Synthesis of Prostacyclin from Platelet-derived Endoperoxides by Cultured Human Endothelial Cells

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ABSTRACT We have previously shown that aspirin-treated endothelial cells synthesize prostacyclin (PGI_2) from the purified prostaglandin endoperoxide PGH_2 (1978. *J. Biol. Chem.* **253**: 7138). To ascertain whether aspirin-treated endothelial cells produce PGI_2 from endoperoxides released by stimulated platelets, [^{3}H]arachidonic acid-prelabeled platelets were reacted in aggregometer cuvettes with the calcium ionophore A 23187, thrombin, or collagen in the presence of aspirin-treated endothelial cell suspensions. This procedure permitted thin-layer radiochromatographic quantitation of [^{3}H]PGI_2 as [^{3}H]6-keto-$PGF_{1\alpha}$ and [^{3}H]thromboxane A_2 (TXA_2) as [^{3}H]TXB_2, as well as analysis of platelet aggregation responses in the same sample. In the presence of aspirin-treated endothelial cells, platelet aggregation in response to all three agents was inhibited. [^{3}H]6-keto-$PGF_{1\alpha}$ was recovered from the supernates of the combined cell suspensions after stimulation by all three agents. The order of PGI_2 production initiated by the stimuli was ionophore > thrombin > collagen. The amounts of platelet [^{3}H]TXB_2 recovered were markedly reduced by the addition of aspirin-treated endothelial cells. In separate experiments, 6-keto-$PGF_{1\alpha}$ and TXB_2 were quantitated by radioimmunoassay; the results paralleled those obtained with the use of radiolabeling. The quantity of 6-keto-$PGF_{1\alpha}$ measured by radioimmunoassay represented amounts of PGI_2 sufficient to inhibit platelet aggregation. These results were obtained when 200,000 platelets/μl were combined with 3,000–6,000 aspirin-treated endothelial cells/μl. At higher platelet levels the proportion of 6-keto-$PGF_{1\alpha}$ to TXB_2 decreased and platelet aggregation occurred. Control studies indicated that aspirin-treated endothelial cells could not synthesize PGI_2 from exogenous radioactive or endogenous arachidonate when stimulated with thrombin. Therefore the endothelial cell suspensions could only have used endoperoxides from stimulated platelets.

Thus, under our experimental conditions, production by endothelial cells of PGI_2 from endoperoxides derived from activated platelets could be demonstrated by two independent methods. These experimental conditions included: (*a*) enhanced platelet-endothelial cell proximity, as attainable in stirred cell suspensions; (*b*) use of increased endothelial cell/platelet ratios; and (*c*) utilization of arachidonate of high specific activity in radiolabeling experiments. Furthermore, when a mixture of platelets and endothelial cells that were not treated with aspirin was stimulated with thrombin, more than twice as much 6-keto-$PGF_{1\alpha}$ was formed than when endothelial cells were stimulated alone. These results indicate that endothelial cells can utilize platelet endoperoxides for PGI_2 formation to a significant extent.

INTRODUCTION

Prostacyclin (PGI_2)[1] (1) and thromboxane A_2 (TXA_2) (2) are the main products of the cyclooxygenase pathways in endothelial cells and platelets, respectively. PGI_2 is a strong vasodilator and inhibitor of platelet aggregation. TXA_2 is a powerful vasoconstrictor and a stimulator of platelet aggregation. PGI_2 and TXA_2 are enzymati-

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[1] *Abbreviations used in this paper*: ASA, acetylsalicylic acid (aspirin); PGE_2, prostaglandin E_2; PGI_2, prostacyclin; PRP, platelet-rich plasma; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; TLC, thin-layer chromatography; TSG, Tris-saline-glucose buffer; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2.

cally derived from a common precursor, the unstable prostaglandin endoperoxide PGH_2. Bunting and associates (3, 4) had suggested that a biochemical interaction took place between platelets and vessel walls in which endoperoxides from stimulated platelets were also used by the vessels to form PGI_2. In this manner a balance might be maintained between the proaggregatory effect of TXA_2 and the antiaggregatory action of PGI_2 (see reference 5 for review).

We previously demonstrated that exogenously added radioactive PGH_2 was converted to PGI_2 by endothelial cell monolayers (6). In the present study we show that stimulated [^{3}H]arachidonate-labeled platelets can serve as a source of endoperoxide for PGI_2 synthesis by endothelial cells. This was demonstrated with the use of [^{3}H]arachidonate-labeled platelets and radio thin-layer chromatography (TLC), as well as with unlabeled platelets and radioimmunoassay.

METHODS

Platelet collection and processing. Platelet-rich plasma (PRP) was prepared in a plastic pack system as previously described (7). Samples of PRP were removed for evaluation of the burst of O_2 consumption after stimulation with collagen (7). The latter was used as a criterion for activity of platelet cyclooxygenase.

An average of 80 ml PRP (calculated by weight) containing ~37–50 × 10^9 platelets was expressed into a satellite bag. 1 mCi (5 μg) [^{3}H]arachidonate (20:4), 61.0 Ci/mmol sp act (New England Nuclear, Boston, Mass.), was initially converted to the sodium salt with 0.2 ml 0.01 M Na_2CO_3 (6) and added to PRP in 3 ml Tris-saline-glucose (TSG) buffer (8) containing 1% albumin. The satellite bag was enclosed in a second plastic bag and incubated in a shaking water bath at 20 oscillations/min for 1 h at 37°C (8).

