Exp. Clin. Endocrinol. (Life Sci. Adv.) 1993, 12: 57 – 64

Control of cell cycle by metabolites of prostaglandin D2 through a non-cAMP mediated mechanism

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ABSTRACT:

The dehydration products of PGD_2, 9-deoxy-9 prostaglandin D_2 (PGJ_2), 9-deoxy-$\Delta^9,\Delta^{12},\Delta^{13}$ dehydro-prostaglandin D_2 (Δ^{12} PGJ_2), and PGA_2 all contain an unsaturated cyclopentenone structure which is characteristic of prostaglandins which effectively inhibit cell growth. It has been suggested that the action of the inhibitory prostaglandins may be through a cAMP mechanism.

In this study, we use S49 wild type (WT) and adenylate cyclase variant (cyc^-) cells to show that PGD_2 and PGJ_2 are not acting via a cyclic AMP mechanism. First, the increase in cyclic AMP in wild type S-49 cells is not proportional to its effects on DNA synthesis. More importantly, when S-49 cyc^- cells were exposed to PGJ_2, the adenylate cyclase (cyc^-) mutant had decreased DNA synthesis with no change in its nominal cAMP content. Short-term (2 hours or less) exposure of the cyc^- cells to prostaglandin J_2 caused an inhibition of DNA synthesis. PGJ_2 caused cytolysis at high concentrations. Long-term exposure (>14 hrs) of the cells to PGJ_2, $\Delta^{12}PGJ_2$ or Δ^{12}, $\Delta^{14}PGJ_2$ caused a cell cycle arrest in G_1 demonstrating a cell cycle specific mechanism of action for growth inhibition by naturally occurring biological products independent of cAMP.

INTRODUCTION:

The direct effect of PDG_2 on cell proliferation was first reported by Fukushima in 1982(a), where they found that PGD_2 inhibits in vivo proliferation of L-1210 mouse leukemia

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cells, as well as several lines of human leukemia cells. Subsequently, other laboratories showed that PGD_2 inhibits growth in over 23 different cancer cell lines (Kawamura, 1983, Okada, 1983 and Simmet, 1983). In the past 2 decades, inhibition of cell growth by prostaglandin A_1, A_2 and E_1 has been reported by several investigators (Johnson, 1971, Fukushima, 1983). In these studies, it was noted that PGA_1, was a dehydration product of PGE_1, and it was postulated that the dehydration product of PGD_2 would be a more effective inhibitor of cell growth than PGD_2. Fukushima et al (1982b). synthesized PGJ_2 and found that it was three times more effective than PGD_2 in stopping cell growth. PGJ_2 possesses a cyclopentenone structure similar to the effective PGA_1 series supporting the hypothesis that the α,β unsaturated ketone group in the ring structure is an important feature for the effective inhibition of DNA synthesis (Turner, 1982). Studies have shown that PGD_2 is converted to $\Delta^{12}PGJ_2$ by human albumin and serum (Fukushima, 1982b, Fitzpatrick, 1983). The anti-growth properties of PGD_2 are probably due to it's dehydration to ΔPGJ_2, $\Delta^{12}PGJ_2$ or Δ^{12}, $\Delta^{14}PGJ_2$.

Prostaglandins stimulate the synthesis of adenosine 3'5' cyclic monophosphate (cAMP), and therefore, it has been suggested that the growth retardation caused by the inhibitory prostaglandin E_1 is mediated by this increase in intracellular cAMP level (Ramwell, et al, 1970, Perry, 1971 and Honn, 1981)). However, in 1983, Wiley (Hughes-Fulford) et al. showed that prostaglandins per se can inhibit DNA synthesis . More recently, Nakahata et al (1990) reported that PGJ_2 and Δ^{12} PGJ_2 inhibited cell growth in parallel with an inhibition of phosphoinositol turnover. They also reported that PGJ_2 and $\Delta^{12}PGJ_2$ disturbed the adenylate cyclase system which might regulate on cell growth. In another related work, it was reported that PGD_2 specifically elevated intracellular cAMP suggesting a cAMP mediated cell cycle arrest by the prostaglandin D_2 and PGJ_2 (Sugama, 1989). Finally, reports of the induction of *gadd153* mRNA in prostaglandin cell cycle arrest (Choi 1992) have shown that protein kinase inhibitor 2-aminopurine decreased PGA_2 induction of *gadd 153* expression. These authors suggested that the PGA_2 mediated growth arrest through a protein kinase since the use of the broad spectrum protein kinase inhibitor reduced the *gadd 153* signal. These observations have caused renewed interest the prostaglandins mechanism of action and their role as growth regulators.

