Bioactivity of Plasma Prolactin in Ovariectomized, Diethylstilbestrol-Treated Long-Evans and Holtzman Rats after Thyrotropin-Releasing Hormone or Bromocriptine Administration (43283)

MICHAEL CANNON, LIMIN HU, JING YE, AND DAVID LAWSON[1]
Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan 48201

Abstract. **The objective of this study was to determine the effects of thyrotropin-releasing hormone (TRH) and bromocriptine on plasma levels of biologically active prolactin in ovariectomized, diethylstilbestrol (DES)-treated rats. Female Long-Evans and Holtzman rats were ovariectomized and each was given a subcutaneous implant of diethylstilbestrol (DES). One week later, groups of DES-treated rats were fitted with indwelling intra-atrial catheters, and 2 days later blood samples were withdrawn before and at 1, 2, 5, 10, and 20 min after intravenous administration of TRH (250, 500, or 1000 ng/rat). Blood samples were obtained from other groups at 4 weeks of DES treatment by orbital sinus puncture under ether anesthesia before and at 30, 60, and 120 min after bromocriptine administration (2.5 mg/rat sc). Plasma was assayed for prolactin by conventional radioimmunoassay (RIA) and by Nb2 lymphoma bioassay (BA). Holtzman rats released significantly more prolactin following TRH than did Long-Evans rats when the RIA was used to measure prolactin. However, when the BA was used to assay prolactin in the same samples, the Long-Evans rats released more prolactin than did the Holtzman rats. In addition, the ratio of the BA to RIA values was significantly increased in both strains following TRH, but the greatest increase was observed in the Long-Evans rats, in which the ratio was 4.5 at the peak of the TRH-induced rise in plasma prolactin. Gel filtration chromatography of plasma obtained at 5 min after TRH treatment in Long-Evans rats revealed large molecular forms of prolactin with BA to RIA ratios of 4–5. In addition, monomeric prolactin had a BA to RIA ratio of 2. Bromocriptine treatment reduced prolactin levels in both strains, but the effect was more rapid in Holtzman than in Long-Evans rats. In addition, bromocriptine treatment of Holtzman, but not Long-Evans, rats significantly reduced the BA to RIA ratio of plasma prolactin. The results indicate that TRH and bromocriptine affect the release of biologically active prolactin to a greater extent than prolactin detected by antibody in the RIA, and that Long-Evans and Holtzman rats respond to these secretagogues differently with regard to BA to RIA comparisons.** [P.S.E.B.M. 1991, Vol 197]

Following the development of the Nb2 lymphoma bioassay for lactogenic hormones by Tanaka and his colleagues in 1980 (1), it has been possible to measure blood levels of biologically active prolactin in a number of different species including humans during various physiological conditions. In humans, growth hormone also stimulates Nb2 cell proliferation. This complicates the use of these cells as a bioassay for prolactin in human serum requiring the absorption of the unwanted hormone by antiserum. In rodents and other nonprimate species, this is not a problem, as growth hormones of these species do not stimulate these cells (1). Several reports of the levels of bioactive prolactin in the plasma or serum of rats have appeared (2–5). These bioassay measurements are particularly interesting when they are compared with measurements obtained by radioimmunoassay. We (2, 3), and Klindt *et al.* (4), have observed that the ratio of bioactive to

[1] To whom correspondence and requests for reprints should be addressed at Department of Physiology, Wayne State University School of Medicine, 540 East Canfield Avenue, Detroit, MI 48201.

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immunoactive hormone to be at or near 1.0 under many conditions. However, there appear to be conditions under which the ratio increases above 1.0 (following suckling in lactating rats, during metestrus and diestrus, and during the proestrus afternoon surge) (3–5). In addition, we have found the ratio to be less than 1.0 in nonsuckled lactating rats (3).

In our laboratory, we have observed the greatest increase in the BA to RIA ratio in plasma obtained under basal conditions from ovariectomized rats treated with diethylstilbestrol (DES) (unpublished data). This effect is not due to a direct effect of DES on the Nb2 cells, nor is it due to other serum factors which we have shown enhance the response of the cells to prolactin (6). The objective of the current study was to examine the effects of thyrotropin-releasing hormone (TRH) and bromocriptine, a stimulator and an inhibitor of prolactin release, respectively, on the plasma levels of prolactin in DES-treated ovariectomized rats, as measured by bioassay and radioimmunoassay. A secondary objective was to compare two strains of rats (Long-Evans and Holtzman Sprague-Dawley) that have been shown to have different susceptibilities to carcinogen-induced mammary cancer.

