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ENDOTHELIAL THROMBOXANE RECEPTORS: BIOCHEMICAL CHARACTERIZATION AND FUNCTIONAL IMPLICATIONS

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We have identified thromboxane specific receptors in membrane preparations of bovine pulmonary artery endothelial cells using a potent thromboxane specific antagonist, [^{125}I]-PTA-OH in a binding assay. The binding was specific and saturable. Neither thromboxane B_2, prostaglandin D_2 nor prostaglandin $F_{2\alpha}$ displaced the ligand (0.1 nM) at concentrations up to 10 μM. However, binding was displaced by IPTA-OH > SQ29548 > U46619. In addition, we observed that thromboxane mimetic U46619 significantly lowered the basal production of prostacyclin and also markedly suppressed bradykinin-stimulated prostacyclin released by endothelial cells. We propose that an important biological effect of thromboxane on vascular endothelial cells may be the suppression of prostacyclin production.

Thromboxane A_2 (TXA_2) is a potent platelet aggregating agent and vasoconstrictor and thought to be involved in the pathophysiology of cardiovascular diseases, such as atherosclerosis, thrombosis and angina (1). It has been implicated in a variety of circulatory disorders (2-4) including unstable angina, coronary artery vasospasm, traumatic and endotoxic shock, and acute myocardial infarction. Although the precise mechanism of TXA_2 in platelet aggregation and vascular effects are not yet known, it is believed that these activities are receptor mediated. Because both prostaglandin H_2, a precursor of TXA_2, as well as TXA_2 itself can aggregate and bind to platelets, the putative receptor(s) has been designated as the TXA_2/PGH_2 receptor. The existence of TXA_2/PGH_2 receptors in platelets is substantiated by a variety of radioligand binding studies and by platelet aggregation bioassay (5-11). However, the presence of the putative receptor(s) in vascular tissues is based mainly on studies of vascular smooth muscle

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contraction. Surprisingly, there have been few direct binding studies of thromboxane receptors on endothelial cells and no direct functional studies have been reported. This communication describes the existence of TXA_2 receptors in cultural vascular endothelial cells using a membrane preparation of bovine pulmonary artery endothelial (BPAE) cells and direct ligand binding of a potent TXA_2 specific antagonist, I-PTA-OH. Furthermore, we have identified a new physiologic endpoint for TXA_2 receptor stimulation in endothelial cells, namely thromboxanes inhibit the endothelial synthesis or release of prostacyclin.

MATERIALS AND METHODS

Reagents and Chemicals

Prostaglandin $F_{2\alpha}$, prostaglandin D_2 and thromboxane B_2 were purchased from Biomol Research Laboratories (Philadelphia, PA). [^{125}I]-PTA-OH (specific activity = 2000 Ci/mmol) was obtained from Amersham Corporation (Arlington Heights, Ill). 9,11-dimethylmethano-11,12-methano-16-(3-iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15αß-ω-tetranor-TXA_2 (I-PTA-OH) was kindly supplied by Dr. Perry V. Halushka. [(1S)1α,2ß(5Z), 3ß,4α]-7-[3-[[2-[(phenylamino)carbonyl]-hydrazino]methyl]-7-oxabicyclo[2,2,1] -hept-2-yl]-5-heptenoic acid (SQ29548) was a gift of the Squibb Institute (Princeton, NJ) and (15S)-hydroxy-11α,9α-(epoxymethano)prosta-5Z,13E-dienoic acid (U46619) was purchased from Upjohn Company (Kalamazoo, MI). 6-keto-prostaglandin $F_{1\alpha}$ [^{3}H] RIA Kit for measurement of stable prostacyclin metabolite were purchased from Du Pont (Wilmington, DE). The Dulbecco's modified Eagle's medium (DMEM) and related culture media were prepared by the Media Preparation Lab of Smith Kline and French Labs using materials from Gibco Laboratories (Grand Island, NY). Fetal calf serum (FCS) was obtained from Biochemical Division, Armour Pharmaceutical Company (Kankakee, Ill). All other chemicals used were reagent grade from commercial sources without further purification.

