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Expression and functional evidence of the prostaglandin $F_{2\alpha}$ receptor mediating contraction in human umbilical vein

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ABSTRACT

Our purposes were to perform the pharmacological characterization of $PGF_{2\alpha}$ receptor (prostanoid FP-receptor) involved in human umbilical vein contraction and confirm its expression in this tissue. Umbilical cords from healthy patients after full-term deliveries were employed. The vein was dissected out of cords and used for either isolated organ bath or reverse transcription-polymerase chain reaction (RT-PCR) and Western blot assays. The natural prostanoid FP-receptor agonist, $PGF_{2\alpha}$, and its selective analogues, latanoprost and bimatoprost free acids are full agonists (produce more than 80% of the maximal contractile response to 5-HT) in human umbilical vein. The agonist potency (pEC_{50}) order was $PGF_{2\alpha}$ (6.01 ± 0.05) > latanoprost free acid (5.65 ± 0.07) = bimatoprost free acid (5.59 ± 0.08). The contractile effects of $PGF_{2\alpha}$ and latanoprost free acid were blocked competitively by the prostanoid FP-receptor antagonist, AL-8810. The antagonist potencies (pK_B) of AL-8810 *vs.* $PGF_{2\alpha}$ (5.93 ± 0.05) and *vs.* latanoprost free acid (6.40 ± 0.08) in human umbilical vein are in good agreement with its ability to antagonize prostanoid FP receptors of rat, mouse and human cells. In all samples, clear signal was detected for cDNA amplification of prostanoid FP receptor and the specific prostanoid FP-receptor antibody recognized a protein of approximately 64 kDa. In conclusion, taking into account the obtained functional and biochemical data, we propose for the first time that human umbilical vein express prostanoid FP-receptors and these receptors could be involved in the vasoconstriction action of $PGF_{2\alpha}$ in this tissue.

1. Introduction

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is a product of arachidonic acid catalyzed by the cyclooxygenase enzyme (Smith et al., 1991). A single $PGF_{2\alpha}$ receptor (prostanoid FP-receptor), which mediates the biological effects of $PGF_{2\alpha}$, has been cloned from human myometrial tissue (Abramovitz et al., 1994). Activation of prostanoid FP-receptors initiated by ligand binding, triggers $G_{\alpha}q$ protein-coupled mechanisms involving Ca^{++} signalling, IP3 turnover and activation of protein kinase C (Toh et al., 1995). $PGF_{2\alpha}$ has been implicated in diverse physiological processes such as luteolysis (Horton and Poyser, 1976), cell proliferation and cardiac myocyte hypertrophy (Adams et al., 1996), contraction of smooth muscle in various tissues, including the respiratory (Coleman et al., 1981) and gastrointestinal tract (Dong et al., 1986), iris sphincter (Ansari et al., 2004a,b) and uterus (Senior et al., 1992, 1993).

The umbilical vein transports the oxygenated blood from the placenta to the foetus; therefore, a normal blood flow is crucial for its growth. Umbilical and placental vessels lack autonomic innervation (Reilly and Russell, 1977; Fox and Khong, 1990) and regulation of its vascular tone depends on the release of vasoactive substances, which are locally produced or conveyed through the blood stream. On the other hand, previous study of our laboratory indicated that $PGF_{2\alpha}$ and selective prostanoid FP-receptor agonist fluprostenol, induce contraction in human umbilical vein. In addition, we have reported that both $PGF_{2\alpha}$ and fluprostenol-mediated contractions in the umbilical vein, are not antagonized either by ICI-192605 (selective TP-receptor antagonist) or by AH 6809 ($DP/EP_1/EP_2$ receptor antagonist), suggesting the presence of putative prostanoid FP-receptor population in this vessel (Daray et al., 2003).

$PGF_{2\alpha}$ is released in the outputs from human umbilical vessels perfused in vitro (Bjøro et al., 1986). In addition, $PGF_{2\alpha}$ is released from cultured human umbilical endothelial cells where $PGF_{2\alpha}$ is secreted with values tenfold higher compared to thromboxane B_2 or prostaglandin E_2 (Watanabe et al., 1997). Consistent with these results, when cultured human umbilical endothelial cells were exposed to plasma from women with normal pregnancies a

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predominant production of $PGF_{2\alpha}$ was observed (De Groot et al., 1998). Furthermore, $PGF_{2\alpha}$ production was significantly greater in cultured human umbilical endothelial cells exposed to plasma from preeclamptic women than by identical cells exposed to plasma from normal pregnant patients (De Groot et al., 1998).

In view of the relevance of this type of pharmacologic information, the main objective of our current study was to evaluate the prostanoid FP-receptors mediating contraction in human umbilical vein employing $PGF_{2\alpha}$ (natural prostanoid FP-receptor agonist), latanoprost and bimatoprost free acids (synthetic and selective prostanoid FP-receptor agonists) and the prostanoid FP-receptor antagonist, AL-8810, to complete the characterization of this receptor. Moreover, the existence of the prostanoid FP-receptor in this tissue was evaluated at mRNA and protein level using reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis.