Materials and Methods

Sexually mature female rats of the Long-Evans and Holtzman Sprague-Dawley strains were purchased from Harlan Sprague Dawley (Indianapolis, IN) and housed two per cage in an environmentally controlled room (lights on 0600-2000 hr; temperature 23–25°C; relative humidity, 40–50%). The two strains were used because they exhibit different degrees of susceptibility to experimentally induced mammary cancer and the current experiments are related to a larger study designed to determine whether differences in prolactin secretion can contribute to these differences in mammary tumor induction. One or 4 weeks before the TRH or bromocriptine experiments, respectively, each rat was bilaterally ovariectomized under ketamine-xylazine (80 mg/kg–4 mg/kg im) anesthesia. At the same time, one 10 mm Silastic capsule of DES (12–15 mg) was placed subcutaneously in each rat. Two days before the TRH experiment was conducted, three groups of rats (8–10 rats of each strain per group) were fitted with indwelling intra-atrial catheters that were filled with heparinized saline, sealed with a brass male Amphenol connector pin, and exteriorized to the nape of the neck for the purpose of drawing blood samples. Following catheterization, the rats were placed in individual cages. The anesthetic for catheterization was ketamine-xylazine. The ovariectomized, DES-treated rats (8–10 rats of each strain) to be used in the bromocriptine experiments were not catheterized.

On the day before the experiment, the catheterized rats were moved to an isolated room for sampling and the next morning (0830–0900 hr) were placed in a chamber containing 100% CO_2 until they became unconscious (10–15 sec). The rats were removed from the chamber and a long piece of polyethylene tubing filled with heparinized saline and attached to a stopclock was quickly connected to the indwelling catheter via a short length of 23-gauge stainless-steel tubing; the catheter was flushed with heparinized saline and the rats were returned to their home cage. The rats were allowed 90 min to acclimate to the sampling environment and to recover from the effects of CO_2 narcosis. Blood samples (0.25 ml) were then withdrawn via the catheters before and at 1, 2, 5, 10, 15, and 20 min after the intravenous administration of TRH (250, 500, or 1000 ng/rat; Sigma Chemical Co., St. Louis, MO) in saline containing 0.1% bovine serum albumin. Rat body weights ranged between 250 and 300 g. The blood volume was maintained by heparinized saline administered intravenously after each sample was withdrawn. At the end of the experiment, the animals were sacrificed by prolonged exposure to 100% CO_2 and bilateral pneumothorax.

The noncatheterized rats, which were used to determine the effects of bromocriptine, were bled by orbital sinus puncture during a brief (1–2 min) period of ether anesthesia at 4 weeks of DES treatment. The orbital sinus method was used so that the data could be compared with those of other studies conducted in this laboratory in which bromocriptine was used to suppress prolactin levels in DES-treated rats. Blood samples (0.5 ml) were obtained, using heparinized glass capillary tubes, before and at 30, 60, and 120 min after a subcutaneous injection of bromocriptine mesolate (2.5 mg/rat; Sigma) dissolved in 50% ethanol and 50% saline.

Plasma obtained by centrifugation of all the blood samples was stored at −70°C until assayed for prolactin by radioimmunoassay (7) or Nb2 lymphoma cell bioassay (1, 2). The standard used for both assays was NIDDK-RP-1 (11 IU/mg). Two or three dilutions of each sample were assayed in duplicate in both assays and parallelism of the plasma dilution curves with the standard prolactin curve was achieved. No more than 1 μl of plasma was added per milliliter of cells in the bioassay to circumvent a potential problem of serum factors potentiating the response, as we have reported previously (6). Plasma remaining after assay in those samples obtained at 5 min after TRH in Long-Evans rats were pooled and this plasma pool was subjected to gel filtration chromatography at 4°C on a polyacrylamide (Bio-Rad P-100) column (2.6 × 100 cm). The column was eluted with phosphate-buffered saline containing 0.1% bovine serum albumin. Dextran 2000 and phenol red were used to mark the void and total volumes of the column, respectively. Fractions (5 ml) collected between the void and total volumes were assayed for prolactin by both assays.

