Vol. 85, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 29, 1978 Pages 827-836

THROMBOXANE A_2 BIOSYNTHESIS IN HUMAN LUNG FIBROBLASTS WI-38

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Received September 22, 1978

SUMMARY:

A fibroblast of human lung origin (WI-38) synthesizes thromboxane A_2 from the prostaglandin endoperoxide PGH_2. Thromboxane A_2 synthesis was demonstrated by radio thin layer chromatography, gas chromatography/mass spectrometry, and by bioassay. This is the first demonstration of thromboxane A_2 biosynthesis in a homogeneous cell population other than the human platelet.

INTRODUCTION:

Considerable attention has been given to the recent discoveries of two new products of arachidonic metabolism, thromboxane A_2 (TXA_2) (1) and PGI_2 or prostacyclin (2-4). TXA_2 and/or PGI_2 biosynthesis has been demonstrated in a number of organs and tissues such as lung, umbilical artery, heart, and kidney (5-12). PGI_2 synthesis has also been demonstrated in cell cultures of human and bovine endothelial cells (13) and indirect evidence for PGI_2 biosynthesis in fibroblast and smooth muscle cell cultures has been reported (14,15). The only homogenous cell population that has been shown to produce TXA_2 is the human platelet (1). In this report we show that a fibroblast, WI-38, derived from human lung, synthesizes thromboxane A_2 from the prostaglandin endoperoxide PGH_2.

METHODS:

Adenosine 3',5'-cyclic monophosphate (cyclic AMP) was measured by an adenine prelabelling technique (16), or by radioimmunoassay

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(RIA) according to Steiner et al. (17), with the incorporation of the acetylation-modifications of Harper and Brooker (18). PGH_2 and ($1-^{14}C$ PGH_2) was prepared according to Gorman et al. (19).

Human lung fibroblasts WI-38 and VA-13 were purchased from Flow Laboratories, Rockville, Maryland. Cells were split 1:5 into Falcon tissue culture bottles (75 cm^2) and grown in Eagles Minimum Essential Medium (Earle's Base) supplemented with 10% bovine serum (inactivated 56°, 30 min) from Reheis Chemical Company. This medium will be referred to as MEM-10 throughout the text. Confluent cells were fed with 25 mls of MEM-10 every 5 days, and passed every 13-14 days. For cyclic nucleotide measurements, 1×10^5 cells were seeded into 35 mm Coster wells, and grown under a humidified atmosphere of 95% air - 5% CO_2 at 37° until confluent (3-4 days, approximately 9×10^5 cells/well).

WI-38 homogenates were prepared by washing the cells twice with 2.0 ml of ice-cold 50 mM Tris-HCl - 0.15 M NaCl buffer pH 7.5, followed by scrapping with a rubber policeman. The pooled cells were centrifuged at 4° for 15 min at 2000 xg, frozen and thawed 3 times in liquid N_2, and further disrupted by 10 strokes of a Dounce homogenizer (glass/glass).

($1-^{14}C$ PGH_2) metabolism by WI-38 homogenates was measured by quantitative radio thin layer chromatography (radio-TLC) according to Sun et al. (20).

The radio-TLC product that co-chromatographed with thromboxane B_2 was eluted from the silica gel with methanol, taken to dryness, and resuspended in diethyl ether. The product was esterified with diazomethane and silanized with a 3:1 mixture of bis (N, O-trimethylsilyl)trifluoroacetamide and dimethylformamide. The mixture was allowed to stand at room temperature for 1 hr before gas chromatography/mass spectrometric analysis (GC/MS). The actual GC/MS analysis was done on an LKB-9000 equipped with a 6 ft column of 1% SE-30 on Gas Chrom Q

operated at 210°. The flash heater and separator were operated at 270° with a helium flow of 30 ml/min. Electronic energy was kept at 22.5 eV, and the trap current was 60 μA.

The superfusion technique of Vane (21) was used to assay RCS (TXA_2) biological activity. Spirally cut strips of rabbit aorta and rabbit pulmonary artery were superfused in cascade with Kreb's solution at a flow rate of 10 ml/min, containing a mixture of antagonists which rendered the system insensitive to acetylcholine, serotonin, catecholamines and histamine (22). Indomethacin (1 μg/ml) was also added to prevent endogenous prostaglandin synthesis by the vessel strips.

