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Potentiation of adrenaline vasoconstrictor response by sub-threshold concentrations of U-46619 in human umbilical vein: Involvement of smooth muscle prostanoid TP_α receptor isoform

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Abstract

Considering the potential physiological, pharmacological and therapeutic relevance of synergistic interaction of thromboxane A_2 with adrenaline at postjunctional receptor sites, we examined whether sub-threshold concentrations of thromboxane A_2 mimetic U-46619 (9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$) could amplify adrenaline-induced contraction in human umbilical vein. The receptor involved in U-46619-induced potentiation of adrenaline contractility was also investigated. Umbilical cords ($n=125$) from healthy patients after full-term vaginal or caesarean deliveries were employed. The vein was dissected out of cords and rings used for isolated organ bath experiments or reverse transcription-polymerase chain reaction (RT-PCR) and Western blot. Presence of endothelium did not modify U-46619-induced contraction in human umbilical vein. Prostanoid TP-selective receptor antagonist, SQ-29548 (7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-[1S(1α,2α(*Z*),3α,4α)]-5-Heptenoic acid), inhibited U-46619-induced contraction ($pA_2=8.22\pm0.11$). U-46619 sub-threshold concentrations (0.1–0.3 nM) potentiated adrenaline-vasoconstriction response in a concentration-dependent manner. SQ-29548 (0.1 μM) abolished this potentiation. Using RT-PCR, we found that human umbilical vein rings with or without endothelium express the prostanoid TP_α, but not the prostanoid TP_β receptor isoform. Western blot allowed the identification of proteins with an electrophoretic mobility (47- and 55-kDa) indistinguishable from human platelet prostanoid TP receptor, a rich source of prostanoid TP_α receptor isoform. Collectively, present results demonstrate that prostanoid TP_α is the major receptor isoform localized on smooth muscle cells which participate in both direct vasoconstriction and potentiating effects of U-46619 on adrenaline contractions in human umbilical vein. These results suggest that thromboxane A_2 may interact synergistically with adrenaline in pathophysiological situations that lead to an increase of its umbilical venous levels (e.g. preeclampsia associated with fetal distress) raising the possibility of vasoconstriction affecting fetal blood flow.

Keywords: Human umbilical vein; Potentiation; Vasoconstriction; Adrenaline; Thromboxane A_2 Analogue U-46619; SQ-29548; RT-PCR (Reverse transcription-polymerase chain reaction); Western blot; Prostanoid TP_α receptor isoform

1. Introduction

Catecholamines measured in umbilical cord blood are of fetal origin and in normal delivery they reach levels (Puolakka et al., 1983; Chow et al., 1984; Paulick et al., 1985) slightly lower than those needed for an effective contraction of human umbilical vein (Altura et al., 1972; Errasti et al., 1999). Production and release of catecholamines are affected by a number of factors such as acute fetal hypoxemia and fetal distress increasing catecholamine levels in human umbilical arterial and venous blood (Chow et al., 1984; Paulick et al., 1985).

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During pregnancy the production of thromboxane A_2 increases in the mother and fetoplacental tissue (Ylikorkala and Mäkilä, 1985). Plasma level of the stable metabolite of thromboxane A_2 (thromboxane B_2) in umbilical venous blood, collected immediately after delivery of normal term pregnancies, reaches values (Mitchell et al., 1978) close to those needed to produce an effective contraction of the human umbilical vein (Daray et al., 2003). In preeclampsia, placenta has been reported to increase thromboxane production (Walsh, 1985) and a remarkable increase of its level was noted in both maternal and umbilical venous blood, reaching levels which were higher than during normal pregnancy (Liu et al., 1998).