After incubation, the labeled PRP was processed as previously described (7). The once-washed platelets were finally suspended in 0.15 M saline and maintained at 4°C. Radioactivity and platelet count (microhematocrit technique) (9) were then determined. The above procedure yielded an average of 38 × 10^9 platelets containing ~600,000–800,000 cpm/10^8 platelets.

Experiments with endothelial cell monolayers. Human endothelial cells derived from umbilical cords were cultured and acetylsalicylic acid (ASA)-treated as previously described (6, 10). Labeled platelets in 5 ml TSG buffer were prewarmed to 37°C (5 min) and then layered over endothelial cells in T-75 flasks. The stimulus was added and the flasks were incubated for 5 min on a rotating platform at 37°C. Experiments were terminated by aspirating the supernate from each flask, acidifying it to pH 3–3.5 with 1 M citric acid, and placing it on ice.

Human thrombin (kindly provided by Dr. John Fenton, II) was used at a concentration of 5 U/ml, and collagen at 30 μg/ml. In thrombin and collagen experiments 3 mM Ca^{++} was also added. The calcium ionophore A23187 in 2.5 μl ethanol was added to a final concentration of 1 μM.

When thromboxane synthetase inhibitors were studied they were added to the platelets during the 5-min preincubation period. Imidazole was added at a concentration of 5 mM (pH of 7.4). Inhibitor U54701 (9,11-iminoepoxyprosta-5,13 dienoic acid, a gift from Dr. Robert R. Gorman, Upjohn Co., Kalamazoo, Mich.) was used at a final concentration of 2 μg/ml and added in 10 μl ethanol.

Experiments with endothelial cell suspensions. Endothelial cell monolayers to be used for suspensions were treated with 1 mM ASA (30 min) and removed with collagenase-EDTA solution (6). The detached cells were pooled, washed twice in Hepes-buffered saline (0.14 M NaCl, 5 mM KCl, 15 mM Hepes, pH 7.4, 11 mM glucose) containing 1 mM ASA, suspended in ASA-free TSG buffer (0.25 ml/flask), and maintained at 22°C.

Labeled platelets and TSG buffer (plus inhibitors when used) were preincubated at 37°C (5 min) in a total volume of 0.25 ml in siliconized aggregometer cuvettes containing stirring bars. A 0.25-ml sample of endothelial cell suspension was added and cuvettes were placed in the aggregometer. 1 min after endothelial cell addition, stimuli were added and aggregation was recorded over a 5-min period. Endothelial cells did not interfere with light transmission in the aggregometry studies. Controls wherein platelets alone were stimulated, were carried out after combined platelet-endothelial cell suspensions were tested.

Reactions were stopped by adding 5 μl 1 M citric acid, cooling the tubes to 4°C, and centrifuging them at 2,000 *g* for 20 min. Supernates were aspirated with siliconized Pasteur pipettes and processed for lipid studies.

For radioimmunoassay studies, reactions were stopped by placing the tubes on ice. Cells were removed by rapid centrifugation in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 3 min at 4°C. Supernates were removed and stored at −20°C.

Lipid analyses. 3.5 vol methanol:chloroform (5:2) were added to the supernates from either monolayer flasks or tube suspensions. Mixtures were vortexed and stored at −60°C. The tubes were later brought to room temperature and a modified Bligh and Dyer lipid extraction was continued (11). The extracts were dried and taken to a volume of 0.1 ml with chloroform:methanol (2:1) and aliquots removed for scintillation counting (1). TLC (double development), scanning, and quantitation techniques have been described previously (1, 6).

Control experiments were carried out to ascertain whether 6,15-diketo $PGF_{1\alpha}$ was being formed in addition to 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) (Wong et al. [12]). Intact and sonicated endothelial cells were incubated (5 min, 37°C) with [^{3}H]PGI_2 (kindly provided by Dr. David Ahern, New England Nuclear). Cofactors were added to the sonicates (12). Radio-TLC analysis indicated that the [^{3}H]PGI_2 was not metabolized to 6,15-diketo-$PGF_{1\alpha}$. In addition, [^{3}H]PGE_2 was used as an alternate substrate to detect 15-hydroxyprostaglandin dehydrogense activity, and no 15-keto-PGE_2 was detected.

6-keto $PGF_{1\alpha}$ was not detected when radiolabeled platelet suspensions were stimulated in the absence of endothelial cells. The major cyclooxygenase product in activated platelet suspensions was TXA_2, which we measured as TXB_2 by both radioactivity and radioimmunoassay.

Studies of ASA inhibition of PGI_2 synthesis. Endothelial cells were treated with 1 mM ASA as outlined above, followed immediately (0 min) by [^{3}H]arachidonate, 10.9 μM (862,049 cpm), Ca^{++} (3 mM), and thrombin (5 U/ml). After a 5-min incubation period samples of the supernates were processed for lipids as described above. The procedure was repeated on samples from the ASA-treated stock suspension 30 and 90 min later. This control protocol was carried out as part of all experiments. Comparable controls were carried out in the radioimmunoassay experiments. The PGI_2 synthetase system of these ASA-treated endothelial cells was functional, as demonstrated by formation of 6-keto-$PGF_{1\alpha}$ (62% yield) fol-

lowing addition of [^{14}C]PGH_2 to the cell suspensions as previously described (6).

