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Research Article

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Bovine Endothelial Cells in Culture Produce Thromboxane as well as Prostacyclin

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ABSTRACT Bovine aortic endothelial and smooth muscle cells in culture were incubated with arachidonic acid or prostaglandin H_2. The amount of prostacyclin and thromboxane A_2 synthesized was then determined by specific radioimmunoassay for 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2. Although smooth muscle cells produced only 6-keto-prostaglandin $F_{1\alpha}$, endothelial cells produced 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2 in a ratio of 5:1 to 10:1. The same ratio of these metabolites of arachidonic acid was also found when prostaglandin production from endogenous arachidonic acid was stimulated in endothelial cells by the ionophore A23187. Cyclooxygenase inhibitors inhibited the production of both metabolites equally, whereas thromboxane synthetase inhibitors selectively inhibited the production of thromboxane B_2. Cells in culture were also incubated with [1-^{14}C]arachidonic acid and the extracted products were identified by two-dimensional thin-layer chromatography. 6-Keto-prostaglandin $F_{1\alpha}$ was the only metabolite produced by smooth muscle cells, but endothelial cells synthesized 6-keto-prostaglandin $F_{1\alpha}$, thromboxane B_2, prostaglandin E_2, and prostaglandin $F_{2\alpha}$.

INTRODUCTION

It has been postulated that a balance between the amount of thromboxane A_2 (TxA_2),[1] the vasoconstrictor and inducer of platelet aggregation formed by platelets, and prostacyclin (PGI_2), the vasodilator and inhibitor of platelet aggregation formed by blood vessels, might be critical for thrombus formation (1). This theory presumes that blood vessels possess PGI_2 synthetase but not Tx synthetase. Evidence is now accumulating that blood vessels can make TxA_2 (2–4). To determine whether endothelial or smooth muscle cells might contribute to the formation of TxA_2 by blood vessels, bovine aortic endothelial and smooth muscle cells were incubated with arachidonic acid (AA), prostaglandin H_2 (PGH_2), or the ionophore A23187. The incubation medium was then assayed for both 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, and TxB_2, the stable metabolite of TxA_2, by specific radioimmunoassays. Our results indicate that bovine endothelial cells but not smooth muscle cells possess Tx synthetase activity.

METHODS

Preparation of cell cultures. Bovine aortic endothelial cells were obtained by mild collagenase digestion of aortae from freshly slaughtered calves (5). At the abattoir, aortae were removed from the animals (10–14 wk old), placed in a sterile container, and submerged in chilled (4°C) Medium 199 (Gibco Laboratories, Grand Island, N. Y.) supplemented with antibiotics (Penicillin-Streptomycin, 50 U/ml–50 μg/ml, and Fungizone, 2.5 μg/ml, E. R. Squibb & Son, Princeton, N. J.). The aortae were then transported to the laboratory, and all subsequent operations carried out in a laminar-flow hood. Adipose tissue was removed from the aorta to free the intercostal vessels, which were then double-tied with 00 braided silk sutures. Cleaned and tied aortae were submerged in fresh Medium 199 supplemented as above and thoroughly washed. The bottom of each aorta was then closed with a sponge clamp and 10–15 ml of collagenase (1 mg/ml, Worthington CLS II in M199 [Worthington Biochemical Corp., Freehold, N. J.]) was added. The top of each aorta was similarly clamped shut, and the preparation was allowed to sit undisturbed for 20–30 min. After the collagenase incubation, the top of each aorta was cut off to open the vessel, and the contents were poured off and discarded. The aorta was washed four times with Medium 199 supplemented with 20% fetal bovine serum (FBS) and antibiotics (Gentamicin, 50 μg/ml, and Fungizone, 2.5 μg/ml). Each wash consisted of filling the vessel with the above medium, gently agitating the contents

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[1] *Abbreviations used in this paper:* AA, arachidonic acid; DPBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; PG, prostaglandin; PGI_2, prostacyclin; TLC, thin-layer chromatography; Tx, thromboxane.

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back and forth, and subsequently collecting the effluent in a beaker. When all washes had been collected, culture plates were inoculated with 1×10^4 cells/cm^2. Medium was changed the following day. Cultures were allowed to grow until confluent (usually 4–7 d) and then used for experimental purposes. For each experiment, a minimum of four cultures were tested.

