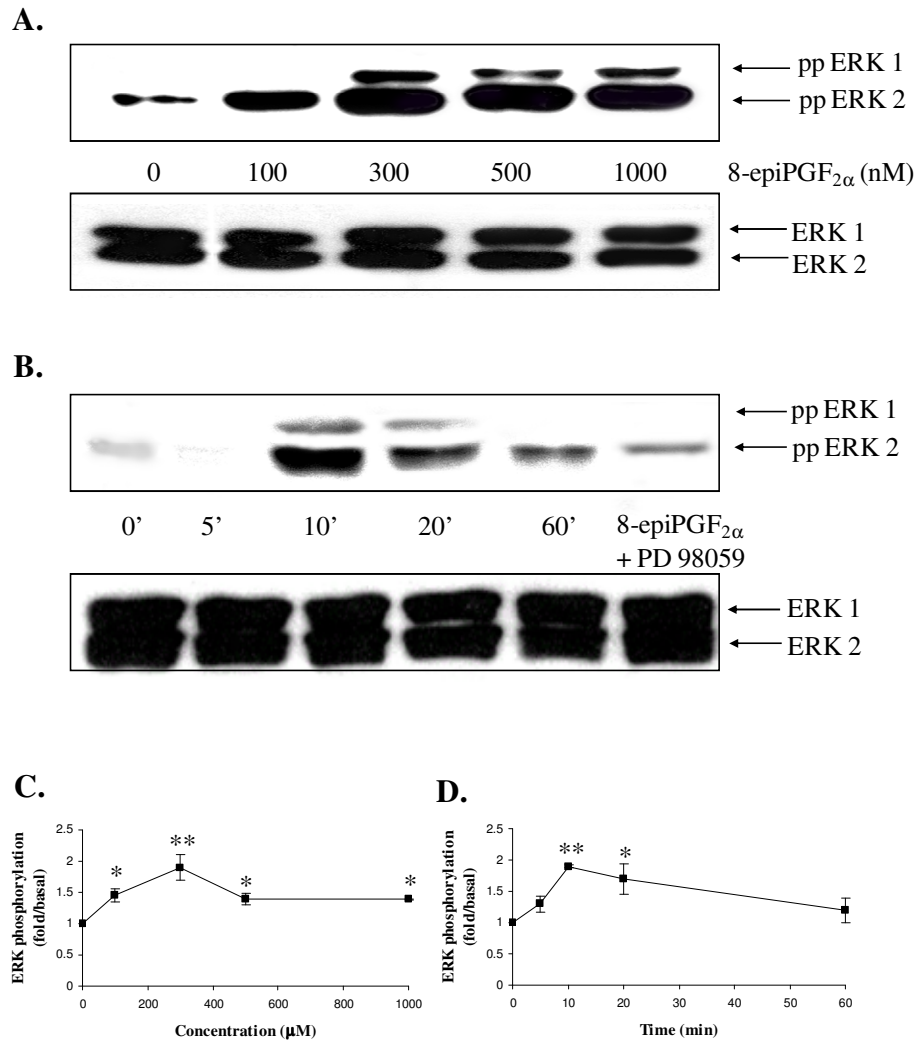
**Figure 2. Modulation of U46619 mediated ERK activation.**

ULTR cells were preincubated with either SQ29,548 (1  $\mu$ M, 1 min; Lane 3), PD 98058 (10  $\mu$ M, 30 min; Lane 4), H-89 (10  $\mu$ M, 5 min; Lane 5), PTX (50 ng/ml, 16 hr; 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. Panel A: Immunoblots were screened with anti-ACTIVE<sup>TM</sup> ERK to detect the phosphorylated, active forms of ERK (pp ERK1/2) whereas in Panel B: blots were screened with anti-ERK antibodies to detect ERK1/2 immunoreactive protein. Results are representative of at least three independent experiments. Panel C: Fold increases in ERK phosphorylation (pp ERK1/2) in panel A are presented as mean fold increases of basal ERK phosphorylation  $\pm$  S.E.M (n = 3-5), where the levels of basal ERK phosphorylation in vehicle-treated cells are assigned a value of 1.0. \* ( $P \leq 0.05$ ) and \*\* ( $P \leq 0.01$ ) indicate that the levels of U46619 mediated pp ERK activation (lane 2) were significantly reduced in the presence of the respective pharmacologic inhibitors (lanes 3 – 8).