Means were compared across time intervals within strains using repeated-measures analysis of variance and Newman-Keuls multiple comparison test. An F test was used to compare means between strains at each time interval. Statistical significance was achieved if $P < 0.05$.

Results

The levels of plasma prolactin in catheterized rats given TRH are shown in Figure 1. Since plasma levels in the groups of rats within each strain treated with 250, 500, or 1000 ng of TRH/rat were not different from each other (perhaps because all doses may have been maximally stimulatory), the data were combined across doses.

When plasma was assayed by radioimmunoassay (RIA) (Fig. 1A), levels of prolactin in Holtzman rats were significantly greater than those in Long-Evans females at 2, 5, and 10 min after TRH, although basal levels and the patterns of release were very similar between strains. Basal levels were approximately 200 ng/ml and increased to a peak by 2–5 min, and then declined linearly to near baseline by 20 min. The elevated basal levels of prolactin probably resulted from the 1 week of DES treatment.

When the bioassay was used to measure the same samples for prolactin (Fig. 1B), an entirely different pattern emerged. Long-Evans rats had levels of prolactin that were significantly greater than Holtzman rats both before and at 5 and 10 min after TRH. In addition, the pattern of release in the Long-Evans rats was more prolonged than was observed using RIA or than was observed in Holtzman rats.

When the ratios of Nb2 lymphomia bioassay (BA) to RIA values were calculated, a pattern similar to that seen by BA was apparent (Fig. 1C). The ratio in the pre-TRH samples from Holtzman rats was only slightly greater than 1.0, while that in Long-Evans rats was approximately 2.5. Following TRH administration, the ratio increased in both strains, but the pattern and magnitude of the increase was different between the strains. Long-Evans rats showed a continual increase in the ratio over the first 10 min following TRH, with the ratio reaching a maximum of 4.5 by 10 min. The ratio then declined by 20 min to a value that was not significantly different from that obtained at 0 min. Holtzman rats, on the other hand, showed an increase in the ratio to approximately 2.25 by 2 min, and then the ratio only slightly decreased from that time to the end of the experiment.

The gel filtration elution profile of plasma prolactin at 5 min after TRH in Long-Evans rats is shown in Figure 2. Most of the prolactin eluted from the gel at a position consistent with prolactin monomer (~25 kDa), but there was some prolactin (approximately 15–20%) that eluted at positions consistent with larger molecular