We used the presence of factor VIII antigen to characterize the isolated cells as endothelium (6). Neither fibroblasts nor smooth muscle cells stain with factor VIII antibody by indirect immunofluorescence microscopy. The isolated endothelial cells also did not overgrow one another, but rather remained as a simple monolayer of squamous epithelium. Furthermore, these calf endothelial cells produced extracellular matrix only beneath their basal surface, and were polarized with respect to it. These characteristics are not found in cultures of other vascular cells such as medial smooth muscle cells or adventitial fibroblasts. It has further been shown that cells isolated using the above methodology synthesize and secrete a basement membrane collagen and not the type I collagen characteristic of both fibroblasts and smooth muscle cells. Thus, while it is impossible to guarantee absolute purity of these cultures, it is realistic to state that the degree of contamination is <1%.

Smooth muscle cells were obtained from explants of the media of the same vessels after removal of endothelial cells and adventitia and were used after 1–20 passages.

Incubations with AA, PGH_2, or A23187. Reagents were diluted in Dulbecco's phosphate-buffered saline (DPBS) and added to cultures washed free of growth medium. Controls included cultures incubated with DPBS alone and substrates incubated in empty petri dishes. Experiments were carried out in 35-mm or 60-mm petri dishes or 24-well plates of cultured cells incubated in a shaking bath at 37°C. Inhibitors were incubated with cultures for 15 min at 37°C before the addition of AA, PGH_2, or A23187.

6-Keto-$PGF_{1\alpha}$ and TxB_2 radioimmunoassays. The supernates from incubations of AA, PGH_2, or A23187 with cell cultures were assayed directly for both 6-keto-$PGF_{1\alpha}$ and TxB_2 using specific antibodies developed in our laboratory (7). The 6-keto-$PGF_{1\alpha}$ antiserum was used at a final dilution of 1:1,500; the TxB_2 antiplasma was used at 1:10,000. There was >70% binding of radiolabeled antigen in all assays, and the limit of detection of unlabeled antigen was 0.5 pmol/ml. The cross-reactivities of other prostaglandins and AA with both antibodies are listed in Table I.

Incubations with [1-^{14}C]AA. Petri dishes (60 mm) of endothelial cells and smooth muscle cells were washed free of medium and incubated with DPBS (3 ml) containing [1-^{14}C]AA and unlabeled AA (final concentration, 10 μM) for 15 min at 37°C. In control experiments [1-^{14}C]AA was incubated in empty petri dishes. The supernates from four dishes were combined, adjusted to pH 3 with concentrated formic acid, and extracted twice with ethyl acetate (12 ml). The extracts were dried under nitrogen and chromatographed on thin-layer chromatography (TLC) plates along with authentic standards of prostaglandin and hydroxy-fatty acid metabolites of AA. The plates were developed in a two-dimensional system, first in the organic phase of a modified A9 system (8) (ethyl acetate: acetic acid:trimethylpentane:water, 110:20:50:100, vol/vol), and then in system C (9) (chloroform:methanol:acetic acid: water, 90:8:1:0.75, vol/vol). Autoradiograms were made by exposing the TLC plates to Ultrafilm ^{3}H (LKB Instruments Inc., Rockville, Md.) for 5 d at −60°C. After autoradiography, the locations of the standards were determined by staining the plates with phosphomolybdate, and these zones were scraped and counted in a Packard scintillation counter (Hewlett-Packard Co., Palo Alto, Calif.).

TABLE I

Specificity of the Radioimmunoassays for TxB_2 and 6-Keto-$PGF_{1\alpha}$

Prostaglandin added	Amount required to inhibit binding by 50%	
	TxB_2 assay	6-Keto-$PGF_{1\alpha}$ assay
	pmol	
TxB_2	0.5	>1,000
PGD_2	900	1,000
6,15-Diketo-$PGF_{1\alpha}$	>1,000	>1,000
PGE_2	>1,000	100
$PGF_{2\alpha}$	>1,000	80
6-Keto-$PGF_{1\alpha}$	>1,000	1.2
AA	1×10^8	4×10^7

Materials. AA (>99% pure, Nu-Chek Prep, Elysian, Minn.) was dissolved under nitrogen in 0.1 M sodium carbonate and stored at −30°C under nitrogen. PGH_2 was obtained by incubation of AA with ram seminal vesicles, extracted into diethyl ether, and purified by TLC. Carbocyclic TxA_2 (10) was synthesized by Dr. K. C. Nicolaou, University of Pennsylvania, Philadelphia, Pa. Indomethacin was a gift of Dr. J. Schrogie, Merck, Sharp & Dohme, West Point, Pa. Ibuprofen was a gift of the Upjohn Company, Kalamazoo, Mich. The ionophore A23187 was a gift of Dr. H. Holmsen, Temple University, Philadelphia, Pa. Prostaglandin standards were a gift of Dr. J. Pike, Upjohn Co. [1-^{14}C]AA (0.05 Ci/mmol) [^{3}H]-6-keto-$PGF_{1\alpha}$ (100 Ci/mmol) and [^{3}H]TxB_2 (150 Ci/mmol) were obtained from New England Nuclear, Boston, Mass. TLC plates were precoated with silica gel 60 without fluorescent indicator (EM Laboratories Inc., Elmsford, N. Y.).