There is evidence that contractile responses mediated by α-adrenoceptors are potentiated by low concentrations of U-46619 in different experimental models: rabbit isolated mesenteric artery (Trachte and Stein, 1989) and portal vein (Stein and Trachte, 1989), porcine isolated ear artery (Bhattacharya et al., 2005) and pressor responses to renal nerve stimulation in rat isolated kidney (Rump and Schollmeyer, 1989). To our knowledge, evidence with regard to human vessels only comes from experiments performed on isolated human saphenous vein, showing that sub-threshold concentrations of U-46619 facilitate sympathetic neurotransmission and potentiate vasoconstrictor effects of exogenous noradrenaline (Vila et al., 2001). In contrast to human saphenous vein, the umbilical vein is under no influence from the sympathetic nervous system (Reilly and Russell, 1977; Fox and Khong, 1990; Kawano and Mori, 1990) and regulation of its vascular tone must depend on the release of vasoactive substances which are locally produced or conveyed through the blood stream. Furthermore, it is known that the vasoconstrictor effects of adrenaline depend on the stimulation of α_{1B}-adrenoceptors in human umbilical vein (Errasti et al., 1999, 2003), while in human saphenous vein these are mediated primarily by postjunctional α_{2C}- (Rizzo et al., 2001; Giessler et al., 2002) and to a lesser degree by $\alpha_{1A/1B}$-adrenoceptor subtypes (Yan et al., 2001). Moreover, the absence of both functional α_2- and β-adrenoceptors has been demonstrated in human umbilical vein (Errasti et al., 1999). Considering the anatomical, physiological and pharmacological differences between both vessels, data obtained by Vila et al. (2001) in human saphenous vein can not be entirely extrapolated to the umbilical vein.

Taking into account potential physiological, pharmacological and therapeutic relevance of synergistic interaction of thromboxane A_2 with adrenaline at postjunctional receptor sites, the present study was undertaken to determine whether sub-threshold concentrations of thromboxane A_2 mimetic U-46619 could amplify the vasoconstrictor responses to adrenaline in isolated human umbilical vein. Furthermore, two different isoforms of thromboxane A_2 (TP) receptor, termed prostanoid TP_α and TP_β, have been recognized as a result of alternate splicing (Hirata et al., 1991; Nüsing et al., 1993; Raychowdhury et al., 1994; Kinsella et al., 1994). At present, the biological effects elicited by distinct prostanoid TP-receptor isoforms activation have not been studied in human vessels.

In this study, we are reporting for the first time in human umbilical vein that sub-threshold concentrations of U-46619 potentiate adrenaline vasoconstrictor responses by an interaction with a specific prostanoid TP-receptor isoform. Taken together, our functional and molecular data suggest that prostanoid TP_α is the major receptor isoform present on isolated human umbilical vein mediating direct vasoconstriction and enhancing effects of U-46619 on adrenaline-induced responses.

2. Materials and methods

2.1. Tissue collection and preparation

Approximately, 15 to 35 cm segments were excised from human umbilical cords ($n=125$) midway between the placenta and newborn, collected from healthy patients after term vaginal or cesarean deliveries. Written informed consent was obtained from each parturient. 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, D-glucose 11 and ascorbic acid 0.11.

Human umbilical vein samples were placed onto dissecting dishes containing Krebs solution; the vein was dissected free from Warthon's jelly and cut into rings of approximately 3 mm width. The endothelium was removed from some of the rings 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).

2.2. Functional studies

Human umbilical vein rings were suspended in 10 ml organ baths and stretched with an initial tension of 3 g as described previously (Errasti et al., 1999). Changes in tension 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 pH 7.4 by constant bubbling with 95% oxygen/5% carbon dioxide, and the bath solution was replaced every 15 min with fresh Krebs solution. After 70 min of equilibration period, each preparation was contracted with 40 mM KCl in order to test the functional state of the tissue. Optimal passive tension was adjusted throughout the equilibration period. Concentration–response curves to the agonists were obtained after 120 min of equilibration by cumulative addition of the agonist to the organ bath in 0.25 log increments. Only one concentration–response curve to the agonist was obtained per each ring.

Concentration–response curves to U-46619 (0.1 nM to 10 μM) were performed after 120 min on human umbilical vein rings with and without endothelium.

Table 1
Oligonucleotide primer sequence information

Primer name	Receptor cDNA sequence	Product size (bp)	Restriction endonuclease enzyme	Digestion products (bp)
TP_α sense	5′gag atg atg gct cag ctc ct 3′	313	*Pvu*II	120/193
antisense[a]	5′ctt cct act gca gcc cgg agc gct g 3′			
TP_β sense	5′gag atg atg gct cag ctc ct 3′	269		
antisense[b]	5′aga ctc cgt ctg ggc cg 3′			
GAPDH sense	5′cgg gaa gct tgt cat caa tgg 3′	357		
antisense[c]	5′ggc agt gat ggc atg gac tg 3′			

[a]Kyveris et al., 2002.
[b]Miggin and Kinsella, 1998.
[c]Errasti et al., 2003.

