Published in final edited form as:

Prostaglandins Other Lipid Mediat. 2008 December ; 87(1-4): 54–61. doi:10.1016/j.prostaglandins.2008.08.002.

Characterization of Endothelial Thromboxane Receptors in Rabbit Aorta

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Abstract

An increased synthesis of thromboxane (TX) A₂ is associated with a number of cardiovascular diseases including atherosclerosis, unstable angina and hypertension. We previously identified a subgroup of NZW rabbits in which isolated arteries failed to contract to the TX agonists, U46619 or I-BOP. In vascular smooth muscle membranes, there was a significant decrease in TX receptors, termed TP. These rabbits are referred to as vTP[−] and those with the TP receptor are called vTP⁺. Because TP receptors are expressed in some types of endothelial cells, the present study was designed to determine whether functional TP receptors are present in endothelial cells cultured from aortas of vTP⁺ and vTP[−] rabbits. Radioligand binding studies were performed with 125I-BOP. Aortic endothelial cells from vTP⁺ rabbits exhibited specific and saturable binding. In contrast, in endothelial preparations from vTP[−] rabbit aortas, no measurable binding to 125I-BOP was detected. Using an anti-TP receptor antibody, we compared the amount of receptor expressed in endothelial cell lysates obtained from vTP⁺ and vTP[−] rabbits. Consistent with the results observed radioligand binding assays, the expression of TP receptor protein was decreased in vTP[−] compared to vTP⁺ endothelial cells. An in vitro wound healing assay was used on confluent monolayers of endothelial cells. In the untreated vTP⁺ cells, the area of the scratch was completely closed by 30 hrs. In the vTP⁺ cells treated with U46619 (3 μM), the rate of closure of the scratch area was reduced with approximately 12% of the scratch area remaining at 30 hrs. Pretreatment with the TP receptor antagonist, SQ 29548 (10 μM) prevented the inhibitory effect of U46619. The rate of closure of the scratch in the vTP[−] was not altered by U46619. In a separate study, U46619 (3 μM) increased the release of 6-keto PGF_{1α}, the stable metabolite of prostacyclin, in vTP⁺ but not vTP[−] endothelial cells. Pretreatment with SQ29548 (10 μM) or the cyclooxygenase inhibitor, indomethacin (10 μM) blocked the increase in vTP⁺ endothelial cells. In vascular reactivity studies in aortas from vTP⁺ rabbits, removal of the endothelium enhanced the vasoconstrictor response to U46619 indicating that activation of endothelial TP receptors may modulate vascular tone via the release of the vasodilator, prostacyclin. The results of this study suggest an important role for endothelial TP receptors in modulating vascular function.

Keywords

thromboxane; endothelial cells; receptor; cell migration

INTRODUCTION

Thromboxane (TX) A₂, the predominant metabolite of arachidonic acid in platelets, promotes platelet aggregation and vascular smooth muscle vasoconstriction via activation of a G-protein coupled receptor referred to as the TP receptor (1,2). Numerous studies have implicated

TXA₂ as an important pathophysiologic mediator of a variety of cardiovascular diseases including atherosclerosis, unstable angina and hypertension (3–5). In addition to platelets and vascular smooth muscle cells, there is evidence that other tissues and cell types express TP receptors. Of particular interest to understanding the role of TXA₂ in cardiovascular disease is the evidence that endothelial cells express TP receptors (6–10). The endothelium has an integral role in the control of vascular tone and most forms of cardiovascular disease are associated with some type of endothelial dysfunction. Exactly what role the endothelial TP receptor may play is controversial. Studies have shown that activation of the endothelial TP receptor can have opposing effects on prostacyclin release, cell migration and cAMP accumulation (6–15).

