ELEVATION OF ADENOSINE 3′, 5′-MONOPHOSPHATE LEVELS IN 3T3 FIBROBLASTS BY ARACHIDONIC ACID: EVIDENCE FOR MEDIATION BY PROSTAGLANDIN I_2

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1. Introduction

Although adenosine 3′,5′-monophosphate (cyclic AMP) has important roles in the control of cell proliferation and cell growth [1], the regulation of cyclic AMP synthesis in cultured cells is relatively unknown. In addition to classical hormones [2], E-type prostaglandins stimulate adenylate cyclase in a variety of tissues [3]. Prostaglandins can be produced by most mammalian cells and might thus serve as local modulators of cyclic AMP formation. This contention is supported by our recent observation that endogenous prostaglandin (PG)E_2 synthesis by polyoma virus transformed 3T3 fibroblasts markedly raises the cellular cyclic AMP levels [4]. In this report we describe a stimulatory effect of arachidonic acid on cyclic AMP concentrations in regular 3T3 fibroblasts which is not mediated by PGE_2. Instead, conversion to the unstable prostaglandin I_2 appears to be involved. PGI_2 (prostacyclin, PGX) is a recently discovered inhibitor of platelet aggregation [5,6] which elevates cyclic AMP levels [7,8]. Although it was described as a product of arachidonic acid metabolism in rat stomach in 1971 [9] the biological properties of PGI_2 were not revealed until 1976 [6].

Abbreviations: EMPA, 9α, 11α-epoxymethano-15(*S*)-hydroxyprosta-5(*cis*), 13 (*trans*)-dienoic acid; MEPA, 9α, 11α-methanoepoxy-15(*S*)-hydroxyprosta-5(*cis*), 13 (*trans*)-dienoic acid; PG, prostaglandin

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2. Materials and methods

[1-^{14}C]Arachidonic acid was obtained from the Radiochemical Centre, Amersham, England and arachidonic acid from Nu Chek Prep Inc., Elysian, Minn., USA. PGG_2 [10] and 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid [11] were prepared as described. Balb/c 3T3 mouse embryo fibroblasts were kindly given by Dr M. M. Burger. 9α, 11α-Epoxymethano- and 9α, 11α-methanoepoxy-15-hydroxy-prosta-5, 13-dienoic acid (EMPA and MEPA, respectively), PGD_2, PGE_2 and $PGF_{2\alpha}$ were provided by the Upjohn Company. Cells were cultivated in Dulbecco's modified Eagle's medium containing 10% calf serum [4]. Three or four days after seeding, media were carefully removed and the cells were rinsed with phosphate-buffered saline (PBS, 3 × 5 ml). After addition of 3 ml PBS/9 cm dish, substances to be tested were added in 0.6–6 μl ethanol or acetone. Incubations were performed at 37°C. Cyclic AMP and DNA were determined on the same dish [4]. Platelet rich plasma (PRP) [12] was prepared as described before. Platelet aggregation was monitored with a Payton dual channel aggregometer.

3. Results

3.1. *Effects of arachidonic acid and PGG_2 on cyclic AMP levels*

Arachidonic acid elevated cyclic AMP levels in 3T3 fibroblasts as measured after 5 min incubations

Table 1
Effect of arachidonic acid on cyclic AMP levels

Arachidonic acid (ng/ml)	Cyclic AMP (pmol/μg DNA)
–	0.26
25	0.55
100	2.12
250	6.97
500	13.30
–[a]	0.15
250[a]	0.23

[a]Indomethacin (final concentration 10^{-6} M) was added 5 min prior to the addition of arachidonic acid

3T3 fibroblasts were incubated for 5 min at 37°C with various concentrations of arachidonic acid in phosphate buffered saline (PBS) prior to cyclic AMP analyses. The values are means of triplicate determinations on each of two separate culture dishes

(table 1). The effect was prevented by indomethacin, suggesting that arachidonic acid had to be converted by prostaglandin endoperoxide synthase (EC 1.14.-99.1) [13] prior to stimulation. In agreement with this, PGG_2 elevated cyclic AMP and this effect was not inhibited by indomethacin (table 2). Two endoperoxide analogs, EMPA and MEPA, which have similar biological effects as PGG_2 [14] did not raise cyclic AMP. This suggested that the endoperoxide had to be converted further to exert its effect. The product which mediated the effect was not PGD_2, PGE_2 or $PGF_{2\alpha}$ (table 2).

