A Role of Guanosine 3′,5′-Monophosphate in Human–Prolactin–Induced Estrogen Production by Feminizing Adrenal Neoplastic Cells

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FURUHASHI, N. and FANG, V.S. A Role of Guanosine 3′,5′-Monophosphate in Human–Prolactin–Induced Estrogen Production by Feminizing Adrenal Neoplastic Cells. Tohoku J. exp. Med., 1980, 132 (1), 87–92 — Using feminizing adrenal neoplastic gland (FANG) cells we showed that hPRL stimulated cGMP biosynthesis. Also we could clearly demonstrate a stimulatory effect of db-cGMP plus theophylline on estrogen production without concomitant stimulation of cell growth. These findings indicate that a stimulatory effect of hPRL on estrogen biosynthesis by Fang-8 cells may act via increased cGMP concomitant with depressed cAMP.

We have recently succeeded in establishing a clonal line of human epithelial cells derived from a feminizing adrenal neoplastic gland (FANG) and designated them Fang-8 cells (Fang 1977; Fang et al. 1978). In continuous culture, Fang-8 cells maintained the specific function of estrogen synthesis. Also, we demonstrated a stimulatory effect of human prolactin (hPRL) on estrogen biosynthesis (Fang et al. 1978). We have carried out experiments to study the stimulatory effect of hPRL on estrogen production and the relationship between hPRL, estrogen production and cyclic nucleotides.

MATERIALS AND METHODS

The cell culture environment was kept at 37°C, 100% humidity, and 5% CO₂-95% air. We transferred 5–10 × 10⁴ cells to each new Falcon 3003 petri dish (100 × 20 mm) and cultured them in 6–10 ml of regular medium (Matalon modified Eagle’s medium containing 10% each calf and fetal calf sera). To maintain the culture, medium was changed every 3 or 4 days as previously described (Fang 1977; Fang et al. 1978).

Experiment 1: Effects of hPRL on the cAMP and cGMP concentrations in Fang-8 cells.
The Fang-8 cells harvested from dishes were divided to tubes (approximately 5–10 × 10⁸ cells/tube). Then, hPRL (0.2 µg) was put in each tube and the cells were incubated with

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Abbreviations: ACTH, adrenocorticotropic hormone; hPRL, human prolactin; cAMP, adenosine cyclic 3′,5′-monophosphate; cGMP, guanosine cyclic 3′,5′-monophosphate; db-cAMP, N⁶,O²-dibutyryl-adenosine-3′,5′-cyclic monophosphate; db-cGMP, N⁶,O²-dibutyryl-guanosine-3′,5′-cyclic monophosphate.
constant shaking. At the end of incubation, the cells were centrifuged down (2,300 rpm, 10 min) and the medium was collected for cAMP and cGMP determinations. The cell pellets were sonicated and protein concentration was determined by Lowry’s method (Lowry et al. 1951). Cyclic AMP and cGMP in the medium were measured by competitive protein assay as previously described (Kobayashi and Fang 1975).

**Experiment 2: Effects of db-cAMP and db-cGMP on estrogen production by Fang-8 cells.** The Fang-8 cells were incubated with regular medium after reprogramming. On the third day, experiments were performed. At the end of each experiment, the cells were collected and washed two times with phosphate buffer saline (pH 7.5) before the determination of cellular protein. Estrogen in the medium and intracellular estrogen were measured by the radioimmunoassay technique as previously described (Fang et al. 1974).

**Isotopes and chemicals.** [3H]cGMP (21 Ci/mmmole) was purchased from Amersham Searle Corp., Arlington Height, Ill., and [3H] cAMP (37.7 Ci/mmmole) was purchased from New England Nuclear, Boston, Mass.; db-cAMP, db-cGMP and dopamine were purchased from Sigma Chemical Co., St. Louis, Mo. and CB-154 (2-Br-ergocryptin) was purchased from Sandoz Ltd, Switzerland. Human PRL (VSL-3) was generously donated by the pituitary hormone distribution program of NIAMDD.

**Statistical Analysis.** Group data were subjected to Student’s t-test for significance of difference.

**RESULTS**

Fig. 1 shows the effect of hPRL (0.2 μg/tube) on concentrations of cAMP and cGMP produced by Fang-8 cells. Cyclic GMP levels in the control system increased progressively whereas cAMP levels in control did not change so much. Cyclic GMP levels in the group which was added with hPRL (0.2 μg/tube) were increased 5 times at 15 min of incubation as compared with those in the control.

![Graph showing the effect of hPRL on concentrations of cAMP, cGMP, and estrogen](image)

Fig. 1. Effects of hPRL (0.2 μg/tube) on concentrations of cAMP, cGMP and estrogen in the culture medium of Fang-8 cells. ▲, cGMP, hPRL 0.2 μg; ●, cGMP-control; ○, cAMP-hPRL 0.2 μg; ▲, cAMP-control; ◀, estrogen-hPRL 0.2 μg; ◐, estrogen-control. Each point represents the mean value of duplicate determinations.
hPRL Stimulates cGMP in Fang-8 Cells

system, then decreased to near a control level at 45 min. Cyclic AMP levels at 15 min in the group incubated with hPRL (0.2 µg/tube) decreased to about 40% of the level at 2 min, then increased to near a control level in 45 min.