Cell Culture

Bovine pulmonary artery endothelial cells (BPAE) were obtained from the American Type Culture Collection (Rockville, Maryland). The cells were grown in T-150 flasks (Corning Glass Works, Corning, NY) containing 50 ml of Dulbecco's modified minimal essential medium supplemented with 20% (vol/vol) heat inactivated fetal calf serum (DMEM-20%FCS) without the addition of antibiotics. They were seeded at $3x10^6$ cells/T-150 flask and incubated under constant humidity (93%) and CO_2 content (5%) at 37°C and harvested 3-4 days later for experimentation or further cultivation. Harvesting and sub-culturing of the cells were performed by mechanical dislodgement of cell sheet and trituration.

TXA_2/PGH_2 Receptor Binding Assay

BPAE cells between 3-9 passages were scraped gently off the culture flask with a disposable cell scraper (Costar, Cambridge,MA). Cells were washed twice with Dulbecco's calcium-free, magnesium-free phosphate buffered saline (DPBS) solution. The washed cells were suspended in a small volume (2-3 ml) of ice-cold 25 mM Tris-HCl buffer, pH 7.4 (buffer) and homogenized with a Brinkman polytron (model PT 10/35 control unit, PTA7 probe, Brinkmann Instruments Co., Westbury, NY), setting at 7, in a small centrifuge tube surrounded with ice in a beaker. Three homogenations of 20 seconds each were needed to obtain uniform homogenates containing less than 5% intact cells. The homogenates were then centrifuged at 50,000xg for 15 minutes at 4°C. The precipitate was washed twice with intermediate rehomogenation in ice cold buffer. At this stage, no intact cells remained in the homogenates. The binding assay was performed in 12 x 75 mm disposable Borosilicate glass tubes (Kimble, Ill) using a vacuum filtration method. The incubation mixture

consisted of 25 mM Tris-HCl buffer, pH 7.4, radiolabelled ligand (0.1 nM) with or without unlabelled ligand (100 nM) and 100-200 µg of membrane protein. The assay was performed at 4°C for 1 hr which was previously shown to yield equilibrium binding conditions. The incubation was initiated by adding 50 µl of membrane preparation. The total assay medium was 0.25 ml.

The binding was stopped by the addition of 4 ml ice cold buffer followed by rapid filtration through GF/B glass fiber filters under reduced pressure using a Brandel Model M-24S cell harvester (Brandel, Gaitherburg, MD). The filters were washed three times with 4 ml each of buffer, and counted for radioactivity in a gamma counter (Beckman Gamma 5500). All assays were carried out in triplicate or quadruplicate. Binding data are presented as specific binding which is defined as the difference between the binding in the absence and presence of unlabelled I-PTA-OH at 1000-fold excess of the highest ligand concentration used. Protein was measured by the method of Lowry et al (13).

Determination of Prostacyclin (PGI_2) Production by BPAE Cells

Cells were grown in 24 well Linbro plates to approximately 80% confluency. The cells were rinsed 3 times with Puck's Saline F containing 10 mM HEPES, pH 7.4 and were incubated with or without test compounds in the same medium in a final volume of 500 µl for 10 min at 37°C. 25 µl aliquots of the cell supernatant were assayed for the stable metabolite of prostacyclin, 6-keto-$PGF_{1\alpha}$, using the procedure recommended in the RIA Kit. Under these conditions, none of the test compounds interfered with the assay.

