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Stimulation of prostaglandin E synthesis in cultured human umbilical vein smooth muscle cells

(angiotensin II/bradykinin/histamine/indomethacin/vascular smooth muscle)

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ABSTRACT **Cultured human umbilical vein smooth muscle cells secreted 1.26 ± 0.20 μg of immunoreactive prostaglandin E (iPGE) per mg of cell protein per 24 hr (mean ± SEM). Indomethacin, an inhibitor of prostaglandin synthesis, abolished greater than 95% of this basal iPGE production (ID_{50} = 1.8 nM). The vasoactive mediators bradykinin, angiotensin II, and histamine stimulated iPGE production in a dose-dependent manner (maximal levels 10-, 3.5-, and 2.3-times basal, respectively) during 2 hr incubations in serum-free medium. Serotonin (5-hydroxytryptamine) elicited small (less than 50%) increases, whereas norepinephrine was inconsistent in stimulating iPGE production. Exogenous prostaglandins are extremely potent vasoactive substances, which can increase or decrease the tone of resistance and capacitance vessels. Endogenous prostaglandin production in vascular smooth muscle cells, therefore, may represent an intrinsic control mechanism affecting basal tone and modulating the response of blood vessels to vasoactive mediators. Cell culture techniques may prove to be useful generally in the study of the biochemical events involved in agonist-vascular smooth muscle receptor interaction.**

Prostaglandins (PG) are a group of vasoactive unsaturated 20-carbon fatty acids, which can be synthesized by virtually all mammalian tissues, and are thought to exert their biological effects primarily near their locus of origin (1). Intravascular infusion of PG can produce profound changes in vascular tone which varies qualitatively and quantitatively depending upon the type of PG, the anatomical site, and the species (2). It has been suggested therefore that synthesis of endogenous PG potentially might attenuate or amplify the response of blood vessels to humoral and neural stimuli, thus serving to modulate local vascular tone (3).

Although PG have been detected in the venous blood from various tissues and organs, the specific cellular origin(s) of these endogenous PG, factors controlling their biosynthesis, and the biochemical and physiologic implications of this biosynthesis have not been clearly defined. It has been suggested that the vascular network itself might be an important source of PG production (4). Recent data on the *in vitro* stimulation of PG synthesis in bovine mesenteric arteries and veins by bradykinin support this view (1). Blood vessels *per se*, however, are heterogeneous tissues consisting of several cell types, including endothelium, smooth muscle, fibroblasts and neural elements. Recently, we have demonstrated angiotensin II stimulation of prostaglandin E production in cultured vascular endothelium (5), and others have suggested that fibroblasts (6) and neural elements (7) also may produce PG. Smooth muscle, however, is the principal structural and contractile component of resistance and capacitance vessels; changes in the synthesis of PG by these cells, therefore, may be important in the local control of vascular tone. Consequently, we have isolated homogeneous populations of human vascular smooth muscle in tissue culture and studied the effect of various vasoactive mediators on prostaglandin E production by this cell type.

Abbreviations: PG, prostaglandins of type A, B, E, or F; iPGE, immunoreactive prostaglandin E.

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MATERIALS AND METHODS

Reagents. [^{3}H]Prostaglandin E_1 (specific activity, 90–110 Ci/mmol), which was shown to be chromatographically pure (>98%), was obtained from New England Nuclear Corp. (Boston, Mass.). Silicic acid (*SILICAR*, CC-4, 200–325 mesh, Mallinckrodt, St. Louis, Mo.) was heat activated at 105° overnight before use. Charcoal and dextran (radioimmunoassay grade) were purchased from Schwarz/Mann Division, Becton-Dickinson (Orangeburg, N.Y.). All organic solvents were spectral grade (Burdick-Jackson Co., Muskegon, Mich.). Prostaglandins were generously provided by the Upjohn Co. (Kalamazoo, Mich.) and indomethacin by Merck, Sharp and Dohme (West Point, Pa.). Vasoactive mediators included: angiotensin II (Hypertensin-CIBA); bradykinin triacetate and histamine diphosphate (SIGMA); (−)norepinephrine bitartrate and serotinin creatinine sulfate (5-hydroxytryptamine) (Calbiochem). Medium 199 and Dulbecco's phosphate-buffered saline were obtained from the N.I.H. media unit. Ten per cent fetal calf serum was obtained from Microbiological Associates (Bethesda, Md.).