RESULTS AND DISCUSSION:

Incubation of WI-38 cells with 2.8 μM PGE_2, PGD_2, PGI_2, or PGE_1 results in the marked stimulation of cyclic AMP levels (Table 1). The order of potency is $PGE_1 = PGE_2 > PGI_2 >> PGD_2$. This cell line is the first system that we have found where prostaglandins of the E series are more potent than PGI_2. PGI_2 in previous work was at least equipotent to and usually 10-100 times more potent than E prostaglandins (23,24). The same qualitative relationships observed in WI-38 cells were also found in the SV-40 virus transformed WI-38, VA-13. PGE_1 and PGE_2 were both more potent stimulators than PGI_2, while PGD_2 was the weakest agonist (Table 1).

The data obtained by the (^3H) adenine prelabelling technique was verified by R.I.A. of cyclic AMP in confluent cultures of WI-38. Dose response curves for PGE_2 and PGI_2 showed PGE_2 to be a more potent stimulator of cyclic AMP accumulation in WI-38 cells (Figure 1). The addition of the prostaglandin endoperoxide PGH_2 to confluent cultures of WI-38 cells also resulted in an elevation in cyclic AMP levels, but this stimulation was less than that obtained with equivalent concentrations of PGE_2 (Figure 1). These data suggested that either PGH_2

Table 1

Stimulation of Cyclic AMP Accumulation in WI-38 and VA-13 Cells by Prostaglandins

Prostaglandin	Percent Conversion ATP to cAMP/15 min WI-38	VA-13
None-Basal Activity	0.12 ± 0.01	0.13 ± 0.01
PGE_2	3.78 ± 0.33	0.50 ± 0.02
PGI_2	0.71 ± 0.02	0.21 ± 0.01
PGE_1	4.60 ± 0.18	0.44 ± 0.03
PGD_2	0.16 ± 0.05	0.12 ± 0.01

Cultures of WI-38 and VA-13 cells were grown in 35 mm plastic wells. At confluency (3-4 days) cells were prelabelled with 5 μCi of (8-^3H) adenine (16). The cells were then challenged with either 2.8 μM PGE_2, PGI_2, PGE_1, or PGD_2 for 15 min at 37°. The cAMP was purified by sequential passage over Dowex AG-50X4, and neutral alumina columns. Data reported as Mean ± S.E.M. of triplicate determinations.

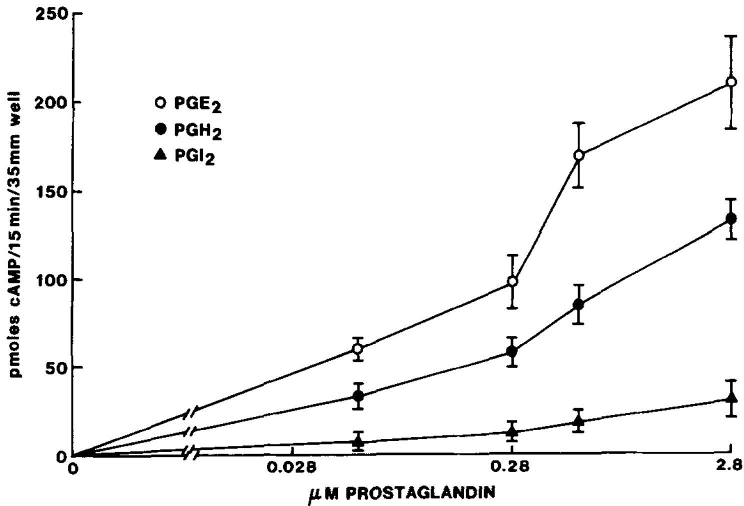

Figure 1. Stimulation of WI-38 Fibroblast Cyclic AMP Levels by PGE_2, PGH_2, and PGI_2.

WI-38 fibroblasts were grown to confluency in 35 mm wells (0.85×10^5 cells) and then challenged with from 140 nM to 2.8 μM PGE_2, PGH_2, or PGI_2 for 15 min at 37°. Cyclic AMP was measured by R.I.A. (17,18). Data presented as pmoles cAMP/15 min/well, and are mean ± S.E.M. of triplicate determinations.