Radioimmunoassays for 6-keto-$PGF_{1\alpha}$ and TXB_2. A radioimmunoassay for 6-keto-$PGF_{1\alpha}$ was developed in our laboratory using antisera raised in rabbits against synthetic 6-keto-$PGF_{1\alpha}$ conjugated to keyhole limpet hemocyanin.[2] PGI_2 in the supernates of cell suspensions was converted to 6-keto-$PGF_{1\alpha}$ by acidification to pH 3 with 1 N HCl and subsequent neutralization, or by incubation for 1 h at 37°C. Radioimmunoassay was performed by incubating 100 μl of diluted test sample (in 50 mM Hepes buffer, pH 7.5, containing 0.2% bovine serum albumin), 100 μl of antiserum at a dilution of 1 to 3,200, and 100 μl of [^{3}H]6-keto-$PGF_{1\alpha}$ (10,000 dpm, 100 Ci/mmol, sp act, New England Nuclear). Following incubation at 20°C for 18 h, the bound radioactivity was separated from residual-free radioactivity by adding 100 μl of a 1% suspension of protein A-containing *Staphylococcus aureus* (Pansorbin, Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.), incubating for 1 h at 20°C, and centrifuging. Aliquots of the supernate were then counted in a liquid scintillation counter. Standard curves with 6-keto-$PGF_{1\alpha}$ were run with each assay and were superimposable with standard curves using PGI_2. Half displacement of labeled [^{3}H]6-keto-$PGF_{1\alpha}$ was achieved at 60 pg/sample. This assay had low crossreactivities with other prostaglandins similar to other assays previously reported.

The radioimmunoassay for TXB_2 was similarly developed in our laboratory using antiplasmas raised in rabbits against TXB_2 conjugated to keyhole limpet hemocyanin. Conditions for the assay were similar to those used in the assay described above, except that the antiplasma was used at a dilution of 1 to 4,800. Half displacement of labeled [^{3}H]TXB_2 (150 Ci/mmol sp act, New England Nuclear) was achieved with 35 pg/sample.

TXB_2, PGI_2, and 6-keto-$PGF_{1\alpha}$ were generous gifts of Dr. John Pike, Upjohn Co.

Studies with platelet lysates. Platelet integrity was monitored by measuring lactic dehydrogenase in supernates as previously described (13). Platelets incubated for 5 min without stimulation demonstrated 6% lysis. Following thrombin- or collagen-stimulation, lactic dehydrogenase values were unchanged. Addition of ionophore resulted in 27% lysis. To assess the effect of lysed platelets on PGI_2 synthesis by endothelial cells, control experiments were carried out in which 5, 10, and 40% of the platelet suspensions added to endothelial cells consisted of lysates. The lysates were prepared by rapidly freezing and thawing platelet suspensions three times. Lactic dehydrogenase measurements on the latter indicated 73% lysis. In no instance did the presence of a platelet lysate increase 6-keto-$PGF_{1\alpha}$ formation as measured by radioimmunoassay. For example, the quantity of 6-keto-$PGF_{1\alpha}$ produced by ASA-treated endothelial cells in the presence of a platelet suspension containing 40% platelet lysate following thrombin stimulation, was 5.5 ng/0.5 ml incubation mixture. In the presence of a normal platelet suspension these endothelial cells produced 8.4 ng 6-keto-$PGF_{1\alpha}$/0.5 ml. In an additional experiment 40% platelet lysate was added to the usual platelet number to compensate for platelet dilution by the lysate. In this instance 6.4 ng of 6-keto-$PGF_{1\alpha}$ was measured per 0.5 ml compared with the 8.4 ng/0.5 ml in the control containing no lysate. Stimulus-associated lysis was studied at two concentrations of ionophore, 1 and 0.3 μM. The higher concentration of ionophore induced a 12-fold increase in 6-keto-$PGF_{1\alpha}$ production, but only a 1.8-fold increase in lactic dehydrogenase release. Thus, PGI_2 production could not be correlated with the degree of platelet lysis induced by this stimulus.

RESULTS

Effects of ASA on prostaglandin production by endothelial cells. When non-ASA-treated endothelial cell suspensions were stimulated with thrombin in the presence of [^{3}H]arachidonic acid (20:4), the major radiolabeled product in the supernate was 6-keto-$PGF_{1\alpha}$. The supernate also contained prostaglandins $F_{2\alpha}$, E_2, D_2, hydroxy acids (including 12L-hydroxy-5,8,10-heptadecatrienoic acid, HHT), and free arachidonate (Table I). 6-keto-$PGF_{1\alpha}$ was also identified in parallel experiments by radioimmunoassay. When ASA-treated endothelial cell suspensions were stimulated with thrombin in the presence of [^{3}H]-arachidonic acid before (0 min), during (30 min), and at the conclusion of each experiment, no radiolabeled 6-keto-$PGF_{1\alpha}$ was found (Table I). Radioimmunoassay similarly demonstrated that ASA treatment of endothelial cells completely inhibited 6-keto-$PGF_{1\alpha}$ formation induced by thrombin or ionophore. Although the final test sample noted in Table I was studied at 90 min, experimental protocols were completed within 20–60 min. Thus, ASA-inhibited endothelial cells did not recover their cyclooxygenase activity in the course of the experiments.

