Prostaglandin I2 is not a major metabolite of arachidonic acid in cultured endothelial cells from human foreskin microvessels.

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Research Article

Prostaglandin I2 (PGI2), a potent vasodilator and inhibitor of platelet aggregation, is a major product of arachidonic acid metabolism in endothelial cells that are derived from large blood vessels (e.g., umbilical veins). We have examined whether PGI2 is also a major product of arachidonic acid metabolism in cultured endothelial cells that are derived from dermal microvessels in human newborn foreskin. Supernatants from confluent monolayers of endothelial cells that had been incubated for 20 min with [3H]arachidonic acid and the calcium ionophore A23187 (10 microM) were assayed for prostaglandin F2 alpha (PGF2 alpha), prostaglandin E2 (PGE2), and 6-keto-prostaglandin F1 alpha (PGF1 alpha) (the stable metabolite of PGI2) by using authentic standards and high performance liquid chromatography. Whereas supernates from stimulated umbilical vein endothelial cells contained 6-keto-PGF 1 alpha much greater than PGF 2 alpha much greater than PGE2, supernates from stimulated foreskin microvessel endothelial cells contained PGF 2 alpha congruent to PGE2 much greater than 6-keto-PGF 1 alpha. Similar results were obtained when supernates from stimulated, unlabeled endothelial cells were analyzed by radioimmunoassay. These data indicate that PGI2 is not a major metabolite of arachidonic acid in cultured endothelial cells from human foreskin microvessels.

Prostaglandin I_2 Is Not a Major Metabolite of Arachidonic Acid in Cultured Endothelial Cells from Human Foreskin Microvessels

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Abstract. Prostaglandin I_2 (PGI_2), a potent vasodilator and inhibitor of platelet aggregation, is a major product of arachidonic acid metabolism in endothelial cells that are derived from large blood vessels (e.g., umbilical veins). We have examined whether PGI_2 is also a major product of arachidonic acid metabolism in cultured endothelial cells that are derived from dermal microvessels in human newborn foreskin. Supernatants from confluent monolayers of endothelial cells that had been incubated for 20 min with [^{3}H]arachidonic acid and the calcium ionophore A23187 (10 μM) were assayed for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin E_2 (PGE_2), and 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) (the stable metabolite of PGI_2) by using authentic standards and high performance liquid chromatography. Whereas supernates from stimulated umbilical vein endothelial cells contained 6-keto-$PGF_{1\alpha} \geqslant PGF_{2\alpha} \gg PGE_2$, supernates from stimulated foreskin microvessel endothelial cells contained $PGF_{2\alpha} \cong PGE_2 \gg$ 6-keto-$PGF_{1\alpha}$. Similar results were obtained when supernates from stimulated, unlabeled endothelial cells were analyzed by radioimmunoassay. These data indicate that PGI_2 is not a major metabolite of arachidonic acid in cultured endothelial cells from human foreskin microvessels.

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Introduction

Endothelial cells, which line the walls of both large and small blood vessels, normally present a nonthrombogenic surface to circulating blood. Prostaglandin I_2 (PGI_2)[1] is a major product of arachidonic acid metabolism in endothelial cells that are derived from large blood vessels (e.g., human umbilical veins) (1, 2). PGI_2 may play a role in preventing thrombus formation by inhibiting both platelet aggregation (1, 2) and attachment of platelets to endothelial surfaces (3). Since microvascular endothelial cells differ from large vessel endothelial cells with respect to their morphology, growth characteristics, and ability to activate prothrombin in vitro (4–6), we have examined whether PGI_2 is also a major product of arachidonic acid metabolism in these cells. We have found that cultured endothelial cells derived from dermal microvessels in human foreskin synthesize almost no PGI_2. Instead, the major products of arachidonic acid metabolism in these microvascular endothelial cells are prostaglandins $F_{2\alpha}$ and E_2 ($PGF_{2\alpha}$ and PGE_2).

Methods

Endothelial cell cultures. Calcium- and magnesium-free Hanks' balanced salt solution (CMF-Hanks), Medium 199, Iscove's medium, fetal calf serum, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (Hepes buffer), penicillin, streptomycin, and trypsin were obtained from Gibco Laboratories, Grand Island, NY. Endothelial cells from freshly obtained human umbilical veins were isolated and cultured by using minor modifications of the methods described previously by Jaffe et al. (7). Cells were detached by incubating umbilical veins for 45 min with sterile CMF-Hanks, pH 7.4, that contained 3.0 mg/ml trypsin and 1.0 mg/ml ethylenediaminetetraacetate (EDTA) (Sigma Chemical Co., St.