RESULTS

Saturable Binding of I^{125}-PTA-OH to BPAE Cell Membranes

Figure 1 illustrates that the specific binding of [^{125}I]-PTA-OH to the membrane preparation of endothelial cells reached a plateau between 5-10 nM. The Scatchard analysis of the binding (Figure 2A) reveals a dissociation constant (k_d) of 1.94 nM and maximal binding (B_{max}) of 26.6 fmol/mg. Figure 2B shows the Hill analysis of I-PTA-OH binding. The Hill coefficient (slope of the fitted line) was found to be 1.095 which was close to unity

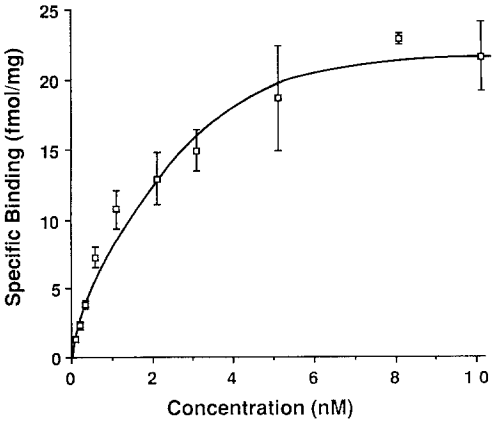

Figure 1. Saturation Curve of [$_{125}$I]-PTA-OH Binding to BPAE Cell Membranes. The binding of a fixed concentration of [^{125}I]-PTA-OH (0.1 nM) to cell membranes of BPAE was measured at equilibrium with increasing concentrations of unlabelled I-PTA-OH up to 1 µM. Bound [^{125}I]-PTA-OH was determined for each point by dividing the cell membrane associated dpm by the calculated specific activity in dpm/mole. Results are mean ± SEM of values obtained from 5 separate experiments done in triplicate.

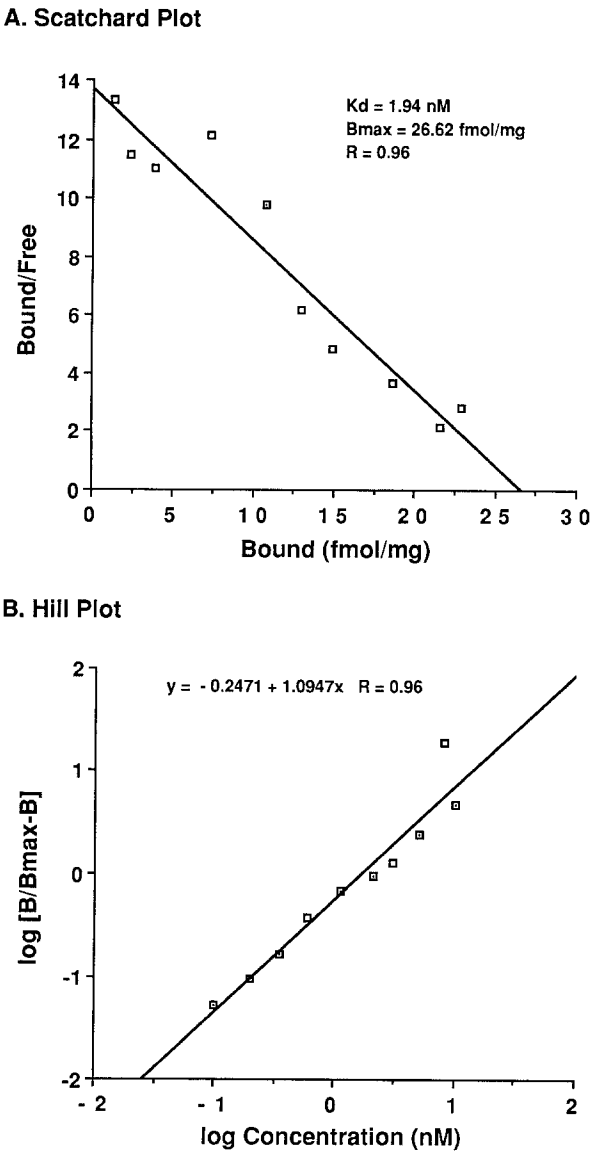

Figure 2. Scatchard Analysis and Hill Plot of [^{125}I]-PTA-OH Binding.
A. Scatchard plot: The line represents a least squares regression of the data (average of 5 separate experiments done in triplicate) from Figure 1.
B. Hill plot: The line was contracted from the data obtained from Scatchard plot (B_{max}) and Figure 1 (specific binding at each concentrations of radio-labelled ligand expressed as B).

suggesting that this ligand binding was to a single class of receptors.