An additional control experiment to evaluate possible endothelial cell recovery from ASA treatment was performed. When ASA-treated radiolabeled platelets were stimulated with thrombin in the presence of ASA-inhibited endothelial cell suspensions, radioactive arachidonic acid was released from the platelets (data not shown). However, no 6-keto-$PGF_{1\alpha}$ was formed by the endothelial cells. This is further evidence that the ASA-inhibited endothelial cells could not metabolize arachidonic acid to PGI_2 during the experimental procedure.

Stimulated platelets as a source of endoperoxide for PGI_2 synthesis. Combined suspensions of radiolabeled platelets (200,000/μl) and ASA-treated endothelial cells (3,000–6,000/μl) were stimulated with either ionophore, thrombin or collagen, in aggregometer cuvettes, and the response was recorded. In radiolabeling experiments cuvette contents were then processed for lipid analysis as described in Methods. When nonlabeled platelets were studied, supernates were processed for radioimmunoassay of 6-keto-$PGF_{1\alpha}$ and TXB_2. Thus, the platelet aggregation response and formation of PGI_2 and TXA_2 were evaluated in the same sample. In the absence of endothelial cells, 6-keto-$PGF_{1\alpha}$ was not detected. Furthermore, incubation of unstimulated radiolabeled platelets with ASA-treated

[2] Levin, R., E. A. Jaffe, B. B. Weksler, and K. Tack-Goldman. Manuscript in preparation.

TABLE I

*TLC-Radiochromatographic Analysis of Effect of ASA on Prostaglandin Production from [^{3}H]Arachidonic Acid by Thrombin-stimulated Endothelial Cells**

Product	No ASA		ASA 0 min		ASA 30 min		ASA 90 min	
	cpm‡	%§	*cpm*	%	*cpm*	%	*cpm*	%
6-keto-$PGF_{1\alpha}$	116,008	(65.5)	103	(0.7)	97	(0.8)	118	(1.0)
$PGF_{2\alpha}$	30,484	(17.2)	132	(0.9)	172	(1.3)	196	(1.7)
PGE_2	6,060	(3.4)	162	(1.1)	132	(1.0)	157	(1.3)
PGD_2	2,119	(1.2)	166	(1.1)	227	(1.8)	253	(2.1)
Hydroxy acids	6,559	(3.7)	1,448	(10.3)	1,351	(10.5)	1,376	(11.6)
Arachidonic acid	3,220	(1.8)	11,168	(79.2)	10,125	(79.0)	8,901	(74.9)

* To 5.5×10^6 endothelial cells, treated or not treated with 1 mM ASA, was added [^{3}H]arachidonic acid (final concentration 10.9 μM; 862,049 cpm), Ca^{++} (3 mM), and thrombin (5 U/ml). Total volume was 0.5 ml.

‡ Total counts in supernate. Note that the supernates from the ASA-treated endothelial cells contain fewer total counts. This is because free and esterified arachidonic acid remains largely associated with the cells, whereas products of the cyclooxygenase pathway (6-keto-$PGF_{1\alpha}$ and other prostaglandins) are more soluble in the supernate.

§ Percentage of recovered counts minus phospholipid and solvent front. Radioactive areas on TLC plates that did not correspond to standards are not included. In the ASA-treated samples, counts obtained in the 6-keto-$PGF_{1\alpha}$ areas do not represent a peak of radioactivity.

endothelial cells did not result in PGI_2 production. Thus, in the absence of an endoperoxide source, ASA-treated endothelial cells were unable to form PGI_2.

Ionophore A23187. Essentially no platelet aggregation occurred when platelets and ASA-treated endothelial cell suspensions were stimulated with ionophore (Fig. 1b), whereas platelets alone responded normally (Fig. 1a). The TLC radioscan (Fig. 2) of supernatant lipids from the sample shown in Fig. 1b indicated formation of 6-keto-$PGF_{1\alpha}$, as did quantitative analysis of the peaks on this TLC plate (Table II). In the absence of ASA-treated endothelial cells (Fig. 1a), ionophore-treated platelets produced 1.9 times more TXB_2 (24,940 cpm) than in their presence (12,997 cpm). In two separate groups of experiments, production of significant quantities of 6-keto-$PGF_{1\alpha}$ and TXB_2 was demonstrated by radioimmunoassay, corroborating the radiolabeling experiments. For example, a mixture of ASA-treated endothelial cells (2×10^6) and platelets (1×10^8) produced 1.7 ng 6-keto-$PGF_{1\alpha}$ and 21.3 ng TXB_2 following stimulation with ionophore (1.0 μM). It is of interest that no platelet aggregation occurred in the presence of endothelial cells (Fig. 1b), despite formation of relatively large quantities of TXB_2.