1. *Abbreviations used in this paper:* CMF-Hanks, calcium- and magnesium-free Hanks' balanced salt solution; FGF, fibroblast growth factor; HPLC, high performance liquid chromatography; PG, prostaglandin; PGE_2, $PGF_{2\alpha}$, and PGI_2, prostaglandins E_2, $F_{2\alpha}$, and I_2, respectively.

Louis, MO). Incubation with trypsin yielded as many viable endothelial cells as incubation with collagenase and was considerably less costly. Detached endothelial cells were sedimented at 250 *g* for 10 min, washed once, and resuspended in Medium 199, supplemented with 20% (vol/vol) fetal calf serum, 25 mM Hepes buffer, pH 7.4, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 ng/ml fibroblast growth factor (FGF) (generously provided by Dr. Denis Gospodarowicz, University of California, San Francisco). Primary cultures were established in 35-mm diam plastic Petri dishes (Falcon Plastics, Oxnard, CA) that had been coated previously with fibronectin (0.05 mg/dish), which was prepared from plasma by the method of Engvall and Ruoslahti (8). Plating density was $\sim 2.5 \times 10^5$ cells/dish. Primary cultures were provided with fresh medium every 48 h, and grown to confluence at 37°C in an environment of 5% CO_2 and 100% humidity (usually 3–5 d). For subculture, cells were harvested by brief treatment (4 min, 37°C) with EDTA (0.2 mg/ml)-trypsin (0.5 mg/ml) in CMF-Hanks, sedimented, washed once, resuspended in fresh medium, and diluted 1:4 before plating ($\sim 2.5 \times 10^5$ cells/dish).

Endothelial cells from the microvessels of newborn human foreskin were isolated as described by Davison et al. (4). Foreskin tissue, obtained after routine circumcision, was collected in sterile 150 mM NaCl that contained 400 U/ml penicillin and 200 μg/ml streptomycin. The epidermis was removed with a Castroviejo keratome (Storz Instrument Co., St. Louis, MO), and the highly vascular region of the dermis was sectioned into 5-mm cubes. These were incubated for 45 min at 37°C with 3.0 mg/ml trypsin in sterile 150 mM NaCl (supplemented with 1.0 mg/ml glucose, 10 mg/ml EDTA, and 0.4 mg/ml KCl, and adjusted to pH 7.3). After washing the tissue sections three times with 150 mM NaCl, endothelial cells were expressed from small blood vessels into modified Iscove's medium (9) that contained 10% (vol/vol) human (maternal) serum and antibiotics. Cells were pooled, sedimented (800 *g* for 1 min), and resuspended in modified Iscove's medium supplemented with 10% (vol/vol) human (maternal) serum, antibiotics, and 0.5 mM dibutyryl cyclic 3′,5′-adenosine monophosphate (cAMP) (Sigma Chemical Co.) before plating onto 35-mm diam culture dishes that had been coated previously with fibronectin (0.05 mg/dish) (4). Because of the relatively small yield of microvessel endothelial cells, plating density for primary cultures was $\sim 3 \times 10^4$ cells/dish. Primary cultures were provided with fresh medium every 72 h and maintained at 37°C (6% CO_2, 100% humidity) until they were confluent (~5–7 d). Modified Iscove's medium containing 10% (vol/vol) maternal serum was as effective as the medium reported previously (i.e., minimal essential medium that contained 50% (vol/vol) pooled human serum) (4) for supporting growth of the microvascular endothelial cells. For subculture, cells were harvested with EDTA-trypsin (see above), sedimented, washed once, resuspended in fresh medium, and diluted 1:4 before plating ($\sim 2.0 \times 10^5$ cells/dish). Only confluent monolayers of either primary or early-passage endothelial cells (5–7 d after isolation or passage) were used in the experiments described below.