2. Materials and methods

2.1. Tissue collection and preparation

Approximately, 15 to 35 cm segments were excised from human umbilical cords ($n=62$) midway between placenta and newborn. All cords were obtained from normotensive and healthy patients after term cesarean or vaginal deliveries (no differences in concentration–response curves to $PGF_{2\alpha}$ were observed between these groups, data none shown). Approval from a local ethics committee and written informed consent were obtained. Cords were immediately placed at 4 °C in modified Krebs solution of the following composition (mM): NaCl 119, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.0, EDTA 0.004 and D-glucose 11. Human umbilical vein samples were placed onto dissecting dishes containing Krebs solution; the vein was carefully dissected out from Wharton's jelly using micro-dissecting instruments and cut into rings of approximately 3 mm width. For some of the rings, the endothelium was removed by gently rolling a rough stick inside the vessel. In most investigations in which vessel preparations are used, it is possible to assess the endothelial cell integrity by evaluating the endothelial-dependent relaxation response to acetylcholine. However, acetylcholine, as well as other agonists that typically induce endothelium-dependent vasorelaxation in different vascular preparations (e.g. bradykinin), induces vasoconstriction in human umbilical vein (Sardi et al., 1997; Pujol-Lereis et al., 2006). Therefore, in the present study the presence or absence of endothelium was determined by histology as described previously (Daray et al., 2006; Nowak et al., 2007). Briefly, intact human umbilical vein rings were cut in order to expose their inner face and washed in dextrose 5%. Silver nitrate ($AgNO_3$, 5%) was applied and left to rest in the dark for 1 min. After removing the excess in dextrose 5%, the samples were covered with glycerine and put under a 300-W UV lamp for 12 min. Then, samples were fixed for 1 min in the following solutions, in this order: fixing solution (alcohol 80%, formaldehyde 37%, and acetic acid), alcohol 70%, alcohol 80%, alcohol 99% and xylene. Finally, samples were prepared for optic microscopic visualization.

2.2. Functional studies

Human umbilical vein rings were suspended in 10 ml organ baths and stretched with an initial tension of 3–5 g as described previously (Errasti et al., 1999). The time from delivery until the tissue was set up in the baths was approximately 3 h. Changes in tension of the vascular tissue were measured with Grass isometric transducers (FT-03C) and displayed on Grass polygraphs (model 7D) (Grass Instruments, Quincy, MA, USA). During the equilibration period, Krebs solution was maintained at 37 °C and at a pH 7.4 by constant bubbling with 95% oxygen/5% carbon dioxide. The bath solution was replaced every 15 min with fresh Krebs solution. Optimal passive tension of the vascular tissue was adjusted throughout the equilibration period.

After 70 min of equilibration, each ring was contracted with 40 mM KCl in order to evaluate the functional state of the tissue, and then washed. Indomethacin (30 μM) was added 30 min before the concentration–response curve was performed in order to avoid any unexpected effects produced by the release of endogenous prostanoids (Daray et al., 2003).

Concentration–response curves to the agonist were obtained after 120 min of equilibration by cumulative addition of the agonist into the bath solution in 0.25 log increments. At the end of each concentration–response curve, 5-HT (10 μM) was applied to determine the tissue maximal contractile response (Altura et al., 1972). All experiments were performed in parallel with rings from the same umbilical cord. Only one agonist concentration–response curve was performed in each ring.

Concentration–response curves to $PGF_{2\alpha}$ (0.01 μM to 30 μM), bimatoprost free acid (0.01 μM to 30 μM) and latanoprost free acid (0.01 μM to 30 μM) were performed after 120 min of equilibration. In other series of experiments, concentration–response curves to $PGF_{2\alpha}$ were also performed on human umbilical vein rings with and without endothelium.

To determine the blocking potency of AL-8810 (prostanoid FP-receptor antagonist) *vs.* $PGF_{2\alpha}$ in human umbilical vein, concentration–response curves to $PGF_{2\alpha}$ were performed in the absence (control tissue) or presence of three increasing concentrations of AL-8810 (1, 3 and 10 μM). The rings were incubated with the antagonist 30 min before the curve was performed.

In other related experiments, the blocking potency of AL-8810 was determined *vs.* latanoprost free (selective prostanoid FP-receptor agonist). Concentration–response curves were performed in the absence (control tissue) or presence of three increasing concentrations of AL-8810 (0.3, 1 and 3 μM). As previously described, rings were incubated with the antagonist 30 min before the curve was performed.

2.3. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Human umbilical veins were carefully dissected out of cords free of surrounding tissues, rinsed in ice-cold Krebs solution, cut in small pieces, blotted dry, and then frozen and stored in liquid nitrogen until processed. The tissue was pulverized to powder under liquid nitrogen, and total RNA was extracted from approximately 500 mg of each frozen tissue by using TRIzol® Reagent method (Invitrogen, Carlsbad, CA, USA) essentially as described by the supplier. Human umbilical vein RNA samples were quantified with a spectrophotometer at 260 to 280 nm and stored at −70 °C for later use.