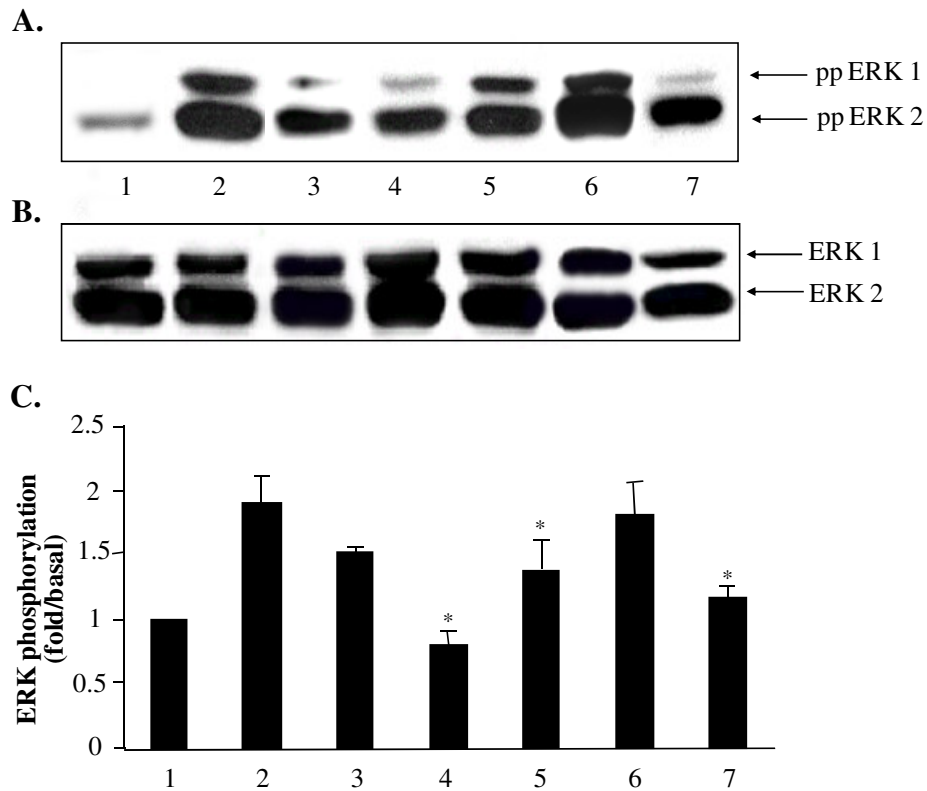
**Figure 3. Effect of tyrphostin AG1478 on U46619 mediated ERK activation.**

ULTR cells were preincubated with tyrphostin AG1478 (125 nM, 30 min; Lanes 3 & 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. Panel A: Immunoblots were screened with anti-ACTIVE™ ERK to detect the phosphorylated, active forms of ERK (pp ERK1/2) whereas in Panel B, blots were screened with anti-ERK antibodies to detect ERK1/2 immunoreactive protein. Results are representative of four independent experiments. Panel C: Fold increases in ERK phosphorylation (pp ERK1/2) in panel A are presented as mean fold increases of basal ERK phosphorylation ± 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 pp ERK activation (lanes 2 and 4, respectively) were significantly reduced in the presence of AG 1748 (lanes 3 and 5, respectively).



