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Estrogen control of prolactin synthesis *in vitro*

(primary pituitary cell culture/immunoprecipitation)

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ABSTRACT **Primary cultures of rat pituitary cells respond to estradiol-17β by increased incorporation of radiolabeled precursors into prolactin but not into the bulk of other cellular proteins. The rate of increase in prolactin synthesis is dose dependent, reaching maximal levels in the physiological range of estradiol. At a concentration of 1 nM, estradiol, diethylstilbestrol, and estriol are stimulatory whereas androgens, progesterone, and corticosterone are without significant effect. Exposure of pituitary cells to 10 nM estradiol resulted in a 500% increase in prolactin synthesis after 7 days of culture. The results indicate that estradiol can stimulate prolactin synthesis through direct action on the pituitary.**

There is an increasing body of evidence implicating estradiol in the regulation of prolactin synthesis and secretion. Recent work from our laboratory demonstrated that treatment of rats with estradiol specifically increased the incorporation of precursors into prolactin (1) and led to the accumulation of preprolactin mRNA in the pituitary (2). The effects of estradiol may be due to either direct effects on the pituitary or indirect effects mediated by another tissue such as the hypothalamus.

Nicoll and Meites (3) first demonstrated increased prolactin production by pituitary explants incubated in unphysiological concentration (2 μM) of estradiol. Conflicting data have been presented regarding the effects of estradiol on GH_3 cells, a clonal strain derived from a rat pituitary tumor (4). Tashjian and Hoyt (5) and Haug and Gautvik (6) reported up to 3-fold increases in prolactin secretion by GH_3 cells in the presence of physiological concentrations of estradiol (10 pM–10 nM). Subsequently, Dannies *et al.* (7) reported that the stimulatory effect could no longer be elicited in later generations of the same cell line and, paradoxically, estradiol behaved as an antagonist. In the above studies, the investigators used either unphysiological concentrations of estradiol or pituitary tumor cells. Such cells may have different functional characteristics from normal pituitary cells and may also exhibit changes in their functional characteristics with time.

The present study uses primary cultures of dispersed pituitary cells from immature rats. Estrogen treatment of these cell cultures results in the stimulation of prolactin production in a manner that is comparable to responses seen *in vivo*.

MATERIALS AND METHODS

Chemicals. Estradiol-17β, estriol, diethylstilbestrol, progesterone, testosterone, 17β-hydroxy-5α-androstan-3-one (5α-dihydrotestosterone), and corticosterone were purchased from Sigma. [4,5-^{3}H]Leucine (50 Ci/mmol) and [*U*-^{14}C]leucine (342 Ci/mol) were purchased from New England Nuclear.

Isolation and Culture of Cells. Anterior pituitaries from 18-day-old female rats (Holtzman) were dispersed by the method of Vale *et al.* (8) with 0.35% (wt/vol) collagenase (CLS, Worthington) and 0.25% (wt/vol) Viokase (GIBCO). Cell viability was assessed by trypan blue exclusion (9). DNA content of the cells was estimated by the method of Burton (10). Cells were plated in Falcon flasks (2–4 × 10^5 cells per 25 cm^2, 87–95% viable) that had been treated with 2 ml of 0.001% (wt/vol) poly(L-lysine) (Sigma) to increase plating efficiency. Culture medium consisted of Dulbecco's modified Eagle's medium buffered with 25 mM Hepes and supplemented with 10 μg of insulin (Sigma) per ml, 15% (vol/vol) horse serum, 2.5% (vol/vol) fetal calf serum, 100 units of penicillin G per ml, 0.5 μg of amphotericin B (GIBCO) per ml, and 50 μg of gentamycin (Schering) per ml. Sera were treated with dextran-coated charcoal (11) to remove free steroids. Steroid hormones were dissolved in ethanol and diluted in the medium so that the final ethanol concentration did not exceed 0.1%. Flasks were loosely capped and placed in a water-jacketed incubator at 37°C in an atmosphere of 95% air/5% CO_2. Media were changed on the third day (day 3) after plating (day 0) and every 2–3 days thereafter.

