Effect of Estradiol and FBS on PRL Cells, GH Cells, and PRL/GH Cells in Primary Cultures of Pituitary Cells from Prenatal Rats

YUTAKA YAMAMURO,[1] TOMOHIRO AOKI, AND NAOTO SENSUI

Department of Animal Science, College of Bioresource Sciences, Nihon University, Fujisawa, Kanagawa 252-8510, Japan

The effects of estradiol (E_2) treatment on prolactin (PRL) cells, GH cells, and PRL/GH cells in immature pituitary cells were determined using primary cultures from prenatal rats and immunocytochemistry with fluorescent antibodies. Anterior pituitaries obtained from fetuses on day 22 of pregnancy were monodispersed and cultured in chemically defined medium or medium containing 10% fetal bovine serum (FBS). After preincubation for 24 hr, E_2 (final concentrations were 0 *M*, 10^{-8} *M*, 10^{-7} *M*, and 10^{-6} *M*) was added into each medium. After 72 hr of incubation, cells were subjected to immunocytochemistry. E_2 stimulated the increase of PRL cells in a dose-dependent manner, and the PRL cell percentage cultured with FBS in all groups was significantly higher than that cultured in chemically-defined medium. PRL/GH cells also responded to E_2 in the same manner as PRL cells. E_2 was not effective in proliferating GH cells, and GH cell percentage significantly decreased with the addition of FBS into the medium. These results suggest that E_2 is dose-dependently capable of increasing immature PRL cells and/or PRL/GH cells *in vitro*. Moreover, there is a factor(s) in FBS that regulates the increase of these cells.

[E.B.M. 2001, Vol 226:140–143]

Key words: estradiol; PRL cell; GH cell; PRL/GH cell; prenatal rat

Prolactin (PRL) is secreted from specific secretory cells called mammotrophs (PRL cells) in the anterior pituitary. It is widely believed that the populations of PRL cells and somatotrophs (GH cells) consist of morphologically and functionally heterogeneous cells. Mammosomatotrophs, which secrete both PRL and GH (PRL/GH cells), were also found in the pituitary of several species (1–3). Previous studies have demonstrated that substantial development of PRL cells in rats occurs in the perinatal period (4–7). It has been proposed that a pituitary-specific transcription factor (Pit-1) is required for the onset of PRL and GH gene expression during the developmental stage of the anterior pituitary (8, 9). However, the factor(s) that regulates Pit-1 *per se*, and finally, proliferation and differentiation of PRL cells in this period is not yet clear.

Estrogen is an important factor in the control of postnatal sexual maturation in female rats. Chronic estradiol (E_2) treatment stimulates not only PRL secretion (10, 11) but also the proliferation of PRL cells (12, 13) in the anterior pituitary of mature rats. However, the estrogen-induced PRL gene expression or PRL cell proliferation in the anterior pituitary *in vivo* is not involved in the expression of Pit-1 mRNA in the mature animals (14), and there have been no reports of investigations of the response to estrogens of immature PRL cells *per se*.

To define the responsiveness of developing these cells to estrogen, we investigated the effect of estradiol treatment on the PRL cells, GH cells, and PRL/GH cells using primary cultures of immature pituitary cells from prenatal rats and immunocytochemistry using antibodies labeled with two different fluorescent markers, and, moreover, the difference between culture in chemically defined medium and medium with fetal bovine serum (FBS).

Materials and Methods

Wistar rats bred in our laboratory were used in this experiment. Anterior pituitaries were obtained from 8 to 10 fetuses of mothers on day 22 of pregnancy (the day before parturition) and placed in 2 ml of D-MEM (Gibco Life Technologies, Inc., NY) containing 0.1% BSA with trypsin (1 mg/ml; Gibco Life Technologies) and incubated at 37°C for 60 min under 95% air–5% CO_2. The monodispersed cells were then rinsed three times with D-MEM without trypsin. The cells were resuspended in basal medium (D-MEM:HAM F-12 [Gibco Life Technologies] = 1:1) containing (a) 2.5 mg/ml BSA, 5 μg/ml insulin, 2 μg/ml transferrin, 0.2 ng/ml 3,3′,5-triiodo-L-thyronine (3,5,3′), 5 ng/ml 1,4-diaminobutane (chemically defined medium), or (b) 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS), and

This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan to Y.Y.