Statistical analysis. Statistical analysis of differences was based on the Student's *t* test. All data are expressed as mean±SD.

RESULTS

Production of 6-keto-$PGF_{1\alpha}$ and TxB_2 by endothelial cells. Incubation of confluent cultures of endothelial cells with DPBS resulted in production of small amounts of 6-keto-$PGF_{1\alpha}$ and TxB_2 (Table II). Addition of AA in DPBS to the same cultures caused an increase in production of both metabolites that was evident at 5 min and complete by 10 min. Although the absolute amounts of prostaglandins produced depended on the culture tested, all cultures produced both metabolites, and the final ratio of 6-keto-$PGF_{1\alpha}$ to TxB_2 was always between 5:1 and 10:1. When cultures were incubated with PGH_2 or A23187, both 6-keto-$PGF_{1\alpha}$ and TxB_2 were produced (Table II). To verify that the material in the endothelial cell culture supernates was indeed TxB_2, supernates from two or more experiments were pooled, and aliquots were diluted with DPBS to obtain a series of solutions of varying concentrations. These solutions were then assayed along with dilutions of authentic TxB_2. The values obtained for the dilutions of the culture supernates followed very closely the standard curve for authentic TxB_2 (Fig. I).

Production of 6-keto-$PGF_{1\alpha}$ by smooth muscle cells.

TABLE II

Synthesis of 6-Keto-PGF$_{1\alpha}$ and TxB$_2$ by Aortic Endothelial Cells

Addition	Synthesis	
	6-Keto-PGF$_{1\alpha}$	TxB$_2$
	pmol/10^6 cells	
No addition	7±8 (4)*	1±0.5 (4)
AA (40 nmol)	584±291 (4)	101±47 (4)
PGH$_2$ (400 pmol)	83±24 [5]‡	9±5 [5]
A23187 (10 μM)	76±47 [5]	14±13 [5]

Primary cultures were washed free of growth medium and incubated with reagents in DPBS for 15 min at 37°C. Data are mean±SD.

* Numbers in parentheses indicate the number of cultures tested in duplicate.

‡ Numbers in brackets indicate the number of cultures tested in quadruplicate.

Smooth muscle cell cultures incubated with either AA or PGH$_2$ produced 6-keto-PGF$_{1\alpha}$ but not TxB$_2$ (Table III). The smooth muscle cells did not produce 6-keto-PGF$_{1\alpha}$ or TxB$_2$ in response to A23187. Smooth muscle cells that had been passed from 1 to 20 times all gave similar results.

Effect of inhibitors on production of 6-keto-PGF$_{1\alpha}$ and TxB$_2$. Endothelial cells were tested in duplicate with DPBS, AA, or AA plus a cyclooxygenase or Tx synthetase inhibitor (Table IV). The cyclooxygenase inhibitors, indomethacin and ibuprofen, equally inhibited production of both 6-keto-PGF$_{1\alpha}$ and TxB$_2$. The selective thromboxane synthetase inhibitor, imidazole, greatly inhibited the production of TxB$_2$ with little effect on 6-keto-PGF$_{1\alpha}$ production. The Tx analog carbocyclic TxA$_2$ caused a marked inhibition of TxB$_2$ production and a smaller decrease in 6-keto-PGF$_{1\alpha}$.