Incubation with Radiolabeled Precursors. Flasks were rinsed with leucine-free Earle's minimal essential medium (GIBCO) and then incubated for 60 min at 37°C in either leucine-free medium to which 5 μCi of [^{14}C]leucine was added per ml or in medium made 30 μM in leucine to which 10 μCi of [^{3}H]leucine was added per ml. For analysis on gels, cells were removed and homogenized in 0.4 ml of 1% (wt/vol) sodium dodecyl sulfate ($NaDodSO_4$)/4 M urea/50 mM Tris·HCl, pH 7.4 (Tris/$NaDodSO_4$/urea), stored at −20°C, and analyzed on 0.8 × 12 cm $NaDodSO_4$/polyacrylamide gels as described (1). For immunoprecipitation, the cells were removed and homogenized in 0.4 ml of ice-cold 0.15 M NaCl/10 mM sodium phosphate, pH 7.4/10 mM leucine/1% Triton X-100/0.5% sodium deoxycholate (P_i/NaCl/Triton/deoxycholate) and stored at −20°C until assayed. Aliquots of medium (0.1 ml) were analyzed on 0.6 × 5 cm, 7.5% acrylamide gels as described (1).

Immunoprecipitation. Cell homogenates were centrifuged at 10,000 × *g* for 10 min, and aliquots of the supernatant (0.1 ml) were immunoprecipitated with an antiserum to prolactin as described (12), except that 2.5 μg of internally labeled [^{14}C]prolactin was included in each sample as carrier and also to monitor immunoprecipitation efficiency. The immunoprecipitate was dissolved in Tris/$NaDodSO_4$/urea and electrophoresed on 0.6 × 5 cm $NaDodSO_4$/12% polyacrylamide/1.2% *N,N'*-diallyltartardiamide gels in a discontinuous Tris/glycine buffer system (13). The gels were sliced and incubated overnight in 2% periodic acid; radioactivity was determined after addition of scintillation fluid.

Analysis of Data. Results are expressed as dpm per culture dish; values are means ± SEM for four cultures at each point. Differences between groups were determined by analysis of variance and Student's *t* test.

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Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

RESULTS

Effects of Estradiol on Prolactin Synthesis. The pattern of proteins synthesized during a 60-min labeling of control ([^{3}H]leucine) or estradiol-treated ([^{14}C]leucine) cultures was assessed by electrophoresis of the combined cell extract on NaDodSO$_4$/polyacrylamide gels (Fig. 1). An increased ratio of radioactivity was seen in the prolactin peak of estradiol-treated compared with control cultures (^{14}C/^{3}H), whereas a relatively constant ratio was obtained in other areas of the gel. Reversal of the isotopes (^{3}H/^{14}C) yielded identical results (not shown). Growth hormone migrates slightly faster than prolactin in this gel system, and a slight change in ratio in the area corresponding to growth hormone was seen in this experiment and occasionally, but not consistently, in others. These results suggest that estradiol specifically stimulates prolactin synthesis in pituitary cell cultures without appreciably affecting the synthesis of other cellular proteins.

In a separate experiment, DNA content per flask of control cultures was 11.6 ± 0.4 μg and that of estradiol-treated cultures was 10.9 ± 1.2 μg (n = 4). In replicate cultures labeled with [^{3}H]leucine, radioactivity in dpm × 10^{-6} of trichloroacetic acid-precipitable material per flask was 2.3 ± 0.4 for control and 2.4 ± 0.1 for estradiol-treated cultures (n = 4). Thus, the increased rate of prolactin synthesis was not accompanied by an overall increase in the synthesis of the bulk of pituitary cell proteins or by increased cell proliferation.

Quantitation of Prolactin Synthesis. Analysis on gels of immunoprecipitates from cells labeled with [^{3}H]leucine showed a single major ^{3}H-labeled peak that comigrates with the [^{14}C]prolactin standard (Fig. 2). Estradiol caused a marked increase in the incorporation of [^{3}H]leucine into prolactin. For both control and estradiol-treated cultures, no labeled prolactin was detected in the medium after 60-min labeling periods (data not shown). Thus, immunoprecipitation of cell homogenates provides an estimate of the total rate of prolactin synthesis.