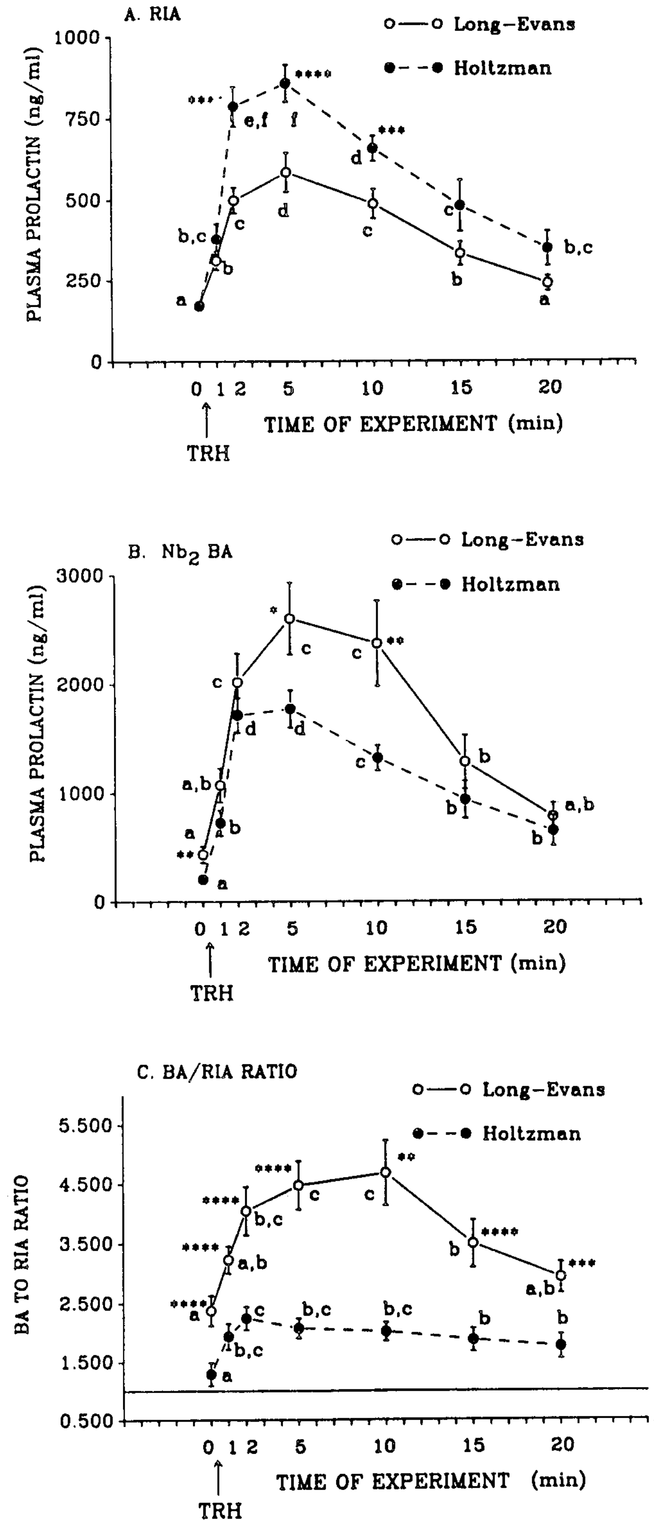


Figure 1. The effect of TRH on plasma levels of prolactin in ovariectomized Long-Evans and Holtzman Sprague-Dawley rats at 1 week of diethylstilbestrol treatment. (A) Radioimmunoassay. (B) Nb2 rat lymphoma cell bioassay. (C) BA to RIA ratio. The solid horizontal line represents a BA to RIA ratio of 1.0 (a-f) Means compared across time within strains with different superscripts are statistically different by repeated-measures analysis of variance and Newman-Keuls multiple comparison test ($P < 0.05$). Means compared between strains at each time interval using an F test were different at $P < 0.05$ (*), $P < 0.025$ (**), $P < 0.01$ (***), or $P < 0.001$ (****) (n = 15–20 rats per strain).

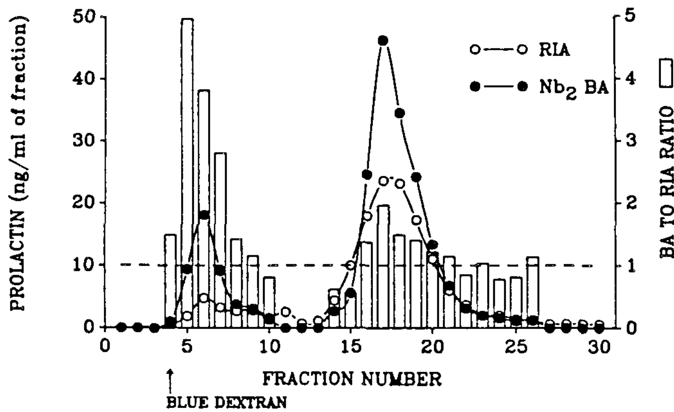
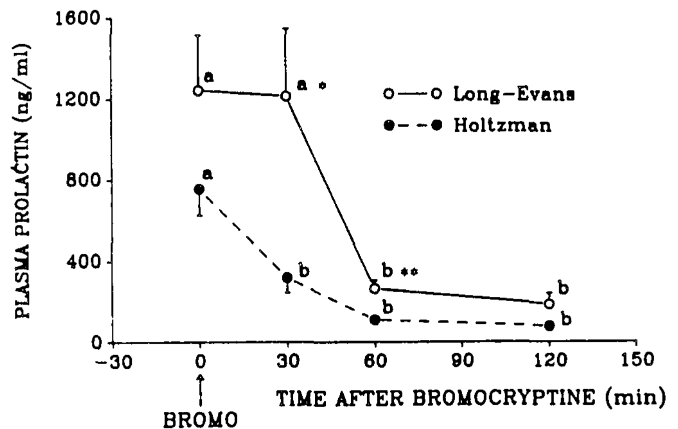
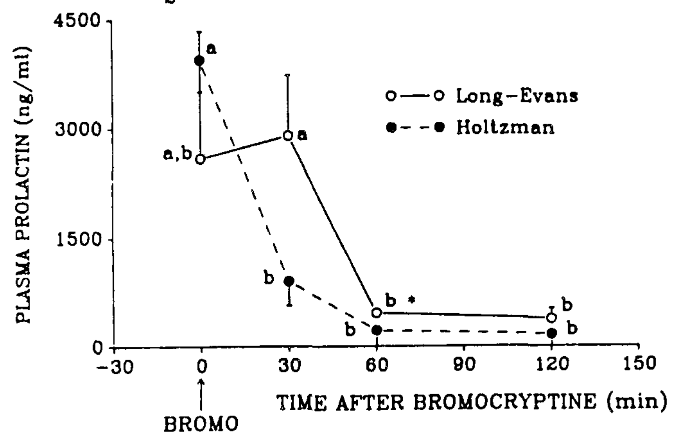


