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STIMULATION OF PROSTACYCLIN SYNTHESIS BY THROMBOXANE A_2-LIKE PROSTAGLANDIN ENDOPEROXIDE ANALOGUES IN CULTURED VASCULAR SMOOTH MUSCLE CELLS

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Summary: In this study, the ability of two chemically stable thromboxane A_2-like PG endoperoxide analogues, 15S-hydroxy-9α,11α-(epoxymethano)-prosta-5Z,13E-dienoic acid and 15S-hydroxy-11α,9α-(epoxymethano)-prosta-5Z,13E-dienoic acid, to stimulate PGI_2 synthesis by cultured vascular smooth muscle cells isolated from rat superior mesenteric arteries was evaluated. The aforementioned analogues, at concentrations of 0.1 to 10μg/ml, stimulated PGI_2 synthesis by 1.5 to 3 fold over basal synthesis. Evoked PGI_2 synthesis was essentially over within 2 to 3 min of incubation, similar to previous findings made in vascular smooth muscle cells incubated with peptide hormones, vasopressin and angiotensin II. The PG-stimulatory activity of 15S-hydroxy-9α,11α-(epoxymethano)-prosta-5Z-13E-dienoic acid appeared to be receptor-mediated inasmuch as it was completely inhibited by (±)5-endo-(6'-carboxyhex-2'Z-enyl)-6-exo-{1"-[N-(phenylthiocarbamoyl)-hydrazono]-ethyl}-bicyclo[2,2,1] heptane, a novel antagonist of PG endoperoxide analogue-provoked smooth muscle contraction and platelet aggregation. The results suggest that thromboxane A_2 and/or PG endoperoxide may stimulate PGI_2 synthesis in vascular smooth muscle by a direct, receptor-mediated, interaction.

Activated platelets synthesize a mixture of two potent vasoconstrictor eicosanoids, thromboxane A_2 and PG endoperoxides. Originally termed rabbit aorta contracting substance (1), these agents, in addition to their ability to cause platelet aggregation are now well established as having potent vasoconstrictive effects on a variety of vascular and non-vascular smooth muscle tissues (2).

We recently communicated that two vasoconstrictor peptides, vasopressin and angiotensin II, stimulate the synthesis of a potent vasodilator eicosanoid, PGI_2, by cultured vascular smooth muscle cells (3). The purpose of the present

Abbreviations : U 44069: 15S-hydroxy-9α,11α-(epoxymethano) prosta-5Z,13E-dienoic acid; U 46619: 15S-hydroxy-11α,9α-(epoxymethano) prosta-5Z,13E-dienoic acid; EP-092: (±)5-endo-(6'-carboxyhex-2'Z'enyl)-6-exo-{1"-[N-(phenylthiocarbamoyl)-hydrazono]-ethyl}-bicyclo[2,2,1] heptane.

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study was to evaluate the abilities of two stable PG endoperoxide/thromboxane A_2 analogues, U 44069 and U 46619, which have thromboxane A_2-like vasoconstrictor activity (4,5), to stimulate the synthesis of PGI_2 by cultured vascular smooth muscle cells isolated from rat superior mesenteric arteries. We also assessed whether the PG-stimulatory effect of the aforementioned vasoconstrictors could be inhibited by EP-092, a pharmacologic antagonist of PG endoperoxide/thromboxane A_2 analogue activity (6).

Materials and Methods

The following were purchased from Sigma Chemical Co. (St. Louis, MO): collagenase (800 U/mg), elastase (120 U/mg), DNAse I(2790 U/mg), purified soybean trypsin inhibitor, 6-keto-$PGF_1\alpha$. EP-092 was obtained from Dr. R.L. Jones (University of Edinburgh, U.K.). U 44069 and U 46619 were purchased from Cayman Chemicals (Denver, CO). Medium 199 and HEPES-supplemented Eagle's medium were purchased from K-C Biologicals (Lenexa, KS). Fetal bovine serum and culture wells were purchased from M.A. Bioproducts (Walkersville, MD) and Costar (Cambridge, MA), respectively. Tritiated 6-keto-$PGF_1\alpha$ (100-200 Ci/mmol) was from New England Nuclear (Boston, MA) or Amersham (Arlington Heights, IL). 6-keto-$PGF_1\alpha$ found in supernatant media was measured by a radioimmunoassay which has been previously described (3). The sensitivity and cross-reactivity characteristics of this assay were as follows: approximately 30 pg of nonradioactive 6-keto-$PGF_1\alpha$ was required to inhibit binding of (^{3}H)-6-keto-$PGF_1\alpha$ by 50%. Standard curves were made linear by a log-logit transformation and calculated by a least-square regression analysis. Each sample was assayed, in triplicate, over an 8-fold concentration range to ascertain that the dilution of immunoreactive material paralleled that of standard 6-keto-$PGF_1\alpha$. Inter- and intraassay coefficient of variation were 10% and 5%, respectively. Cellular protein was estimated by the method of Lowry et al (7), using bovine serum as standard.