MATERIALS AND METHODS

Reagents. [^{3}H]-methyl thymidine (20 μCi/mmol), and cAMP radioimmunoassay kits were obtain from New England Nuclear. Chromomycin A3 was purchased from Calibiochem, San Diego, CA.

Dulbecco's modified media (DME) with Earle's salts was supplemented with 10% v/v fetal calf sera (Hy Clone).

Cell Culture. S-49 wild type and variant cells were grown in suspension while maintained in a humidified incubator (5% CO_2) at 37°C in 250 ml flasks containing from 20-40 ml media. Each experiment was carried out in duplicate or triplicate in individual tubes. All prostaglandins were added in ethanolic solutions. The controls were treated with the same concentration of ethanol that never exceeding 0.1%.

Measurements of DNA synthesis. At the specified time points, cells were incubated with [3H]methyl thymidine (2 μCi ml) for 15 minutes at 37°C. After incubation, cells were centrifuged, the solution removed, and 1 ml of cold tricholoroacetic acid (TCA) (5%) was added to each tube; the samples were washed twice more with TCA solution and then solubilized with 0.2 ml of sarcocyl buffer. The solution was analyzed for tritiated thymidine incorporation in mini-vials using 5 ml of Ultrafluor (National Diagnostics, Sommerville, NJ) in a Beckman LS-330 Counter.

Protein determination. Samples were assayed for protein using the method of Lowry (1951).

Measurement of cAMP content. Cyclic AMP levels were measured according to the modified method of Rapoport (1970) on the same cells in which the DNA synthesis was determined. Briefly, after centrifugation the tritiated thymidine solution was rapidly aspirated and 1.0 ml of cold 5% TCA was added. After 30 minutes at 0-4°C, the TCA solution containing cAMP from the disrupted cells was collected, frozen, and later extracted and assayed for cAMP by radioimmunoassay.

Cell counts and flow cytometry. All cell counts were made on a ZBI Coulter Counter using Isotone II as the diluent. For flow cytometry, cells were fixed and stained as described by Gray (1979). Briefly, cells ($1x10^6$) were centrifuged and washed one time with PBS. After centrifugation, the pellet was re-suspended in 1 ml of 15 mM $MgCl_2$ in 95% EtOH and stored at 4°C. To stain, the cells were centrifuged and re-suspended in 2 ml chromomycin A3 (10 mg + 1.5 g $MgCl_2$ in 500 ml H_2O) filtered through 37 micron nylon mesh, capped, and measured for DNA content at the University of California Brain Tumor Research Center. The flow cytometry data were graphically interpreted by the method of Gray (1979).

Dye exclusion. After centrifugation media was aspirated and replaced by an equal volume of .04% trypan blue in PBS for 10 minutes and counted using a hemocytometer.

RESULTS AND DISCUSSION

As seen in Table I, in S-49 wild

	cAMP pm/mg protein @ 15 Minutes -	DNA SYNTHESIS % Inhibition @ 2 HRS
CONTROL	1.6 ± .1	0
1 M	5.4 ± .3	0
5 M	4.1 ± .5	17±3
10 M	4.1 ± .3	44±2

TABLE 1
EFFECT OF PGJ_2 ON cAMP CONTENT AND DNA SYNTHESIS IN S-49 WILD TYPE CELLS

S-49 wild type cells were exposed to various concentrations of PGJ2 (or vehicle). After 15 minutes, cAMP sample were taken at 1.5 hours, tritiated thymidine was added, and its incorporation into DNA was determined over the following 30 min. Thymidine incorporation into DNA ranged in controls from 1,200 cpm/mg to 600 cpm/μg protein. All values are given in ± S.E. of the mean.