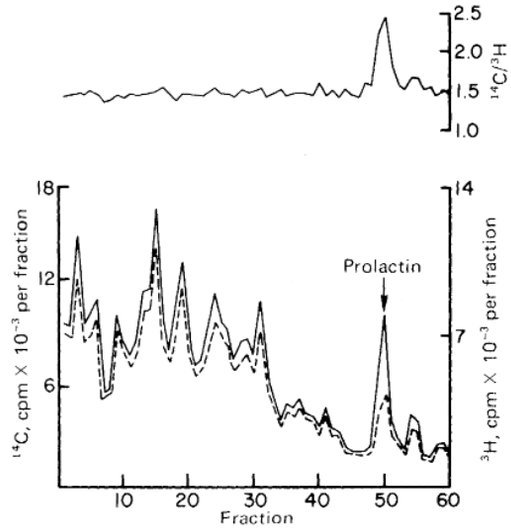

FIG. 1. Comparison between radiolabeled proteins from control and estrogen-treated cells. Pituitary cells were cultured for 5 days in control medium or in medium containing 10 nM estradiol. Cells were pulse labeled (60 min) with either [^{3}H]leucine, 10 μCi/ml (control), or [^{14}C]leucine, 5 μCi/ml (estrogen-treated). An aliquot of the cell extract from a control and an estrogen-treated culture was combined and electrophoresed on a NaDodSO$_4$/polyacrylamide gel. Dashed line, migration of radioactive proteins from the control culture (^{3}H); solid line, radioactive proteins from the estrogen-treated culture (^{14}C). Ratio of ^{14}C to ^{3}H is plotted at the top of the figure. The position of prolactin was determined with authentic prolactin from the National Institute of Arthritis, Metabolism and Digestive Diseases.

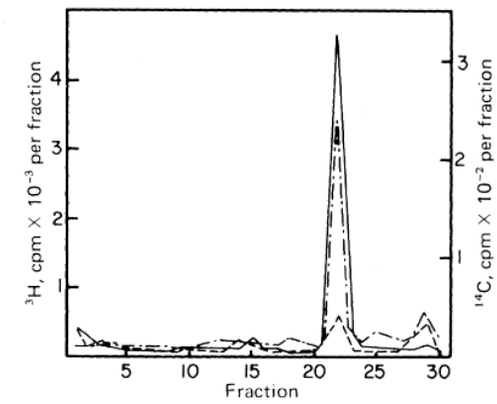

FIG. 2. Comparison between immunoprecipitable prolactin from control and estrogen-treated cells. Pituitary cells were cultured for 5 days in control medium or in medium containing 10 nM estradiol. Cells were pulse labeled for 60 min with [^{3}H]leucine (10 μCi/ml). Aliquots of the 10,000 × g supernatant of cell homogenates were immunoprecipitated with [^{14}C]prolactin carrier and antiserum to prolactin and electrophoresed on NaDodSO$_4$ gels. (—) Profile of ^{3}H-labeled immunoprecipitate from an estrogen-treated culture. The peak of radioactivity coincides with the position of [^{14}C]prolactin carrier (- · -). (- - -) Migration of ^{3}H-labeled immunoprecipitate from a control culture run in parallel.

Dose Response and Steroid Specificity. A dose-dependent increase in the rate of prolactin synthesis was obtained up to physiological concentration of estradiol (Fig. 3). Near-maximal induction was achieved at a concentration of 10^{-9} M, reaching a plateau at 10^{-8} M estradiol. At a concentration of 10^{-9} M, estradiol, estriol, and diethylstilbestrol stimulated prolactin synthesis in a comparable manner (Fig. 4), whereas, at the same dose level, testosterone, 5α-dihydrotestosterone, progesterone, or corticosterone had minimal effects. Of the three steroids tested at 10^{-7} M, testosterone was partially stimulatory, while progesterone or corticosterone had no appreciable effect.

Time Course of Prolactin Induction. After 3 days' exposure

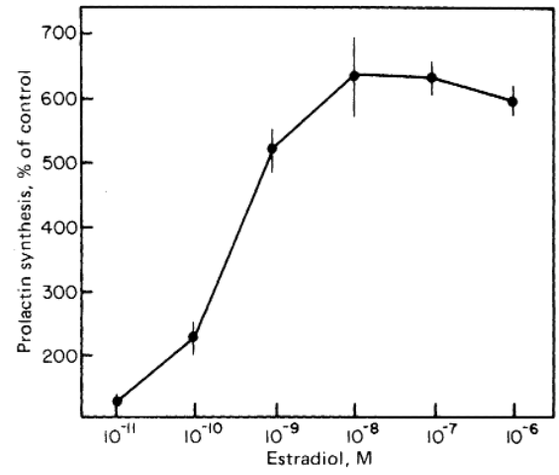

FIG. 3. Rate of prolactin synthesis as a function of estradiol concentration. Pituitary cells were cultured for 5 days in control medium or in medium containing estradiol at the concentration indicated. The rate of incorporation of [^{3}H]leucine into immunoprecipitable prolactin was quantitated. Each point represents the mean ± SEM of four independent determinations.