Vascular Smooth Muscle Cultures. Morphologically homogeneous populations of smooth muscle cells, free of contamination by other elements, such as leukocytes, fibroblasts, or endothelium, were cultured from the walls of term umbilical cord veins, as previously described (8). The identity of cultured cells was monitored by electron microscopy, which demonstrated bundles of myofilaments and other ultrastructural features characteristic of vascular smooth muscle *in vivo* (9). Cultures were maintained in Medium 199 supplemented with 10% fetal calf serum (M199 + 10% FCS) under a humid atmosphere consisting of 5% CO_2 in air; each liter of medium also contained 15 mmol of Hepes buffer [(*N*-2-hydroxy)ethylpiperazine-*N*′-2-ethanesulfonic acid, Calbiochem Corp., San Diego, Calif.] adjusted to pH 7.40 with NaOH, 60 mg of penicillin and 120 mg of streptomycin. In all experiments, cells at the second to fourth passage were replicate plated in Linbro plastic culture trays (FB-16-24-TS, Bellco Glass, Inc., Vineland, N.J.) and allowed to grow to confluent densities (4 to 6 × 10^4 cells per cm^2). Before and after incubation with test media, all cultures were examined for signs of cytotoxicity by phase contrast microscopy. For prostaglandin determinations, media samples from duplicate or triplicate culture wells were removed and kept frozen until assayed. For protein determinations, the cells in each culture well were rinsed twice with Dulbecco's

phosphate-buffered saline, resuspended by scraping into 1.0 ml of distilled H_2O, and disrupted by sonication (Branson Cell Disruptor, Ultrasonics, Inc., Plainview, N.Y.). Proteins were measured by the Lowry *et al.* method (10).

Prostaglandin Determinations. Immunoreactive prostaglandin E (iPGE) was measured by a radioimmunoassay method previously described in detail (11). Briefly, PG were extracted from culture media after acidification to pH 3.5 with formic acid into ethyl acetate. Group separation of prostaglandins type A, E, and F (PGA, PGE, and PGF, respectively) was achieved by silicic acid column chromatography. The PGE fraction was quantitatively converted to PGB by treatment with 0.1 M methanolic-KOH and assayed using an antiserum generated in sheep against PGA_1. At a final dilution of 1:75,000 this antiserum bound [^{3}H]PGB_1 and gave a standard curve ranging from 0.008 to 2.0 ng of PGB_2. Fifty percent inhibition of binding by PGB_2, PGB_1, PGE_2, PGE_1, and $PGF_{2\alpha}$ occurred with 0.5, 0.09, 12.0, 5.5, and greater than 100 ng, respectively. Recoveries of known amounts of PGE_2, ranging from 0.8 to 5.6 ng, added to plasma and handled in this manner, were 104 ± 19.8% (mean ± 1 SD), with a mean absolute error of 0.22 ng over the entire range. This assay procedure measures both PGE_1 and PGE_2, which we refer to as iPGE.

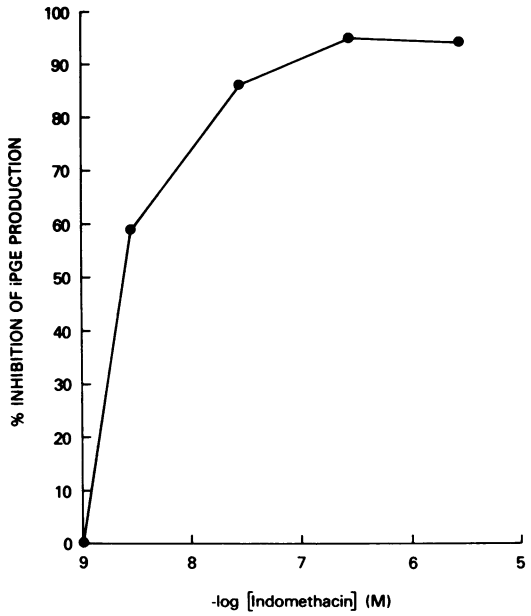

FIG. 1. Inhibition of iPGE production by indomethacin. Mean values from two separate experiments are plotted.