By using only the highly vascular region of the dermis as a source of microvessel endothelial cells, contamination of primary cultures with either fibroblasts or keratinocytes (easily recognized by their characteristic morphology) was minimal. Substantially longer and more vigorous treatment of dermal tissue with trypsin is required to release fibroblasts. Furthermore, proliferation of fibroblasts in primary cultures is inhibited in medium that contains cAMP (10). When contamination was observed in primary cultures, microvessel endothelial cells were separated from fibroblasts and keratinocytes by brief treatment with EDTA-trypsin and replated. Only confluent cultures containing cells that exhibited a homogeneous cobblestone morphology were used in the experiments described below (Fig. 1). In representative cultures, >95% of the umbilical vein and dermal microvessel endothelial cells stained positively for Factor VIII antigen by indirect immunofluorescence (4) (Fig. 2) and also contained characteristic Weibel-Palade bodies (11) when examined by transmission electron microscopy (not shown).

Incubations with [^3H]arachidonic acid. Confluent monolayers of umbilical vein and foreskin microvessel endothelial cells were incubated for 2 h in fresh medium without FGF or cAMP, and washed before

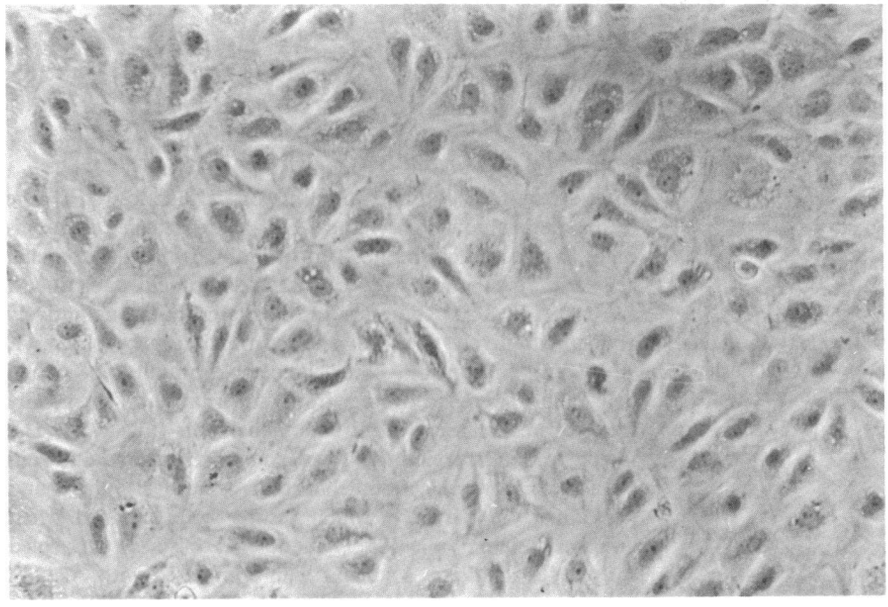

Figure 1. Morphology of cultured microvessel endothelial cells at confluence (phase microscopy). × 300.

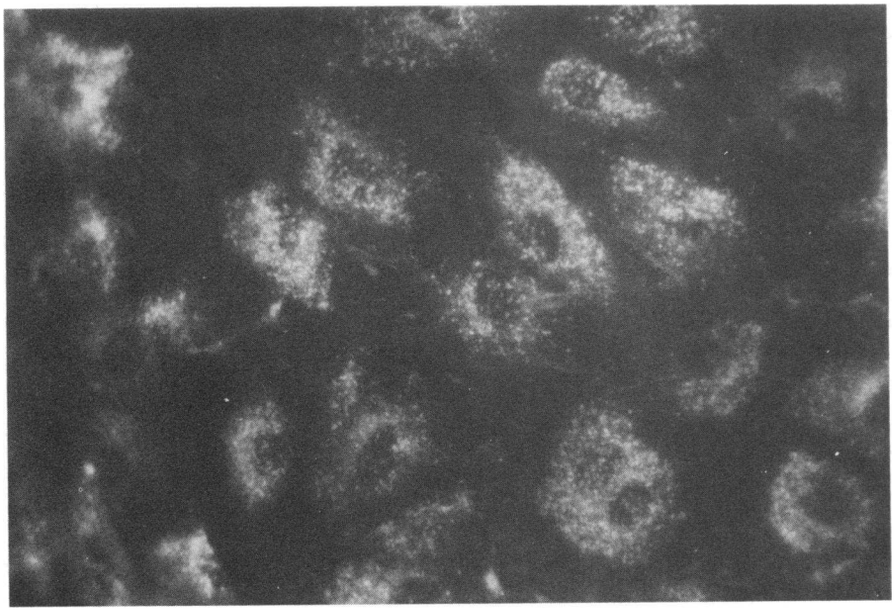

Figure 2. Indirect immunofluorescent staining for Factor VIII antigen in primary culture of foreskin microvessel endothelial cells × 1,300.