Complementary DNA (cDNA) synthesis was attained by using ThermoScript RT kit (Invitrogen, San Diego, CA, USA) for first strand cDNA synthesis. Five μg of total RNA and 50 pmol of oligo(dT) 20 primers were incubated at 65 °C for 5 min. Samples were then incubated at 50 °C for 60 min after adding a reaction buffer that provided a final concentration of 50 mM KCl, 20 mM Tris–HCl (pH

Table 1
Oligonucleotide primer sequence information.

Primer name	Receptor cDNA sequence	Product size	Restriction endonuclease enzyme	Digestion products
		bp		bp
[a]FP sense	5′tgg tgt ttc tac aac aca gaa gac3′			
Antisense	5′ata gag att ctt aag gac agc ctt 3′	396	Bgl II	169/227
[b]GAPDH sense	5′tga agg tca gag tca acg gat ttg gt 3′			
Antisense	5′cat gtg ggc cat gag gtc cac cac 3′	983		

[a,b]Kyveris et al., 2002.

8.3), 2.5 mM $MgCl_2$, 0.5 mM dNTPs mix, 0.1 mM dithiothreitol, and 15 U of RT in 20 µl of total reaction volume. Reactions were terminated at 85 °C for 5 min. RNase H (2 U) was added, and tubes were incubated for 20 min at 37 °C. Reactions in the absence of RT were also included for each RNA tested to check for genomic contamination.

PCR was performed to amplify prostanoid FP-receptor mRNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. Primer sequence information and predicted amplification sizes are summarized in Table 1. PCR amplifications were carried out at a final volume of 50 µl of buffer containing 50 mM KCl, 20 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer, 5 U AmpliTaq DNA polymerase (Invitrogen, Carlsbad, CA, USA), and 5 µl of cDNA. Samples were cycled in the Progene (Techne, Cambridge) with the following parameters: 1 cycle of denaturation at 94 °C, 5 min, followed by 40 cycles of denaturation at 94 °C, 30 s; annealing at 60 °C (prostanoid FP-receptor, GAPDH), 30 s; extension at 72 °C, 45 s. A final extension at 72 °C for 7 min was performed for all samples. PCR conditions used in order to amplify prostanoid FP-receptor cDNA were as described by Kyveris et al. (2002). PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide and photographed under fluorescent ultraviolet illumination. Initial product identification was made by comparison to the molecular weight 100 bp ladder (Promega, Madison, WI, USA). Endonuclease digestion was used to confirm product identity. Digestion of prostanoid FP-receptor mRNA amplification product was performed using the appropriate restriction endonuclease enzyme (Table 1).

2.4. Protein isolation and Western blot

Human umbilical veins (500 mg approximately) were pulverized to powder essentially as described above for RNA isolation. In the next step, tissues were homogenized for 1 h in ice-cold rapid immunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors (phenylmethyl sulphonyl fluoride (PMSF), 1 mM; leupeptin, 1 µg/ml; pepstatin A, 1 µg/ml; phenanthroline, 1 mM; benzamidine, 1 mM) and centrifugated at 14,000 $\times g$ for 15 min to remove cellular debris. The supernatant was recovered and protein concentration was determined by Bradford method (Bradford, 1976) using Bio-Rad Kit (Bio-Rad Laboratories, Hercules, CA, USA).

Proteins (100 µg) were resuspended on loading buffer and boiled at 100 °C for 5 min. Samples were loaded to 10% sodium dodecyl sulphate (SDS)-polyacrylamide electrophoresis gel and ran at 120 V for 1 h. After running, proteins were transferred by electrophoresis to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA) at 140 mA for 1 h (Towbin et al., 1979). Ponceau Red staining of the membrane verified equal gel loading.

Membranes were blocked at 4 °C overnight in Tris-buffered saline (TBS) containing 0.5% Tween 20 (TTBS) and 5% non-fat dried milk, and then incubated at room temperature for 2 h with the prostanoid FP-receptor rabbit polyclonal antibody (0.5 µg/µl; Cayman Chemical Company, Michigan, USA) at a dilution 1:500 in TTBS with 1% non-fat dried milk. Membranes were washed four times for 15 min each in TTBS prior to incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Bio-Rad Laboratories, Hercules, CA, USA) at a dilution 1:1000 in TTBS with 1% non-fat dried milk for 1 h. Membranes were washed three times for 15 min each in TTBS and then given a final 15 min wash with TBS. The molecular sizes of the proteins were determined by comparison with standard proteins (Bio-Rad Laboratories, Hercules, CA, USA) in an adjacent lane.

Immunoreactive bands were visualized by using enhanced chemiluminescence reagent (ECL Western blotting detection Reagents, Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA) and then exposed to autoradiographic double-emulsion film (Agfa Ortho CP-G plus 100 NIF, Agfa-Gevaert S.A., Argentina). Each gel contained the positive (human placenta) control plus four samples from each human umbilical vein. Negative controls were carried out in the absence of the primary antibody.