RESULTS

Basal iPGE production

Complete medium (M199 + 10% FCS) incubated with confluent vascular smooth muscle cultures for 24 hr contained 66.5 ± 11.3 ng (mean ± SEM, seven experiments) iPGE per ml (1.26 ± 0.02 μg of iPGE per mg of cell protein). Fresh culture medium and medium incubated in the absence of cells contained less than 0.2 ng of iPGE per ml.

Inhibition of iPGE synthesis

Prostaglandins do not appear to be stored in tissues; therefore, release is taken as evidence of *de novo* synthesis (12). To confirm that the extracellular accumulation of iPGE in our vascular smooth muscle cultures reflected synthesis, rather than release of preformed material, indomethacin, a potent, and relatively specific, inhibitor of prostaglandin synthesis (12), was added to the culture medium (M199 + 10% FCS) at the beginning of a 24 hr incubation. Basal iPGE production was inhibited in a dose-dependent manner (Fig. 1), and virtually abolished by 0.28 μM indomethacin (infective dose, ID_{50} = 1.8 nM). Ethanol, used initially to dissolve indomethacin before dilution in culture medium, had no effect on basal iPGE production. Cytotoxic changes were not observed by phase-contrast microscopy in cultures exposed to indomethacin at any of the concentrations tested.

Effects of vasoactive mediators on iPGE production

To study the effects of certain vasoactive mediators on iPGE production by vascular smooth muscle, confluent cultures plated in replicate were incubated with varying concentrations of each agonist for 2 hr at 37°. Serum-free medium 199 was utilized in order to minimize potential degradation and/or protein binding of added agonists and secreted prostaglandins, as well as to avoid the introduction of any unknown vasoactive substances present in serum. Serum-free medium 199, before and after incubation at 37° in the absence of cells, did not contain detectable levels of iPGE.

Angiotensin II and histamine both stimulated iPGE production in a dose-dependent manner (Fig. 2). Thus, angiotensin II increased iPGE production from control values of 0.094 ± 0.01 to 0.337 ± 0.079 ng/μg of protein per 2 hr at 0.5 μM (five experiments). Histamine increased iPGE release from a basal level of 0.102 ± 0.01 to 0.233 ± 0.032 ng/μg of protein per 2 hr at 1.5 μM (three experiments). In preliminary experiments, bradykinin appeared to be extraordinarily potent in stimulating iPGE production. As seen in Fig. 3, up to 10-fold increases resulted at bradykinin concentrations ranging from 4 nM to 0.4 μM.

Serotonin (5-hydroxytryptamine) elicited small increases in iPGE production, from basal levels of 0.118 ± 0.007 to 0.169 ± 0.006 ng/μg of cell protein per 2 hr (three experiments) at 1.2 μM concentrations. In three experiments norepinephrine, over the dose range of 0.01 to 1 μM, did not produce consistent stimulation of iPGE production.

None of the above agonists, at any of the concentrations tested, significantly suppressed the basal production of iPGE or produced phase microscopic evidence of cytotoxicity.