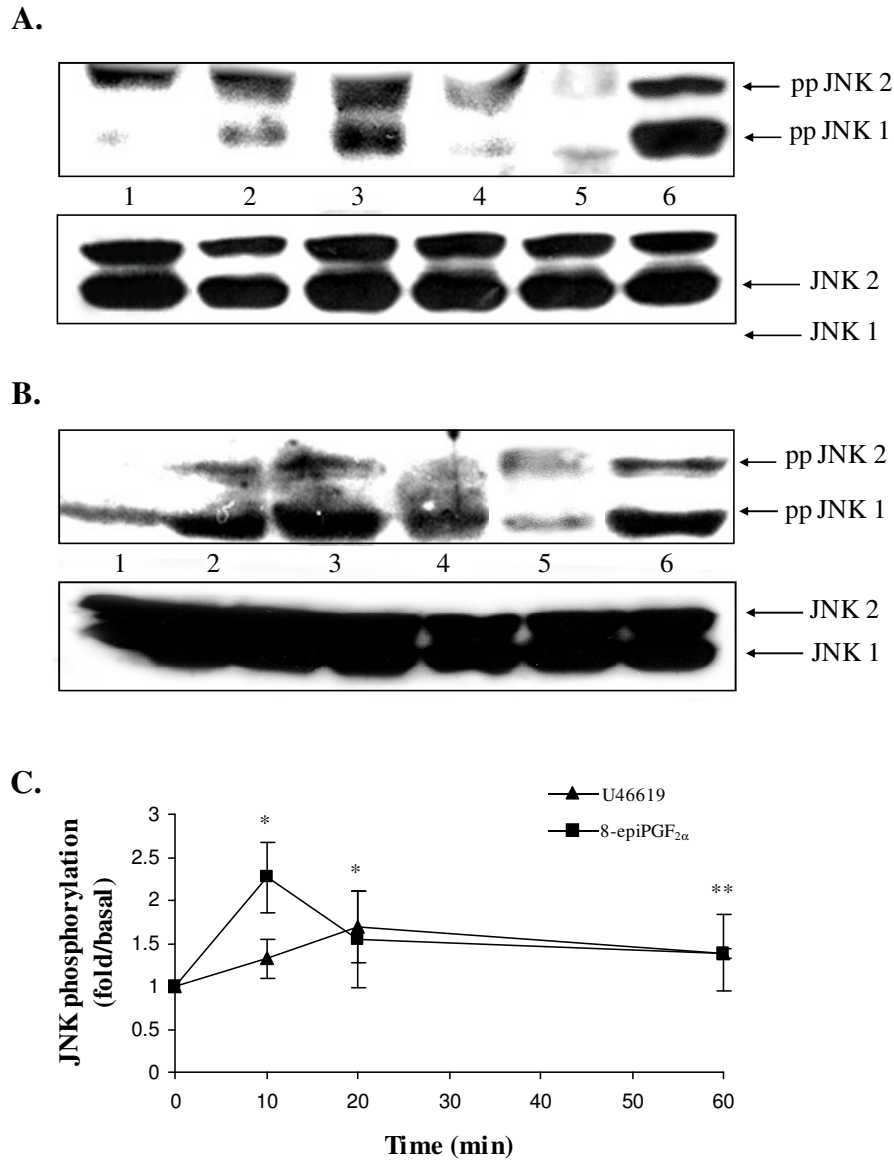
**Figure 4. 8-epiPGF<sub>2α</sub> mediated ERK activation in ULTR cells.**

Panel A: Dose dependent (0 – 1000 nM, 10 min) and Panel B, time dependent (300 nM, 0 – 60 min) activation of ERK 1/2 in response to 8-epiPGF<sub>2α</sub>. Alternatively, in Panel B (8-epiPGF<sub>2α</sub> and PD 98059), cells were preincubated with PD 98059 (10 μM, 30 min) prior to stimulation of cells with 8-epiPGF<sub>2α</sub> (300 nM, 10 min). Upper panels, blots were screened with anti ACTIVE™ ERK to detect the phosphorylated, active forms of ERK (pp ERK1/2) whereas in the lower panels, blots were screened with anti-ERK antibodies to detect ERK1/2 immunoreactive protein. Results are representative of at least three independent experiments. Panels C and D: Fold increases in ERK phosphorylation (pp ERK1/2) in panels 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 ≤ 0.05) and \*\* (P ≤ 0.01) indicate that the levels of 8-epiPGF<sub>2α</sub> mediated ERK activation (pp ERK) were significantly greater relative to basal levels.