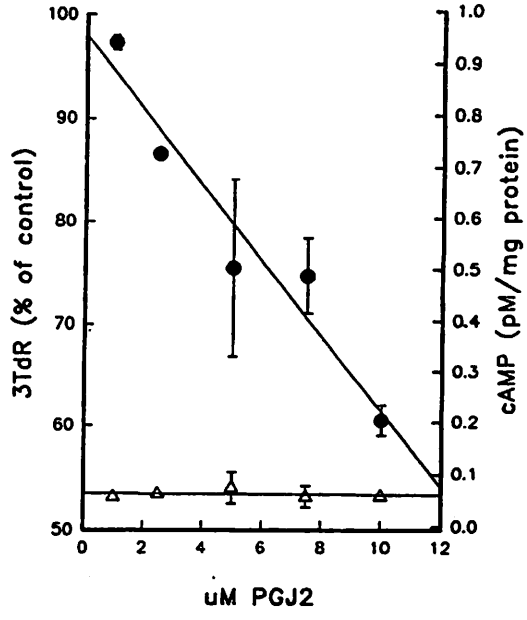

Figure 1
Effect of incubation with PGJ_2 on DNA synthesis and cAMP generation in cyc^- cells. DNA incorporation (●–●) was measured after 2 hours and cAMP (Δ–Δ) samples taken after 15 minutes exposure to PGJ_2 or vehicle. Tritiated thymidine incorporation into DNA range from 1150 cpm/μg to 700 cpm/μg. There was no difference in cAMP levels in treated and untreated cells. All values are reported +/- S.D. of the mean.

type lymphoma cells, PGJ_2, caused an inhibition of DNA synthesis, and a rise in cAMP content.
Subsequent studies used a cyc^- cell variant derived from the S-49 mouse lymphoma cell line. This cyc^- variant possesses the catalytic unit of adenylate cyclase, but lacks the protein component needed for coupling the hormone receptor to the adenylate cyclase (Bourne, 1975).

As illustrated in Figure. 1, the inhibition of DNA synthesis in the cyc cells is proportional to the concentration of PGJ_2. By contrast, cAMP concentration is completely unaffected by PGJ_2. These data demonstrate that the PGJ_2 inhibition of DNA synthesis is not dependent or cAMP concentration.

Although PGJ_2, Δ^{12}-PGJ_2 and $\Delta^{12},\Delta^{14}PGJ_2$ differ only slightly in structure there is a marked difference in the effectiveness of growth and DNA synthesis inhibition. When the compounds are added acutely for two hours and DNA synthesis is then measured, the concentration needed

for half maximal inhibition of DNA synthesis in S49 cyc⁻ cells for PGJ_2 is 15 μM, and for Δ^{12}PGJ2 and $\Delta^{12},\Delta^{14}PGJ_2$ is 5.6 and 5.0 μM respectively. As a point of reference, we found the concentration needed for maximal inhibition of DNA synthesis in the same system is 2 μM for actinomycin D and 6.2 μM for adriamycin (unpublished observation by authors).

In addition to the acute effect on DNA synthesis, these prostaglandins have a second and profound effect of blocking the cell cycle. Prolonged exposure to 10 μM or lower concentrations of the J_2 prostaglandins cause the cells to become arrested in G_1. A typical flow cytometric graph is shown in Fig. 2.