2.5. Expression of results and statistical analysis

All data are expressed as mean ± S. E. M. The number of experiments (n) is denoted as r/v, were r represents the number of rings and v the number of veins. Each vein was obtained from a different umbilical cord and typically, four to eight rings of each vein were employed. The experiments were carried out in parallel. Only one concentration–response curve to the agonist was obtained per ring.

Contractile responses are expressed as a percentage of the tissue maximal response elicited by 10 µM 5-HT (Sardi et al., 1997).

The concentration–response curves were fitted to a four-parameter logistic model, where estimates of EC_{50} value, the agonist concentration that produces 50% of the maximal response, the maximal response (E_{max}) and the slope factor (n_H), were obtained using the software program GraphPad Prism (GraphPad Software, San Diego, CA, USA). Briefly, responses obtained for each agonist concentration in each ring tested in the same group were averaged and then fitted to a four-parameter logistic model expressed as follow: $Y = a - E_{max} / 1 + (X / EC_{50})^{nH} + E_{max}$; where Y is the response, X is the arithmetic dose, and a is the response when $X = 0$. The EC_{50} values were transformed into pEC_{50} ($-\log_{10}$ EC_{50}). Agonist log concentration ratio (r) was determined by subtracting the pEC_{50} value of the agonist in the presence of the antagonist from the pEC_{50} in the control preparations. When criteria for competitive antagonism were satisfied, that is the antagonist produced a parallel rightward shift of the agonist curve without attenuation of the maximal response, antagonist pA_2 values and slopes of Schild's regressions were calculated as described by Arunlakshana and Schild (1959). If the slope of the Schild's plot was not significantly different from unity, the regression was recalculated with Schild's slope constrained to unity (Kenakin, 1982; Neubig et al., 2003) and the affinity value obtained was then referred as pK_B.

Statistical analysis was performed by means of two-tailed unpaired Student's t test or one-way analysis of variance (ANOVA) followed by Tukey's post-test, when appropriate. P-values lower than 0.05 were considered to indicate significant differences.

Terms and equations are as recommended by the IUPHAR Committee on Receptor Nomenclature and Drug Classification (Neubig et al., 2003).

2.6. Chemicals and solutions

$PGF_{2\alpha}$ (9α,11α,15S-trihydroxy-prosta-5Z,13E-dien-1-oic acid), bimatoprost (free acid) (17-phenyl trinor prostaglandin $F_{2\alpha}$),

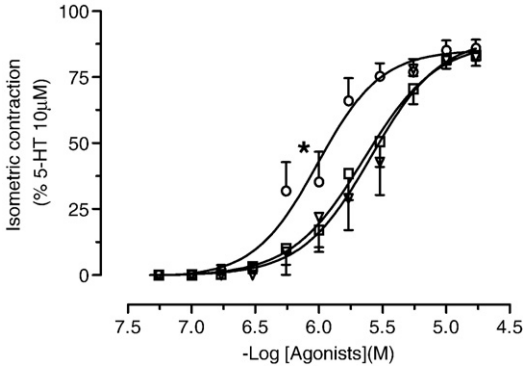

Fig. 1. Concentration–response curves to $PGF_{2\alpha}$ (○, $n = 11/11$), latanoprost free acid (□, $n = 14/14$) and bimatoprost free acid (▽, $n = 11/11$) on human umbilical vein rings. Each *symbol* represents the mean of n separate experiments and the *vertical lines* show S. E. M. Contractile responses are expressed as a percentage of the tissue maximal response elicited by 10 µM 5-HT. * The pEC_{50} of $PGF_{2\alpha}$ was significantly different ($P<0.05$) from latanoprost free acid and bimatoprost free acid, respectively.

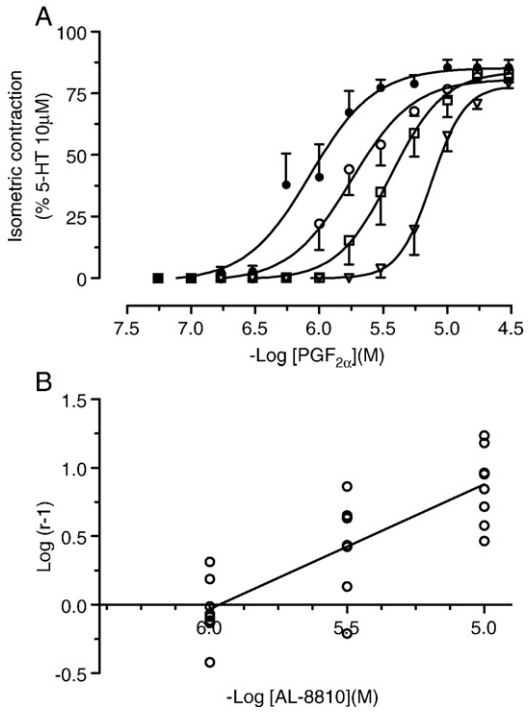

Fig. 2. Antagonism of $PGF_{2\alpha}$ by AL-8810 in human umbilical vein. (A) Concentration–response curves to $PGF_{2\alpha}$ on control rings (●, $n=8/8$) and on tissues previously exposed to AL-8810 (○, 1 μM, $n=8/8$; □, 3 μM, $n=8/8$; ▽, 10 μM, $n=8/8$). Each *symbol* represents the mean and *vertical lines* show S. E. M. The pEC_{50} of $PGF_{2\alpha}$ in tissues exposed to AL-8810 (1, 3 and 10 μM) were significantly different from control ($P<0.05$). (B) Schild's plot for AL-8810 *vs.* $PGF_{2\alpha}$ was constructed with concentration-ratios from individual experiments. The slope parameter was found to be not significantly different from unity and it was subsequently constrained to unity to estimate a pK_B of 5.93 ± 0.05 ($n=32/8$).