In order to avoid any effect induced by the release of endogenous prostanoids, the blocking potency of selective prostanoid TP-receptor antagonist SQ-29548 versus U-46619 was previously assessed in human umbilical vein by our group (Daray et al., 2003) in the presence of 30 μM indomethacin (cyclooxygenase inhibitor). In the present study, we tested this antagonist without indomethacin to determine its blocking potency in human umbilical vein under control conditions. Therefore, concentration–response curves to U-46619 were performed after 120 min in the absence (control) and in the presence of three increasing concentrations of SQ-29548 (0.03, 0.1 and 0.3 μM). Human umbilical vein rings were incubated with this antagonist for 30 min before the U-46619-concentration–response curve was performed.

In other series of experiments, concentration–response curves to adrenaline (1 nM to 10 μM) were performed at 120 min in the absence (control) and in the presence of either increasing sub-threshold vasoconstrictor concentrations of U-46619 (0.03, 0.1 and 0.3 nM) or SQ-29548 (0.1 μM) plus U-46619 (0.3 nM). Tissues were challenged with U-46619 for 10 min before adrenaline concentration–response curves were started. When SQ-29548 was employed, tissues were incubated with the antagonist for 30 min prior to the start of incubation with U-46619.

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. RNA samples obtained from human umbilical veins were then 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 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 (2U) 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 TP_α and TP_β receptor mRNA isoforms. Glyceraldehyde 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, 5U 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 61 °C (TP_α, 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 TP_β cDNA were as described by Miggin and Kinsella (1998). 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 with the molecular weight 100 bp ladder (Promega, Madison, WI, USA). Endonuclease digestion was used to confirm product identity. Digestion of prostanoid TP_α 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. Platelet rich plasma was obtained by centrifugation at 150 ×*g* for 15 min at room temperature from whole blood obtained by venipuncture from healthy volunteers (Klein, 1996). Then, platelets were pelleted by centrifugation at 1000 ×*g* for 10 min at 4 °C. In the next step, tissues were homogenized for 1 h in ice-cold rapid immunoprecipitation assay (RIPA) lysis

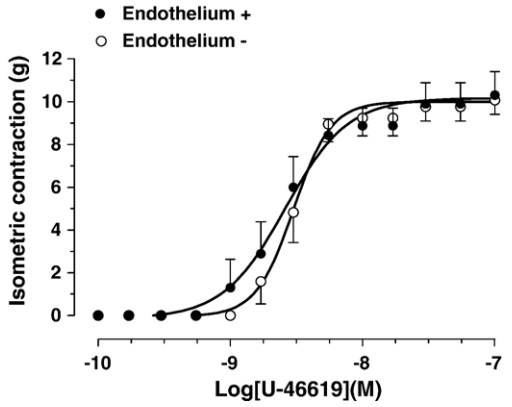

Fig. 1. Concentration–response curves to U-46619 on human umbilical vein rings with endothelium (●, $n=8/8$) and without endothelium (○, $n=8/8$). The pEC_{50} of U-46619 in tissues without endothelium was not different from tissues with endothelium ($P>0.05$). Each symbol represents the mean of n separate experiments and the vertical lines show S. E. M. The responses are expressed in grams of developed contraction.

buffer containing protease inhibitors (phenylmethyl sulfonyl fluoride (PMSF), 1 mM; leupeptin, 1 μg/ml; pepstatin A, 1 μg/ml; phenanthroline, 1 mM; benzamidine, 1 mM) and centrifugated at 14,000 ×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 sulfate (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) and stained with Ponceau S to determine uniformity of electrophoretic transfer.