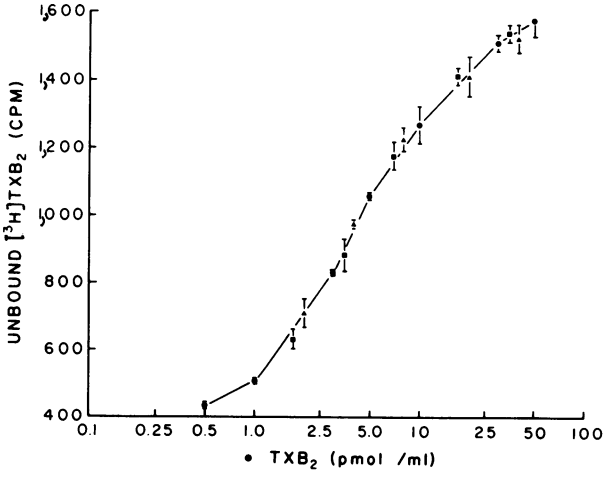

FIGURE 1 Displacement of [^{3}H]TxB$_2$ by authentic TxB$_2$ standards and by dilutions of supernate from endothelial cell cultures incubated with arachidonic acid. Aliquots of pooled endothelial cell culture supernates (unknown No. 1 [▲] and No. 2 [■] were diluted to 50, 20, 10, and 5% of their original concentration. The original supernate and dilutions were then assayed for TxB$_2$. Each point represents the mean±SD of quadruplicate determinations.

TABLE III

Synthesis of 6-Keto-PGF$_{1\alpha}$ by Aortic Smooth Muscle Cells

Addition	Synthesis	
	6-Keto-PGF$_{1\alpha}$	TxB$_2$
No addition	5.6±3.8 (4)*	<1.0 (4)
AA (40 nmol)	136±118 (4)	<1.0 (4)
PGH$_2$ (400 pmol)	64±11 [5]‡	<1.0 [5]
A23187 (10 μM)	<1.0 [5]	<1.0 [5]

Cultures (1st to 20th passage) were washed free of growth medium and incubated with reagents in DPBS for 15 min at 37°C. Data are mean±SD.

* Numbers in parentheses indicate the number of cultures tested in duplicate.

‡ Numbers in brackets indicate the number of cultures tested in quadruplicate.

Identification of radiolabeled products of [1-^{14}C]AA metabolism. Two-dimensional chromatography of authentic standards of prostaglandin, thromboxane, and hydroxy-fatty acid metabolites of AA is illustrated in Fig. 2. Standards were spotted on each TLC plate with the products extracted from the culture supernates. Autoradiography revealed areas of intense radioactivity corresponding to 6-keto-PGF$_{1\alpha}$, PGF$_{2\alpha}$, TxB$_2$, PGE$_2$, hydroxy-fatty acids, and AA in extracts of endothelial cell supernates. Autoradiography of smooth muscle cell products showed spots corresponding to 6-keto-PGF$_{1\alpha}$, hydroxy-fatty acids, and AA. Table V lists the amount of radioactivity found in the zones corresponding to these standards and the percent conversion of AA to each product based on the total amount of radioactivity in each extraction. These values have been corrected for recovery based on extraction and TLC of [^{3}H]-6-keto-PGF$_{1\alpha}$ and [^{3}H]TxB$_2$. The major metabolite synthesized by both endothelial and smooth muscle cells was 6-keto-PGF$_{1\alpha}$. However, endothelial cells also synthesized significant quantities of PGE$_2$, TxB$_2$, PGF$_{2\alpha}$, and hydroxy-fatty acids, whereas TLC of smooth muscle cell extracts revealed a very low amount of radioactivity in these zones. Total conversion of [1-^{14}C]AA by smooth muscle cells was very much lower than by endothelial cells, whereas conversion of PGH$_2$ was about the same for both cell types (Tables II and III).

DISCUSSION

TxA$_2$ induces platelet aggregation and causes constriction of blood vessels, whereas PGI$_2$ inhibits platelet

TABLE IV

Effect of Inhibitors on Production of 6-Keto-PGF$_{1\alpha}$ and TxB$_2$ by Aortic Endothelial Cells

Addition	6-Keto-PGF$_{1\alpha}$	Inhibition	TxB$_2$	Inhibition
	pmol/well	%	*pmol/well*	%
No addition	11±4*	—	<0.5	—
AA (10 nmol)	168±53	—	17±7	—
Plus indomethacin (10 μM)	6±4‡	96	<0.5‡	97
Plus ibuprofen (5 μM)	54±33‡	68	5±3§	70
Plus imidazole (1 mM)	132±49	21	3±2§	82
Plus carbocyclic TxA$_2$ (10 μM)	100±52‖	40	1.5±1‡	91

Inhibitors were incubated with cultures in DPBS for 15 min at 37°C before addition of AA and further incubation for 15 min at 37°C.

* Mean±SD of results from four cultures tested in duplicate (3 × 10^5 cells/well, 24-well plate).

‡ Difference from AA alone, $P < 0.001$ (paired t test).

§ Difference from AA alone, $P < 0.01$.