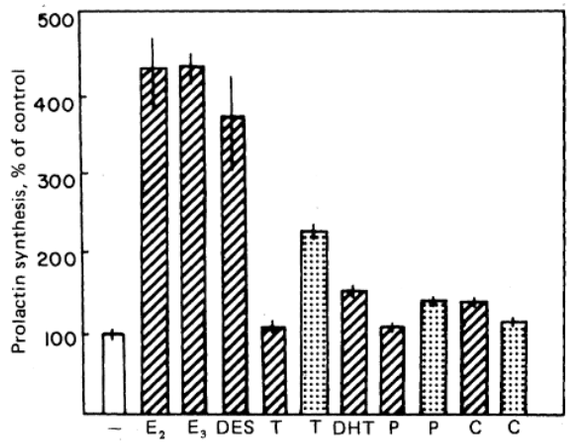

FIG. 4. Effects of estrogens, androgens, progesterone, and corticosterone on prolactin synthesis. Pituitary cells were cultured for 5 days in control medium or in medium containing one of the following steroid hormones: estradiol (E_2), estriol (E_3), diethylstilbestrol (DES), testosterone (T), 17β-hydroxy-5α-androstan-3-one (DHT), progesterone (P), and corticosterone (C). Concentration of steroids is indicated by hatched columns (10^{-9} M) and dotted columns (10^{-7} M). Empty column indicates the control. The rate of incorporation of [^{3}H]leucine into immunoprecipitable prolactin was quantitated. Each bar represents the mean ± SEM of four independent determinations.

of the cells to estradiol there was a significant rise in prolactin synthesis (Fig. 5). Prolactin synthesis continued to increase, reaching a 500% increase over the control rate by day 7 of culture.

Addition of estradiol on day 3 rather than at the start of culture resulted in a 50% increase by day 4 ($P < 0.05$; Student's t test) and in a highly increased rate of prolactin synthesis by day 7 (Fig. 5). Conversely, cells exposed to estradiol for the initial 3 days continued to synthesize increased amounts of

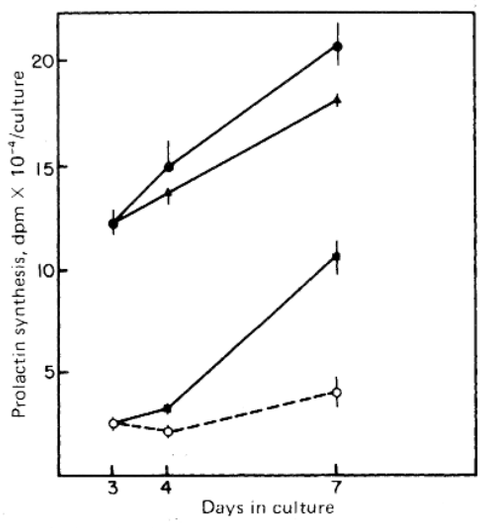

FIG. 5. Delayed induction of prolactin synthesis. Pituitary cells were cultured up to 7 days in control medium ($-E_2$) or in medium containing 10 nM estradiol ($+E_2$) according to the following scheme: O- - -O, $-E_2$, day 0–7; ■—■, $-E_2$, day 0–3, and $+E_2$, day 3–7; ▲- -▲, $+E_2$, day 0–3, and $-E_2$, day 3–7; ●—●, $+E_2$, day 0–7. The rate of incorporation of [^{3}H]leucine into immunoprecipitable prolactin was determined. Each point represents the mean ± SEM of four independent determinations.

prolactin when the steroid was removed, although at a somewhat reduced rate ($P < 0.001$; Fig. 5).

DISCUSSION

The results indicate that estradiol stimulates prolactin synthesis through direct action on the pituitary. A direct effect of estrogen on explanted pituitary fragments (3, 14) and on a clonal strain of pituitary cells (5, 6) has been previously demonstrated. The significance of the present study rests on the use of primary cultures of normal pituitaries. This approach overcomes the limitations inherent in organ cultures, which require unphysiological concentrations of estradiol, and in tumor-derived cells, which may exhibit loss of responsiveness to estradiol (7).