[1] To whom requests for reprints should be addressed at Department of Animal Science, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-8510, Japan. E-mail: yamamuro@brs.nihon-u.ac.jp

Received December 13, 1999.
Accepted September 18, 2000.

0037-9727/01/2262-0000$15.00/0

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330 µl of each was pre-incubated for 24 hr in chamber slides coated with poly-D-lysine. After pre-incubation, 1 µl of 0, 0.3 × 10^{-8}, 0.3 × 10^{-7}, and 0.3 × 10^{-6} mol/ml E_2 (β-estradiol 3-benzoate; Sigma Chemical Co., St. Louis, MO) prepared with saline was added into the medium; final concentrations were 0 *M*, 10^{-8} *M*, 10^{-7} *M*, and 10^{-6} *M*, respectively. After 72 hr of incubation, cells were subjected to immunocytochemistry as described in detail by Yamamuro *et al.* (7). After fixation by 4% paraformaldehyde and blocking, anti-rat PRL mouse IgG_1k (Chemicon International Inc., Temecula, CA) and anti-rat GH rabbit antiserum (Biogenesis, Poole, UK) as primary antibodies and FITC-conjugated anti-mouse IgG (Fc) goat IgG (American Qualex, San Clemente, CA) and RITC-conjugated anti-rabbit IgG goat IgG (Chemicon International Inc.) as second antibodies were applied to the cells. The immunostaining was visualized with a microscope using a mercury lamp with a 490 nm or 570 nm excitor filter for FITC or RITC, respectively. The percentage of PRL, GH, and PRL/GH cells (cells positive against both antibodies) per all anterior pituitary cells in a visual field at four different locations was calculated, and the mean of the percentage was used as the representative value.

Another experiment was performed to examine whether the increase in percentage of cells depends on the proliferation. The monodispersed cells prepared by the same procedures as described above were suspended in basal medium with 10% FBS, and 330 µl of each was pre-incubated for 24 hr. After pre-incubation, 1 µl of 0 or 0.3 × 10^{-6} mol/ml E_2 was added into medium. After 72 hr of incubation, cell culture medium was aspirated and 150 µl of BrdU (5-bromo-2′-deoxy-uridine; Boehringer Mannheim, Germany) labeling reagent diluted with culture medium (final concentration 10 µM BrdU) was added. The cells were incubated at 37°C for 60 min. After being washed three times with PBS and fixation by 70% ethanol in 50 m*M* glycine buffer (pH 2.0) for 20 min at −20°C, anti-BrdU mouse IgG_1 (Boehringer Mannheim) and anti-rat PRL antiserum (NIDDK-anti-rPRL-IC-5, rabbit) or anti-rat GH rabbit antiserum as primary antibodies and FITC-anti-mouse IgG and RITC-anti-rabbit IgG as second antibodies were applied to the cells. The percentage of BrdU-positive cells per PRL- or GH-positive cells was calculated, and the mean of the percentage was used as the representative value from five separate experiments.

Statistical significance was determined by two-way analysis of variance (ANOVA) and Duncan's new multiple range test. The criterion for significance was $P < 0.05$ in all cases.