Thrombin. When compared with controls, plate-

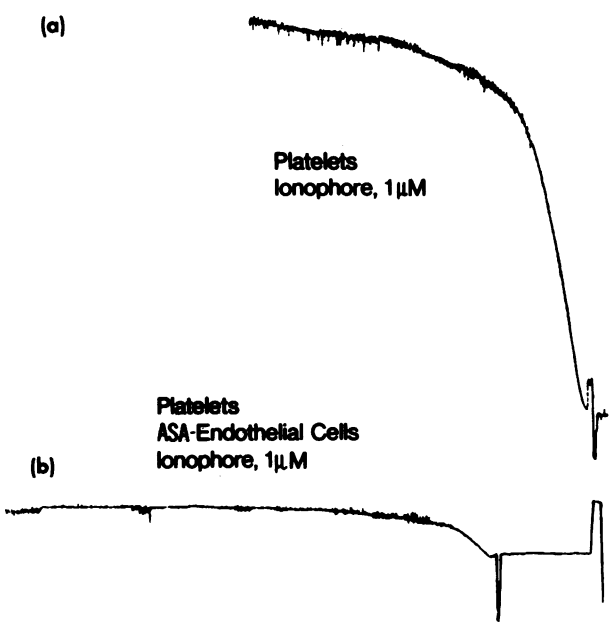

FIGURE 1 (a) Aggregation response of platelets alone to ionophore. (b) Response of platelets to ionophore when ASA-treated endothelial cells were included in the incubation mixture. As shown in Fig. 2 and Table II, PGI_2 synthesis was demonstrable in the supernate derived from this suspension.

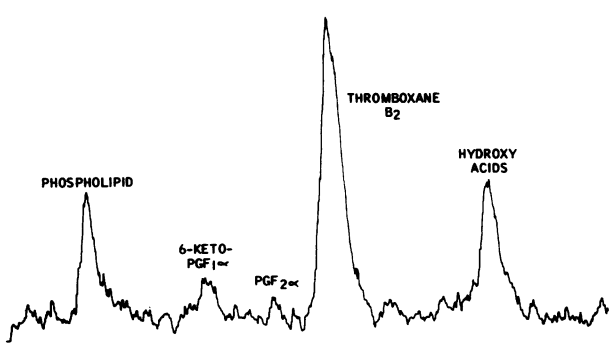

FIGURE 2 Platelets, ASA-treated endothelial cells, and ionophore, 1 μM. Scan of TLC radiochromatogram of lipids in supernate from the experiment depicted in Fig. 1(b) and quantified in Table II. In contrast, the supernate from the aggregation experiment shown in Fig. 1a contained 1.9 times more TXA_2 than did that from Fig. 1b, and no PGI_2.

TABLE II

*TLC-Radiochromatographic Analysis of Products in the Supernate of Combined Suspensions of Radiolabeled Platelets and ASA-treated Endothelial Cells after Stimulation with Ionophore, Thrombin, and Collagen**

Product	Ionophore		Thrombin		Collagen	
	cpm‡	%§	*cpm*	%	*cpm*	%
6-keto-$PGF_{1\alpha}$	1,261	(5.4)	444	(4.1)	60	(3.0)
$PGF_{2\alpha}$	895	(3.9)	356	(3.3)	73	(3.7)
TXB_2	12,997	(56.1)	6,624	(61.5)	1,008	(51.0)
PGE_2	845	(3.6)	247	(2.3)	58	(2.9)
PGD_2	372	(1.6)	111	(1.0)	58	(2.9)
Hydroxy acids	4,209	(18.2)	1,713	(15.9)	451	(22.8)
Arachidonic acid	142	(0.6)	253	(2.3)	126	(6.4)

* Total of 1×10^8 platelets (783,678 cpm) = 200,000/μl. Total of 3.1×10^6 endothelial cells = 6,238/μl. Total volume was 0.5 ml. Ionophore (1 μM), thrombin (5 U/ml), collagen (30 μg/ml), Ca^{++} (3 mM).

‡ Total counts in supernate.

§ Percentage of recovered counts minus phospholipid and solvent front. Radioactive areas on TLC plates that did not correspond to standards are not included. In all samples counts obtained in the 6-keto-$PGF_{1\alpha}$ areas represented a peak of radioactivity.

let aggregation responses to thrombin were always markedly reduced in the presence of ASA-treated endothelial cell suspensions (Fig. 3), and production of 6-keto-$PGF_{1\alpha}$ was demonstrable. Table II shows the TLC-radiochromatographic analysis of supernatant lipids from the experiment shown in Fig. 3b. By radioimmunoassay, combined suspensions of ASA-treated endothelial cells (2×10^6) and platelets (1×10^8) generated 0.5 ng 6-keto-$PGF_{1\alpha}$ and 7.6 ng TXB_2, respectively, after thrombin addition. As noted with ionophore, inhibition of thrombin-induced platelet aggregation (Fig. 3b) occurred, even in the presence of thromboxane production (Table II). Thrombin-treated platelets generated 1.4 times more thromboxane (9,555 cpm) in the absence of ASA-treated endothelial cells than in their presence (6,624 cpm). When measured by radioimmunoassay in a separate experiment the quantities were 16.3 ng and 7.6 ng, respectively. When both platelets and endothelial cells were pretreated with ASA and then stimulated with thrombin, no 6-keto-$PGF_{1\alpha}$ was measurable.