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 anti-human prostanoid TP-receptor rabbit polyclonal antibodies (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. Immunoreactive bands were visualized by using enhanced chemiluminescense 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 SA, Argentina). The molecular sizes of the proteins were determined by comparison with standard proteins (Bio-Rad Laboratories, Hercules, CA, USA) in an adjacent lane. Each gel contained the positive control (proteins isolated from human platelets) plus four samples from each human umbilical vein.

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, where r represents number of rings and v number of veins. Each vein was obtained from a different umbilical cord and typically four or eight rings of each vein were employed. Responses are expressed as g of developed contraction.

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 maximum and the slope factor (n_H), were obtained using the software program GraphPad Prism (GraphPad Software, San Diego, CA, USA). 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 preparation. When criteria for competitive antagonism were satisfied, which means that the antagonist produced a parallel rightward shift of the agonist curve without attenuation in the maximum response, antagonist pA_2 value and slope of Schild's regression were calculated as described by Arunlakshana and Schild (1959).

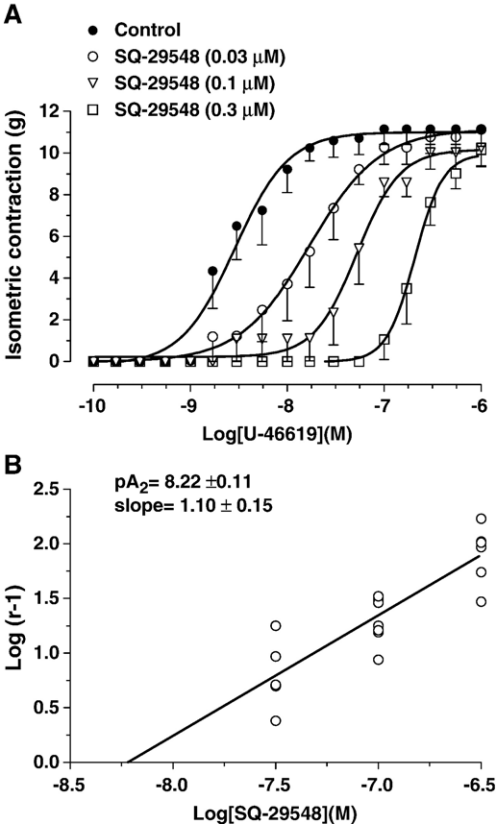

Fig. 2. (A). Concentration–response curves to U-46619 on human umbilical vein rings in the absence (●, $n=9/9$) or in the presence of the prostanoid TP-receptor antagonist SQ-29548 (○, 0.03 μM, $n=9/9$), (▽, 0.1 μM, $n=9/9$) and (□, 0.3 μM, $n=9/9$). Each symbol represents the mean and the vertical lines show S. E. M. of n separate experiments. (B) Schild plot for SQ-29548 versus U-46619 was constructed with concentration-ratios from individual experiments ($n=36/9$).