DISCUSSION

Human umbilical vein smooth muscle, cultured free of contamination by other cell types, generates an immunoreactive prostaglandin E-like material which accumulates in the extracellular medium. This iPGE production is inhibited by indomethacin, a noncompetitive blocker of prostaglandin synthesis, at concentrations comparable to those which are effective in other isolated cell systems (5, 13). Basal iPGE production is stimulated by histamine, angiotensin II, and bradykinin, in order of increasing potency (molar basis), while serotonin and norephinephrine are relatively ineffective. The presence of heterologous serum did not appear to influence the basal rate of iPGE production. In comparison to endothelial cells cultured from the same source (5), the basal production of iPGE by smooth muscle cells is approximately 20 times greater (per mg of cell protein). Since the smooth muscle cell is a major structural component of blood vessels, PG production by this cell type may be an important determinant of PG concentration within the vascular wall.

Recently, much attention has been directed to the role of PG generated within blood vessel walls in modulating vascular tone.

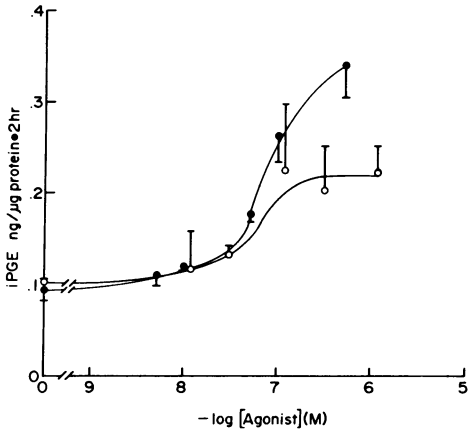

FIG. 2. Stimulation of iPGE production by angiotensin II in five experiments (●—●), and histamine in three experiments (O—O). Stimulation by both agonists was statistically significant (t = 0.03). Each experimental point was paired with the control point and the sign test was utilized. Half-bars indicated 1 SEM.

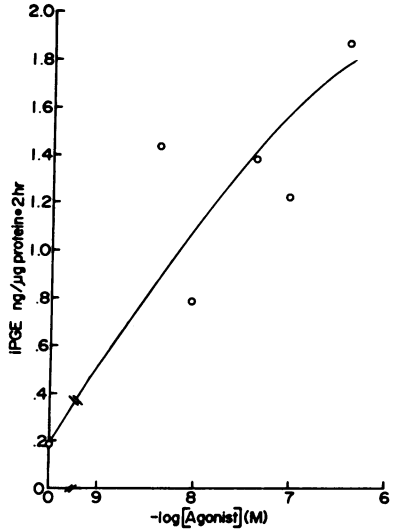

FIG. 3. Effect of bradykinin on iPGE production. Each point represents results obtained from duplicate smooth muscle cultures.

Aiken has shown that indomethacin can reverse angiotensin II induced tachyphylaxis in isolated rabbit celiac arteries (14). Subsequently, he also demonstrated that angiotensin II will cause a partial relaxation of norepinephrine induced contraction in the same preparation (15). Indomethacin blocked this effect, as well as relaxation directly induced by bradykinin. Although PG were not measured, the following points can be inferred from these data: (*1*) angiotensin II and bradykinin both stimulate the production of a vasodilator PG (presumably PGE_1 or PGE_2) in rabbit celiac arteries; (*2*) this PG appears to antagonize the vasoconstrictor action of angiotensin II, and mediates or amplifies the vasodilator action of bradykinin; (*3*) norepinephrine, a potent vasoconstrictor, does not significantly stimulate the production of a vasodilator PG.

Terragno and her coworkers have directly measured PG production in isolated bovine mesenteric blood vessels (1). Basal production of PGE was greater than PGF in both arteries and veins. However, bradykinin selectively stimulated PGE production in arteries and PGF production in veins. Since exogenous PGE and bradykinin both dilate arteries, whereas exogenous PGF and bradykinin constrict veins, they concluded that the effects of bradykinin on vascular contractility may be mediated, at least in part, by the stimulation of endogenous PG production within blood vessel walls.

Implicit in the conclusions of the above studies is the assumption that intramurally generated PG have direct access to the smooth muscle cell, which is the functional contractile component of the vessel wall. However, as emphasized previously, blood vessels are heterogeneous tissues consisting of various cell types. We have demonstrated the capacity of the smooth muscle cell for PG synthesis in culture, as well as for stimulated production in response to certain vasoactive mediators. The concept of endogenous PG synthesis as a determinant of local blood flow in organs such as the heart (11, 16), kidney (17), and uterus (18) can now be extended to control systems at the level of the vascular smooth muscle cell.