incubation for 20 min at 37°C in serum-free medium that contained [^{3}H]arachidonic acid (1.0 μCi/10^6 cells) (87.4 Ci/mmol) (New England Nuclear, Boston, MA) and the calcium ionophore A23187 (10 μM) (Sigma Chemical Co.). In some experiments, endothelial cells were prelabeled by incubation for 20 h with [^{3}H]arachidonic acid (0.5 μCi/10^6 cells) before exposure to A23187. No changes in endothelial cell morphology or viability (trypan blue exclusion) were observed after prelabeling.

Supernates from quadruplicate culture dishes were pooled, extracted with ice-cold acetone (1 vol) and petroleum ether (3 vol) (Burdick & Jackson Laboratories, Muskegon, MI), acidified to pH 3.0 with 1.0 N HCl, and then extracted twice with ethyl acetate (1 vol) (Burdick & Jackson Laboratories) (12). The ethyl acetate phases were combined and evaporated to dryness under vacuum. Extracted material was dissolved in acetonitrile (Burdick & Jackson Laboratories)/water/acetic acid (Sigma Chemical Co.) (65:35:1). Recoveries of radiolabeled (tritiated) PG standards (New England Nuclear) using this extraction procedure were >90%.

High performance liquid chromatography (HPLC). Reverse-phase HPLC was performed by using an Ultrasphere-ODS C18 column (5 μM, 4.7 mm × 25 cm) and precolumn (4.7 mm × 3 cm) (Rainin Instrument Co., Emeryville, CA). Lipids were eluted isocratically at a flow rate of 1.0 ml/min with acetonitrile/water/acetic acid (65:35:1). HPLC fractions (1.0 ml) were analyzed for radioactivity by liquid scintillation counting after mixing with 10 ml Aquasol 2 (New England Nuclear). In experiments performed to calibrate the column, recoveries of tritiated PG standards were >95%.

Radioimmunoassays (RIA). Confluent monolayers of umbilical vein and foreskin microvessel endothelial cells (8–9 × 10^5 and 7—8 × 10^5 per dish, respectively) were preincubated for 10 min with and without 10 μM indomethacin (Sigmal Chemical Co.) before exposure for 20 min to either A23187 (10 μM), human α-thrombin (5.0 U/ml) (provided by Dr. J. W. Fenton, II, Dept. of Health, Albany, NY), or unlabeled arachidonic acid (10 μM) (Nu-Chek Prep, Inc., Elysian, MN). Supernates were assayed for 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, and PGE_2 by RIA as described previously (13), by using antisera from Seragen Inc., Boston, MA, and tritiated PG standards. There was >35% binding of radiolabeled antigen in all assays, and the lower limit of detection was 50 pg (0.5 ng/ml). Total cell DNA was measured by the method of Labarca and Paigen (14).

In some experiments, culture supernates were extracted and subjected to HPLC before RIA. RIA was performed on aliquots of 1.0-ml HPLC fractions diluted 1:5 with 0.05 M Tris buffer, pH 7.4. Authentic, unlabeled PG standards (Upjohn Co., Kalamazoo, MI) were dissolved in the HPLC mobile phase and included in the usual standard curves to control for possible effects of the solvents on the RIA.

Results

Products of exogenous arachidonic acid formed by endothelial cells

Analysis by HPLC. Large amounts of [^{3}H]6-keto-$PGF_{1\alpha}$ (the stable metabolite of PGI_2) and $PGF_{2\alpha}$ were detected in supernates from confluent monolayers of umbilical vein endothelial cells after incubation with [^{3}H]arachidonic acid and 10 μM A23187 (Fig. 3 *A*). These results are similar both qualitatively and quantitatively to those reported previously by others (1, 2, 15, 16). In contrast, barely detectable amounts of [^{3}H]6-keto-$PGF_{1\alpha}$ were recovered from supernates of identically treated foreskin microvessel endothelial cells (Fig. 3 *B*). The major products formed from exogenous [^{3}H]arachidonic acid by the microvessel endothelial cells were [^{3}H]$PGF_{2\alpha}$ and [^{3}H]PGE_2. Qualitatively similar results were obtained when umbilical vein and microvessel endothelial cells were prelabeled with [^{3}H]arachidonic acid and then stimulated with A23187 (not shown). In control experiments, >95% of [^{3}H]6-keto-$PGF_{1\alpha}$ added to confluent monolayers of microvessel endothelial