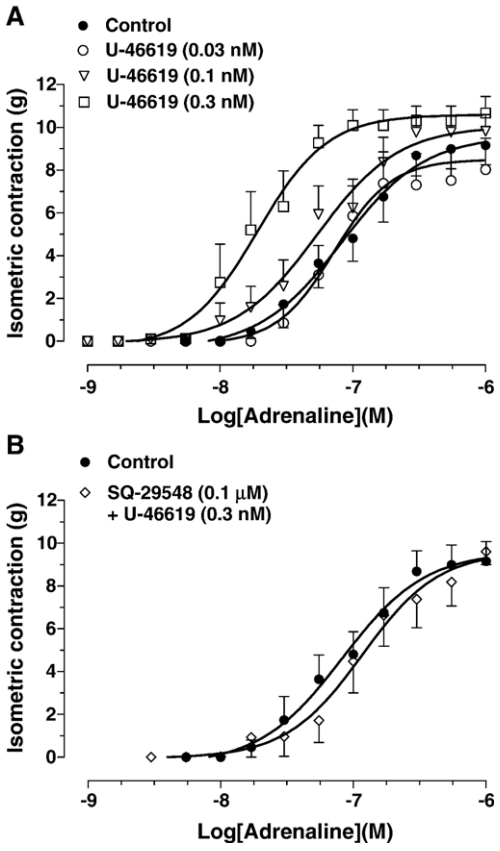

Fig. 3. (A). Concentration–response curves to adrenaline on human umbilical vein rings in the absence (●, $n=15/15$) or in the presence of U-46619 (○, 0.03 nM, $n=5/5$; ▽, 0.1 nM, $n=10/10$; □, 0.3 nM, $n=10/10$). The pEC_{50} of adrenaline in tissues exposed to U-46619 (0.1 and 0.3 nM) were different from control ($P<0.05$). (B) Concentration–response curves to adrenaline on human umbilical vein rings in the absence (●, $n=15/15$) or in the presence of prostanoid TP-receptor antagonist SQ-29548 0.1 μM plus 0.3 nM of U-46619 (◊, $n=8/8$). Each symbol represents the mean and the vertical lines show S. E. M. of n separate experiments. The responses are expressed in grams of developed contraction.

Statistical analysis was performed by means of one-tailed unpaired Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey's post-test. 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

Adrenaline bitartrate was purchased from Research Biochemical Incorporated (Natick, MA, USA). U-46619 (9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$) and SQ-29548 (7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1] hept-2-yl]-[1S(1α,2α(Z),3α,4α)]-5-Heptenoic acid) were purchased from Biomol Research laboratories (Plymouth Meeting, PA, USA).

Adrenaline was initially dissolved in HCl (0.01 N) to give a stock solution and their subsequent dilution was prepared in ascorbic acid Krebs' solution to avoid oxidation. U-46619 and SQ-29548 stock solutions were made up with ethanol and subsequent dilutions were prepared in bidistilled water. Control trials were performed in the presence of the corresponding concentration of ethanol in order to rule out any possible non-specific action of this solvent on tonus or contractility of the tissue.

All stock solutions were stored in frozen aliquots, thawed, and diluted daily. All concentrations of drugs are expressed as a final concentration in the organ bath.

3. Results

3.1. U-46619 concentration–response curves in human umbilical vein rings with and without endothelium

In order to assess the role of endothelium on U-46619-induced contraction, concentration–response curves to U-46619 were performed on human umbilical vein rings with and without endothelium. The pEC_{50} and maximum response for U-46619 on rings with endothelium (8.58±0.06 and 10.66±1.15 g, $n=8/8$) were not significantly different from rings without endothelium (8.52±0.03 and 10.61±0.97 g, $n=8/8$, $P>0.05$), respectively (Fig. 1).

3.2. Antagonism of SQ-29548 on U-46619 concentration–response curves in human umbilical vein

Concentration–response curves to U-46619 were performed in the absence (control, $n=9/9$) and presence of SQ-29548 (0.03 μM, $n=9/9$; 0.1 μM, $n=9/9$ and 0.3 μM, $n=9/9$;

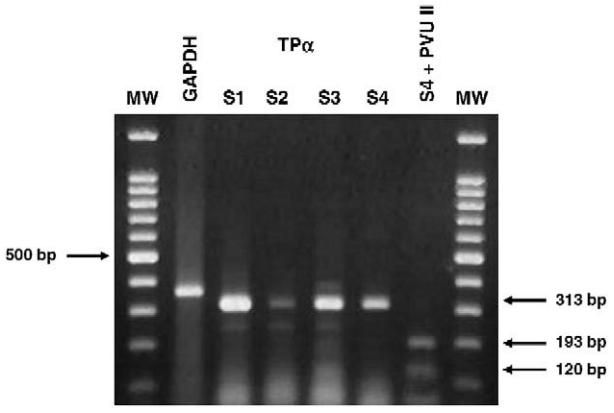

Fig. 4. Detection of prostanoid TP_α receptor mRNA isoforms by reverse transcription-polymerase chain reaction (RT-PCR) in human umbilical vein. Total RNA from different human umbilical veins was isolated, reverse transcribed to construct cDNA, amplified by PCR and analyzed on a 2% agarose gel and ethidium bromide staining. (Lanes 1 and 8) molecular weight (MW) is a 100 bp DNA standard ladder, and the arrow indicates the 500 bp band. (Lane 2) Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal positive control. (Lanes 3 to 6) positive amplification of TP_α receptor transcripts was evident in all intact umbilical veins (S1–S4). (Lane 7) fragments generated after restriction enzyme digestions with *Pvu*II for TP_α transcript from S4. The predicted sizes of the PCR products are as follow: GAPDH, 357 bp; TP_α, 313 bp; fragments generated after *Pvu*II digestion, 193 bp plus 120 bp.