latanoprost (free acid) (17-phenyl-13,14-dihydro trinor prostaglandin $F_{2\alpha}$) and AL-8810 (9α,15R-dihydroxy-11β-fluoro-15-(2,3-dihydro-1H-inden-2-yl)-16,17,18,19,20-pentanor-prosta-5Z,13E-dien-1-oic acid) were purchased from Cayman Chemical (An Arbor, MI, USA). 5-hydroxytryptamine (5-HT) creatine sulphate complex was purchased from Research Biochemical Incorporated (Natick, MA, USA).

$PGF_{2\alpha}$, bimatoprost and latanoprost free acids were initially dissolved in ethanol to give a stock solution, and subsequent dilutions were prepared in bidistilled water (maximal final concentration in the bath was 0.1 v/v %). AL-8810 was diluted in dimethylsulphoxide (maximal final concentration in the bath was 0.1 v/v %). 5-HT stock solution and subsequent dilutions were performed in bidistilled water. All stock solutions were stored in frozen aliquots, thawed and diluted daily.

All concentrations of drugs are expressed as a final concentration at the organ bath. Preliminary experiments in the presence of the corresponding concentrations of ethanol and dimethylsulphoxide were performed in order to rule out any possible non-specific action of these solvents on tonus or contractility of the preparation.

3. Results

3.1. Effects of prostanoid FP-receptor agonists in human umbilical vein

After a 120 min incubation period, all agonists produced a concentration-related contraction of human umbilical vein rings (Fig. 1). Contractions induced by $PGF_{2\alpha}$, the natural agonist of prostanoid FP-receptor, were more potent than contractions induced by the selective prostanoid FP-receptor agonists, latanoprost free and bimatoprost free acids. The pEC_{50} of $PGF_{2\alpha}$ (6.01 ± 0.05, $n=11/11$) was significantly ($P<0.05$) greater than that of latanoprost free acid (5.65 ± 0.07, $n=14/14$), and bimatoprost free acid (5.59 ± 0.08, $n=11/11$). The maximal responses were not significantly different: 12.22 ± 1.09 g (E_{max} $85.98\pm3.25\%$) for $PGF_{2\alpha}$, 11.91 ± 0.85 g (E_{max} $82.44\pm1.89\%$) for latanoprost free acid and 12.26 ± 0.92 g (E_{max} $82.42\pm3.14\%$) for bimatoprost free acid. The slopes of the concentration–response curves obtained for $PGF_{2\alpha}$ ($n_H=1.74\pm0.32$), latanoprost free acid ($n_H=1.66\pm0.32$) and bimatoprost free acid ($n_H=1.60\pm0.38$) were nearly identical.

In other series of experiments, to assess the role of endothelium on $PGF_{2\alpha}$-induced contraction, concentration–response curves to $PGF_{2\alpha}$ were performed on human umbilical vein rings with and without endothelium. The pEC_{50} and maximal responses for $PGF_{2\alpha}$ on rings with endothelium (6.09 ± 0.01 and $78.3\pm5.5\%$, $n=4/4$) were not significantly different from rings without endothelium (6.03 ± 0.02 and $80.6\pm6.4\%$, $n=4/4$, $P>0.05$), respectively (data none shown).

3.2. Effect of prostanoid FP-receptor antagonist, AL-8810, on $PGF_{2\alpha}$ concentration–response curves in human umbilical vein

$PGF_{2\alpha}$ on control tissues gave a pEC_{50} of 6.07 ± 0.05, with an n_H of 1.73 ± 0.31 and the maximal response was 12.46 ± 0.94 g (E_{max} $85.51\pm3.06\%$, $n=8/8$; Fig. 2A). Increasing concentrations of the antagonist produced a competitive rightward shift of $PGF_{2\alpha}$ concentration–response curves. Data analysed by Schild's regression gave a slope ($0.91+0.14$), which was not significantly different from unity, and a pA_2 value of 5.97 yielding a pK_B value from constrained Schild's plots of 5.93 ± 0.05 ($n=32/8$, Fig. 2B).

