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# Effects of High Doses of Estrogen on Prolactin-binding Activity and Growth of Carcinogen-induced Mammary Cancers in Rats[1]

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## SUMMARY

Specific binding sites for prolactin (PRL) were present in membrane preparations from 7,12-dimethylbenz(a)- anthracene-induced rat mammary tumors. The specific binding of PRL was time and temperature dependent. A significant negative correlation was noted between administered doses of estrogen and the subsequent binding of PRL to tumor cell membranes. Injections of 10 or 25 μg estradiol benzoate daily for 10 days effectively inhibited mammary tumor growth and significantly reduced specific PRL binding to mammary tumor cell membranes.

## INTRODUCTION

The 2 most important hormones in mammary tumorigenesis in mice and rats are believed to be PRL[2] and estrogen (11). Growth of mammary tumors in rats can be maintained at least temporarily by PRL even in the absence of the ovaries and adrenals (15), but estrogen has no growth-promoting action on mammary tumors in the absence of the pituitary (18). Whereas low doses of estrogen can be stimulatory to mammary tumor development and growth in the intact rat (5), large doses of estrogen have an inhibitory effect despite their ability to increase blood PRL levels (5, 11). Recent studies have suggested that high doses of estrogen may interfere directly with the stimulatory action of PRL on mammary tumor tissue (12). Welsch *et al.* (21) reported that, whereas PRL stimulated DNA synthesis in rat mammary tumor organ cultures, high doses of estrogen inhibited DNA synthesis and also suppressed PRL-induced DNA synthesis.

PRL receptors have been shown to be present in carcinogen-induced mammary adenocarcinomas, and a direct relationship has been reported between the growth response of these cancers to PRL and the number of PRL receptors in the cancer tissue (7). Since the binding of PRL to its receptor is believed to initiate PRL-dependent cellular events, it was of interest to determine the effect of large doses of estrogen on PRL-binding activity in mammary tumor tissue.

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[2] The abbreviations used are: PRL, prolactin; DMBA, 7,12-dimethylbenz(a)anthracene; EB, estradiol benzoate; oPRL, ovine prolactin.

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## MATERIALS AND METHODS

Virgin female Sprague-Dawley rats (Spartan Research Animals, Inc., Haslett, Mich.) were housed in a light (14 hr/day)- and temperature (24 ± 1°) controlled environment and fed a diet of Purina rat chow (Ralston Purina Co., St. Louis, Mo.) and tap water *ad libitum*. At 55 to 60 days of age, each rat received a single i.v. injection of a lipid emulsion containing 5 mg of DMBA. Approximately 2.5 months later, when each rat had developed at least 1 mammary tumor 1 cm in diameter or larger, the rats were randomly divided into groups and given daily s.c. injections for 10 days as follows: Group 1, 0.1 ml corn oil (controls); Group 2, 2.0 μg EB in 0.1 ml corn oil; Group 3, 25.0 μg EB in 0.1 ml corn oil. Immediately prior to and at 5-day intervals during the treatment period, each mammary tumor was measured with calipers to the nearest mm for length, width, and depth. The sum of these measurements was determined for each tumor. Differences between diameter sums before and after the treatment period were recorded as the growth index. Each rat also received 3 injections of 100 μg ergocornine per 100 g body weight during a 24-hr period after the 10 days of treatment in order to reduce circulating levels of PRL and thereby minimize competition for PRL-binding sites by the endogenous PRL. We have previously determined that this dose of ergocornine can counteract estrogen stimulation of PRL release (10) and has no significant effect on the binding of PRL to a variety of target tissues including mammary tumors (8). All rats were killed approximately 4 hr after the last ergocornine injection, and blood was collected from the decapitated trunk. Each palpable mammary tumor was excised, frozen on dry ice, and stored at −20° until assayed 2 weeks later for PRL-binding activity. PRL-binding activity has been reported to be stable at −20° for as long as 6 months (17). The blood was allowed to clot at 4°, and the serum was separated and stored at −20° until radioimmunoassayed for circulating PRL. The entire experiment was subsequently repeated with the following treatments: Group 1, 0.1 ml corn oil (controls); Group 2, 0.2 μg EB in 0.1 ml corn oil; Group 3, 10 μg EB in 0.1 ml corn oil; Group 4, 25 μg EB in 0.1 ml corn oil.