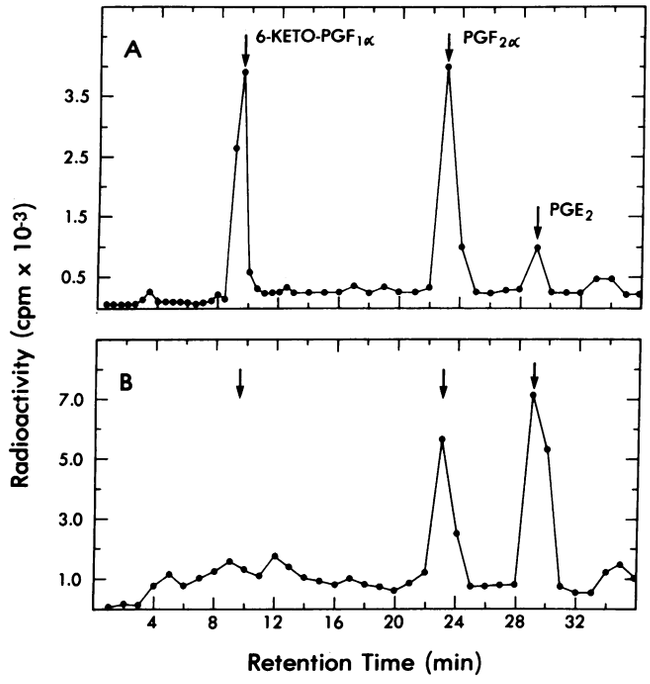

Figure 3. Products formed by endothelial cells from exogenous arachidonic acid. Confluent monolayers of 1.0×10^5 human umbilical vein endothelial cells (*A*) and 1.0×10^6 human foreskin microvessel endothelial cells (*B*) were incubated for 20 min at 37°C with [^{3}H]arachidonic acid (1.0 μCi/10^6 cells) and the calcium ionophore A23187 (10 μM). Supernates were extracted and analyzed by HPLC as described in Methods. Arrows indicate retention times of authentic standards (6-keto-$PGF_{1\alpha}$, 9.4 min; $PGF_{2\alpha}$, 23.0 min; and PGE_2, 29.0 min). Data shown are from a representative experiment (n = 5).

cells was recovered intact after incubation for 20 min with or without A23187. Thus, our inability to detect 6-keto-$PGF_{1\alpha}$ in supernates from stimulated microvessel endothelial cells most likely was due to failure of these cells to synthesize this compound, and not simply due to either binding or catabolism of 6-keto-$PGF_{1\alpha}$ by the endothelial cells.

Analysis by RIA. Supernates from confluent monolayers of umbilical vein endothelial cells that had been incubated for 20 min with either A23187, α-thrombin, or arachidonic acid contained 6-keto-$PGF_{1\alpha} \cong PGF_{2\alpha} \gg PGE_2$ (measured by RIA) (Table I). Identical results were obtained with umbilical vein endothelial cells that had been grown in Iscove's medium that contained human serum and cAMP (not shown). In contrast, only very small amounts of 6-keto-$PGF_{1\alpha}$ were detected by RIA in supernates from similarly treated foreskin microvessel endothelial cells. Rather, $PGF_{2\alpha}$ and PGE_2 were the major products of arachidonic acid metabolism in these stimulated cells. In response to A23187, for example, microvessel endothelial cells generated approximately the same amount of PGE_2 as umbilical vein endothelial cells, but less than $^1/_{15}$th as much 6-keto-$PGF_{1\alpha}$. Levels of 6-keto-$PGF_{1\alpha}$, PGE_2, and $PGF_{2\alpha}$ were undetectable (<0.5 ng/ml) in supernates from both umbilical vein and microvessel endothelial cells that had been stimulated in the presence of 10 μM indomethacin (not shown).