In previous studies, we observed that blood vessels obtained from approximately 25% of New Zealand White (NZW) rabbits did not contract to TXA₂ agonists (16,17). Using a combination of physiological, biochemical and pharmacological studies, this subgroup of rabbits was found to be deficient in the vascular smooth muscle cell TP receptor, but not the platelet TP receptor. These rabbits are referred to as vTP[−]. Rabbits which express the vascular smooth muscle cell TP receptor are called vTP⁺. Radioligand binding showed a significantly reduced density of TP receptors in vascular smooth muscle cells ($B_{\max} = 397 \pm 59$ vs 157 ± 59 fmol/10⁶ cells, vTP⁺ vs vTP[−]) (17). Platelet rich plasma obtained from vTP⁺ and vTP[−] rabbits aggregated to the TP receptor agonist, U46619 and the maximum aggregation was not significantly different in the two groups (maximum response; $39 \pm 10\%$ vs. $52 \pm 7\%$, vTP⁺ vs. vTP[−]) (16). Platelet lysates from vTP[−] and vTP⁺ rabbits were analyzed by Western immunoblotting using a polyclonal TP receptor antibody and there was no difference in the TP receptor protein expression between the vTP⁺ and vTP[−] rabbits (17). Thus, these studies indicated that the defect in TP receptors was specific to the vasculature.

Since the vascular smooth muscle cell TP receptor had been well characterized in our previous studies, the purpose of the present study was to determine if endothelial cells derived from vTP⁺ and vTP[−] rabbits express TP receptors using a radioligand binding assay and Western immunoblotting. Because the functional significance of the endothelial cell TP receptor is unclear, an important aspect of the described studies is to compare the effects of a TP receptor agonist on cell migration and prostacyclin synthesis in cells from vTP⁺ and vTP[−] rabbits.

MATERIALS and METHODS

Animals

Studies were conducted in accordance with NIH guidelines for the care and use of laboratory animals. Two month old male NZW rabbits obtained from New Franken Rabbitry were housed in the Medical College of Wisconsin Animal Care Facilities. The animals were maintained on a standard rabbit chow diet and given tap water ad libitum. Rabbits were anesthetized with sodium pentobarbital (120 mg/kg, iv), aorta removed and placed in Krebs-bicarbonate buffer of the following composition (mM): NaCl 118, KCl 4, CaCl₂ 3.3, NaHCO₃ 24, KH₂PO₄ 1.4, MgSO₄ 1.2, glucose 11, pH 7.4. Aortas were dissected from the aortic valve to approximately 0.5 cm distal to the iliac bifurcation and carefully cleaned of adhering fat and connective tissue. A portion of the abdominal aorta was used for the vascular reactivity protocols and the remainder was used for isolation of endothelial cells for establishment of cell cultures.

Vascular Reactivity

The criteria to determine if a rabbit has the vTP⁺ or vTP[−] phenotype is to measure vascular responses to U46619 in isolated aortas. Rings of abdominal aorta (3–4 mm) were suspended in 6 ml organ baths containing Krebs bicarbonate buffer that was warmed to 37° C and continuously aerated with a 95% O₂/5% CO₂ mixture. Isometric tension was measured as

previously described (18). Contractions were produced by increasing the KCl concentration of the bath to 40 mM and repeated until maximal, reproducible responses were obtained. Because KCl contractions remain stable throughout the experiment, results were expressed as a percent of the KCl contraction. Responses to the TXA₂ mimetic, U46619 (10⁻⁷ M) were obtained. vTP⁻ rabbits are identified by aortas which contracted to KCl but not to U46619 while vTP⁺ rabbits contract to both KCl and U46619 (figure 1). In some aortas, the endothelium was carefully removed prior to addition of vasoactive agents.