**Hormones and Drugs.** The following hormones and drugs were used: oPRL (NIH-S-10, 25.6 IU/mg); ovine growth hormone (NIH-S-11, 0.56 IU/mg); ovine luteinizing hormone (NIH-S-15, 0.99 NIH-LH-S-1 unit/mg); ovine follicle-stimulating hormone (NIH-S-7, 1.15 NIH-FSH-S-1 units/mg); ovine thyroid-stimulating hormone (NIH-S-6, 2.47 USP

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units/mg); EB (Nutritional Biochemicals Corp., Cleveland, Ohio) and ergocornine methanesulfonate (Sandoz Pharmaceuticals, Hanover, N. J.). The DMBA was kindly provided by Dr. Paul Schurr, The Upjohn Co., Kalamazoo, Mich.

**Assays.** Mammary tumor membranes were isolated by a method previously described by Shiu *et al.* (17). Individual tumors were homogenized in 0.3 M sucrose using a Brinkman Polytron Type PT10 (setting of 8 for 2 × 15-sec pulses). The homogenates were centrifuged in a Sorvall RC-2B centrifuge at 14,500 × *g* for 20 min, and the resultant supernatants were centrifuged in a Sorvall OTD-2 ultracentrifuge at 105,000 × *g* for 90 min. Each particulate membrane pellet obtained was resuspended in Tris buffer (25 mM Tris-10 mM $CaCl_2$, pH 7.6) so that 100 µl contained 300 µg of protein as determined by the method of Lowry *et al.* (9) using BSA as the standard.

oPRL was radioiodinated by a lactoperoxidase method as described by Gelato *et al.* (4). The specific activity of [$^{125}$I]iodoprolactin was determined to be approximately 60 µCi/µg. The $^{125}$I-labeled PRL was diluted in Tris buffer containing 1% bovine serum albumin to give approximately 100,000 cpm/100 µl in a Nuclear Chicago automatic γ counter. Individual tumor samples were incubated in quadruplicate in 12- x 75-mm disposable culture tubes. Each tube contained 100 µl $^{125}$I-labeled PRL, 100 µl of the membrane preparation containing 300 µg protein, and Tris buffer for a final volume of 0.5 ml. Parallel incubations were performed containing the same reactants together with excess unlabeled oPRL (1 µg/tube). Again the final incubation volume was 0.5 ml. The incubations were terminated by the addition of 3 ml Tris buffer, and the bound and free [$^{125}$I]iodoprolactins were separated by centrifugation at 800 × *g* for 30 min. The resulting pellets were counted for 60 sec each in a Nuclear Chicago γ counter. Specific binding, expressed as a percentage of the total radioactivity used in each incubation, is the difference between cpm bound in the absence of excess unlabeled PRL and that bound in its presence. Hence, 1% change in specific binding represents approximately 1000 cpm. Specificity of binding of $^{125}$I-labeled PRL to mammary tumor membranes was determined by incubating various concentrations of unlabled PRL and other pituitary hormones with 300 µg of membrane protein and [$^{125}$I]iodoprolacitn.

Serum PRL was radioimmunoassayed by the method of Niswender *et al.* (14) using National Institute of Arthritis, Metabolism and Digestive Diseases rat Prolactin-RP-1 as the reference preparation.

## RESULTS

Analysis of covariance disclosed that the data obtained from the 2 experiments could be combined for further statistical treatment, and hence the results are presented as from a single experiment. Chart 1 shows that mammary tumors in control rats increased 0.46 ± 0.09 (S.E.) cm in their growth index during the 10-day treatment period. Daily injections of 0.2 µg EB resulted in a gain in tumor growth index of 0.63 ± 0.70 cm, and rats given 2.0 µg EB daily showed an increase of 0.39 ± 0.10 cm. By contrast, rats given injections of 10.0 or 25.0 µg EB daily showed a significant decrease in tumor growth index of 0.31 ± 0.13 and 0.39 ± 0.11 cm, respectively, during the treatment period.