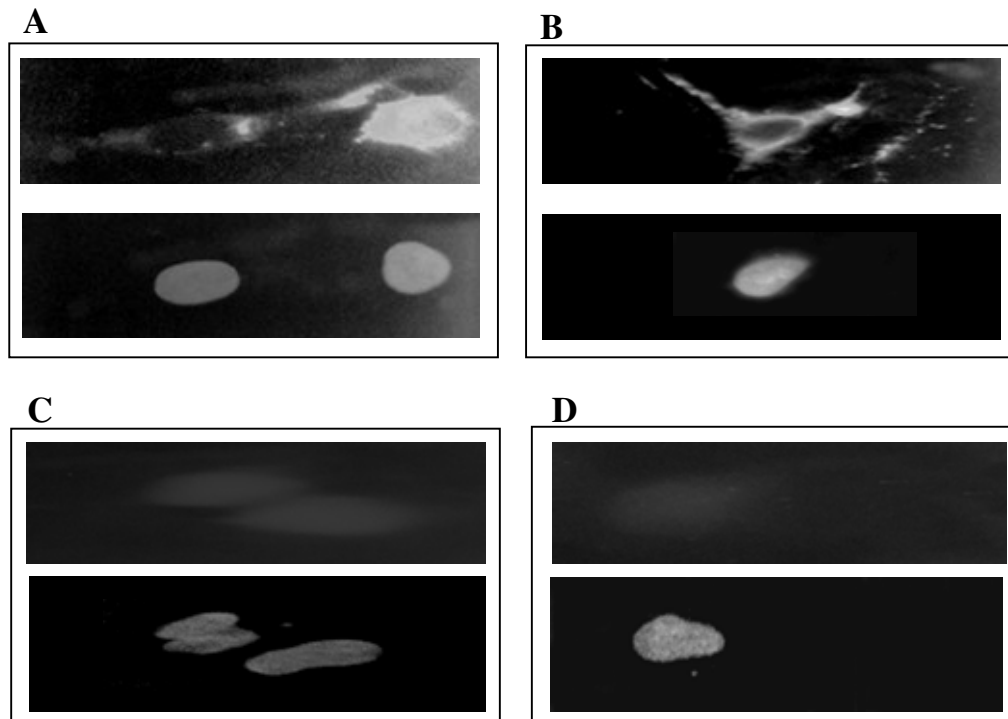
**Figure 5. Modulation of 8-epiPGF<sub>2α</sub> mediated ERK activation.**

ULTR cells were preincubated with either SQ29,549 (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 hr; Lane 6) or GF109203X (500 nM, 30 min; Lane 7). Subsequently, 8-epiPGF<sub>2α</sub> (300 nM) was added for 10 min (Lanes 3 –7), with cells exposed to 8-epiPGF<sub>2α</sub> alone (300 nM for 10 min; Lane 2) or with vehicle alone (Lane 1) serving as references. Panel A: Immunoblots were screened with anti-ACTIVE™ ERK to detect the phosphorylated, active forms of ERK (pp ERK1/2). Panel B: Immunoblots were screened with anti-ERK antibodies to detect ERK1/2 immunoreactive protein. Panel C: Fold increases in ERK phosphorylation (pp ERK1/2) in panels 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<sub>2α</sub> mediated pp ERK activation (lane 2) were significantly reduced in the presence of the respective pharmacologic inhibitors (lanes 3 – 7).