Endothelial Cell Isolation

Endothelial cells were isolated from aortas of vTP⁺ and vTP⁻ rabbits as previously described (19). The vessels were placed into medium containing antibiotics for approximately 15 min and then cleaned of connective tissue and placed on damp sterile gauze in petri dishes in a laminar flow hood. Collagenase (0.1%) was introduced into the vessel lumen with a polyethylene-tipped syringe, and the luminal surface gently pressed together. Aortas were incubated at 37°C in an atmosphere of 5% CO₂ in air for a minimum of 30 min. The vessels were rinsed through the lumen with RPMI culture medium, and the detached cells collected into a centrifuge tube. The cells were sedimented by centrifugation, washed once with medium and resuspended in RPMI containing 20% rabbit serum. The endothelial cells were plated in 25 cm² culture flasks and remain undisturbed for 24 h. The medium was changed daily for the first few days and then twice weekly thereafter. Endothelial cells start growing from small clumps of cells and spread to confluence within 5–10 days. The presence of specific endothelial cell markers were used to confirm the purity of the cultures. Incorporation of acetylated low density lipoprotein and the presence of Factor VIII antigen was determined by immunofluorescence as previously described (20).

Receptor Binding

The equilibrium binding studies were performed on cells that were grown to approximately 80% confluency in 6-well culture plates (16,17). Cells were incubated for 30 min at room temperature with 2×10⁴ cpm of the TXA₂ agonist, ¹²⁵I-BOP, and varying concentrations of unlabeled I-BOP (0.1 to 20 nM) in a final volume of 0.6 ml. The reaction was terminated by washing the cells twice with ice cold buffer of the following composition (mM): N-2-hydroxyethyl-piperazine-N'-ethanesulfonic acid (HEPES) 10, NaCl 150, KCl 5, CaCl₂ 2, MgCl₂ 1, and glucose 6, pH 7.4, followed by the addition of 0.5 N NaOH (1 ml) to solubilize the cells. Bound radioactivity was counted in a gamma counter. Non-specific binding was determined in the presence of the TP receptor antagonist, SQ 29548 (10 μM). Specific binding was approximately 80% of the total binding and in all cases, less than 10% of the radioligand was bound to the cells. The binding data were analyzed using the nonlinear regression one-site binding program of PRISM (GraphPad Software, Inc., San Diego, CA). Total protein content was determined in the extracts using the Bradford technique (Bio-Rad, Melville, NY) with albumin as a standard.

Western Immunoblot

Endothelial cell lysates from vTP⁺ and vTP⁻ rabbits were incubated on ice in 75-cm² plastic flasks for 10 minutes in lysis buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, and protease inhibitors). The protein lysates (40 μg/lane) were resolved by SDS-PAGE using a 4% acrylamide stacking gel and 10% resolving gel (Bio-Rad). The proteins were electrophoretically transferred to nitrocellulose and the nitrocellulose membrane was blocked for overnight at 4° C with 2% nonfat dry milk in Tris-buffered saline (20 mM TRIZMA hydrochloride; 500 mM NaCl, pH 7.5) with Tween-20 (TTBS) before incubation with a polyclonal TP receptor antibody. Incubation with the primary antibody was at a dilution of 1:1000 for 1 hour at room temperature. Following washing, the

blot was incubated for 1 hour at room temperature with the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG antibody) at a dilution of 1:3000. After again washing with TTBS, the blot was incubated for 1 minute with DuPont Renaissance Chemiluminescent reagents. The membrane was subsequently exposed to Kodak Biomax MR scientific imaging film to visualize protein separation. Prestained protein markers (Sigma) were used for molecular mass determination. Membranes were re-probed with mouse anti- β -actin as a loading control. Bands were quantitated by ImageJ software (NIH, Bethesda, MD), and the optical density values were expressed as arbitrary units (AU).

In Vitro Wound Healing Scratch Assay

Aortic endothelial cells from vTP⁺ and vTP⁻ rabbits were seeded on 12-well multiplates plates and grown to confluence. Once cells reached 100% confluency, the cell layer of each well was scratched with a 200 μ l pipette tip to create a wound. After washing 5–6 times with serum free media to remove floating cellular debris, cells were incubated in fresh media containing U46619 (3 μ M). In some cases, the TP receptor antagonist SQ29548 (10 μ M) was included. Cells were photographed at different times (0, 2, 4, 8, 12, 24 and 30 hours) by a phase contrast microscope equipped with a digital camera. The size of the wound (area of the scratch) was determined using ImageJ software. The percent wound closure at the various time points was calculated and values are the average wound closure rates for 4 representative experimental protocols.