The time course of specific binding of [$^{125}$I]iodoprolactin to tumor membranes at 37°, 24°, and 4° is shown in Chart 2. Because the highest level of specific binding observed was between 45 and 69 hr at 4° as well as at 14 hr at 24°, a convenient time of 48 hr at 4° was selected for subsequent incubations. Chart 3 shows that, of the various polypeptide hormones tested, only unlabeled oPRL readily competed with [$^{125}$I]iodoprolactin for binding to tumor membranes. A Scatchard plot (16) of a competitive inhibition curve revealed the presence of high-affinity PRL binding sites in tumor membranes with an apparent association constant ($K_a$) of 2.5 × $10^8$ moles$^{-1}$ and a binding capacity of 130 fmoles/300 µg protein (Chart 4).

The effects of the administered doses of EB on specific PRL-binding activity in the DMBA-induced mammary tumors is shown in Chart 5. A linear regression analysis of all

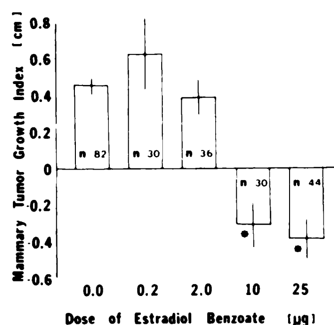


Chart 1. Effects of estrogen treatment on tumor growth. The growth index is the difference between the sums of tumor length, width, and depth before and after treatment. Analysis of variance was used to determine variations among groups, and the least-significant-difference test was used for all comparisons between treated and control groups; ★, significant difference *versus* controls at $p < 0.01$. *Vertical lines* at each dose, S.E.

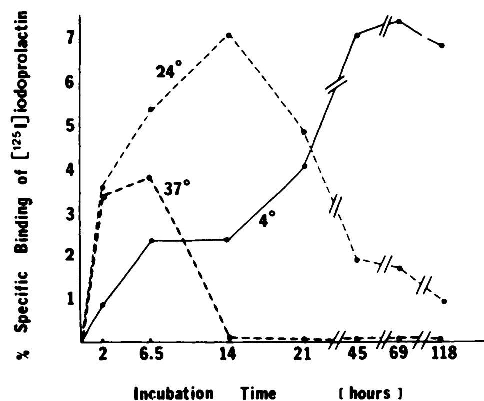


Chart 2. Time course of specific binding of [$^{125}$I]iodoprolactin to tumor membranes at 37°, 24°, and 4°. The membrane preparations used for incubations were derived from a pooled source of 38 untreated DMBA-induced mammary tumors. Each reaction tube, incubated in quadruplicate, contained 300 µg membrane protein and approximately 100,000 cpm [$^{125}$I]iodoprolactin. Parallel incubations were performed in the presence of excess (1 µg/tube) unlabeled oPRL. Specific binding, expressed as a percentage of total radioactivity used in each incubation, is the difference between cpm bound in the absence of excess unlabeled PRL and that bound in its presence. Since all values were replicates of a common membrane source, the S.E. at each point was negligible (<0.1%).