Results

$Estradiol_2$ stimulated the increase in PRL cell percentage in a dose-dependent manner in cultures in both chemically defined medium ($F_{(3,32)}$ = 4.860, $P < 0.01$) and with 10% FBS ($F_{(3,32)}$ = 3.476, $P < 0.05$). When compared between the groups with the same dose of E_2, PRL cells cultured with FBS were significantly higher than those cultured in chemically defined medium. No dose of E_2 altered the GH cell percentage. In contrast to PRL cells, GH cell percentage significantly decreased by FBS addition into the medium. PRL/GH cells tended to be increased by E_2 in the same manner as PRL cells, but a significant difference compared to the control was not seen except at the highest dose (10^{-6} *M*) in medium with FBS (Fig. 1).

The ratio of proliferating cells in PRL- and GH-positive cells was determined by immunocytochemistry using anti-BrdU antibody and anti-PRL or anti-GH antibody. There was no significant difference between the control and E_2 treatment groups in both PRL- and GH-positive cells (Table I).

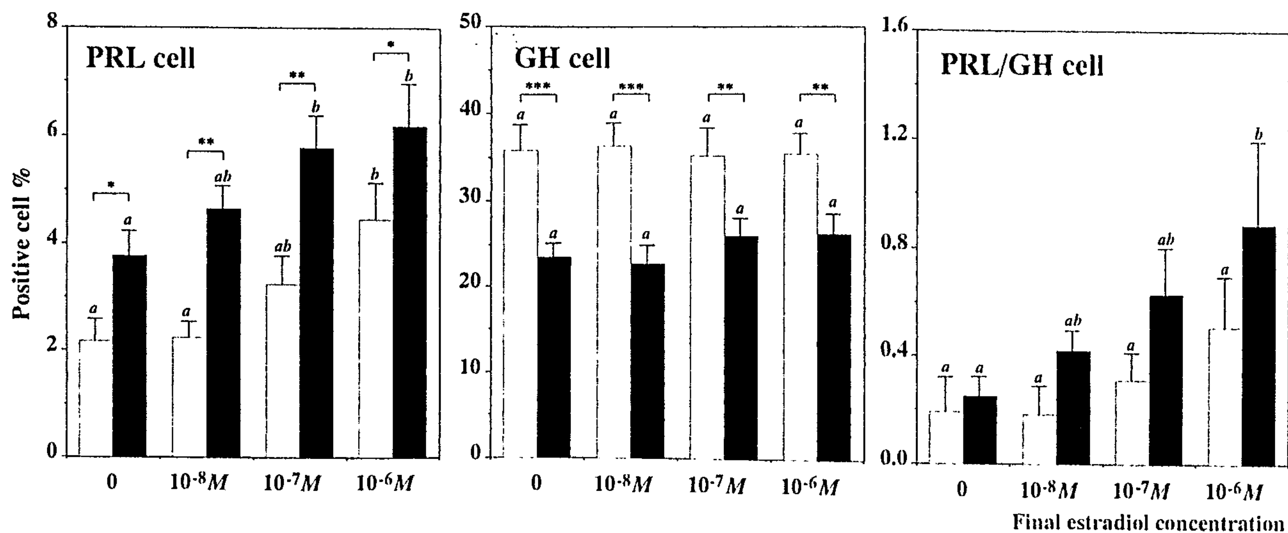

Figure 1. Effect of estradiol treatment on the percentage of PRL-, GH-, and PRL/GH-containing cells in primary cultures of pituitary cells from prenatal rats. Different small letters indicate a significant difference between the treatments ($P < 0.05$). *, **, ***Values are significantly different between chemically defined medium (□) and medium with 10% FBS (■) treated with the same dose of estradiol ($P < 0.05$, 0.01, and 0.001, respectively). Data were obtained from 9 separate experiments.

Table I. Percentages of BrdU-Positive Cells in PRL and GH Cells in Primary Cultures of Pituitary Cells from Prenatal Rats

Groups	$(n)^a$	E_2 treatments	
		0 *M*	10^{-6} *M*
BrdU/PRL	5	22.2 ± 10.1	24.2 ± 5.3
BrdU/GH	5	33.7 ± 2.3	34.6 ± 6.2

Note. Values are mean ± SEM.
[a] Data were obtained from 5 separate experiments.