6-keto PGF_{1 α} Production

Aortic endothelial cells from vTP⁻ and vTP⁺ rabbits were grown in 24-well plates. When cells were near confluency, they were incubated in HEPES buffer at 37° C for 30 min with and without U46619 (3 μ M). In some cases the endothelial cells from vTP⁺ rabbits were pretreated with the TP receptor antagonist, SQ 29548 or the cyclooxygenase inhibitor, indomethacin (10 μ M) for 10 min prior to the addition of U46619. The reaction was stopped by removing the media and freezing in a methanol/dry ice bath. Samples were stored at -20° C until 6-keto PGF_{1 α} , the stable metabolite of prostacyclin, was measured by specific RIA according to the method of Campbell and Ojeda (21).

Materials

U46619, I-BOP, ¹²⁵I-BOP, SQ29458, 6-keto PGF_{1 α} and anti-TP receptor antibody were from Cayman Chemical Company (Ann Arbor, MI). Indomethacin and mouse anti- β actin antibody was from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was from Zymed (San Francisco, CA). All cell culture reagents were purchased from GIBCO (Grand Island, NY). Flasks used in cell culture were from Corning (Corning, NY). ³H-6-keto PGF_{1 α} was from Amersham (Arlington Heights, IL). All other chemicals were of reagent grade. All drugs were diluted in ethanol and given in a volume that gave a final ethanol concentration of the bath or incubation buffer of less than 0.07 %.

Statistical Analysis

Data are expressed as the mean $\pm$ SEM for observations in cells obtained from different animals. [¹²⁵I]-BOP saturation data and competition data were determined using a computer-assisted (PRISM) nonlinear regression analysis two-way analysis of variance followed by the Bonferroni multiple-comparison test and Student's t test for paired observations were used for statistical evaluation. A value of P < 0.05 was considered statistically significant.

RESULTS

Ligand binding

The affinity and density of TXA₂ receptors in cultured aortic endothelial cells obtained from both vTP+ and vTP− rabbits was assessed using ¹²⁵I-BOP. The binding of ¹²⁵I-BOP to cultured vTP+ rabbit aortic endothelial cells was saturable and specific (figure 2). In those vTP− endothelial cell preparations where there was measurable binding, it was saturable (figure 2). A representative analysis of the equilibrium binding studies including saturation curves (top panel) and the Scatchard analysis (bottom panel) is shown in figure 2. Table 1 shows the averaged results for vTP+ and vTP− endothelial cells. The K_d values were not different. However, there was a significant decrease in the density of receptors from endothelial cells of vTP− rabbits (B_{max} = 56.8 ± 9.7 fmol/mg vs 10.7 ± 5.7 fmol/mg, vTP+ vs vTP−, p < 0.01).

Western Blots

Utilizing a specific polyclonal anti-TP receptor antibody, Western blot analysis showed the presence of a immunoreactive band in lysates prepared from vTP+ and vTP− endothelial cells (figure 3, top panel). These bands corresponded to the 55 kDa receptor protein previously described by Le Breton and coworkers (22). Blots were reprobed with β-actin as a loading control. Expression of β-actin did not change in vTP+ and vTP− endothelial cells. TP receptor expression normalized to β-actin showed that the endothelial cell expression of the receptor was significantly reduced in vTP− compared to vTP+ rabbits (figure 3, bottom panel). This experiment was repeated 3 times and similar results were observed.