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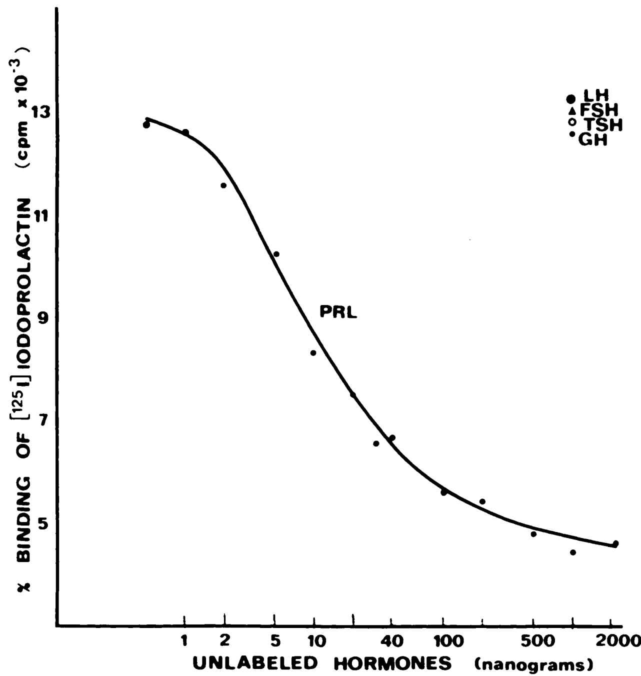


Chart 3. Competition of [125I]iodoprolactin and unlabeled hormones for binding to tumor membranes obtained from a pooled source. Luteinizing hormone (*LH*), follicle-stimulating hormone (*FSH*), thyroid-stimulating hormone (*TSH*), and growth hormone (*GH*) were tested only at 1000 ng/reaction tube. Incubations were carried out in quadruplicate at 4° for 48 hr.

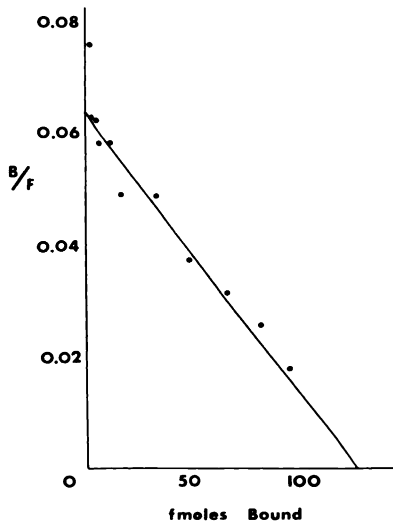


Chart 4. Scatchard analysis derived from a competitive inhibition curve. *Ordinate*, ratio of bound (*B*) to free (*F*) oPRL; *abscissa*, fmoles oPRL bound to tumor membranes; *abscissa intercept*, total oPRL-binding capacity for 300 μg of membrane protein.

tumors assayed indicated a significant negative correlation ($p < 0.01$) between estrogen dose and the specific binding of [125I]iodoprolactin to tumor membranes. However, further statistical analysis showed that only the 2 largest doses of EB significantly reduced specific PRL-binding activity as compared to controls ($p < 0.01$). The circulating levels of PRL in the ergocornine-treated rats at the time of killing were determined to be consistently low (<20 ng/ml) in all groups.

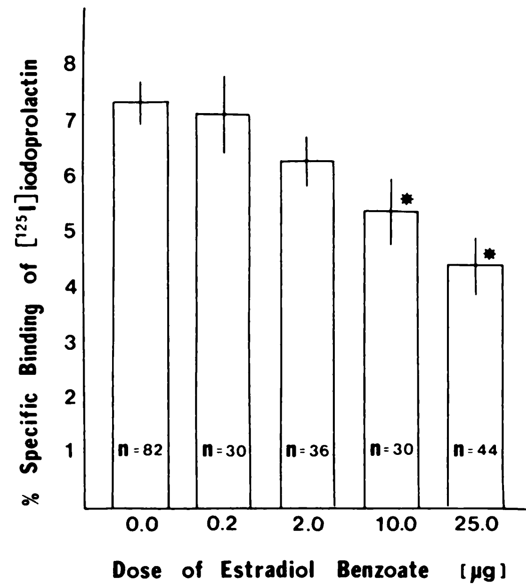


Chart 5. Effects of graded doses of EB on the specific binding of [125I]iodoprolactin to mammary tumor membranes. Binding ranged from low (<2%) to high (>10%) in each group, with no single tumor showing more than 18% specific binding. Variations among groups and mean comparisons were statistically analyzed as described in Chart 1. ★, significant difference *versus* controls at $p < 0.01$; *vertical lines* at each dose, S.E.