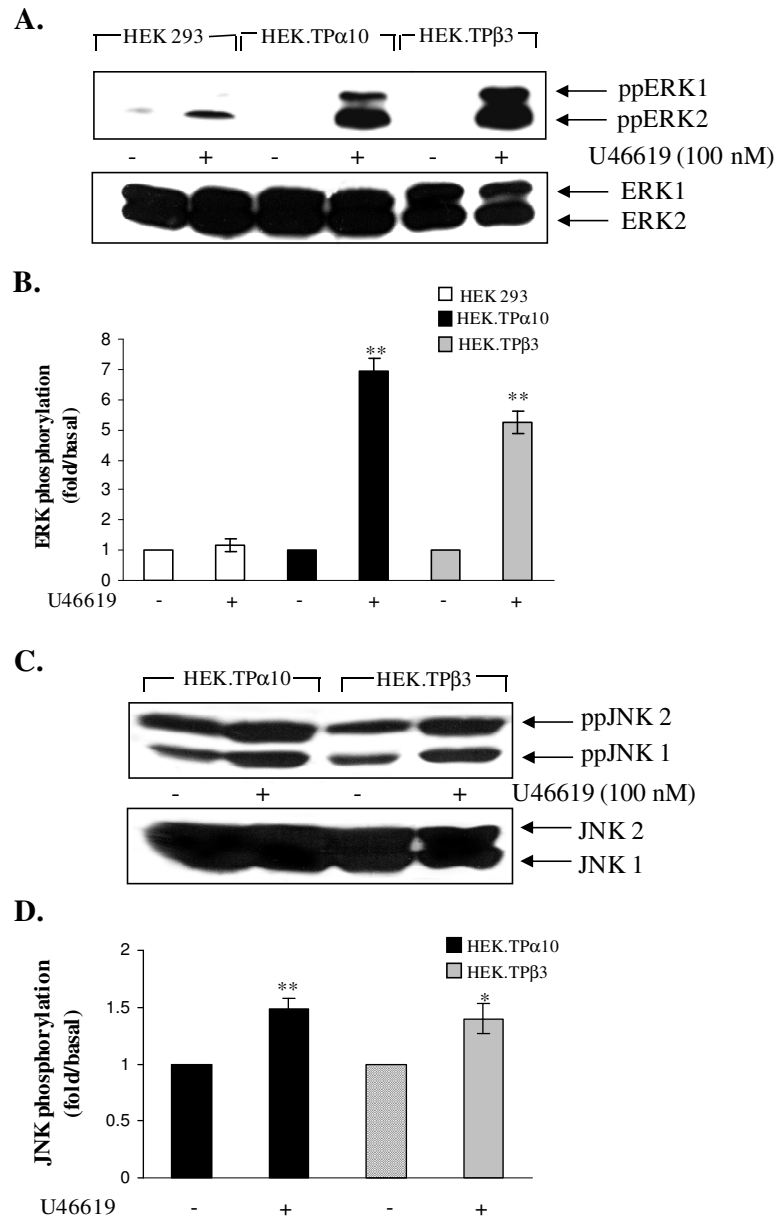
**Figure 6. Effect of U46619 and 8-epiPGF<sub>2α</sub> on JNK activation in ULTR cells .**

ULTR cells were stimulated with either U46619 (100 nM; Panel A; Lanes 2-4) or 8-epiPGF<sub>2α</sub> (300 nM; Panel B; Lanes 2-4) for 10, 20 and 60 min, respectively, or with vehicle alone (control; Panels A & B, Lane 1) or were preincubated with SQ29,548 (1 μM, 1 min) prior to stimulation with 100 nM U46619 (Panel A; Lane 5) or 300 nM 8-epiPGF<sub>2α</sub> (Panel B; Lane 5) for 10 min. As positive control, cells were incubated with anisomycin (10 μg/ml) for 60 min (Panels A and B, Lane 6). Upper panels, blots were screened with anti ACTIVE<sup>TM</sup> JNK to detect the phosphorylated, active forms of JNK (pp JNK1/2) whereas in the lower panels, blots were screened with anti-JNK antibodies to detect JNK1/2 immunoreactive protein. Results are representative of three independent experiments. Panel C: Fold increases in U46619 and 8-epiPGF<sub>2α</sub> mediated pp JNK1/2 in panels 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<sub>2α</sub> mediated JNK activation (pp JNK) were significantly greater relative to basal levels.