Figure 2. Gel filtration pattern of plasma prolactin at 5 min after TRH treatment of Long-Evans rats. A 2.6 × 100-cm column of BioGel P-100 polyacrylamide gel was eluted at 4°C using phosphate-buffered saline containing 0.1% bovine serum albumin at a flow rate of 20 ml/hr.

weight forms of prolactin. The BA to RIA ratio was much greater than 1.0 (3.0–5.0) in the fractions near the void volume. In addition, the prolactin eluting as monomer also had a ratio approaching 2.0.

The levels of prolactin in ovariectomized rats treated for 4 weeks with DES and then given a single, acute injection of bromocriptine are shown in Figure 3. When samples were assayed by RIA (Fig. 3A), prolactin levels prior to bromocriptine treatment were higher in both strains than the basal levels seen in the TRH study. In addition, the basal levels in Long-Evans rats were slightly greater than those in Holtzman rats, but these differences were not significant. Following bromocriptine administration, plasma prolactin levels significantly decreased in both strains, but the time course was different. Long-Evans rats did not show a significant inhibition until 60 min after bromocriptine, while levels of prolactin in Holtzman rats were significantly inhibited by 30 min. The two strains were significantly different at both 30 and 60 min after bromocriptine treatment. Levels remained low in both strains up to 120 min of the experiment. When BA was used to assay the samples (Fig. 3B), a pattern of response similar to that seen with RIA was seen, except that the absolute levels of prolactin were much higher. This difference between assays was more evident when the BA to RIA ratio was calculated (Fig. 3C). In Long-Evans rats, the BA to RIA ratio was approximately 3.0 before bromocriptine and did not change significantly after bromocriptine. In contrast, the BA to RIA ratio in Holtzman rats was 5.5 before bromocriptine and decreased significantly to 2.0 by 60 min after the dopamine agonist was given.