Inhibition of I^{125}-PTA-OH Binding by TXA_2/PGH_2 analogs and Other Prostanoids

Some TXA_2/PGH_2 analogs were examined for their ability to displace the specific binding of [^{125}I]-PTA-OH (0.1 nM). Figure 3 shows that while unlabelled I-PTA-OH, SQ29,548 and the TXA_2 mimetic, U46619 effectively displaced [^{125}I]-PTA-OH binding at low concentrations, $PGF_{1\alpha}$ displaced

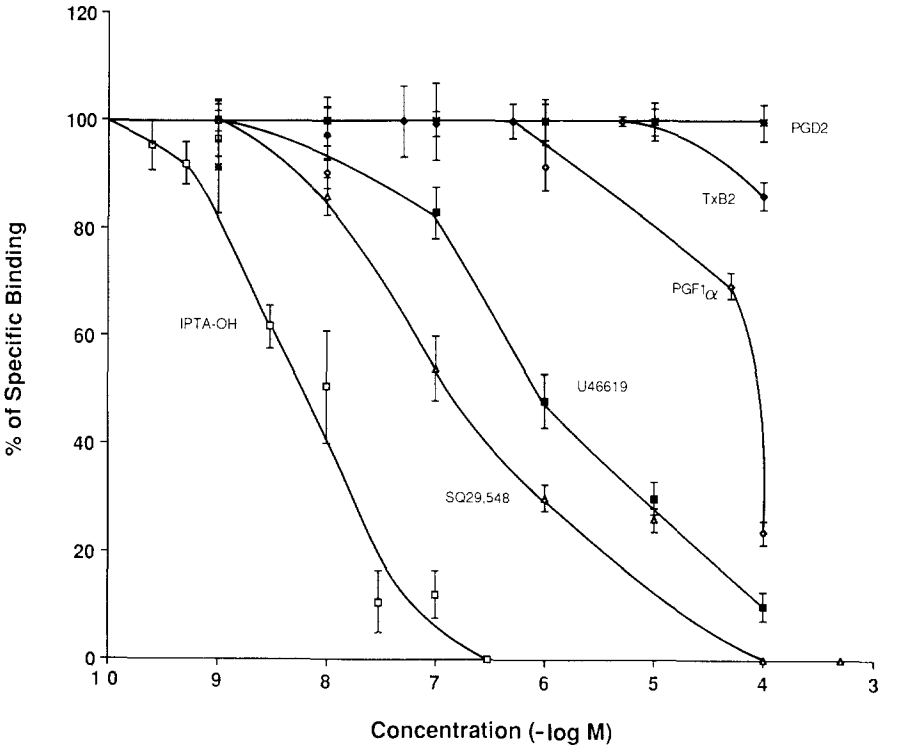

Figure 3. Displacement of the Specific [^{125}I]-PTA-OH Binding by Various Compounds.

The BPAE cell membranes were incubated with 0.1 nM [^{125}I]-PTA-OH in the presence of various concentrations of test compound. The specific binding was obtained by substracting non-specific binding obtained with 1 μM I-PTA-OH from each total binding. Results are mean ± SEM from the data obtained from 6 experiments done in triplicate for I-PTA-OH; 3 experiments for SQ29,548 and U46619 and a single experiment for $PGF_{1\alpha}$, TXB_2 and PGD_2.

ligand binding only at much higher concentrations. PGD_2 and TXB_2 were ineffective at concentrations up to 10^{-4}M. The estimated IC_{50} and K_i values of I-PTA-OH, SQ29,548 and U46619 are summarized in Table 1. The