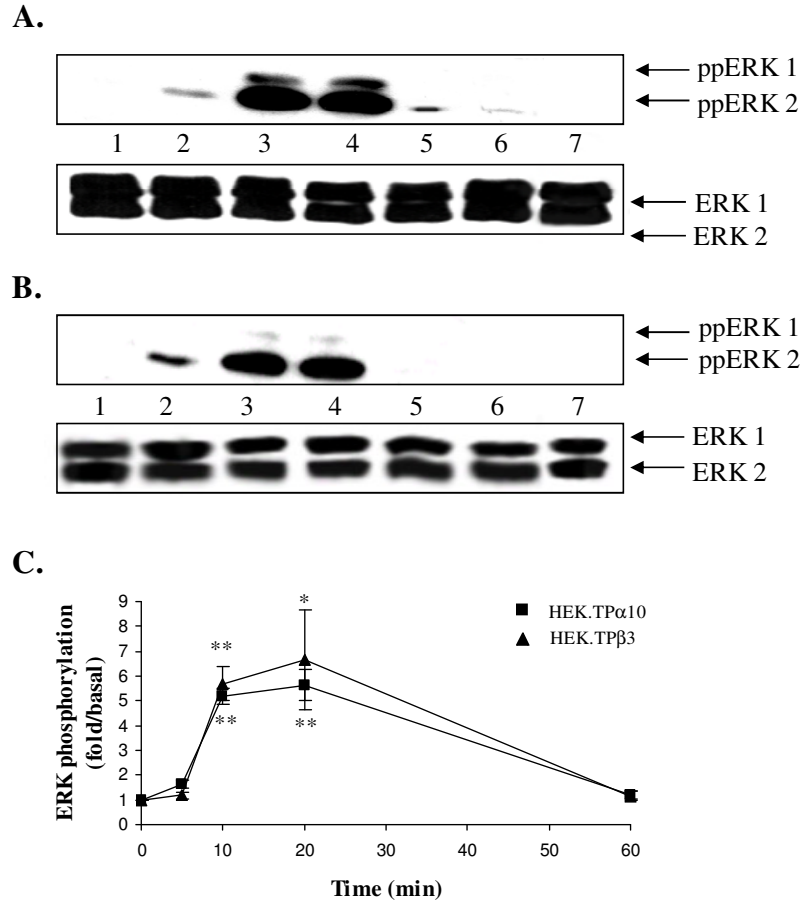
**Figure 7. Expression of TP $\alpha$  and TP $\beta$  in ULTR cells.**

Indirect immunofluorescent staining of TP $\alpha$  and TP $\beta$  in permeabilised ULTR cells was performed as described under Materials and Methods. Within each panel, the upper photograph represents the FITC immunofluorescence and the lower photograph illustrates the localisation of nuclei following DAPI staining. (A) anti-TP $\alpha$  antiserum; (B) anti-TP $\beta$  antiserum; (C) anti-TP $\alpha$  antiserum pre-incubated with a TP $\alpha$ -specific peptide; (D) anti-TP $\beta$  antiserum preincubated with a TP $\beta$ -specific peptide. Results presented are representative of at least 3 independent experiments.



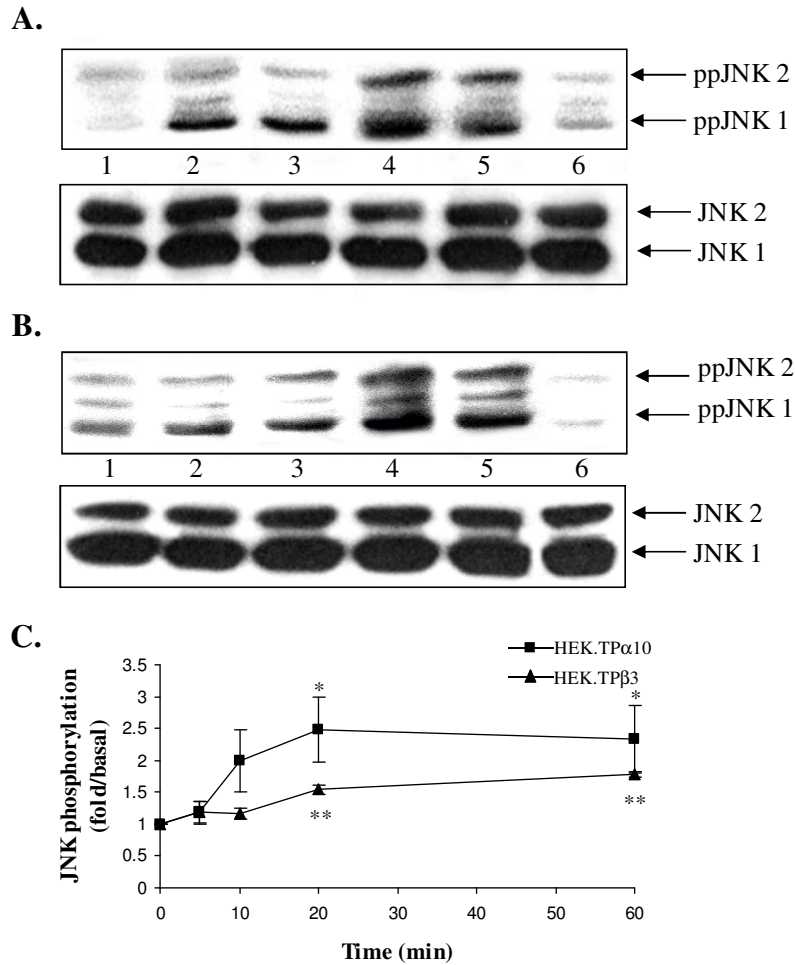
**Figure 8. Effect of U49919 on ERK 1/2 and JNK 1/2 activation in HEK.TPα10 and HEK.TPβ3 cells.**