To confirm the specificity of the RIA and to increase the likelihood of detecting synthesis of 6-keto-$PGF_{1\alpha}$, supernates from stimulated microvessel endothelial cells were analyzed by RIA after extraction and HPLC. None of the HPLC fractions at or near the retention time of authentic 6-keto-$PGF_{1\alpha}$ contained detectable amounts of this arachidonic acid metabolite (Fig. 4). Again, the major products detected were $PGF_{2\alpha}$ and PGE_2.

Discussion

Results of our studies indicate that, unlike endothelial cells derived from large blood vessels, human foreskin microvessel endothelial cells synthesize little, if any, PGI_2 in vitro. Whereas umbilical vein endothelial cells synthesized abundant amounts

Table I. Products of Arachidonic Acid Metabolism by Endothelial Cells: Analysis by RIA

Stimulus	Source of Cells*	(n)	Prostaglandins (*ng/μg DNA*) (mean±SE)		
			$PGF_{2\alpha}$‡	PGE_2§	6-keto-$PGF_{1\alpha}$‖
None	Umbilical vein	(3)	2.3±0.1	1.3±0.7	3.0±2.5
	Foreskin microvessel	(3)	0.3±0.1	0.3±0.1	0.7±0.2
A23187 (10 μM)	Umbilical vein	(3)	17.1±2.9	3.6±0.3	18.7±1.8
	Foreskin microvessel	(8)	4.2±0.7	3.2±1.0	1.1±0.4
Arachidonic acid (10 μM)	Umbilical vein	(4)	12.3±0.1	4.6±0.4	15.7±0.7
	Foreskin microvessel	(4)	3.5±0.8	1.3±0.5	0.9±0.3
α-Thrombin (5.0 U/ml)	Umbilical vein	(4)	10.4±2.1	2.3±0.1	14.2±0.1
	Foreskin microvessel	(4)	0.9±0.1	0.4±0.2	0.8±0.1

* Confluent monolayers of umbilical vein and dermal microvessel endothelial cells ($8–9 \times 10^5$ and $7–8 \times 10^5$ per dish, respectively) were incubated with or without stimuli for 20 min at 37°C in serum-free media. ‡ The antiserum to $PGF_{2\alpha}$ cross-reacted with 6-keto-$PGF_{1\alpha}$ (0.5%), PGE_2 (1.1%), 6-keto-$PGE_{1\alpha}$ (0.1%), and thromboxane B_2 (0.3%). § The antiserum to PGE_2 cross-reacted with 6-keto-$PGF_{1\alpha}$ (1.0), $PGF_{2\alpha}$ (<1.0%), 6-keto-$PGE_{1\alpha}$ (1.3%), and thromboxane B_2 (<0.1%). ‖ The antiserum to 6-keto-$PGF_{1\alpha}$ cross-reacted with PGE_2 (0.6%), $PGF_{2\alpha}$ (2.2%), 6-keto-$PGE_{1\alpha}$ (6.8%), and thromboxane B_2 (<0.1%).