It was clear that hPRL (0.2 µg/tube) stimulated an estrogen production of Fang-8 cells (Fig. 2). Cyclic AMP levels were not changed; however, cGMP level responded to concentration of hPRL. Cyclic GMP level in the group treated with hPRL (0.2 µg/tube) increased 2 times as compared with that in the control system.

Fig. 2. Production of cAMP (●), cGMP (▲), and estrogen (○) in the culture medium of Fang-8 cells which were incubated 15 min in the presence of 0 to 0.2 µg of human prolactin. Each point represents the mean value of duplicate determinations.

**TABLE 1. Estrogen production of Fang-8 cells and effects of theophylline and cyclic nucleotides**

<table>
<thead>
<tr>
<th>Compound/dish</th>
<th>Estrogen production (pg/µg Protein)</th>
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<tbody>
<tr>
<td></td>
<td>Incubation time</td>
</tr>
<tr>
<td></td>
<td>4 hr</td>
</tr>
<tr>
<td>Control</td>
<td>1.89±0.05*</td>
</tr>
<tr>
<td>Theophylline, 0.5 mM</td>
<td>1.65±0.27</td>
</tr>
<tr>
<td>Theophylline, 1 mM</td>
<td>1.25±0.13</td>
</tr>
<tr>
<td>db-cAMP, 10⁻⁴M</td>
<td>1.74±0.49</td>
</tr>
<tr>
<td>Theophylline, 1 mM+db-cAMP, 10⁻⁴M</td>
<td>1.37±0.15</td>
</tr>
<tr>
<td>db-cGMP, 10⁻⁴M</td>
<td>2.03±0.33</td>
</tr>
<tr>
<td>Theophylline, 1 mM+db-CGMP, 10⁻⁴M</td>
<td>2.69±0.13</td>
</tr>
</tbody>
</table>

* Mean±s.d. of 4 cases.
† Comparison with each control group.
‡ Not significant.
Incubation with theophylline, db-cAMP, db-cGMP or combinations affected the total estrogen production of Fang-8 cells (Table 1). Addition of 0.5 mM theophylline alone did not exhibit significant effect at 4 hr and 24 hr of incubation; however, 1 mM theophylline suppressed the production significantly at 4 hr of incubation. Addition of $10^{-6}$ M db-cAMP alone exerted no effects, but a combination of 1 mM theophylline and $10^{-6}$ M db-cAMP inhibited estrogen production of Fang-8 cells significantly. db-cGMP of $10^{-6}$ M alone stimulated estrogen production of Fang-8 cells at 24 hr of incubation. A combination of 1 mM theophylline and $10^{-6}$ M db-cGMP stimulated estrogen production of Fang-8 cells significantly; especially the stimulation at 4 hr of incubation was tremendous.

When the time courses of estrogen production of Fang-8 cells which were cultured with 1 mM theophylline plus $10^{-6}$ M db-cAMP or 1 mM theophylline plus $10^{-6}$ M db-cGMP were compared with that of the control system, the response to stimulatory effect of theophylline plus db-cGMP was found to be rapid and continuous. On the other hand, the inhibitory effect of theophylline plus db-cAMP was also rapid and continuous (Fig. 3). The difference in effects of db-cGMP and db-cAMP on the total estrogen production of Fang-8 cells at the incubation of 4 hr and 24 hr was significant ($p<0.05$).

DISCUSSION

The present studies confirm the previous observation (Fang et al. 1978) that hPRL had stimulated the estrogen production by Fang-8 cells (Fig. 2). Millington et al. (1976) reported that in a primary tissue culture of feminizing adrenocortical carcinoma, both PRL and ACTH stimulated the synthesis of estrogens whereas GH, LH and FSH were more effective than PRL in stimulating androgen synthesis. However, many adrenal tumors had failed to respond to ACTH (Saez et al. 1975;
Larson et al. 1976). It is conceivable that Fang-8 cells are of more specific type compared with other cell lines.

In the present studies, we showed that hPRL stimulates the synthesis of cGMP rather than cAMP (Fig. 1). Honn and Chavin (1975) described that ACTH may act via increased intracellular cGMP concomitant with depressed cAMP. Recently, Sharma et al. (1974, 1976) and Perchellet et al. (1978) suggested that cGMP rather than cAMP is the physiological mediator of ACTH-induced adrenal steroidogenesis. These reports indicated that cGMP had physiological regulatory role for ACTH-induced adrenal steroidogenesis. On the other hand, prolactin, LH and FSH were reported to be more effective than ACTH in stimulating the adenylyl cyclase activity in feminizing adrenocortical carcinoma (Millington et al. 1976). This reports suggested that under pathological conditions, PRL rather than ACTH may act via cyclic nucleotide on adrenal steroidogenesis. These findings strongly suggest that PRL may act on adrenal steroidogenesis under pathological conditions.

Our results show that theophylline plus db-cGMP stimulates estrogen production of Fang-8 cells and theophylline plus db-cAMP inhibits estrogen production of Fang-8 cells (Fig. 3, Table 1). An earlier report of Glinsmann et al. (1969) described that exogeneous cGMP stimulated steroidogenesis in rat adrenal slices. Our results suggest that cGMP itself has stimulatory effect for steroidogenesis in adrenal cells. It is conceivable that the feminizing adrenal cortex cells have the capacity to respond to cGMP in estrogen biosynthesis and the stimulatory effect of hPRL may act via cGMP.

References