Panels 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 A and C, blots were screened with anti ACTIVE<sup>TM</sup> ERK (panel A) or anti ACTIVE<sup>TM</sup> JNK (panel C) to detect the phosphorylated, active forms of ERK (pp ERK1/2), and JNK (pp JNK1/2), respectively. In the lower panels A and C, blots were screened with anti-ERK and anti-JNK antibodies to detect ERK 1/2 and JNK1/2 immunoreactive protein, respectively. Results are representative of three independent experiments. Panels B and D: Fold increases in ERK (pp ERK1/2) or JNK (pp JNK) phosphorylation in panels 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 (pp ERK or pp JNK) were significantly greater relative to basal levels.



**Figure 9. 8-epiPGF<sub>2α</sub> mediated ERK activation in HEK.TPα10 and HEK.TPβ3 cells.**

Time dependent effect of 8-epiPGF<sub>2α</sub> (300 nM; 0, 5, 10, 20 and 60 min) on ERK 1 and ERK 2 activation in HEK.TPα10 cells (Panel A; Lanes 1-5) and in HEK.TPβ3 cells (Panel B; Lanes 1-5). Additionally, in Panels A (HEK.TPα10 cells) and B (HEK.TPβ3 cells), cells were preincubated with SQ29, 548 (1 μM, 1 min; Lane 6) or PD 98059 (10 μM, 30 min; lane 7) prior to stimulation of cells with 8-epiPGF<sub>2α</sub> (300 nM, 10 min). Upper panels A & B, blots were screened with anti ACTIVE™-ERK to detect the phosphorylated, active forms of ERK (pp ERK1/2) whereas in the lower panels A & B, blots were screened with anti-ERK antibodies to detect ERK1/2 immunoreactive protein. Results are representative of three independent experiments. Panel C: Fold increases in ERK (pp ERK1/2) phosphorylation in panels 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 ≤ 0.05) and \*\* (P ≤ 0.01) indicate that the levels of 8-epiPGF<sub>2α</sub> mediated ERK activation (pp ERK) were significantly greater relative to basal levels.



**Figure 10. 8-epiPGF<sub>2α</sub> mediated JNK activation in HEK.TPα10 and HEK.TPβ3 cells.**

Time dependent effect of 8-epiPGF<sub>2α</sub> (300 nM; 0, 5, 10, 20 and 60 min) on JNK 1 and JNK 2 activation in HEK.TPα10 cells (Panel A; Lanes 1 - 5) and in HEK.TPβ3 cells (Panel B; Lanes 1 - 5). Additionally, HEK.TPα10 cells (Panel A, Lane 6) and HEK.TPβ3 cells (Panel B, Lane 6) were preincubated with SQ29, 548 (1 μM, 1 min) prior to stimulation with 8-epiPGF<sub>2α</sub> (300 nM, 10 min). Upper panels A & B, blots were screened with anti ACTIVE™-JNK to detect the phosphorylated, active forms of JNK (pp JNK1/2) whereas in the lower panels A & B, blots were screened with anti-JNK antibodies to detect JNK1/2 immunoreactive protein. Results are representative of three independent experiments. Panel C: Fold increases in JNK (pp JNK1/2) phosphorylation in panels A (HEK.TPα10 cells) and B (HEK.TPβ3 cells), respectively, are presented as mean fold increases of basal JNK phosphorylation ± S.E.M (n = ), where the levels of basal JNK phosphorylation in vehicle-treated cells are assigned a value of 1.0. \* (P ≤ 0.05) and \*\* (P ≤ 0.01) indicate that the levels of 8-epiPGF<sub>2α</sub> mediated JNK activation (pp JNK) were significantly greater relative to basal levels.