Wound Healing Scratch Assay of vTP+ and vTP− Endothelial Cells: An in vitro scratch assay was used to assess the effect of TP receptor activation on endothelial cell migration. In the untreated vTP+ cells, the wound was healed by 30 hours. If the cells were treated with U46619, wound healing was delayed (figure 4, top panel). The % of the scratch area was significantly less in U46619-treated when compared to control cells (98.6 ± 1.1, 94.7 ± 1.6, 78.9 ± 3.4, 20.7 ± 6.7, 11.8 ± 5.3 vs 90.5 ± 1.9, 78.5 ± 2.4, 53.8 ± 4.1, 6.2 ± 3.7, 0 ± 0 for 2, 4, 8, 24 and 30 hrs, respectively; U46619 vs control). Pretreatment with the TP receptor antagonist SQ29548 had no effect on the rate of wound healing in the control cells but prevented the inhibitory effect of U46619. When endothelial cells from vTP− rabbits were scratched, there was no significant difference in the rate of cell migration or wound closure either in the presence or absence of U46619 (figure 4, bottom panel) supporting the ligand binding data. However, it did appear that the rate of cell migration was greater in the vTP− cells compared to vTP+ cells as the scratch area was completely healed by 24 hours in all of the vTP− treated cells.

6-keto PGF_{1α} production in vTP+ and vTP− endothelial cells: Results showed that stimulation of the endothelial TP receptor with U46619 (3 μM) increases 6-keto PGF_{1α} production (figure 5) in the vTP+ aortic endothelial cells. In the vTP− rabbits which lacked the endothelial TP receptor there was no increase observed with U46619. Furthermore, blockade of TP receptors with SQ29548 or inhibition of cyclooxygenase with indomethacin, prevented the ability of U46619 to increase 6-keto PGF_{1α} production in the vTP+ cells (figure 6).

U46619-induced contractions in denuded vTP+ aortas: In order to assess the role of the endothelium on U46619-induced contractions, concentration-response curves to U46619 were performed in aortic rings from vTP+ rabbits with and without endothelium. The maximal response for U46619 on rings with endothelium was not different from rings without endothelium (149 ± 13% vs 143 ± 11%; intact vs denuded). However, removal of the endothelium shifted the concentration-response curve to the left. The results are shown in figure 7. The EC₅₀ value for U46619 was decreased in the denuded vTP+ aortas (3.5 ± 0.7 nM vs 1.5 ± 0.3 nM; intact vs denuded, * p < 0.05).

DISCUSSION

It is well established that platelets synthesize and release TXA₂ which elicits platelet aggregation and vascular smooth muscle cell contraction by activating a TP receptor (2). Disturbances in this pathway contributes to vascular diseases such as atherosclerosis and hypertension (3–5). While endothelial dysfunction is a common characteristic in these disorders, much less is known about the role of the endothelial TP receptor in vascular homeostasis and disease. Others showed that cultured endothelial cells from various species express TP receptors (6,7,9) but this is the first report characterizing the endothelial TP receptor in rabbits. This study also provides the first evidence that a subgroup of rabbits are deficient in endothelial cell TP receptors. We showed previously that these rabbits were deficient in vascular smooth muscle cell TP receptors (16,17). In the present study, aortic endothelial cells were cultured from rabbits that were first identified by the ability of aortic segments to contract to the TP agonist, U46619 (figure 1). Ligand binding using the radiolabeled TP agonist, I-BOP, was compared in endothelial cells obtained from vTP+ and vTP– rabbits. Results indicated that the affinity of I-BOP for the TP receptor was not different between vTP+ and vTP– cells but there was a significant decrease in receptor number in the vTP– endothelial cells (Table 1). Protein expression using an antibody specific to the TP receptor confirmed the radioligand binding results by showing a reduced protein expression in vTP– compared to vTP+ (figure 3). Because the decrease in receptor number occurred in cultured endothelial cells, these data would suggest that the deficiency in receptors is related to a defect in the cell. Regulation of TP receptor expression has been studied in some cell types (23–28). In vascular smooth muscle cells, treatment with testosterone increases TP receptor expression (24,25) whereas dexamethazone was found to decrease TP receptor density (23). Our earlier studies reported no differences in steroid concentrations between the vTP+ and vTP– rabbits suggesting that some other factor(s) is involved in regulating TP receptor expression (16). Additional studies are needed to determine the exact mechanisms that contribute to the deficiency of TP receptors in some rabbits.