Fig. 2). Concentration responses-curves to U-46619 were significantly shifted to the right in a parallel manner without affecting the maximum response, indicative of competitive antagonism (Fig. 2A). Analysis of the data by Schild's regression showed a slope (1.10±0.15), which was not significantly different from unity, and a pA_2 value of 8.22±0.11 (n=36/9, Fig. 2B).

3.3. Effect of sub-threshold vasoconstrictor concentrations of U-46619 on adrenaline concentration–response curves in human umbilical vein

U-46619 (0.03 nM) neither caused any contractions nor modified the concentration–response curve to adrenaline (control: pEC_{50}, 7.08±0.09, max, 9.58±0.74 g, n=15/15; treated: pEC_{50}, 7.14±0.07, max, 8.80±1.0 g, n=5/5, P>0.05; Fig. 3A).

U-46619 (0.1 nM) did not induce contractions but potentiated the concentration–response curve to adrenaline without modifying maximal contraction (treated: pEC_{50}, 7.24±0.08, max, 10.01±0.93 g, n=10/10, P<0.05; Fig. 3A).

U-46619 (0.3 nM) did not induce contractions but further potentiated the concentration–response curve to adrenaline without modifying maximal contraction (treated: pEC_{50}, 7.61±0.08, max, 10.54±0.71 g, n=10/10, P<0.05; Fig. 3A).

The prostanoid TP-receptor antagonist SQ-29548 (0.1 μM) abolished potentiation elicited by 0.3 nM of U-46619 (treated: pEC_{50}, 6.93±0.07, max, 9.60±0.60 g, n=8/8, P>0.05; Fig. 3B). On the other hand, concentration–response curve to adrenaline was not modified in the presence of SQ-29548 (0.1 μM, data not shown).

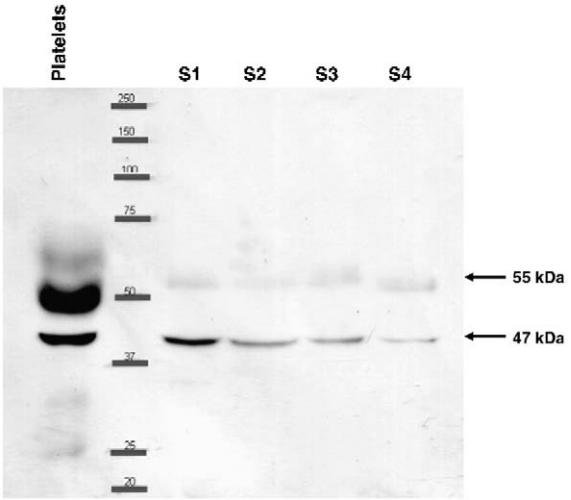

Fig. 5. Detection of prostanoid TP-receptor protein in human umbilical vein by Western blot. Total proteins (100 μg) from different human umbilical veins and human platelets analyzed in parallel as a positive control were isolated, subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis gel and transferred onto polyvinylidene fluoride (PVDF) membrane as described under Methods. Distinct immunoreactive bands of 47- and 55-kDa were detected using anti-human prostanoid TP-receptor polyclonal antibodies (0.5 μg/μl; Cayman Chemical Company, Michigan, USA) at a dilution 1:500. (Lane 1) human platelets. (Lane 2) molecular weight markers. (Lanes 3 to 6) umbilical vein samples S1–S4.

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

In all intact tissue samples, clear signal was detected for cDNA amplification product of prostanoid TP_α isoform of the predicted size of 313 bp (Fig. 4). Prostanoid TP_α isoform mRNA was also consistently detected when the endothelium of the umbilical vein was resected (data not shown). Expression of prostanoid TP_β isoform has also been investigated, but no signals could be detected in human umbilical vein (data not shown).