3.2. *Conversion of arachidonic acid to 6-keto $PGF_{1\alpha}$*

[1-^{14}C]Arachidonic acid (55 Ci/mol, 3 μg in 3 ml PBS/9 cm plate) was incubated at 37°C for 5 min with 3T3 fibroblasts. The PBS was removed and mixed with 5 vol. ethanol. After dilution with water and diethyl ether extraction at pH 3 products were analyzed by thin-layer radiochromatography (solvent system: organic phase of ethyl acetate/acetic acid/2,2,4-trimethylpentane/water, 9:2:5:10 v/v/v/v). 40% of the radioactivity had the same R_F (0.95) as arachidonic acid. The remainder had been converted to more polar compounds. The major product (40% of the radioactivity) cochromatographed with PGE_2 (R_F 0.30), one product (7% of the radioactivity, R_F 0.88) moved immediately behind arachidonic acid and another

Table 2
Effects of PGG_2, EMPA, MEPA, PGD_2, PGE_2 and $PGF_{2\alpha}$ on cyclic AMP levels

Additions (μg/ml)	Cyclic AMP (pmol/μg DNA)
None	0.21
PGG_2 (0.1)	3.24
PGG_2 (0.25)	11.18
PGG_2 (0.25)[a]	12.00
PGG_2 (0.5)	19.19
EMPA (1)	0.19
MEPA (1)	0.15
PGD_2 (1)	0.19
PGE_2 (1)	0.23
$PGF_{2\alpha}$ (1)	0.20

[a]Indomethacin (final concentration 10^{-6} M) was added 5 min prior to the addition of PGG_2

3T3 cells were incubated for 5 min at 37°C with various compounds in PBS prior to cyclic AMP analyses. The values are means of triplicate determinations on each of two separate culture dishes

product (5% of the radioactivity, R_F 0.12) moved behind $PGF_{2\alpha}$ (R_F 0.20). This latter compound chromatographed in the same region as reported for 6-keto $PGF_{1\alpha}$ [6]. The products were converted to methyl ester, methoxime-trimethylsilyl derivatives and analysed by gas–liquid radiochromatography and by gas–liquid chromatography/mass spectrometry. Five radioactive compounds had the following C-values: 19.2, 21.3, 23.8, 24.3 and 25.2 on 1% SE-30. The third and fourth compounds had mass spectra identical to those of the *syn*- and *anti*-isomers of PGE_2 methyl ester, *O*-methoxime-di-*O*-trimethylsilyl derivative, except that all ions containing the carboxyl group were shifted 2 m.u. upwards by the ^{14}C-label. The mass spectrum of the component with the longest retention time showed ions at *m/e* 600, 560, 541, 510, 470, 451, 420 and 380 corresponding to M-15, M-31, M-71, M-90, M-90-31, M-90-71, M-2 X 90, M-2 X 90-31 and M-2 X 90-71-31 of 6-keto-[1-^{14}C] $PGF_{1\alpha}$ methyl ester, *O*-methoxime-tri-*O*-trimethylsilyl derivative [15].

3.3. *Formation of an inhibitor of platelet aggregation*

3T3 fibroblasts were incubated with 3 ml PBS containing arachidonic acid (7 μg/ml) or PGG_2 (2 μg/ml). At different times after the addition, 50 μl of the buffer was removed and immediately added together with 120 μg arachidonic acid to 0.5 ml PRP.

Aliquots removed within 1 min inhibited platelet aggregation. Maximal inhibition (80–100%) was observed 3–6 min after the addition whereas aliquots removed later than 12 min had no inhibitory effect. These results show that 3T3 fibroblasts produce a factor which has the characteristics of PGI_2 on platelet aggregation [6].

3.4. *Inhibition of the effects of arachidonic acid and PGG_2 on cyclic AMP levels*

It has been reported that 15-hydroperoxy-5, 8, 11, 13-eicosatetraenoic acid inhibits the conversion of PGG_2 to PGI_2 [16]. Therefore 3T3 cells were preincubated for 15 s with 15-hydroperoxy-5, 8, 11, 13-eicosatetraenoic acid (9 μg/ml) prior to the addition of arachidonic acid (250 ng/ml) or PGG_2 (250 ng/ml) (table 3). The stimulatory effect of either compound was completely inhibited, indicating that conversion to PGI_2 was involved.