If the prostaglandins are removed, the effect on the cell cycle is reversible;

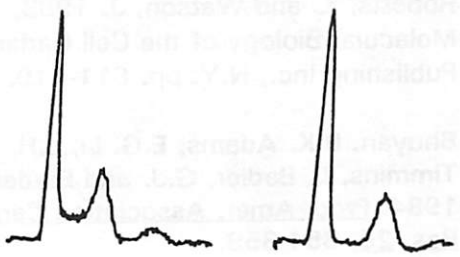

FIGURE 2: Effect of PGJ_2 on cell cycle Cyc⁻ cells were incubated with 10 μM PGJ_2 or vehicle control for 40 hours in DME media with 10% FCS before flow cytometric analysis as described in Methods.

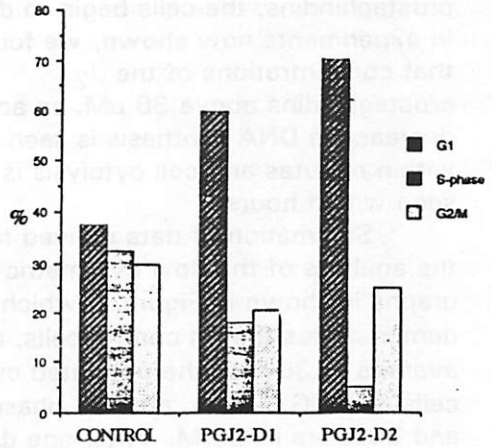

FIGURE 3; Distribution of cell population in 4 phases of the cell cycle: 5x10 cells were grown as previously described in 10 uM prostaglandin J_2 for 48 hours. Cell cycle cell population was determined using flow cytometry. Samples were taken at 0. 24 and 48 hours.

however as early as sixteen hours after exposure to 10μM PGJ_2, $\Delta^{12}PGJ_2$ or $\Delta^{12},\Delta^{14}PGJ_2$, the cell population as determined by low cytometry significantly enriched in G_1 of the cell cycle (39 ± 12 vs. 44 ± 2) $p<.05$. As seen in Figure 3, 24 hours of exposure to the J_2 prostaglandins caused the cell population in G_1 to increase to 54 ± 5 vs. 39 ± 2 ($p<.05$). This increase in the G_1 population is a result of the number of cells in S-phase decreasing to 16 ± 2 from $32\pm3\%$ ($p<.01$).

After 48 hours of exposure, the cells are essentially quiescent and after 60 hours of exposure to these

prostaglandins, the cells begin to die. In experiments now shown, we found that concentrations of the J_2 prostaglandins above 30 μM, an acute decrease in DNA synthesis is seen within minutes and cell cytolysis is seen within hours.

Summation of data derived from the analysis of the flow cytometric graphs is shown in Figure. 3 which demonstrates that in control cells, an average of 38% of the untreated cyc$^-$ cells are in G_1, 32% are in S-phase and 30% are in G_2/M. After one day of treatment, the cell population is greatly enriched to 60% in G_1 with cells in S-phase and G_2/M reduced to almost half. By the second day, 70% of the cells are enriched in G_1 with S-phase reduced to 5%. At this time, many of the cells that had been in S-phase at the time of prostaglandin addition, have completed the cell cycle and are resting in G_1 at the point of the prostaglandin growth block.

Early hypothesis of cell growth regulation suggested that cells have a "restriction point" in G_1 that controls entry into S-phase DNA synthesis as described by Pardee (1983). Others described a "trigger" point or molecule that would allow entry into S-phase DNA synthesis Alberto, (1983). Today it is apparent that regulation of cell cycle is a multi-phasic action of combined pathways which can be receptor mediated, ion flux facilitated and/or regulated at the level of gene expression. The inhibitory prostaglandins like PGD_2 and PGJ_2 are probably interfering with one or more of these regulatory pathways. The data presented here suggests that their mechanism of action is not mediated through cAMP or cAMP dependent protein kinases.

These compounds regulate cell growth by inhibiting entry into S-phase near the G_1/S boundary. These naturally occurring compounds offer a powerful tool for the study of control of the cell cycle and regulation of initiation of DNA synthesis.

ACKNOWLEDGMENTS

This work was supported by NASA grant NAGW-1244 and in part by Veterans Administration Research Grant. We thank Sasha Blaug for his excellent technical assistance.

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