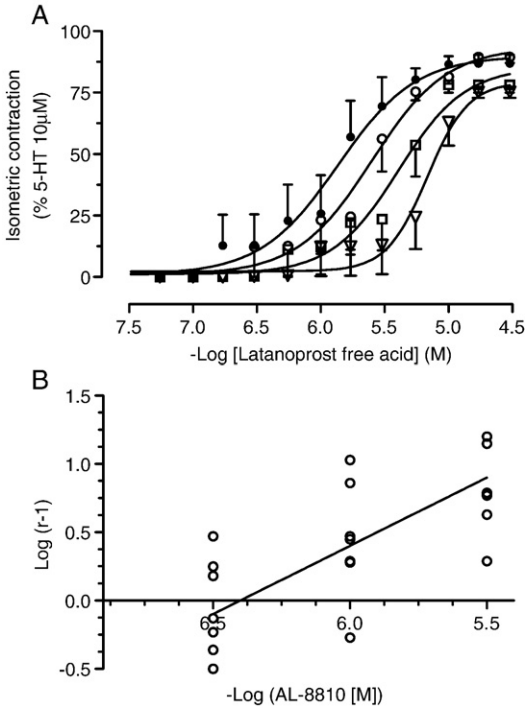

Fig. 3. Antagonism of latanoprost free acid by AL-8810 in human umbilical vein. (A) Concentration–response curves to latanoprost free acid on control rings (●, $n=7/7$) and on tissues previously exposed to AL-8810 (○, 0.3 μM, $n=7/7$; □, 1 μM, $n=7/7$; ▽, 3 μM, $n=7/7$). Each *symbol* represents the mean and *vertical lines* show S. E. M. The pEC_{50} of latanoprost free acid in tissues exposed to AL-8810 (0.3, 1 and 3 μM) were significantly different from control ($P<0.05$). (B) Schild's plot for AL-8810 *vs.* latanoprost free acid was constructed with concentration-ratios from individual experiments. The slope parameter was found to be not significantly different from unity and it was subsequently constrained to unity to estimate a pK_B of 6.40 ± 0.08 ($n=28/7$).

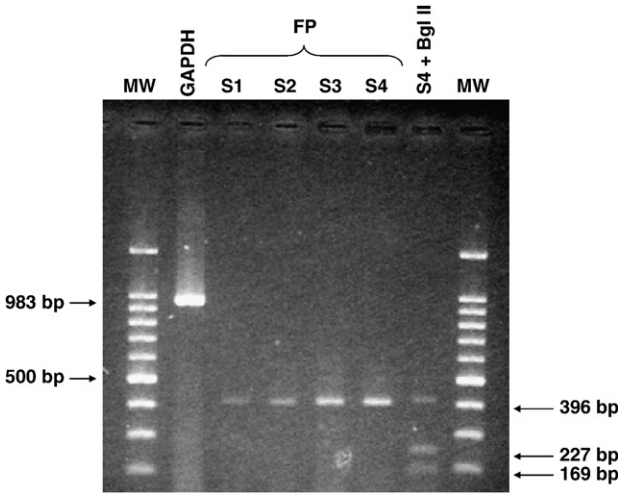

Fig. 4. Detection of prostanoid FP-receptor mRNA in human umbilical vein by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from different veins was isolated, reverse transcribed to construct cDNA, amplified by PCR and analysed on a 2% agarose gel and ethidium bromide staining. Positive amplification of prostanoid FP-receptor transcripts was evident in all umbilical veins (*lanes* 3–6). GAPDH (*lane* 2) was used as an internal positive control. Fragments generated after restriction enzyme digestions with Bgl II for prostanoid FP-receptor transcript (*lane* 7). Molecular weight (*MW*) is a 100 bp DNA standard ladder, and the *arrow* indicates the 500 bp band. The predicted sizes of the PCR products are as follow: GAPDH, 983 bp; FP, 396 bp; fragments generated after Bgl II digestion, 227 bp plus 169 bp.

3.3. Effect of prostanoid FP-receptor antagonist, AL-8810, on latanoprost free acid concentration–response curves in human umbilical vein

Latanoprost free acid on control tissues gave a pEC_{50} of 5.87 ± 0.09, with an n_H of 1.43 ± 0.41 and the maximal response was 12.87 ± 1.13 g (E_{max} $86.92 \pm 3.45\%$, $n = 7/7$; Fig. 3A). Increasing concentrations of the antagonist produced a competitive rightward shift of latanoprost free acid concentration–response curves. Data analysed by Schild's regression gave a slope (0.85 ± 0.19), which was not significantly different from unity, and a pA_2 value of 6.47 yielding a pK_B value from constrained Schild's plots of 6.40 ± 0.08 ($n = 28/7$, Fig. 3B).

3.4. Detection of prostanoid FP-receptor expression by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot in human umbilical vein

The presence of mRNA for the prostanoid FP receptor was assessed in whole cell extract preparations from fresh tissues of human umbilical vein by RT-PCR. In all tissue samples, clear signal was detected for cDNA amplification product of prostanoid FP receptor of the predicted size of 396 bp (Fig. 4).