The time course and magnitude of the response in primary pituitary cultures agree with *in vivo* data demonstrating that treatment of rats with estradiol increased 3- to 5-fold the incorporation of precursors into prolactin (1) and the accumulation of preprolactin mRNA (2). The rate of prolactin synthesis in cultured cells increases as a function of estradiol concentration, reaching a peak at 10 nM estradiol. In view of the presence of steroid-binding proteins in animal sera, the amount of estradiol available to cells in medium containing 17.5% serum is substantially reduced (15, 16). The maximal effective dose in this system is probably 1–2 nM, a reasonable estimate in light of the recent study by Pavlik and Katzenellenbogen (17). Wilfinger *et al.*[‡] reported that 1 nM estradiol enhanced prolactin secretion by human pituitary cells when cultured in media containing 5% rather than 20% serum.

The affinity of serum albumin for estradiol is severalfold greater than the affinity of the protein for estriol (18, 19). This phenomenon is reflected by our finding that equimolar concentrations of estradiol and estriol elicited a comparable increase in prolactin synthesis even though the affinity of the latter for the cytoplasmic estrogen receptor is lower (20). Androgens bind to the uterine estrogen receptor and elicit the synthesis of the uterine-induced protein (21). In that system, approximately 300 times more testosterone than estradiol was required to achieve 50% nuclear accumulation of the receptor (21). This suggests that the partial stimulation of prolactin synthesis obtained with 100 nM testosterone (compared to 1 nM estradiol) may represent binding of the androgen to pituitary estrogen receptors.

Altered incorporation of precursors into a protein could be due to changes in its rate of synthesis or degradation. The increased incorporation of precursors into prolactin observed in the present study is most likely due to an increased rate of synthesis. We have previously shown that incorporation of radiolabeled amino acids into prolactin is linear for 6 hr (1), suggesting that relatively little degradation would occur in the 1 hr labeling period used in this study. Also, Dannies and Tashjian (22) found that there was no detectable degradation of prolactin in the GH_3 pituitary cell line. In ovine pituitary cultures, estrogen induces similar changes in the incorporation of precursors into prolactin and in the levels of prolactin mRNA.[§] This latter study clearly demonstrates that estradiol treatment results in an increased capacity of the pituitary to synthesize prolactin.

The increased rate of prolactin synthesis may be due to an increase in the number of prolactin-producing cells or to an

[‡] Wilfinger, W. W., Davis, J. A., Asawaroengchai, H., Augustine, E. C. & Hymer, W. C. (1977) *59th Annual Meeting of the Endocrine Society, Chicago, IL*, Abstr. 426, p. 273.

[§] Vician, L., Shupnik, M., Baxter, L. A. & Gorski, J. (1978) *60th Annual Meeting of the Endocrine Society, Miami, FL*, Abstr. 555, p. 352.

increased rate of production per cell. In the present study, we were unable to detect an effect of estradiol on cellular proliferation, nor was this evident in cultures derived from postlactational female or adult male pituitaries, which also respond to estradiol by increased prolactin synthesis (unpublished data). However, the culture preparations contain a variety of secretory cells as well as rapidly proliferating fibroblast-like cells, and a specific increase in the number of lactotrophs would not likely be detected by monitoring cellular DNA.

The highly increased ratio of radioactivity in the prolactin peak and the relatively constant ratio in other areas of the gel (Fig. 1) suggest a specific effect of estradiol on prolactin synthesis. However, caution should be exercised in the interpretation of data generated by this type of experiment. Cultured pituitary cells synthesize a large variety of proteins, and minor ratio changes may be masked by high base line levels of radioactivity. Thus we cannot exclude the possibility that the induced rate of prolactin synthesis may be accompanied by synthesis of other proteins or be partially mediated by products of other estrogen-responsive pituitary cells. Indeed, several pituitary cell types were shown to localize estradiol (23), and in ovine pituitary cultures, estradiol increases the production of thyroid-stimulating hormone (24) as well as prolactin[§] and inhibits the production of luteinizing hormone and follicle-stimulating hormone (25).

The stimulatory effect of estradiol on prolactin synthesis may be mediated by selective proliferation of a discrete pituitary cell population, by increased transcription, processing or stability of prolactin mRNA, or by a combination of these processes. Further studies with this system may afford an opportunity to distinguish between these possibilities.

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