Prostanoid TP-receptor protein was further established in human umbilical vein by Western blot where distinct immunoreactive bands of approximately 47- and 55-kDa were observed (Fig. 5). Furthermore, the protein electrophoretic mobility exhibited in human umbilical vein was indistinguishable from that obtained in human platelet preparation, which was immunoblotted in parallel as a positive control (Fig. 5).

4. Discussion

Adrenaline measured in umbilical cord venous blood is of fetal origin and in normal delivery it reaches levels (5.4 nM, Chow et al., 1984) slightly lower than those needed for an effective vasoconstriction (Fig. 3A) of isolated human umbilical vein. Plasma levels of the stable metabolite of thromboxane A_2 (thromboxane B_2) in umbilical venous blood, collected immediately after delivery of normal term pregnancies, reach values close to 1 nM (Mitchell et al., 1978). These values are 3- to 10-fold higher than sub-threshold vasoconstrictor concentrations employed in the present study (0.3 and 0.1 nM) of thromboxane A_2 mimetic U-46619, which is equipotent with thromboxane A_2 (Coleman et al., 1981). In preeclampsia, placenta has been reported to increase thromboxane A_2 production (Walsh, 1985) and its level in the mother and fetal–placental circulation is higher than in normal pregnancy (Liu et al., 1998). On the other hand, fetal plasma catecholamines may reach extremely high levels during deliveries in pregnancies associated with fetal distress (severe hypoxia, asphyxia, acute maternal hemorrhage, maternal smoking, etc.) in the antenatal period or during labour (Puolakka et al., 1983; Chow et al., 1984; Paulick et al., 1985).

In the present study, U-46619 potentiated adrenaline-induced contractions in a concentration-dependent manner (Fig. 3A) with substantially lower concentrations (0.1–0.3 nM) than those required to produce a direct contractile response of the isolated human umbilical vein (Fig. 1). We have previously demonstrated that U-46619 contracts human umbilical vein through prostanoid TP receptors (Daray et al., 2003). Therefore, herein, we examined the involvement of prostanoid TP receptor in the potentiating effects of sub-threshold vasoconstrictor concentration of U-46619 on adrenaline-induced responses. Our present results show that SQ-29548, a selective prostanoid TP-receptor blocker, prevents U-46619 potentiation of adrenaline vasoconstriction (Fig. 3B). The sensitivity of this potentiating effect to a TP-receptor blocker indicates a receptor-mediated event probably

through the prostanoid TP receptor (Ogletree et al., 1985; Coleman et al., 1994). This conclusion is further supported by the fact that SQ-29548 blocked this potentiating effect with a concentration (0.1 μM) only 10-fold higher than the pA_2 value (8.22) obtained in the present study (Fig. 2B). Furthermore, prostanoid TP receptor involved in this potentiation could be localized on smooth muscle cells based on the lack of differences of U-46619 vasoconstrictor effects between human umbilical vein rings with or without endothelium (Fig. 1).

The mechanisms involved in the adrenergic-vasoconstrictor responses potentiated by sub-threshold concentrations of prostanoid TP-receptor agonist U-46619 have been investigated in some vascular tissues by other authors. In the porcine ear artery, the α_2-adrenoceptor-mediated vasoconstriction is enhanced by pre-contraction with the thromboxane A_2 mimetic U-46619. This enhancement occurs through an increased activation of extracellular signal-regulated mitogen-activated protein (ERK-MAP) kinase, and is also dependent upon influx of extracellular calcium (Bhattacharya and Roberts, 2003). In the human saphenous vein, Vila et al. (2001) conclude that U-46619 facilitates sympathetic neurotransmission and potentiates constrictor effects of exogenous noradrenaline through stimulation of prostanoid TP receptors independently of extracellular calcium entry. In contrast, Fabi et al. (2004) demonstrated that the Ca^{2+} channel antagonist, verapamil, blocked the noradrenaline hyperresponsiveness produced by sub-threshold concentrations of U-46619. In human umbilical vein, our previous studies demonstrated that adrenaline vasoconstriction is mediated by α_{1B}-adrenoceptor subtype (Errasti et al., 1999, 2003) and vasoconstrictor effects of U-46619 are mediated by prostanoid TP receptor (Daray et al., 2003). The mechanism of the enhancement of the α_{1B}-adrenoceptor-mediated vasoconstriction by U-46619 through prostanoid TP receptor is unknown, and further work is required to identify the nature of this interaction in human umbilical vein.