In the present study absolute confirmation of the identity of PCR bands by DNA sequence analysis was not performed. However, in order to confirm the identity of the PCR products, each of them was incubated with restriction endonucleases to determine whether predicted mobility shifts occur. We employed the same protocol of Kyveris et al. (2002). Restriction digest of the FP-receptor PCR product with Bgl II yielded the expected 227- and 169-bp fragments in human umbilical vein (Fig. 4).

Prostanoid FP-receptor protein was established by Western blot using rabbit polyclonal anti-FP-receptor antibody on whole cell extract preparations from human umbilical vein, where a distinct immunoreactive band of 64 kDa was observed (Fig. 5). Furthermore, the protein band of 64 kDa exhibited in human umbilical vein was identical from that obtained in human placenta preparation, which was immunoblotted in parallel as a positive control (Fig. 5).

4. Discussion

Previous study of our laboratory indicated that the natural prostanoid FP-receptor agonist, $PGF_{2\alpha}$, and the selective synthetic prostanoid FP-receptor agonist fluprostenol, induce contraction in human umbilical vein. In addition, we have reported that $PGF_{2\alpha}$ and fluprostenol-mediated contraction in human umbilical vein are not antagonized either by ICI-192605 (selective prostanoid TP-receptor antagonist) or by AH 6809 (prostanoid $DP/EP_1/EP_2$ receptor antagonist) suggesting the presence of putative prostanoid FP-receptor population in this tissue (Daray et al., 2003).

In the present study, we have extended these functional observations and provide pharmacological evidence of the involvement of prostanoid FP-receptors mediating vasoconstriction in human umbilical vein by using prostanoid FP-receptor agonists and an antagonist. Moreover, the detection of mRNA and protein of prostanoid FP-receptors in human umbilical vein support our current functional data on these receptors.

Prostaglandin analogues that exhibit a significant selectivity for prostanoid FP-receptors have been synthesized as latanoprost free acid (Abramovitz et al., 2000; Sharif et al., 2003a) and bimatoprost free acid (Sharif et al., 2001, 2003a). The results of the present study show that latanoprost and bimatoprost free acids as well as $PGF_{2\alpha}$ are full agonists (produce more than 80% of the maximal response to 5-HT) inducing vasoconstriction in human umbilical vein rings. The potency order was $PGF_{2\alpha}$ (6.0) > latanoprost free acid (5.7) = bimatoprost free acid (5.6).

The prostanoid FP-receptor antagonist, AL-8810, is considered to be of great utility as a pharmacologic tool for investigating prostanoid FP-receptor mediated functional responses in a complex biological systems (Griffin et al., 1999). In fact, AL-8810 has previously been shown by Griffin et al. (1999) to selectively inhibit fluprostenol-mediated phospholipase C activity in rat thoracic aorta smooth muscle A7r5 cells ($pA_2 = 6.7$) and Swiss mouse 3T3 fibroblasts ($pA_2 = 6.3$). Moreover, at 10-μM concentration, AL-8810 did not significantly inhibited functional responses of prostanoid TP, DP, EP_2 and EP_4 receptor subtypes stimulation in various cell lines (Griffin et al., 1999). In human umbilical vein, the contractile effects of $PGF_{2\alpha}$ and latanoprost free acid were blocked competitively by AL-8810 in a concentration-dependent manner. The antagonist potencies of AL-

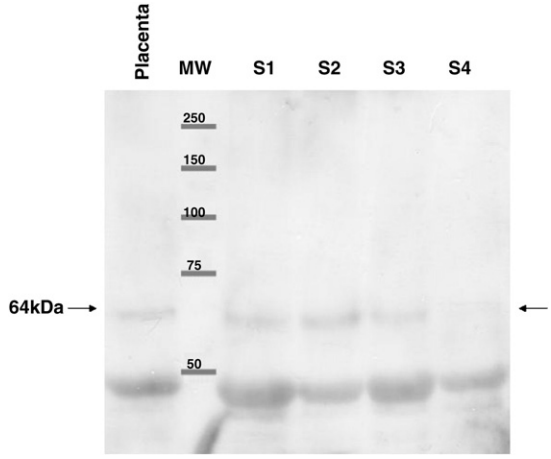

Fig. 5. Detection of prostanoid FP-receptor protein in human umbilical vein by Western blot. Total proteins (100 μg) from different human umbilical veins and placenta analysed in parallel as a positive control were isolated, subjected to 10% sodium dodecyl sulphate (SDS)-polyacrylamide electrophoresis gel and transferred onto polyvinylidene fluoride (PVDF) membrane as described under Materials and methods. A distinct immunoreactive band of 64 kDa was detected using rabbit polyclonal anti-FP-receptor antibody (0.5 μg/μl; Cayman Chemical Company, Michigan, USA) at a dilution 1:500. (*Lane* 1) human placenta. (*Lane* 2) molecular weight markers. (*Lanes* 3–6) human umbilical vein samples S1 to S4.