## DISCUSSION

Specific binding sites for PRL were detected in membrane preparations obtained from DMBA-induced mammary tumors in this study, in agreement with a previous report (7). The binding of [125I]iodoprolactin was time and temperature dependent and sensitive to competition by as little as 0.5 ng unlabeled oPRL. In contrast, levels of ovine luteinizing follicle-stimulating, thyroid-stimulating, or growth hormones as high as 1000 ng each were unable to displace the binding of [125I]iodoprolactin to tumor membranes.

When compared to an earlier study (7), the $K_a$ for high-affinity binding sites presently reported is an order of magnitude lower. The cause of this divergence in $K_a$ is not clear, nor is it clear which value is the more reliable. More knowledge about the nature of the hormone-receptor interaction than the current state of the art permits will be required to settle this question. Until then, observed $K_a$'s should be regarded as apparent $K_a$'s, and they should be considered only relatively accurate at best. The results of Kelly *et al.* (7) and our analysis seem to focus attention upon the advantage that Scatchard analysis has (in principle) over simple binding isotherms, in that Scatchard analysis provides a quantitative estimate for the number of receptor sites present. This parameter is independent of $K_a$, and both the present and earlier study (7) agree on the value for this parameter despite the differences in affinity. Since the number of binding sites, as well as the specific activities of the labeled hormones, were similar the bound:free ratios had to be one-tenth of that previously reported (7) as a result of the 10-fold less $K_a$ seen in our study.

This study shows that large doses of estrogen can effectively inhibit mammary tumor growth in rats, in agreement





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with earlier observations (5, 12), and also can significantly decrease the PRL-binding activity in these tumors. In recent years, the existence of high-affinity estrogen receptor proteins in mammary cancers has been demonstrated (6), and the presence of such receptors appears to be of value in determining estrogen dependency of such cancers. Although Braunsberg (2) has indicated that tumor regression induced by high estrogen therapy cannot be correlated with estradiol uptake and retention in human mammary tumor tissue, other workers (13) have suggested a good correlation with tumor estrogen receptor values. Because the other important hormone in rat mammary tumor development and growth is PRL, it was assumed for many years that the effectiveness of high estrogen treatment was mediated by an inhibition of pituitary PRL release. However, such an assumption was shown to be incorrect when Chen and Meites (3) demonstrated that large as well as small doses of estrogen elevated serum PRL levels in rats. Subsequently, Meites *et al.* (12) suggested that high estrogen doses may inhibit mammary tumor growth by interfering with the peripheral action of PRL on the tumor tissue. The action of PRL on target tissues is currently believed to begin with specific binding to membrane receptors (19). The present results clearly indicate that large doses of estrogen significantly reduce PRL binding to tumor membranes. Previous work in our laboratory also suggested that high doses of estrogen *in vitro* can decrease PRL binding to slices of DMBA-induced mammary tumor (1). Thus, despite elevated circulating PRL levels which normally result from high estrogen administration, the growth-promoting action of PRL on mammary tumors appears to be diminished.

PRL-binding activity in DMBA-induced mammary tumors previously has been shown to be correlated with the growth response to administered PRL (7). In general, the tumors with the greatest amount of PRL receptor activity exhibited the greatest growth response to administered PRL, and *vice versa*. If PRL receptors are reduced in the mammary tumors by administration of high doses of estrogen, then the circulating prolactin would be expected to be less effective in promoting growth of the tumors, as actually observed in the present study. This could then result in less tumor DNA synthesis from labeled thymidine, as reported by Welsch *et al.* (21). However, we also have observed that administration of relatively large doses of PRL can overcome the inhibition by high doses of estrogen of DMBA-induced mammary tumor growth (12). The mechanism(s) involved is not readily apparent at present. It is possible that large doses of administered PRL increase PRL receptors or favorably alter estrogen receptor activity in the mammary tumor tissue. PRL has been reported to increase estrogen receptors in DMBA-induced mammary tumors in rats (20). These possibilities are being explored.

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