Discussion

The present study demonstrated that E_2 is dose-dependently capable of stimulating immature PRL cells in the anterior pituitary.

When cultured in chemically defined medium, PRL cells tended to respond to E_2 at concentrations above 10^{-7} *M*. Concerning the possibility that the increase in cell number by FBS is attributable to its content of estrogens, it is assumed that the concentration of E_2 in FBS was in the range of 10^{-7} to 10^{-6} *M*. However, the percentage of PRL cells cultured in FBS tended to increase with 10^{-8} *M* of E_2 compared to that of the control without E_2. In the absence of FBS, this dose of E_2 had no effect. Customarily, serum is added in cell culture medium in order to maintain cell survival and/or mitosis, but these results suggest that there is a factor(s) in FBS that directly stimulates the cell growth or synergistically enhances the action of estrogens in stimulating the immature PRL cells *in vitro*. PRL cell differentiation and proliferation are stimulated by IGF-I (15) and FGF (16), which are candidates contained in FBS. At present, the direct action of these factors on PRL cell growth in the neonatal period is not known.

The ratio of proliferating or proliferated cells in PRL-positive cells did not increase with E_2 treatment (Table I), although E_2 increased the percentage of PRL cells. This result suggests that PRL synthesis in pre-PRL cells is induced by E_2 or the E_2 converts GH-secreting cells into PRL cells. However, the fact that 20–30% of PRL cells in the control (FBS without E_2) are proliferating *in vitro* might obscure the action of estrogen to immature PRL cells.

PRL/GH cells also responded to E_2 in the same manner as PRL cells. Despite the fact that plasma E_2 concentration throughout the postnatal period is higher as compared in adult rats (17), no particular change in PRL/GH cell percentage in the anterior pituitary was recognized until the middle stage of the suckling period *in vivo* (7). Steroid hormones are rendered biologically inactive while bound to plasma protein. A high concentration of estrogen-binding protein or α-fetoprotein, which binds to estrogens, is shown in the perinatal period, and gradually declines in concentration during the first 4–5 weeks of postnatal life (18, 19). The relationship between the decline in plasma level of these proteins and the maturation of PRL and PRL/GH cells is of interest. However, this remains to be tested. At the present time, another candidate that interferes with the effect of estrogen on the growth of PRL cells and PRL/GH cells in the neonatal period is TGF-β_1, which is known to inhibit the growth of many estrogen-responsive cells. TGF-β_1 is produced in the pituitary gland and inhibits the secretion of PRL and growth of PRL cells in an autocrine and/or paracrine fashion (20), and has a biphasic effect, depression in higher dose (10^{-9} *M*) and stimulation in lower dose (10^{-13} *M*), on the proliferation of PRL cells *in vitro* (21). However, the dose-dependent increase in the percentage of these cells by E_2 in chemically defined medium was not, at least, concerned with factors as described above.

In contrast to PRL cells and PRL/GH cells, FBS added to culture medium significantly reduced GH cell percentage in all groups. Our prevous *in vivo* study (7) confirmed that GH cells, about 10% of anterior pituitary cells in the rat fetus of Day 20, increase up to Day 1 after birth and reach the level of adult rats (>30%) by Day 7. FBS added to culture medium obviously suppressed the increase in GH cells since GH cell percentage in culture with chemically defined medium was above 30%. The same result was obtained in each cell type when we tried to culture using another lot number of FBS (data not shown). It is not clear whether a factor(s) that stimulates PRL cells and PRL/GH cells is identical with a factor(s) that inhibits GH cell growth in FBS. However, the levels of estradiol in FBS do not seem to produce this decrease in GH cell percentage (Fig. 1).

We thank Dr. A. F. Parlow, Scientific Director, National Hormone & Pituitary Program, Harbor-UCLA Medical Center, for providing antiserum for rat PRL (NIDDK-anti-rPRL-IC-5).

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