The mechanism(s) of stimulation of PG synthesis by vasoactive mediators is unclear. Human umbilical veins are strongly contracted *in vitro* by serotonin, bradykinin, and histamine, but only weakly by norepinephrine and angiotensin II (19). When the contractile efficiency of each of these agonists is compared with its ability to stimulate iPGE production in smooth muscle cultures, no correlation is apparent. This suggests that the observed iPGE stimulation is not a concomitant of the contractile process *per se*. Data from other sources support this conclusion. Inhibition of PG synthesis does not enhance the contractile action of norepinephrine on rabbit celiac arteries (15); these vessels are normally relaxed by PGE. When spirally cut rat aortic strips are exposed to equipotent contractile doses of norepinephrine and angiotensin II, only the latter agonist induces a significant increase in basal iPGE production (R. W. Alexander, P. V. Halushka, and J. Fleisch, unpublished data). However, the dose-dependent stimulation of iPGE production observed with certain of the agonists in the current study does indicate that specific receptor systems in the smooth muscle cell may be associated with control points in PG biosynthetic pathways.

The availability of free fatty acid precursors is thought to represent a rate limiting step in PG synthesis (20). Recent studies (21) have suggested that activation of an acylhydrolase and release of arachidonic acid from endogenous triglyceride may be involved in angiotensin II stimulation of PGE_2 synthesis in the rat renal papilla. In the present study, stimulation of iPGE synthesis by certain vasoactive mediators was observed in serum-free media which contained no free fatty acids. The substrate for PG synthesis therefore must have been derived from sources within the smooth muscle cell, presumably through the action of a phospholipase or acylhydrolase. The nature of the association between receptors for vasoactive hormones and the lipolytic enzymes which control the availability of substrate for PG synthesis warrants further investigation. It is also possible that bradykinin, angiotensin II and histamine act at other points in the PG biosynthetic and metabolic pathways to control PG release.

The present data indicating the failure of norepinephrine to stimulate iPGE synthesis in cultured vascular smooth muscle cells are in apparent conflict with *in vivo* data suggesting that norepinephrine infusion stimulates PGE release from several vascular beds (22–24). As emphasized previously, however, the cellular origins of the PG in venous blood cannot be determined. PG release by many cell types could be induced secondary to hemodynamic and metabolic changes, and cannot be related necessarily to agonist–vascular smooth muscle receptor interactions. This point further illustrates the unique advantages of studying the biochemical consequences of vascular smooth muscle receptor activation in cell culture.

The physiologic consequences of iPGE stimulation by va-

soactive agonists in umbilical vein smooth muscle are not clearly defined by the present study. Exogenous E-type prostaglandins can produce profound changes in vascular tone which vary with the species and anatomic site. In general, PGE_1 and PGE_2 promote vasodilation. In human umbilical vessels, however, PGE_1 vasodilates, whereas PGE_2 vasoconstricts (25). The methodology used in this study does not differentiate between PGE_1 and PGE_2.

Basal endogenous PG synthesis by vascular smooth muscle cells, and its stimulation by vasoactive substances, may represent an intrinsic mechanism within the vascular wall for modulating the contractile state and thus the resistance of arteries or the capacitance of veins. Application of this concept to a given blood vessel requires that three kinds of data be obtained: First, the direct action of a given nonprostaglandin agonist (contraction or relaxation); second, the direct action of exogenous prostaglandins of varying types; and third, the type(s) of endogenous prostaglandins stimulated by a given agonist. The net effect of a nonprostaglandin agonist on vascular tone will reflect, at least in part, the physiologic antagonism or synergism of endogenously generated prostaglandins. Further studies on smooth muscle cells isolated from different blood vessels are necessary to clarify this concept.

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