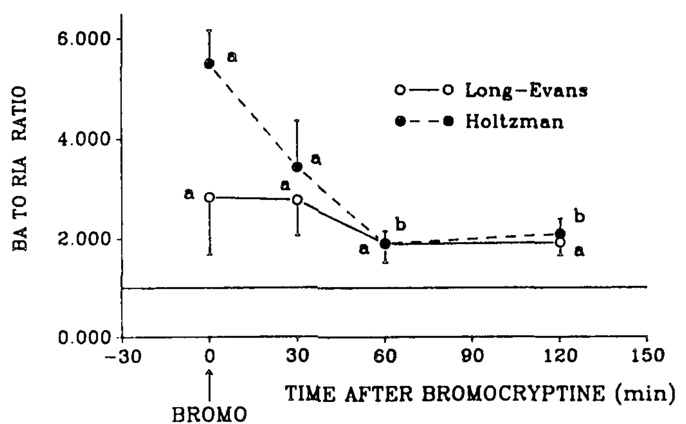


Figure 3. The effect of bromocriptine administration on plasma levels of prolactin in ovariectomized Long-Evans and Holtzman rats treated with diethylstilbestrol for 4 weeks. (A) Radioimmunoassay. (B) Nb2 rat lymphoma cell bioassay. (C) BA to RIA ratio. The solid horizontal line represents a ratio of 1.0. (a,b) Means compared across time within each strain with different superscripts are statistically different at $P < 0.05$ (repeated-measures analysis of variance and Newman-Kuels multiple comparison test). Means between strains within each time interval were compared using an F test and were statistically different at $P < 0.05$ (*) or $P < 0.025$ (**) (n = 8–10 rats/strain).

Discussion

The data in this study show that ovariectomized, DES-treated Long-Evans and Holtzman Sprague-Dawley rats release prolactin that is more bioactive than it is immunoactive, and that this difference is magnified when TRH is given and reduced or not changed when bromocriptine is administered. Furthermore, the two strains did not respond the same with respect to these changes.

That TRH administration caused the release of prolactin that was much more bioactive than that released under basal conditions suggests that the pituitary lactotrophs store a form of prolactin that can be rapidly released by TRH and that is intrinsically much more active in the bioassay than in the RIA. This conclusion, if true, is puzzling, since we have unpublished data which show that the BA to RIA ratio of prolactin in the pituitary of ovariectomized, DES-treated rats was 1.0 from 2 days until 6–8 weeks of DES treatment, after which it increased to above 2.0. One possible explanation for this apparent disparity between plasma and pituitary prolactin is that pituitary prolactin may change such that is becomes more bioactive upon release into blood. A second possibility is that the most bioactive form(s) of prolactin may represent only a small fraction of the total pool of prolactin in the pituitary and may not be readily detected. This small but very bioactive pool may be subject to release by TRH yielding a large shift in the plasma BA to RIA ratio. A third possibility is that there is some other molecule(s) in the plasma, but not in the pituitary, that potentiates prolactin bioactivity. We believe that such molecules do exist, based on our earlier findings (6), but that we have technically circumvented the effects of these substances by using small volumes of plasma in the bioassay. In addition, it seems unlikely that such molecules would augment basal prolactin 2-fold while enhancing TRH-released prolactin 5-fold (Fig. 1C).

The large BA to RIA ratio observed under basal conditions and its increase by TRH must be affected specifically by DES, since we have reported earlier that the BA to RIA ratio of plasma prolactin in ovariectomized, estradiol-treated rats is near 1.0 before and after TRH treatment (2). On the other hand, we have shown that neither DES nor estradiol directly affects the bioassay across a wide dose range (6). It is of interest to note that TRH has been shown to increase the BA to RIA ratio of plasma prolactin in women (8).

That the largest molecular forms of plasma prolactin were by far the most bioactive (Fig. 2) indicates that the synthesis and selective release of these large forms may account for the large increase in the plasma BA to RIA ratio following TRH administration. However, inspection of the data shows that these larger forms accounted for only 15–20% of the total hormone eluted from the gel filtration column. To totally account for the 5:1 ratio seen in the original plasma samples, these forms should have had a BA to RIA ratio of 25:1. Clearly, there must be additional explanations of the large increase in the BA to RIA ratio of plasma prolactin following TRH. One explanation is that a greater proportion of the large molecular forms were retained on the column compared with the monomer. The recovery of prolactin from the column was 75%.

At this point, it is not exactly clear what the largest molecular forms of prolactin are, but one distinct possibility is that they represent disulfide-linked polymers of monomeric prolactin. If this is true, then the high BA to RIA ratio may be due to a disruption of these prolactin polymers by the 2-mercaptoethanol present in the medium in which the bioassay is conducted, yielding many molecules of monomeric prolactin. Since 2-mercaptoethanol is not present in the RIA buffers, the polymers may stay intact during RIA and be recognized as a single prolactin molecule by the antibody. This concept is supported by the report of Farrington and Hymer (9), in which the activities of large molecular weight (57–150 kDa) forms of growth hormone measured by RIA were increased approximately 3 to 5-fold following the acute treatment with 2-mercaptoethanol. Such technical differences between assays do not explain, however, the BA to RIA ratio of 2.0 in the monomeric form of prolactin seen following TRH in the current study (Fig. 2).