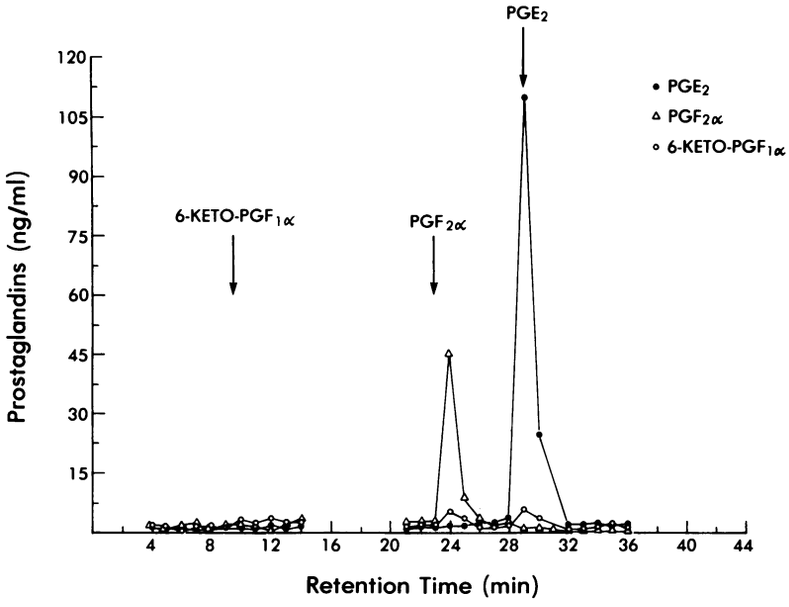

Figure 4. Arachidonic acid metabolism by microvessel endothelial cells: analysis of HPLC fractions by RIA. A confluent monolayer (6×10^6 cells) of microvessel endothelial cells was incubated for 20 min at 37°C with 10 μM A23187. The supernate was then extracted and subjected to HPLC. RIA was performed on individual (1.0 ml) HPLC fractions for 6-keto-$PGF_{1\alpha}$ (○), $PGF_{2\alpha}$ (△), and PGE_2 (●). Analysis of the same culture supernate before HPLC confirmed that the ratio of PGE_2 to $PGF_{2\alpha}$ was 2.2:1.

of [^{3}H]6-keto-$PGF_{1\alpha}$ and [^{3}H]$PGF_{2\alpha}$ when incubated with [^{3}H]arachidonic acid and the calcium ionophore A23187, identically treated foreskin microvessel endothelial cells synthesized only [^{3}H]$PGF_{2\alpha}$ and [^{3}H]PGE_2 (Fig. 3). Similar results were obtained when supernates from confluent monolayers of endothelial cells were analyzed by RIA. Umbilical vein endothelial cells responded to A23187, α-thrombin, and arachidonic acid by synthesizing immunoreactive 6-keto-$PGF_{1\alpha} \cong PGF_{2\alpha} > PGE_2$. Capillary endothelial cells, however, synthesized primarily $PGF_{2\alpha}$ and PGE_2, and only after stimulation with A23187 and arachidonic acid (Table I). Microvessel endothelial cells apparently did not respond to α-thrombin. Failure to detect significant amounts of 6-keto-$PGF_{1\alpha}$ in these supernates was not due to either binding or degradation of this arachidonic acid metabolite by the microvessel endothelial cells, and could not be attributed to effects of either the isolation procedure or the culture medium. Both types of endothelial cells were isolated by using trypsin and the medium used to culture the microvessel endothelial cells did not affect the metabolism of arachidonic acid by umbilical vein endothelial cells. Finally, to increase the likelihood of detecting synthesis of 6-keto-$PGF_{1\alpha}$ by microvessel endothelial cells, supernates obtained after incubating these cells with A23187 were extracted, subjected to HPLC, and then analyzed by RIA. Although these procedures concentrated the $PGF_{2\alpha}$ and PGE_2 in the original culture supernates approximately fourfold, none of the HPLC fractions at or near the retention time of authentic 6-keto-$PGF_{1\alpha}$ contained measurable amounts of this arachidonic acid metabolite (Fig. 4). Furthermore, since the antiserum used to measure 6-keto-$PGF_{1\alpha}$ appeared to cross-react with $PGF_{2\alpha}$ and PGE_2, it is likely that analyses of whole culture supernates by RIA (Table I) somewhat overestimated the amounts of 6-keto-$PGF_{1\alpha}$ formed by foreskin microvessel endothelial cells.

Microvascular endothelial cells from experimental animals also synthesize less PGI_2 than do large vessel endothelial cells. Gerritsen and Cheli (17), for example, reported that endothelial cells from rabbit coronary microvessels synthesize similar amounts of PGE_2 and $PGF_{2\alpha}$, but considerably less 6-keto-$PGF_{1\alpha}$, as compared with endothelial cells from rabbit aorta. Isolated capillaries from guinea pig and rat brain also synthesize much less 6-keto-$PGF_{1\alpha}$ than either PGE_2, $PGF_{2\alpha}$, or prostaglandin D_2 (18). Finally, Coughlin et al. (19) detected only very small amounts of 6-keto-$PGF_{1\alpha}$ in supernates of stimulated bovine adrenal microvessel endothelial cells (as compared with bovine aortic endothelial cells).

Although it has been proposed (20) that synthesis of PGI_2 by endothelial cells is crucial for preventing platelet adherence to blood vessel walls and platelet aggregation, this remains unproven. Indeed, recently summarized evidence (21) suggests that this may not be the case. Our findings that cultured microvessel endothelial cells have only a limited capacity to synthesize PGI_2 suggest that this arachidonic acid metabolite may not play a critical role in regulating hemostasis in the microvasculature.

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