Mesenteric arterial smooth muscle cells were isolated essentially as described by Ives et. al. (8). Briefly, male Sprague-Dawley rats weighing between 200 and 400 g were anesthetized with nembutal, the superior mesenteric artery dissected out, freed of adventitia and incubated for 15-20 min at 37°C with shaking in a modified Krebs-Ringer buffer having the following composition (in mM): NaCl, 105; KCl, 5; KH_2PO_4, 1; HEPES, 25; $MgSO_4$, 1; glucose, 14; $CaCl_2$, 0.2; $NaHCO_3$, 25. This medium was further supplemented with collagenase (360 U/ml), elastase (96 U/ml), DNAse (56 U/ml) and soybean trypsin inhibitor (1 mg/ml). Cells which had dissociated during the first 15 to 20 min were discarded because they were likely to constitute a fraction enriched in adventitial fibroblasts as well as endothelial cells. The medial smooth muscle cells were harvested by a second 15 to 20 min incubation of the remaining portion of blood vessel followed by centrifugation and filtration of centrifuged cells through a 150 µm nytex filter before the cells were allowed to attach to culture wells in the presence of medium 199 lacking fetal bovine serum. After 2 hr, most cells were adherent and could be further cultured in antibiotic-free medium 199 which had typical smooth muscle morphology by light and electron microscopy (results not shown). Cells were subcultured by treatment with 0.08% trypsin-EDTA. For the present experiments, we used different cell strains which were in subcultures 3 to 7. We routinely changed culture media 1 day before the experiment and used cells at less-than-confluent densities of 150 to 300 µg cell protein per 35 mm diameter culture well (corresponding approximately to $3x10^5$ to $6x10^5$ cells per well). We also found it important to wash the cells in the gentlest possible way as vigorous washing increased basal PG synthesis and resulted in lower vasoconstrictor-elicited PG synthesis. We used Eagle's minimal medium supplemented

with 25 mM HEPES for all experiments. All incubations were done at 37°C in media previously equilibrated with an atmosphere of 5% CO_2-95% air.

Statistical analyses of data were done using paired Student's t-test. $p<0.05$ was considered as statistically significant.

Results and Discussion

PGI_2, a PG made primarily in blood vessels is a potent eicosanoid vasodilator of mesenteric arteries as well as numerous other blood vessels (9,10). In addition, it is an effective inhibitor of platelet aggregation (11). On the other hand, TxA_2 made predominantly by platelets is a potent eicosanoid vasoconstrictor and platelet aggregator (2,12). However, because of its extreme lability, a common approach to the study of TxA_2 has been to utilize thromboxane-mimetic compounds rather than the parent compound itself. Derivatives of PGH_2 which have the chemically stable 9,11- or 11,9-epoxymethano moiety (U 44069 and U 46619) have been established to have thromboxane A_2-like constrictor activity in vascular and non-vascular smooth muscle (5). We therefore chose to use these stable thromboxane-like agents to establish whether they stimulated PG synthesis in cultured vascular smooth muscle cells isolated from rat mesenteric arteries. In this communication we present evidence which shows that PG endoperoxide analogues U 44069 and U 46619 have the ability to elicit PGI_2 synthesis from cultured vascular smooth muscle cells.

As observed for peptide vasoconstrictors, vasopressin and angiotensin II (3), U 44069 elicits a rapid burst of PGI_2 synthesis (measured as its stable product, 6-keto-$PGF_{1\alpha}$) which is mostly over by 2 min of incubation (Figure 1). The stimulation of PG synthesis is dose-dependent, reaching a maximum of about 1.5 to 3 fold over basal at 0.1 to 1 µg/ml of U 44069 or U 46619 (Figure 2). An increase of U 44069 concentrations from 1 to 10 µg/ml elicits a decrease of PG synthesis, which nevertheless remains statistically different from basal synthesis (Figure 2). In addition, U 44069 appears more effective than U 46619 as a stimulator of PG synthesis (Figure 2). In comparison, U 46619 was found to be slightly more effective than U 44069 as a constrictor of rabbit aorta *in vitro* (13).

The PG-stimulatory effect of U 44069 appears to be receptor-mediated inasmuch as a novel PG endoperoxide analogue, EP-092, recently established as having