The cDNA for the TP receptor was originally cloned from human placenta and the platelet-like MEG-1 cell line (29). A second isoform of the TP receptor was isolated from a human umbilical vein endothelial cell cDNA library (30). The endothelial cell TP receptor was called the β isoform and the TP receptor originally cloned from platelets was called the α isoform. Genomic cloning of the human TP gene confirmed the existence of a single gene, located on chromosome 19p13.3 (31). Both TP β and TP α are encoded by the same gene but arise by a novel differential splicing event. The two isoforms are identical for the first 328 amino acids but differ in their carboxyl-terminal cytoplasmic tail. In the splice variant, the β isoform, the terminal 15 amino acids are replaced with 79 amino acids. The two isoforms show similar ligand-binding characteristics and phospholipase C (PLC) activation but regulate adenylyl cyclase activity in an opposite manner (12,13). TP α increases cAMP accumulation and TP β inhibits cAMP accumulation (12,13). There is also evidence in transfected cells that the carboxyl-terminal tail of TP α and TP β play a role in G-protein-coupling specificity (32) and confer different desensitization characteristics (12,33–38). While the present study was not designed to identify the TP receptor isoforms expressed in the rabbit endothelial cells, it is predicted that both isoforms exist. This conclusion is based on evidence showing the expression of both isoforms in all tissues, including platelets and vascular smooth muscle and endothelial cells (39). In preliminary studies we see evidence of both receptor subtypes in whole aortic lysates (unpublished observations, 2008).

A key objective of the present study was to determine if the deficiency in receptor density that occurred between the aortic endothelial cells from vTP+ and vTP– rabbits was coupled to differences in functional responses. A number of different laboratories have made notable contributions to characterizing TP receptor signaling in endothelial cells (for review), (2). In

one study, Hunt et al. (9) reported that U46619 (10 μ M) increased the release of 6-keto $\text{PGF}_{1\alpha}$ in a bovine aortic endothelial cell line. In agreement with these studies, Kent et al. (6) investigated the role of the TP receptor in human venous endothelial cells either cultured from saphenous or umbilical veins and also showed that U46619 increased the release of 6-keto $\text{PGF}_{1\alpha}$. The present study measured an increase in 6-keto $\text{PGF}_{1\alpha}$ production in response to TP receptor stimulation only in the cells obtained from the vTP+ rabbits. The formation of prostacyclin is regulated by two different steps, the calcium-mediated activation of phospholipase A_2 which releases arachidonic acid from the cell membrane phospholipids and conversion of arachidonic acid by cyclooxygenase to endoperoxide intermediates which are further metabolized by prostacyclin synthase. Pretreatment of cells with a cyclooxygenase inhibitor prevented U46619-induced 6-keto $\text{PGF}_{1\alpha}$ release in the vTP+ endothelial cells. Based on the results from other studies using other cell types it is likely that activation of the endothelial TP receptor induces a Gq-dependent phospholipase C activation to evoke calcium-dependent activation of phospholipase A_2 (2).

An additional experiment was performed to explain the biological significance of the vasoconstrictor, TXA_2 , activating an endothelial TP receptor to increase in the release of the vasodilator, prostacyclin. Vascular reactivity studies were performed in vTP+ rabbits in the presence and absence of an intact endothelium. U46619-induced contractions were enhanced in the denuded aortas. A number of well characterized vasoconstrictor compounds, including angiotensin II and histamine, have been shown to increase the release of a vasodilator presumably as a counterbalance to the vasoconstriction (40–42). The data from the rabbit which shows that TP receptor stimulation of endothelial cells increases prostacyclin release gives further support to the importance of the endothelial TP receptor in the regulation of vascular tone.