Whatever accounts for the increase in the BA to RIA ratio of plasma prolactin in DES-treated rats following administration of TRH, it appears to be more significantly expressed in Long-Evans rats than in Holtzman rats, at least early in the DES treatment (Fig. 1C). Perhaps Long-Evans rats clear prolactin differently than do Holtzman rats, such that more biologically active hormone is retained. On the other hand there may be systemic factors in the plasma that augment the bioactivity of prolactin, and these may be more prevalent in the Long-Evans rat. These explanations are possible; however, they are not supported by the observation that later in DES treatment, Holtzman rats appear to release a more biologically active prolactin than Long-Evans rats under "basal" conditions (Fig. 3C). The differences observed in prolactin bioactivity between strains may be related to genetic differences in prolactin secretion that these strains exhibit under physiological conditions (e.g., proestrus) and to differences in susceptibility to mammary cancer known to occur between these two strains.

That bromocriptine treatment inhibited the release of bioactive prolactin more than release of prolactin detected by RIA, as indicated by the decrease in the BA to RIA ratio in Holtzman rats (Fig. 3C), indicates that the pituitary mechanisms responsible for the selective release of bioactive forms of prolactin are sensitive to

dopamine inhibition, as well as to TRH stimulation. If this is true, then it is troublesome that bromocriptine did not also affect the ratio in Long-Evans rats. It is interesting that the BA to RIA ratio of prolactin under "basal" conditions was much higher in Holtzman than in Long-Evans rats. This is different than was seen in the TRH study and it may be due, at least in part, to the duration of DES treatment (1 vs 4 weeks). That a decrease in the BA to RIA ratio was seen in the strain with the largest initial ratio, but not in the strain with a lower ratio, suggests that the pituitary mechanisms regulating the release of the most bioactive pool of prolactin may be quite sensitive to dopamine.

These data indicate that the pituitaries of DES-treated rats are capable of secreting prolactin that is more bioactive than immunoactive and that these secretory mechanisms can be stimulated by TRH and inhibited by dopamine. Furthermore, it appears that the discrepancy between BA and RIA may be related to the presence and detection of large molecular forms of prolactin. Additional experiments are needed to completely resolve these issues.

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1. Tanaka T, Shiu RPC, Gout PW, Beer CT, Noble RL, Friesen HG. A new sensitive and specific bioassay for lactogenic hormones: Measurement of prolactin and growth hormone in human serum. J Clin Endocrinol Metab **51**:1058–1063, 1980.
2. Lawson DM, Sensui N, Haisenleder DJ, Gala RR. Rat lymphoma cell bioassay: Observations on its use and comparison with radioimmunoassay. Life Sci **31**:3063–3070, 1982.
3. Lawson DM, Sensui N, Gala RR. Inhibition of suckling-induced release by estrogen in ovariectomized lactating rats: Bioassay versus radioimmunoassay. Proc Soc Exp Biol Med **173**:130–136, 1983.
4. Klindt J, Robertson MC, Friesen HG. Episodic secretory patterns of rat prolactin determined by bioassay and radioimmunoassay. Endocrinology **111**:350–352, 1982.
5. Blank MS, Ching MC, Dufau ML. Bioactivity of serum and pituitary prolactin during the rat estrous cycle. Endocrinology **118**:1886–1891, 1986.
6. Moy JA, Lawson DM. Rat serum specifically effects the mitogenic effect of prolactin on Nb_2 lymphoma cells. Endocrinology **123**:1314–1319, 1988.
7. Ku EYH, Gala RR. Radioimmunoassay of rat prolactin comparing prolactin obtained from anterior pituitary organ culture with that distributed by the National Institute of Arthritis and Metabolic Diseases. Biochim Biophys Acta **264**:462–471, 1972.
8. Love RR, Rose DP. Elevated bioactive prolactin in women at risk for familial breast cancer. Eur J Cancer Clin Oncol **21**:1553–1554, 1985.
9. Farrington M, Hymer WC. Growth hormone aggregates in the rat adenohyphophysis. Endocrinology **126**:1630–1638, 1990.