‖ Difference from AA alone, $P < 0.05$.

aggregation and dilates blood vessels. These properties, and the facility with which platelets make TxA$_2$ and endothelial cells make PGI$_2$, implicate these AA metabolites in both hemostasis and thrombosis. Our results suggest that the role of endothelial cells is more complex than originally postulated. We have shown that bovine aortic endothelial cells produce both PGI$_2$ and TxA$_2$ in vitro. Our evidence that TxA$_2$ is produced enzymatically by endothelial cells is based on (*a*) the specificity of the radioimmunoassay for TxB$_2$, (*b*) the preferential inhibition obtained with selective inhibitors of thromboxane synthetase, and (*c*) the comigration of radiolabeled products with authentic TxB$_2$ in two chromatography systems.

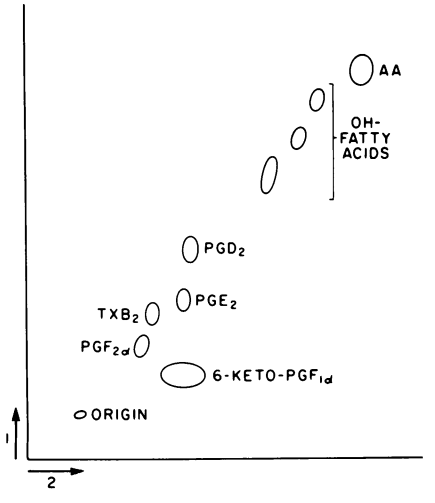

FIGURE 2 Location of authentic standards of prostaglandin, thromboxane, and hydroxy-fatty acid metabolites of AA after two-dimensional TLC. Standards were stained with phosphomolybdate. Arrows indicate the direction of movement in the first (A9) and second (system C) solvent systems.

Although the ratio of PGI$_2$ and TxA$_2$ production by normal cells appears to favor PGI$_2$ under the conditions that we have employed in vitro, it is possible that this ratio may differ in vivo, especially in response to dietary influences and in pathological states. For instance, atherosclerotic vessels and vessels from individuals with diabetes or uremia might produce a greater

TABLE V

Metabolism of [1-^{14}C]AA by Bovine Cells in Culture

Standard	Cell type	cpm × 10^{-4}	Percent converted
6-Keto-PGF$_{1\alpha}$	EC*	10.6±3.8	3.60±0.8
	SM	2.6±0.6	1.10±0.3
TxB$_2$	EC	2.4±0.6	0.80±0.2
	SM	0.2±0.07	0.09±0.04
PGE$_2$	EC	5.4±0.6	1.85±0.09
	SM	0.5±0.2	0.20±0.1
PGF$_{2\alpha}$	EC	1.1±0.5	0.40±0.2
	SM	0.1±0.1	0.06±0.06
PGD$_2$	EC	0.3±0.1	0.16±0.02
	SM	0	0
OH-fatty acids	EC	3.0±2.3	1.40±0.9
	SM	0.7±0.3	0.30±0.2

Mean±SD, corrected for recovery, of three experiments. Cells in culture were incubated with [1-^{14}C]AA in DPBS at 37°C for 15 min. Supernates from four petri dishes (2 × 10^6 cells/dish) were pooled before extraction and chromatography. Pooled supernates from endothelial and smooth muscle cells contained 2.7–3.4 × 10^6 and 2.1–2.5 × 10^6 cpm, respectively. The amount of radioactivity found in each zone after extraction and TLC of the [1-^{14}C]AA control has been subtracted.

* EC, endothelial cells; SM, smooth muscle cells.

amount of TxA_2 or PGI_2, leading to an increased risk of thrombosis or bleeding. Vessels damaged repeatedly by hypertension, hormone imbalance, or noxious chemicals might also show an altered AA metabolism. Our results suggest that the ability of endothelial cells to make TxA_2 as well as PGI_2 should be considered when the mechanisms of hemostasis or thrombosis are examined.

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ADDENDUM

While this manuscript was in review, two papers were published that have a bearing on this work (11, 12). The first reports that bovine endothelial cells in culture synthesized PGI_2 but not TxA_2. This work differs from ours in that the cells tested were cloned lines of adult and fetal (not calf) bovine aortic endothelial cells. These cells are highly selected, have been passed many times, and require fibroblast growth factor in the growth medium. No effort was made by the authors to ascertain whether a primary culture of such cells would synthesize the same products as the cloned cells. The second paper reports that rabbit intrapulmonary arteries synthesize TxA_2 as well as PGI_2. Both products were identified by bioassay, radioimmunoassay, and chromatography.

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