In humans, thromboxane A_2 signals through two receptor isoforms termed prostanoid TP_α and TP_β that arise by alternative splicing (Hirata et al., 1991; Nüsing et al., 1993; Raychowdhury et al., 1994; Kinsella et al., 1994). Prostanoid TP-receptor isoforms do not exhibit any difference in their affinity (K_d) for SQ-29548 and show efficient U-46619-mediated mobilization of intracellular Ca^{2+} in their respective stable cell lines exclusively over- expressing the prostanoid TP_α or TP_β isoform (Kinsella, 2001). The physiological significance for two prostanoid TP receptors in humans remains to be determined, but is now evident that they exhibit distinct patterns of mRNA and protein expression (Miggin and Kinsella, 1998; Kinsella, 2001; Huang et al., 2004). To our knowledge, there are no studies in isolated human vessels demonstrating both vasomotor activity induced by stimulation of prostanoid TP receptors and the existence of specific prostanoid TP-receptor isoform in the same vessel. Therefore, taking into account the functional results described above, the expression of the mRNA isoforms and proteins for the prostanoid TP receptor was assessed in intact human umbilical vein by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot, respectively. All veins analyzed express consistently the mRNA of prostanoid TP_α isoform (Fig. 4). In addition, prostanoid TP_α isoform mRNA was also consistently detected when the endothelium of the human umbilical vein was resected (data not shown). Prostanoid TP-receptor expression was further established in human umbilical vein using Western blot analysis (Fig. 5), where distinct bands of 47- and 55-kDa, expected sizes for the glycosyled human prostanoid TP_α receptor isoform (Zhou et al., 2001), were observed. Similarly, other authors, employing non-specific isoform antibodies, have reported similar protein bands in human neuronal cells (Blackman et al., 1998), human umbilical endothelial cells (Caughey et al., 2001) and human platelets (Caughey et al., 2001). Furthermore, in the present study, the apparent molecular weights of 47- and 55-kDa exhibited in human umbilical vein were indistinguishable from those obtained in human platelets (Fig. 5). In this regard, although mRNA detection for the two recognized human prostanoid TP-receptor isoforms has been reported in human platelets (Hirata et al., 1996), prostanoid TP_α was the only receptor isoform detectably translated in these cells (Habib et al., 1997, 1999; Huang et al., 2004). In the present study, expression of mRNA of prostanoid TP_β isoform was also investigated in intact human umbilical vein, but no signals could be detected in our experimental conditions. In this sense, a previous report (Miggin and Kinsella, 1998) indicates that primary human umbilical endothelial cells (1° HUVECs) express mRNA for prostanoid TP_β isoform, albeit at very low levels (approximately 6-fold lower levels of prostanoid TP_β relative to TP_α). Therefore, the difference between expression in cultured 1° HUVECs and the absence of prostanoid TP_β mRNA expression in freshly isolated human umbilical vein, may be explained by the fact that when cells are transferred into culture, they acquire an activated state, especially when grown on plastic dishes (Jaffe et al., 1973; Herren et al., 1997). Collectively, results obtained from RT-PCR and Western blot demonstrate that prostanoid TP_α is the major isoform present in isolated human umbilical vein.

In conclusion, the present study constitutes pharmacological and molecular evidence that prostanoid TP_α receptor isoform localized on human umbilical vein smooth muscle cells participates in both direct vasoconstriction and potentiating effects of U-46619 on adrenaline contractions. To our knowledge, we have demonstrated for the first time both, the expression and functional significance of a prostanoid TP_α receptor isoform in an isolated human vessel. Furthermore, our results in human umbilical vein suggest that vasoconstriction could occur in those pathophysiological situations in which thromboxane A_2 and adrenaline umbilical venous blood levels are increased as in preeclampsia associated with fetal distress. Therefore, this raises the possibility that thromboxane A_2 may interact synergistically with adrenaline in concurrent pathophysiological conditions, promoting constriction of human umbilical vein affecting fetal blood flow.

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