Table 1. Comparison of Binding Parameters for TXA_2 Analogs

Compound	IC_{50} (μM)[a]	K_i (μM)[b]
I-PTA-OH	0.008	0.007
SQ29,548	0.29	0.263
U46619	1.4	1.270

a IC_{50} is the concentration of inhibitor required to reduce receptor specific binding by 50% and was estimated from the data shown in Figure 3 by a computer program for relative potency problems (Bus P57) documented in the Biostatistic Library.

b Ki is the dissociation constant of the inhibitor and was calculated using the IC_{50} value and dissociation constant (K_d) for [^{125}I]-PTA-OH derived from Scatchard analysis (2 nM) by the Cheng-Prusoff relationship:

$K_i = IC_{50}/[1 + (^{125}\text{I-PTA-OH Conc.}/^{125}\text{I-PTA-OH } K_d)]$

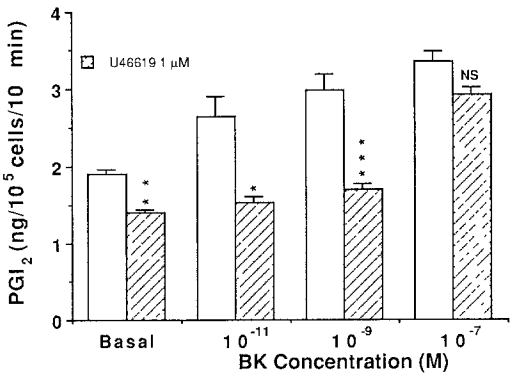

Figure 4. The Effect of U46619 on Basal and BK-Stimulated PGI_2 Production by BPAE Cells.

The BPAE cells were grown and washed as described in the Materials and Methods. They were incubated for 10 min at 37°C with or without bradykinin (BK) in the presence of 1 μM U46619. 25 μl of aliquots of cell supernatant were assayed in duplicate for stable metabolite of PGI_2. Results are mean ± SEM of values obtained from 3-4 separate determinations. * $P<0.05$, ** $P< 0.01$, *** $P< 0.001$ determined by non-paired Student's T-test.

potency of these compounds were I-PTA-OH > SQ29,548 > U46619.

Biological Activity of Thromboxane A_2 Receptor Activation in BPAE Cells

In searching for a possible biological activity of receptor activation, we used the TXA_2 mimetic, U46619 to stimulate the BPAE cells. Figure 4 shows that U46619 (1 μM) significantly lowered the basal production of prostacyclin (PGI_2) by BPAE cells and also inhibited bradykinin-stimulated PGI_2 production. At higher concentrations of BK (10^{-7}M), U46619 was however unable to lower PGI_2 to the control level. The suppression of PGI_2 by U46619 could be effectively blocked by TXA_2 specific antagonists (Table 2). Thus, I-PTA-OH (Expt. 1) and SQ29,548 (Expt. 2) blocked the suppression of PGI_2 by U46619. These data suggest that U46619 suppressed PGI_2 production by the cells via TXA_2/PGH_2 receptor activation.

DISCUSSION

It is well known that TXA_2 is a potent platelet aggregating agent and vasoconstrictor (14). Despite the fact that the receptors for TXA_2 have been identified and characterized extensively in platelets using both bioassay (platelet aggregation) and direct radio-ligand binding assay, the identification of TXA_2 receptors by a direct binding assay in vascular tissues was not reported until very recently by Hanasaki et al (12). Endothelial cells are located at a pivotal site in the vascular wall and may therefore play an important role in the control of vascular tone by the release of vasodilators such as endothelium derived relaxing factor (EDRF) (15) and prostacyclin (16), vasoconstrictors (17,18) including endothelin (19). In order to understand the mechanism of action of TXA_2 in vascular

Table 2. The Suppression of Endothelial Cell Production of Prostacyclin by U46619 Was Blocked by Thromboxane A_2 Antagonists