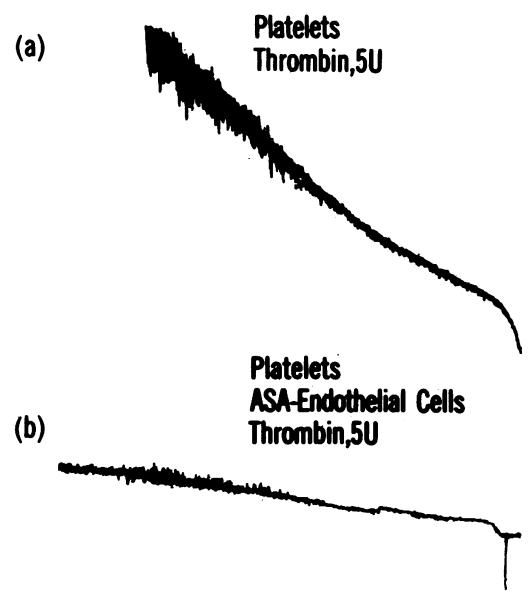

FIGURE 3 (a) Aggregation response of platelets stimulated with thrombin (5 U/ml). (b) Platelet response to thrombin in the presence of ASA-treated endothelial cells. The supernate from this mixture contained PGI_2, as shown in Table II. Total volume of test mixture was 0.5 ml.

Collagen. In the presence of ASA-treated endothelial cells inhibition of aggregation responses to collagen was comparable to that observed with thrombin (Fig. 4). However, smaller quantities of 6-keto-$PGF_{1\alpha}$ and TXB_2 were detected following collagen stimulation than in the case of thrombin or ionophore (Table II). By radioimmunoassay collagen stimulation of the platelet-endothelial cell mixtures resulted in production of 0.1 ng 6-keto-$PGF_{1\alpha}$ and 2.5 ng TXB_2. As can be seen in Table II and from the radioimmunoassay results, the quantitative profile of endothelial cell PGI_2 production was ionophore > thrombin > collagen, and the pattern of thromboxane production by platelets in the combined suspensions followed the same order. In the absence of ASA-treated endothelial cells the quantity of platelet thromboxane recovered in the supernate following collagen stimulation was 5.1 times higher (5,128 cpm) than in their presence (1,008 cpm). When measured by radioimmunoassay in a separate experiment the quantities were 9.9 and 2.5 ng, respectively.

Evaluation of the platelet contribution to PGI_2 formation by endothelial cells. This series of experi-

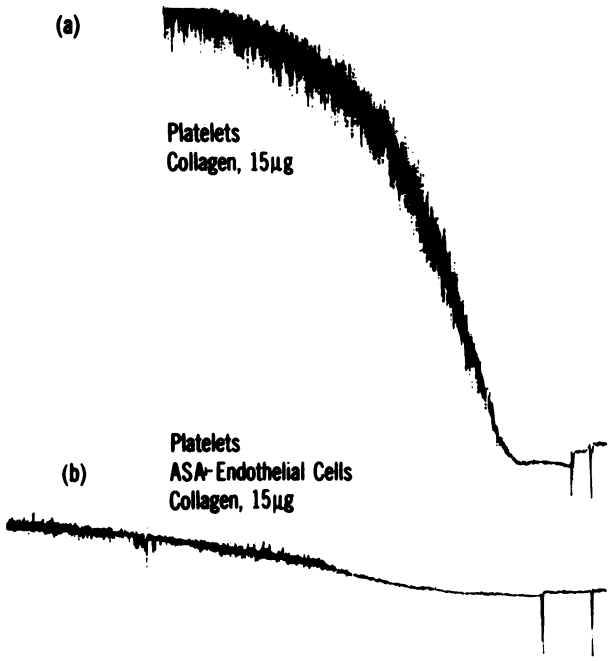

FIGURE 4 (a) Control aggregation curve with collagen (15 μg/cuvette) as the stimulus. (b) Effect of ASA-treated endothelial cells on the platelet response to collagen. The pattern of inhibition was similar to that observed with thrombin. PGI_2 formation was demonstrable, as shown in Table II. However, the quantity of radiolabeled 6-keto-$PGF_{1\alpha}$ detected was lower than in the thrombin and ionophore experiments. Similar results were obtained in radioimmunoassay experiments. Total volume of test mixture was 0.5 ml.

ments was carried out in the radioimmunoassay system. When endothelial cells (3×10^6) were stimulated with thrombin in the absence of ASA, 7.7 ng 6-keto-$PGF_{1\alpha}$ were formed. If a mixture of platelets (1×10^8) and non-ASA-treated endothelial cells were stimulated with thrombin, 19.2 ng of 6-keto-$PGF_{1\alpha}$ were formed (i.e., a 2.5-fold increase).

Endothelial cells from this batch were treated with ASA and added to platelets, which were then the sole source of endoperoxide. Subsequent stimulation with thrombin yielded 8.4 ng 6-keto-$PGF_{1\alpha}$, which was derived from platelet endoperoxides. This was similar to the quantity produced by non-ASA-treated endothelial cells alone (7.7 ng). Thus, in this system approximately half of the total 6-keto-$PGF_{1\alpha}$ produced by endothelial cell-platelet mixtures was derived from platelet endoperoxides.