Table 2

(1-^{14}C) PGH_2 Metabolism in WI-38 Homogenates

	CPM/Radioactive Zone				
Additions	$PGF_{2\alpha}$	PGE_2	TXB_2	PGD_2	HHT
(1-^{14}C) PGH_2	965 ± 84 (6.5)	3729 ± 272 (25.0)	3389 ± 222 (22.5)	1292 ± 79 (8.5)	5650 ± 572 (37.5)
(1-^{14}C) PGH_2 +5.6 μM Azo analog I	2108 ± 162 (14)	7070 ± 437 (47)	318 ± 64 (2.1)	1793 ± 92 (11.9)	3790 ± 382 (25.0)

Homogenates of WI-38 cells (0.61 mg protein/ml) were incubated with 0.60 μM PGH_2 for 15 min at 22°. One preparation was preincubated with 5.6 μM azo analog I for 5 min at 4° before the addition of the PGH_2. The reactions were stopped by acidification with 1N HCl, followed by ether extraction, and silicic acid thin layer chromatography in a 1% acetic acid/99% ethyl acetate solvent system. Zones of radioactivity that corresponded to standard prostaglandins were scrapped and quantitated by liquid scintillation counting (20). Data reported as Mean ± S.E.M of triplicate determinations. Numbers in brackets represent the percent of total recoverable counts.

was a direct stimulator of cyclic AMP accumulation, or was converted by the WI-38 cells to some other stimulatory prostaglandin.

To establish which product(s) PGH_2 was converted to in WI-38 cells, 0.60 μM (1-^{14}C) PGH_2 was incubated with homogenates of WI-38 cells at 22° for 15 min (Table 2). Quantitative radio thin layer chromatography gave evidence for the synthesis of $PGF_{2\alpha}$, PGE_2, PGD_2, the C-17 hydroxy fatty acid HHT, and unexpectedly, a zone of radioactivity that co-chromatographed with thromboxane B_2 (Table 2). Additional evidence that thromboxane B_2 was formed in WI-38 cells from PGH_2 was obtained with the thromboxane synthetase inhibitor 9,11 azoprosta-5,13 dienoic acid (azo analog I) (24). Incubation of WI-38 homogenates with 5.6 μM azo analog I for 5 min prior to the addition of PGH_2 inhibited the formation of the product that co-chromatographed with authentic thromboxane B_2 (Table 2). The inhib-

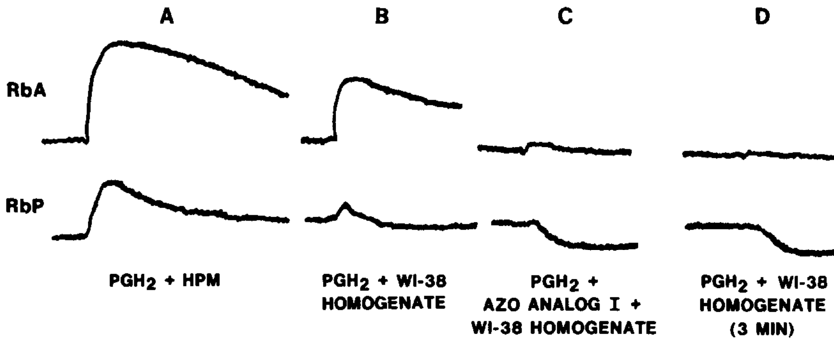

Figure 2. Bioassay of Rabbit Aorta Contracting Substance Synthesized by WI-38 Cells.

Homogenates were prepared from WI-38 fibroblasts and incubated for 15 sec at 22° with PGH_2. The incubate was then added to a muscle cascade consisting of a rabbit aorta, and a rabbit pulmonary artery. (A) PGH_2 + human platelet microsomes (HPM) elicits contraction of both strips (B) PGH_2 + WI-38 membranes also contracts both vessels (C) azo analog I completely blocks the contraction of both muscles with marked relaxation of the pulmonary artery (D). If the preparation used in B above was allowed to sit on the bench top for 3 minutes and then tested there was no contraction of either vessel, but relaxation of the pulmonary artery.

ition of thromboxane synthesis resulted in an increase in the PGE_2 peak, analogous to our original work with thromboxane synthetase inhibitors in human platelets (25). This spectrum of biosynthetic products indicates that the increase in cyclic AMP after PGH_2 was probably due to PGE_2 synthesis.

We have used the Vane cascade to measure a rabbit aorta contracting (RCS) activity generated from PGH_2 by WI-38 homogenates (Figure 2). Two spirally cut vessel strips were used. The upper trace is a rabbit aorta, and the lower trace a rabbit pulmonary artery. Reading left to right: A. Incubation of 30 ng of PGH_2 with 68 μg of human platelet microsomal protein (50 μl) for 15 sec at 22° resulted in the synthesis of a substance that contracted both the rabbit aorta and the rabbit pulmonary artery, a typical RCS activity. B. A similar incubation with 30 ng of PGH_2 and 31 μg (50 ml) of

WI-38 homogenate protein resulted in the generation of a substance that also contracted the rabbit aorta and the pulmonary artery.