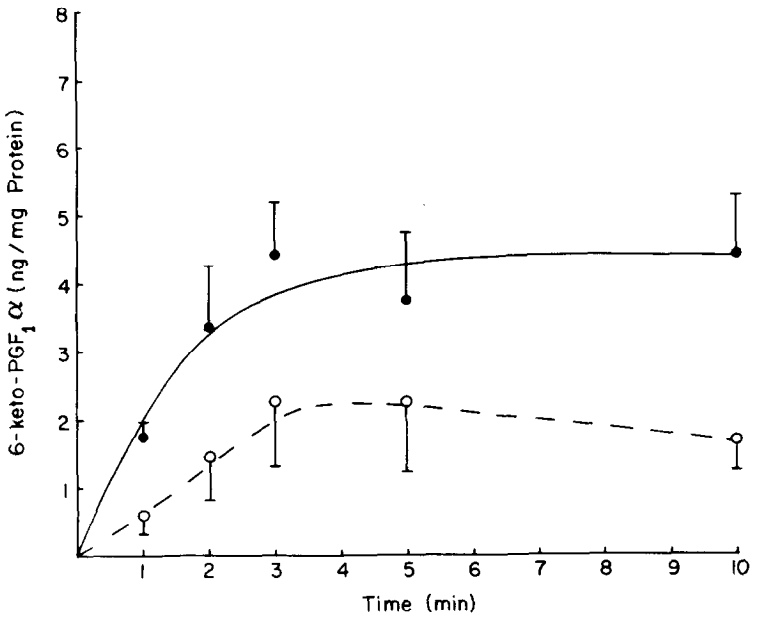

Figure 1. Time-course of U 44069-evoked PG synthesis by cultured vascular smooth muscle cells. The concentration of U 44069 was 1 µg/ml. Closed circles: U 44069-containing incubations. Open circles: control incubations. Results are mean ± S.E. of 4 separate experiments in duplicate. All U 44069-containing incubations were statistically different from equivalent control incubations.

thromboxane antagonistic effects in platelets and bronchial smooth muscle (6), completely inhibits U 44069-evoked PG synthesis while having no effect on basal PGI_2 synthesis (Table 1).

Some years ago, it was proposed by Bunting et al (14) that platelet-derived PG endoperoxides could be transformed into PGI_2 by endothelial cells and that so-formed PGI_2 would then limit further platelet aggregation. Although several subsequent studies did not concur with the concept of shunting of PG endoperoxides from platelets to endothelial cells (15,16), more recent evidence obtained by Schafer et al using cultured endothelial cells does support the aforementioned hypothesis (17). Our study suggests that a second homeostatic mechanism involving vascular smooth muscle cells may be operating as well. We speculate that the ability of TxA_2 analogues to stimulate PG synthesis by vascular smooth muscle cells is part of a vascular homeostatic mechanism in which enhanced PGI_2 synthesis may buffer vasoconstriction and/or platelet aggregation. However, in addition to the PG endoperoxide shunting mechanism, our results suggest that TxA_2 and/or PG

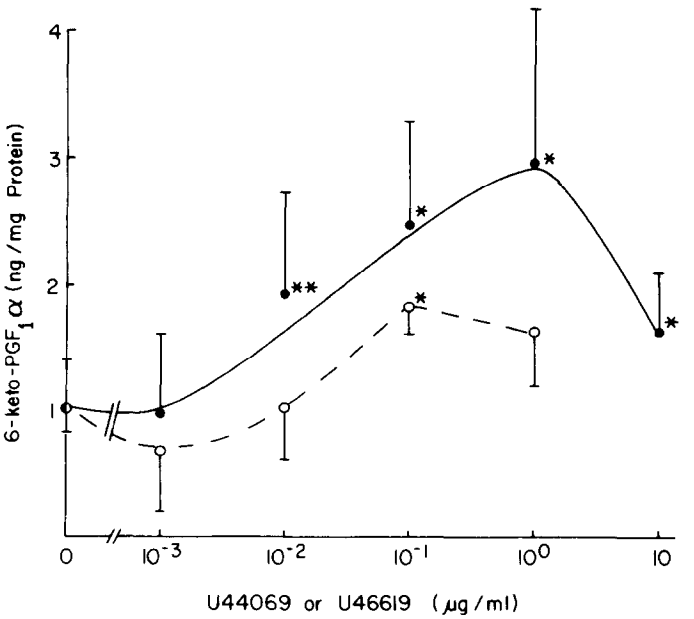

Figure 2. Dose dependence of U 44069 (closed circles) and U 46619-evoked PG synthesis by cultured vascular smooth muscle cells. Incubation time was 2 min. Results are mean ± S.E. for 4 to 5 separate experiments, in duplicate. *p<0.01; **p<0.05.

endoperoxides also have the ability to directly stimulate endogenous vascular smooth muscle cell PG synthesis in what appears to be a receptor-mediated mechanism. The end-result would of course be the same as in the endoperoxide shunting hypothesis, that is the generation, in response to TxA_2 and/or PG endoperoxides, of anti-constrictor and anti-aggregatory PGI_2 by a component of the blood vessel, in this case smooth muscle rather than endothelium.

Table 1. Inhibition of U-44069-provoked PGI_2 synthesis in cultured vascular smooth muscle cells by endoperoxide antagonist, EP-092

Effector	6-keto-PGF$_{1\alpha}$ (% of control)
U 44069	293 ± 38 (p<0.01)
U 44069 + EP-092	128 ± 28 (p>0.05)
EP-092	88 ± 7 (p>0.05)

Cells were preincubated for 5 min with or without EP-092 (1 μg/ml), followed by incubation for 2 min in the presence or absence of U 44069 (0.1 μg/ml) and EP-092 (1 μg/ml). Results of 3 experiments in duplicate (mean ± S.E.) are given. Statistical comparisons were made by paired t-test, relative to control incubations lacking effectors.

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