Relation of platelet concentration to PGI_2 production by ASA-treated endothelial cells. Experiments were carried out wherein 1.45×10^6 endothelial cells (2,900/μl) were combined with either 1×10^8 or 15.5×10^8 platelets (2 or 31×10^5/μl) and then stimulated with ionophore, thrombin, or collagen. The increase in platelet concentration resulted in formation of greater amounts of both 6-keto-$PGF_{1\alpha}$ and TXB_2 (Table III). However, the production of TXB_2 was increased much more than that of 6-keto-$PGF_{1\alpha}$, as shown by the four- to sevenfold increase in the ratio TXB_2/6-keto-$PGF_{1\alpha}$ (Table III). In another experiment the effect of platelet concentration on the aggregation response was examined. Combining 200,000 platelets/μl with 3,850 ASA-treated endothelial cells/μl, followed by stimulation with thrombin (5 U/ml), resulted in inhibition of aggregation comparable to that depicted in Fig. 3b. However, when the platelet concentration was raised to 500,000/μl the inhibitory effect of ASA-treated endothelial cells was lost and a normal aggregation response comparable with that in Fig. 3a ensued.

Studies with ASA-treated endothelial cell monolayers and suspensions. Ionophore stimulation of radiolabeled platelets added to ASA-treated endo-

TABLE III

TLC-Radiochromatographic Analysis of Effect of Platelet Concentration on Production of PGI_2 and TXA_2 by Combined Suspensions of Platelets and ASA-treated Endothelial Cells

Stimulus*	Platelet concentration/μl	6-keto-$PGF_{1\alpha}$		TXB_2‡		TXB_2/6-keto-$PGF_{1\alpha}$
	μl	cpm§	%‖	cpm	%	
Ionophore	2×10^5¶	2,023	(8.6)	9,958	(42.3)	4.9
	31×10^5**	5,586	(2.2)	193,272	(74.5)	34.6
Thrombin	2×10^5	561	(6.1)	4,724	(51.1)	8.4
	31×10^5	3,167	(2.0)	114,311	(72.5)	36.1
Collagen	2×10^5	79	(5.6)	422	(29.9)	5.3
	31×10^5	125	(1.6)	4,576	(61.3)	36.6

* Ionophore (1 μM), thrombin (10 U/ml), collagen (30 μg/ml), Ca^{++} (3 mM). Total volume was 0.5 ml.
‡ Includes a small quantity of PGE_2 that overlapped with TXB_2 on these TLC plates.
§ Total counts in supernate.
‖ Percentage of recovered counts minus phospholipid and solvent front.
¶ Total of 1×10^8 platelets (623,107 cpm); total of 1.45×10^6 endothelial cells.
** Total of 15.5×10^8 platelets (9,346,605 cpm); total of 1.45×10^6 endothelial cells.

thelial cell monolayers resulted in less PGI_2 production than that observed in the same experiment with platelets and ASA-endothelial cell suspensions (491 vs. 782 cpm). A similar effect was noted when thrombin was the stimulus. Monolayer experiments carried out in the presence of the thromboxane synthetase inhibitors imidazole and U54701 resulted in an increase in PGI_2 production as reported by others (14–16). In the case of ionophore stimulation, counts in the 6-keto-$PGF_{1\alpha}$ TLC peak increased from 491 to 3,826 cpm with imidazole, and to 4,177 cpm with U54701. The effect of imidazole and U54701 was also apparent when platelets and ASA-treated endothelial cell suspensions were used. With ionophore, the counts in the 6-keto-$PGF_{1\alpha}$ area rose from 782 to 12,030 cpm in the presence of imidazole, and to 10,847 cpm with U54701. Results with thrombin in the presence of TXA_2 synthetase inhibitors were comparable to those with ionophore in that PGI_2 production increased 10-fold in the ASA-treated endothelial cell monolayers and 14-fold in the suspensions.

DISCUSSION

Although endothelial cells can synthesize PGI_2 from endogenous precursors (17–19) or from exogenously provided endoperoxides (6) or arachidonate (1, 6, 16), PGI_2 synthesis by endothelial cells from platelet-derived endoperoxides has been demonstrated only in the presence of thromboxane synthetase inhibitors (14–16). These results indicated to us that utilization of platelet endoperoxides for PGI_2 synthesis by ASA-treated endothelial cells might be possible even in the absence of thromboxane synthetase inhibitors.

We used two independent methods for detection of 6-keto-$PGF_{1\alpha}$, radiolabeling and radioimmunoassay. The radiometric experiments made it possible to follow the metabolism of platelet-derived endoperoxides by ASA-treated endothelial cells. The radioimmunoassay provided quantitative information on the total amount of 6-keto-$PGF_{1\alpha}$ generated. As previously reported (18), addition of 50 pg PGI_2 to 0.5 ml PRP inhibited aggregation induced by 0.3 mM sodium arachidonate. In addition, the radioimmunoassays allowed us to compare the quantity of 6-keto-$PGF_{1\alpha}$ generated endogenously by the endothelial cells alone with that derived from platelet endoperoxides.