C. Preincubation of the WI-38 homogenate for 5 min with 140 nM azo analog I, followed by incubation with 30 ng of PGH_2 attenuated the RCS activity and induced a marked relaxation of the pulmonary artery.

D. If the incubation of PGH_2 and WI-38 homogenates is allowed to proceed for 3 min before testing, there is no RCS activity, and again the pulmonary artery relaxes (Figure 2). These tracings show that WI-38 homogenates produce a labile RCS from PGH_2, and the synthesis of the RCS is blocked by the thromboxane synthetase inhibitor azo analog I. The relaxation of the pulmonary artery when thromboxane synthesis is blocked, or after decay of the RCS activity, is due to PGE_2 that is formed during the incubation. This relaxation is masked by the RCS activity.

The unequivocal proof that WI-38 cells produce thromboxane B_2 from PGH_2 is shown in Figure 3. An homogenate was prepared from ten 100 mm plates of WI-38 cells in a total volume of 2.0 ml of 50 mM Tris-HCl-NaCl pH 7.5. The homogenate was then incubated for 15 min at 22° with 56 μM PGH_2. The incubation mixture was acidified, extracted with diethylèther, and subjected to preparative silicic acid TLC. The zone corresponding to thromboxane B_2 was eluted with methanol, and derivatized as described in the Methods. The derivatized product was then analyzed by GC/MS. The spectrum of the unknown gave a base peak at m/e 256 and other major ions at m/e 510, 439, 420, 366, 295, 225, and 217 (Figure 3). This pattern is identical to authentic thromboxane B_2 (26), and the published fragmentation pattern for thromboxane reported by Hamberg and Samuelsson (27).

We have now looked at many different cell lines, and although several of them have been found to produce PGI_2, WI-38 cells are the only cell line thus far that synthesizes thromboxane B_2. We have

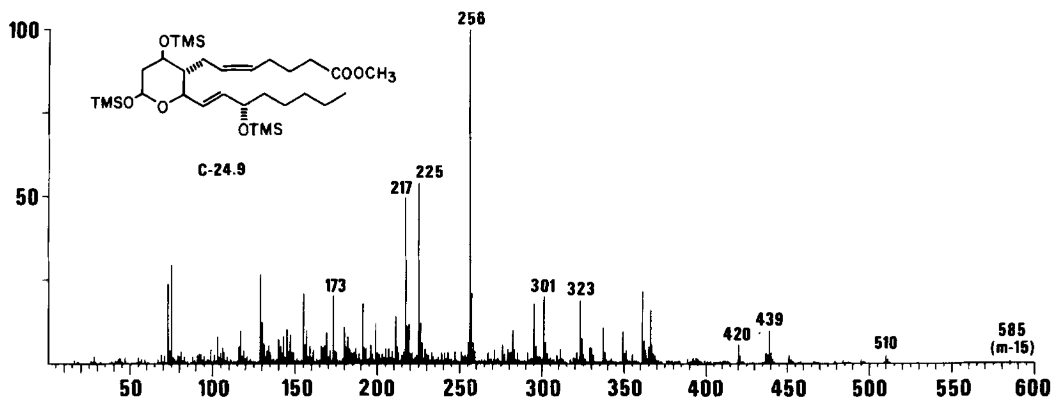

Figure 3. Mass Spectrum of TXB_2 Obtained from Incubation of WI-38 Homogenates with PGH_2.

A homogenate of WI-38 cells was prepared from ten 100 mm plates. The homogenate was then incubated for 15 min at 22° with 56 μM PGH_2. The mixture was purified and derivatized for GC/MS analysis as described in the text. The spectrum obtained from the unknown sample was identical to authentic TXB_2.

found no evidence of PGI_2 production in WI-38 cells. We have never found a cultured cell that is capable of synthesizing both thromboxane B_2 and PGI_2 simultaneously.

We do not know what function(s) thromboxane A_2 may serve in WI-38 cells or the significance, if any, of the dominance of PGE_2 over PGI_2 in this cell. Thromboxane A_2 synthesis has been associated with an inhibition of adenylate cyclase, and as an opposing activity to the stimulation of adenylate cyclase by PGI_2 or E prostaglandins in human platelets (28), and a similar mechanism may occur in WI-38 cells. We plan to use thromboxane synthetase inhibitors such as azo analog I, to study the growth characteristics and adenylate cyclase regulation of WI-38 cells when thromboxane synthesis is blocked. Perhaps these studies will expand our understanding of the role thromboxane A_2 may play in diploid cell growth and metabolism.

ACKNOWLEDGEMENT:

The authors thank Dr. J. W. Aiken for access to the superfusion cascade.

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