Conditions	PGI_2 Production ($ng/10^5$ cells/10 min)	Effect of U46619 (% of Suppression)
Expt. 1		
Basal	2.017 ± 0.135 (18)	
+ U46619	1.641 ± 0.096 (14)	(19 ± 4)*
+ SQ29548	2.155 ± 0.057 (4)	
+ I-PTA-OH	2.295 ± 0.095 (4)	
+ U46619 + SQ29548	2.078 ± 0.138 (4)	
+ U46619 + I-PTA-OH	2.585 ± 0.117 (4)	
BK (10^{-9}M)	3.286 ± 0.196 (7)	
+ U46619	2.105 ± 0.143 (7)	(36 ± 4)***
+ I-PTA-OH	2.961 ± 0.084 (4)	
+ U46619 + I-PTA-OH	3.024 ± 0.157 (4)	
Expt. 2		
BK (10^{-8}M)	5.082 ± 0.471 (4)	
+ U46619	1.844 ± 0.147 (4)	(64 ± 3)***
+ U46619 + SQ29548 (10^{-5}M)	5.348 ± 0.514 (4)	

All compounds were used at final concentrations of 10^{-6}M except those specified in parenthesis. Expt. 1: Cells were pretreated with and without antagonist for 5 min, BK or BK plus U46619 was then added. They were further incubated for 10 min. Expt. 2: Cells were pretreated with or without antagonist for 10 min, medium removed, and fresh medium containing either U46619 or U46199 plus antagonist was added. They were further incubated for 10 min. 25 µl aliquots of cell supernatant were assayed (in duplicate) for 6 keto $PGF_{1\alpha}$. Results are mean ± SEM (n=number of observations).
* $P<0.05$, *** $P< 0.001$ determined by non-paired Student's T- test

tissues it is important to identify TXA_2 specific receptors in endothelial cells. In addition it is helpful to use a tissue source readily available and widely used by other investigators so we utilized bovine pulmonary artery endothelial cells. This communication describes the presence of TXA_2 receptors in endothelial cells by radio-ligand binding of the potent TXA_2 specific antagonist ^{125}I-PTA-OH to BPAE membranes. The binding of [^{125}I]-PTA-OH to membranes of BPAE cells has the following characteristics: (1) it was time- and membrane concentration-dependent (data not shown); (2) it has relatively high affinity (Kd = 2 nM, Figure 2A); (3) it was saturable (Figure 1) and has relatively high specific binding (60-70 % of total binding when the radio-ligand was used at 0.1 nM (data not shown) and (4) the binding was selective, displaceable only by unlabelled I-PTA-OH and the TXA_2 specific antagonist SQ29,548 and TXA_2 mimetic, U46619 but not by $PGF_{1\alpha}$, PGD_2 or TxB_2 at comparable concentrations (Figure 3).

To our knowledge, the biological implications of TXA_2 receptor activation in vascular endothelial cells have not yet been elucidated. We have tested the hypothesis that TXA_2 may suppress the release of vasodilators from vascular endothelial cells, resulting in an enhanced vasoconstriction of blood

vessels. We found that the TXA_2 mimetic U46619 did not affect BPAE cells to release EDRF in response to BK or A23187 (unpublished observations) using the superfusion method for the detection of EDRF described previously by Shikano and Berkowitz (20). In contrast, U46619 suppressed the basal as well as BK-stimulated PGI_2 production by cultured endothelial cells (Figure 4). Furthermore, the suppression of PGI_2 by U46619 could be blocked by TXA_2 specific antagonists, I-PTA-OH or SQ29,548 (Table 2). Thus, thromboxanes may contract vascular smooth muscle not only by a direct effect on muscle cells, but also by inhibiting the synthesis and release of the vasodilator prostacyclin. In addition, these data may explain the great efficacy of thromboxanes to cause platelet aggregation and vascular smooth muscle contraction, as the ameliorating effects of prostacyclin on these phenomena would be reduced as a result of its diminished release from the endothelium.

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