8810 *vs.* $PGF_{2\alpha}$ ($pK_B = 5.9$) and *vs.* latanoprost free acid ($pK_B = 6.4$) in human umbilical vein, compared well with its ability to antagonize phosphoinositide turnover and calcium mobilization induced by different prostaglandin analogs on the endogenous prostanoid FP-receptor of rat A7r5 cells ($pK_i = 5.9–6.2$, Kelly et al., 2003), mouse 3T3 cells ($pK_i = 5.9–6.2$, Kelly et al., 2003), human ciliary muscle cells ($pK_i = 5.2$, Sharif et al., 2003b), human travecular meshwork cells ($pK_i = 5.4–6.0$, Sharif et al., 2003c), cloned prostanoid FP-receptor derived from the human ciliary body expressed in human embryonic kidney (HEK)-293 cells ($pK_i = 5.7–6.1$, Sharif et al., 2002) and rat uterus contraction ($pK_i = 5.5–6.7$, Sharif, 2008). Furthermore, AL-8810 (10 μM), also significantly reduced bovine epididymal duct contraction (Mewe et al., 2006) and guinea pig airways smooth muscle contraction (Schaafsma et al., 2005, 2007).

To the best of our knowledge, no studies in human vessels evaluating the selectivity of AL-8810 have been published and most of the data regarding the selectivity of AL-8810 for the prostanoid FP-receptor comes from cultured cell studies. Nevertheless, there are some studies in animal tissues. One of them, in isolated porcine ciliary arteries, evaluated the effects of AL-8810 *vs.* $PGF_{2\alpha}$ and *vs.* U-46619 (prostanoid TP-receptor agonist) induced contractions. In those experiments AL-8810 induced a rightward displacement of both agonists; suggesting the lack of selectivity of this compound (Vysniauskiene et al., 2006). Other study, in bovine airway smooth muscle, found that AL-8810 inhibited the cholinergic neurotransmission mediated through FP-receptors, whereas prostanoid TP and EP selective blockers did not (Paredes et al., 2007); suggesting the selectivity of this compound on prostanoid FP-receptors.

In conclusion, the pK_B values obtained for AL-8810 against responses mediated by $PGF_{2\alpha}$ and latanoprost free acid in human umbilical vein were similar and close to the affinity values expected for an interaction at the prostanoid FP-receptor subtype in various cells and tissues. Nevertheless, considering data regarding the selectivity of AL-8810 on vascular isolated tissues is scarce and not definite; our results with AL-8810 suggest that prostanoid FP-receptors mediate vasoconstriction in this tissue.

To extend the conclusion reached from the pharmacological studies, we have used reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. In all tissue samples, clear signal was detected for cDNA amplification product of predicted size indicating that prostanoid FP-receptor mRNA is expressed in human umbilical vein. In this sense, studies on the distribution of prostanoid FP-receptors over a wide range of human tissues reveals its mRNA to be abundant in gestational and reproductive tissues such as placenta (Kunapuli et al., 1997; Makino et al., 2007), uterus (Kunapuli et al., 1997; Matsumoto et al., 1997), corpus luteum (Anderson et al., 2001), fallopian tube (Wanggren et al., 2006) and endometrium (Sales et al., 2004, 2005; Milne and Jabbour, 2003). On the other hand, the specific FP-antibody recognized a protein of approximately 64 kDa on Western blot analysis in human umbilical vein. Using the same antibody, similar results were observed in several human tissues and cells such as human uterine smooth muscle cells (Mandal et al., 2005), human airway epithelial cell line (Joy and Cowley, 2008), human endometrial adenocarcinoma (Sales et al., 2005), human travecular meshwork cells (Thieme et al., 2006) and human decidua (Makino et al., 2007).

In summary, taking into account the obtained functional and biochemical data, we propose for the first time that human umbilical vein express prostanoid FP-receptors, and these receptors could be involved in the vasoconstriction action of $PGF_{2\alpha}$ in this tissue.

At this time, the physiological and/or physiopathological role of prostanoid FP-receptors in the human umbilical vein is unknown as well as if endogenous $PGF_{2\alpha}$ has a local action in the umbilical vein via these receptors. Under control conditions measurements of $PGF_{2\alpha}$ synthesis was performed by Bjøro et al. (1986) in the outputs from human umbilical vessels perfused in vitro. $PGF_{2\alpha}$ is also released from cultured human umbilical endothelial cells where it is secreted with values tenfold higher compared to thromboxane B_2 or prostaglandin E_2 (Watanabe et al., 1997). Consistent with these results, when cultured human umbilical endothelial cells were exposed to plasma from women with normal pregnancies a predominant production of $PGF_{2\alpha}$ was observed (De Groot et al., 1998). Furthermore, $PGF_{2\alpha}$ production was significantly greater in cultured human umbilical endothelial cells exposed to plasma from preeclamptic women than by identical cells exposed to plasma from normal pregnant patients (De Groot et al., 1998). Taking together, these data and our results related to the prostanoid FP-receptors functionally present in human umbilical vein suggest that endogenous prostanoid FP-receptor stimulation may be a possible vasoconstrictor mechanism in pregnancies complicated by preeclampsia, producing a diminished foetal perfusion. In view of that hypothesis, the prostanoid FP-receptor blockade could constitute a new pharmacological approach for this pathological condition.

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