4. Discussion

Arachidonic acid elevated cyclic AMP levels in 3T3 fibroblasts. Inhibition of the effect by indomethacin suggested that transformation to PGG_2, PGH_2 or a prostaglandin endoperoxide metabolite was required. Although the cells efficiently converted arachidonic acid to PGE_2, this prostaglandin had no effect on cyclic AMP levels. Neither did PGD_2, a potent stimulator of platelet adenylate cyclase [17], $PGF_{2\alpha}$,

Table 3
Inhibition of the effects of arachidonic acid and PGG_2 on cyclic AMP levels

Additions	Cyclic AMP (pmol/μg DNA)
None	0.19
15 - OOH 20 : 4	0.10
20 : 4	8.89
20 : 4 + 15 - OOH 20 : 4	0.13
PGG_2	11.18
PGG_2 + 15 - OOH 20 : 4	0.17

15-Hydroperoxy-5,8,11,13-eicosatetraenoic acid (15 - OOH 20 : 4, 9 μg/ml) was added to 3T3 cultures 15 s prior to the addition of arachidonic acid (250 ng/ml) or PGG_2 (250 ng/ml). 5 min later the cells were analyzed for cyclic AMP. The values are means of triplicate determinations on each of two separate culture dishes

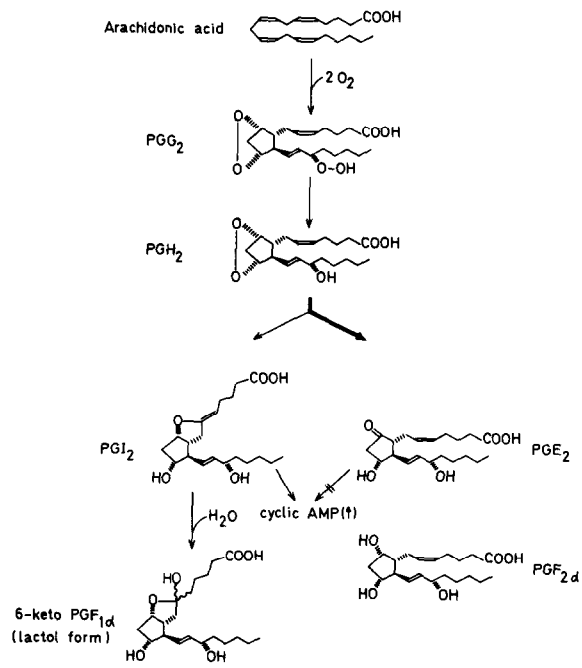

Fig.1. Arachidonic acid transformations and effects on cyclic AMP levels in 3T3 fibroblasts.

nor the endoperoxide analogs EMPA and MEPA. Incubations of arachidonic acid with 3T3 cells showed that in addition to PGE_2 and $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$ was formed. The latter compound is derived from arachidonic acid as outlined in fig.1. Two enzymes are required for the transformation: prostaglandin endoperoxide synthase which forms PGH_2 from arachidonic acid and PGH → PGI isomerase. These enzymes are inhibited by indomethacin [13] and 15-hydroperoxy-5, 8, 10, 13-eicosatetraenoic acid [16], respectively. In addition to arachidonic acid, PGG_2 and 13-dehydro PGI_2 methyl ester [18] raised cyclic AMP levels in 3T3 fibroblasts. On a molar basis 13-dehydro PGI_2 methyl ester was twice as potent as arachidonic acid in raising cyclic AMP levels. Indomethacin prevented the effect of arachidonic acid but not that of PGG_2, whereas 15-hydroperoxy-5,8,11, 13-eicosatetraenoic acid also inhibited the effect of PGG_2. These results suggest that the novel prostaglandin endoperoxide metabolite, PGI_2, has significance as a modulator of cyclic AMP concentrations in 3T3 fibroblasts.

It has recently been reported that arachidonic acid elevates cyclic AMP levels in epithelial cells [19] and in human synovial fibroblasts [20]. Since these effects on cyclic AMP could not be readily explained by conversion to PGE_2 it is possible that PGI_2 formation is involved also in these cells.

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