The high specific activity of [^{3}H]arachidonate for radiolabeling permitted studies of platelets in the range of 200,000/μl and increased the sensitivity of the TLC detection system. Use of suspensions of endothelial cells in preference to monolayers enhanced platelet-endothelial cell proximity by concentrating the components in a small volume and permitted adequate mixing. Under these conditions both aggregometry and analysis of products could be performed on the same sample.

When low endothelial cell-to-platelet ratios (~1:1,000) were used, production of 6-keto-$PGF_{1\alpha}$ relative to TXB_2 was low and the platelets aggregated upon stimulation. The ratio of endothelial cells to platelets was then increased to ~1:50 in order to approach that which occurs in vivo. The surface area of an endothelial cell is ~30 × 50 μm (1,500 μm^2) (10) and the area of the capillary vasculature in a 70-kg man is ~1,000 m^2 (20). Thus the capillary vasculature of the average subject is lined by ~7×10^{11} endothelial cells. Assuming a blood volume of 5,000 ml and a platelet count of ~300,000/μl, the vasculature would contain 1.5×10^{12} platelets. Therefore in vivo the ratio of endothelial cells to platelets, at least in capillaries, approaches 1:1. At our endothelial cell to platelet ratio of 1:50, PGI_2 was produced and platelet aggregation was inhibited.

ASA treatment of endothelial cells. Experiments initially carried out with non-ASA-treated endothelial cell suspensions stimulated with thrombin in the presence of [^{3}H]arachidonate indicated that 6-keto-$PGF_{1\alpha}$ accounted for 66% of the [^{3}H]arachidonic acid metabolites formed (Table I). Following treatment with ASA the capacity of the endothelial cells to synthesize PGI_2 from arachidonate was lost and was not recovered during the experiments. This was ascertained by both radiolabeling and radioimmunoassay studies. However, the PGI_2 synthetase system of ASA-treated endothelial cells remained intact, as evidenced by 6-keto-$PGF_{1\alpha}$ production (~62%) when [^{14}C]PGH_2 was used as substrate (6; see also Methods). That the aspirin-treated endothelial cells did not recover their cyclooxygenase activity during the experiment was also borne out by the absence of detectable 6-keto-$PGF_{1\alpha}$ when [^{3}H]arachidonate was released from thrombin-stimulated, ASA-treated platelets.

PGI_2 synthesis from platelet endoperoxides. Ionophore is a strong inducer of platelet aggregation and TXA_2 production as well as of endothelial cell PGI_2 synthesis (18). In the experiments reported here ionophore was the most potent stimulus of PGI_2 production. Inhibition of platelet aggregation in mixtures of ASA-treated endothelial cells and platelets was also the most pronounced with ionophore (Fig. 1). While thrombin was a stronger stimulus for both PGI_2 and TXA_2 production than was collagen, the inhibitory effects of ASA-treated endothelial cells on thrombin- and collagen-induced aggregation were similar (Figs. 3 and 4).

When a mixture of platelets and endothelial cells was stimulated by thrombin in an ASA-free system, approximately twice as much 6-keto-$PGF_{1\alpha}$ as measured by radioimmunoassay formed than when platelets were omitted. A further experiment indicated that the increase in 6-keto-$PGF_{1\alpha}$ noted in the presence of platelets was due to transfer of platelet endoperoxides to the endothelial cells. Endothelial cells stimulated by thrombin to produce PGI_2 from endogenous sources in the absence of ASA produced no more PGI_2 than when ASA-treated endothelial cells were combined with

platelets as the sole source of endoperoxides. Thus, in our system endothelial cells formed approximately half of their PGI_2 from platelet-derived endoperoxides.

In our experiments stimulated platelets synthesized less thromboxane when admixed with ASA-treated endothelial cells than when present in the incubation system alone. A possible explanation is that some of the available platelet endoperoxides were diverted to PGI_2 synthesis when ASA-treated endothelial cells were present with the platelets. An additional possibility is that the PGI_2 formed by the ASA-treated endothelial cells led to inhibition of platelet aggregation and therefore to a decrease in further TXA_2 production.

Inhibition of TXA_2 synthesis with imidazole or U54701 presumably resulted in accumulation of endoperoxides (14–16), thereby facilitating PGI_2 synthesis by ASA-treated endothelial cells in both monolayers and suspensions. In the presence of TXA_2 synthetase inhibitors, ASA-treated endothelial cells generated PGI_2 from endoperoxides formed in thrombin-treated platelets, even when albumin was included in the medium. However, in the presence of albumin the quantities of PGI_2 formed were lower than in its absence, and there was an increase in PGD_2 formation. The latter observations are in agreement with the reported enhanced isomerization of endoperoxides in albumin (15, 21). Thus, in order to maximize the sensitivity of the assay systems, albumin was not included in the incubation medium in the remainder of the experiments.

There therefore appear to be two mechanisms for PGI_2 synthesis by cultured human endothelial cells. The first involves synthesis of PGI_2, from endogenous precursors, and the second occurs from endoperoxides derived from stimulated platelets. Specific conditions under which one or both mechanisms may be operative have not as yet been established.

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