Endothelial cells are also actively involved in vascular repair and remodeling that occurs in certain cardiovascular diseases. Cell migration is one process that contributes to new vessel growth in diseases such as myocardial ischemia and diabetes (43). Endothelial cell migration is complex and involves multiple signaling pathways including but not limited to the activation of small GTPases of the Rho family, PI3K and eNOS; SAPK2/p38; and phosphorylation of FAK (43). A large number of compounds are known to induce angiogenesis and endothelial cell migration. Factors have also been identified which inhibit endothelial cell migration and these include angiostatin, endostatin and thrombospondin. More recently, there is evidence that drugs that inhibit cholesterol biosynthesis also inhibit endothelial cell proliferation through a mechanism related to inhibition of RhoA activation (44). A functional role for TXA_2 in regulating endothelial cell migration has been suggested (14,15). Daniel and coworkers (14) used human renal microvascular endothelial cells and the wound healing assay and showed that phorbol ester treatment increased endothelial cell migration. This effect was blocked by pretreatment with a cyclooxygenase-2 inhibitor and only the TP agonist U46619 was able to restore the response. A study by Nie and coworkers (15) utilized endothelial cell lines established from rat brain resistance vessels, human umbilical vein endothelial cells and human dermal microvascular endothelial cells and found that U46619 stimulated endothelial cell migration in response to growth factor. Pretreatment with a TP receptor antagonist, SQ29548, inhibited growth factor-stimulated endothelial cell migration.

Contrary to these studies are the reports that TP ligands inhibit endothelial cell migration (8, 10). For example, the TXA_2 agonist, I-BOP inhibited endothelial cell in vitro tube formation in cultured human umbilical vein endothelial cells. Additional studies from these investigators then examined cell migration in cells transfected with either $\text{TP}\alpha$ or $\text{TP}\beta$ (10). In cells expressing $\text{TP}\alpha$, I-BOP had no effect on cell migration. However, if cells expressed $\text{TP}\beta$, I-BOP inhibited VEGF-induced cell migration. The authors suggested that the signaling mechanism to explain $\text{TP}\beta$ -specific inhibition may involve a disturbance in both nitric oxide release and

decreased activation of Akt, eNOS and PDK1. Studies showed that I-BOP inhibited the phosphorylation of Akt kinase, a cell survival mediator (11). Our studies support those of Ashton et al in that in the endothelial cells cultured from the vTP+ rabbits, U46619 inhibited cell migration. The cellular mechanism for this effect was not investigated in the rabbit cells. More recently, Kinsella and coworkers examined the differential regulation of RhoA-mediated signaling in HEK cells or primary human aortic smooth muscle cells expressing either TP α or TP β (45). Their results suggested that both TP α and TP β independently activated RhoA. Interestingly, incubation with prostacyclin impaired the ability of the TP α -expressing cells to activate RhoA but had no effect on the ability of TP β -expressing cells to activate RhoA. Based on these results, we would predict that the rabbit endothelial cells may express TP α that when activated releases prostacyclin and somehow interferes with TP-mediated RhoA activation. This in turn may explain the decrease in endothelial cell migration in the vTP+ cells stimulated with U46619. In future studies it will be important to measure the relative expression of the TP isoforms in the vTP+ endothelial cells to determine if differential expression explains the effect on cell migration. It is also interesting to note that in the endothelial cells in which we measured a reduced density of TP receptors (vTP- cells), the rate of closure of the wound was greater when compared to the vTP+ aortas suggesting that there may be some endogenous regulation of cell migration by TXA₂.

In summary, the present study identifies the presence of functional TP receptors on endothelial cells of vTP+ rabbits, whereas a deficiency in TP receptor number and functions occurs in cells of vTP- rabbits. The functional studies confirm a role of endothelial TP receptors in the inhibition of cell migration and also indicate that activation of endothelial TP receptors can release prostacyclin that counteracts the vasoconstrictor effect of TX-mimetics. Because endothelial dysfunction has implications in a number of cardiovascular disease states, these results provide additional support that endothelial TP receptor are important modulators in the vasculature.

Acknowledgments

We thank Mrs. Renee Penoske, Mr. Nicolas Helderma and Ms. Brittany Wade for technical assistance. Support was provided by a grant from the National Heart, Lung and Blood Institute # HL-57895.

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Figure 1.

Contractile effects of U46619 in aortas from vTP+ and vTP- rabbits. Vessels were suspended in organ baths at a resting tension of 2 grams. Concentration-response curves were obtained by the cumulative addition of U46619. Values are mean $\pm$ SEM expressed as % response to KCl (40 mM)

Figure 2.

Representative equilibrium binding analysis of the binding of ^{125}I -BOP to endothelial cells cultured from vTP+ and vTP- rabbit aortas. The top panel is the saturation binding data and the bottom panel depicts the Scatchard analysis for vTP+ and vTP- endothelial cells. Assay conditions are described in the Methods.

Figure 3.

Protein expression of endothelial TP receptors. A representative Western blot of endothelial cell lysates from vTP+ and vTP- rabbits is shown in the top panel. Western blot is representative of three independent experiments. The bottom panel shows the band intensity when the TP receptor expression is normalized to the β-actin expression. Data are the mean ± SEM for three experiments. * $p < 0.05$; vTP+ compared to vTP-

Figure 4.

U46619 inhibits endothelial cell migration of vTP+ but vTP- cells in an in vitro scratch (wound) assay. Wound was applied to cell monolayer treated with U46619 (3 μ M) or vehicle control as described in detail under "Methods". Graphs show a time course of the percentage of scratch area from the initial wound area of 100% at time zero for vTP+ endothelial cells (top panel) and vTP- endothelial cells (bottom panel). Data is presented as the mean $\pm$ SEM for 4 independent experiments. * p < 0.01 U46619-treated vs control

Figure 5.

Release of 6-keto PGF_{1α} from vTP+ and vTP- endothelial cells. Endothelial cells were grown in multiwell plates and incubated with U46619 (3 μM) as described in the Methods. Production of 6-keto PGF_{1α} was measured in the incubation media by radioimmunoassay. Data points are the mean ± SEM for n = 4. * p < 0.05; U46619-stimulated vs control.

Figure 6.

Release of 6-keto PGF_{1α} from vTP+ endothelial cells. Endothelial cells were grown in multiwell plates and incubated with U46619 (3 μM) as described in the Methods. In some cases, the cells were pretreated with the TP receptor antagonist, SQ29548 (10 μM) or the cyclooxygenase inhibitor, indomethacin (10 μM). Production of 6-keto PGF_{1α} was measured in the incubation media by radioimmunoassay. Data points are the mean ± SEM for n = 4. * p < 0.05; U46619-stimulated vs control. # p < 0.05 inhibitor-treated vs U46619 alone.

Figure 7.

Enhanced U46619-induced contractions in denuded aortas from vTP+ rabbits. Vessels with and without an intact endothelium were suspended in organ baths at a resting tension of 2 grams. Concentration-response curves were obtained by the cumulative addition of U46619. Values are mean $\pm$ SEM expressed as % response to KCl (40 mM) for $n = 6$. * $p < 0.05$

TABLE 1

COMPARISON OF EQUILIBRIUM BINDING DATA OF [125 I]-BOP IN AORTIC ENDOTHELIAL CELLS FROM vTP+ AND vTP- RABBITS

	K_d (nM)	B_{max} (fmol/mg)
vTP+	1.6 ± 0.53	56.8 ± 9.7
vTP-	0.79 ± 0.56	$10.7 \pm 5.7^*$

The affinity (K_d) and density (B_{max}) of TXA₂ receptors in cultured endothelial cells were obtained from both vTP+ and vTP- rabbits by incubating tissue with [125 I]-BOP as described in the Methods. Each value represents the mean $\pm$ SEM for n = 5–8.

* p < 0.01; vTP- compared to vTP+.