Micromethod for the determination of free and total prolactin receptors: Measurement of receptor levels in normal and malignant mammary and prostate tissues

(prolactin binding sites/mammary cancer/prostate cancer)

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ABSTRACT A sensitive micromethod for the determination of free and total prolactin receptors in normal or malignant tissues has been developed. Positive and negative quality controls are incorporated in the procedure. Either whole tissue or the pellet fraction remaining from tissue that had undergone processing for estrogen receptors can be used. Crude microsomal and plasma membrane fractions obtained by homogenization and differential centrifugation are incubated with labeled prolactin in the presence or absence of increasing amounts of unlabeled hormone. The labeled ligand is prepared by a stoichiometric iodination procedure in which one atom of iodine-125 is incorporated into one molecule of the hormone, resulting in an intact labeled prolactin with a high specific activity of 170–186 μCi/μg (1 Ci = 37 GBq). Human prolactin labeled by this procedure has much greater specific binding capacity to various rat tissues than does iodinated rat prolactin. This technique permits an accurate measurement of prolactin receptors in as little as 50 μg of membrane protein. Highest levels of free and total prolactin receptors were found in the liver of 60-day-old female rats that served as a positive control. Liver of immature 21-day-old male rats, devoid of prolactin receptors, was used as a negative control. The amount of detectable free receptors was dependent on the level of circulating plasma prolactin. In 3-day postpartum lactating rats with high prolactin levels in plasma, all prolactin receptors in the mammary glands were found to be occupied, and no free receptors could be detected. When these receptors were desaturated from the endogenous prolactin by exposure to 3 M MgCl2, one class of receptors in a high quantity (1.75 nmol/mg of protein) and with a moderate affinity (Kd = 6.41 × 10−5 M) was detected. A similar type of receptor was found in the mammary glands of rats at midpregnancy and of cycling adult female rats. In malignant rat mammary tissue, however, fewer receptors (27 pmol/mg of protein) but with a very high affinity (Kd = 6.8 × 10−14 M) were detected. Normal ventral and dorsolateral rat prostate contained two classes of prolactin receptors (Kd = 3.46 × 10−10 M and 1.93 × 10−8 M). In the cancerous rat prostate, however, only one of these two classes of receptors was detected, and the number was smaller.

Prolactin (PRL) is one of the principal hormones regulating the alveolar function of the mammary gland (1, 2). PRL is also implicated in mammary tumorigenesis of experimental animals (3–5). Furthermore, there is growing evidence that PRL may be involved in the development and progression of some human breast cancer (6–8), although the PRL-dependency of mammary carcinoma in women is not yet well understood. PRL first binds to specific receptors located in the plasma membrane of the cell. This is the first event in the biological action of PRL and other protein and polypeptide hormones on the target tissues. Membrane receptors for PRL have been demonstrated in mammary tumors in rats (9–12) and in the normal and cancerous prostate of rat and man (13, 14). Detection of receptors for hormones in a malignant tissue is of great value for determining its hormonal dependence and the course of future treatment. Jensen et al. (15) suggested that estrogen receptor assays could be used to predict the estrogen dependency of breast tumors, and this has been confirmed by numerous clinical studies. Since estrogens are well known to stimulate PRL secretion (16, 17) and since estrogens and PRL may interact in promoting mammary tumorigenesis (18, 19), the determination of receptor levels of both estrogens and PRL could predict the responsiveness to endocrine manipulation of serum levels of these hormones more accurately than the measurement of either receptor alone (20).

We have succeeded in preparing human PRL (hpPRL) from an amniotic fluid (21, 22) with a molecular composition identical to that found in the human pituitary and plasma (23). The gentle conditions of our isolation procedure maintain the molecular integrity of the isolated hPRL. Following a stoichiometric iodination performed under mild and controlled conditions, iodinated hPRL (I-hPRL) also preserves its full biological activity for an extended time. This I-hPRL is greatly superior to labeled rat prolactin (rPRL) as the ligand used in rPRL receptor experiments (24).

In the present study, we describe a highly sensitive micromethod for PRL receptor measurement that can be performed in conjunction with the determination of estrogen receptors. The changes in the quantity and type of PRL receptors of rat mammary and prostate tumors are also reported.

MATERIALS AND METHODS

Hormones. NIADDK-hPRL-I-7 (batch number AFP-9900) and NIADDK-rPRL-I-5 (batch number AFP-4459B) were kindly provided by the National Hormone and Pituitary Program and by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (NIADDK; Bethesda, MD). hPRL from amniotic fluid source was prepared as reported earlier (21–23).

Buffers. The wash buffer was composed of 25 mM Tris base, 10 mM MgCl2, 1.0 mM EGTA, 10 mM monothioglycerol, 0.25 mM phenylmethylsulfonyl fluoride, and Trasylol (aprotinin) at 100,000 kallikrein inactivator units per liter. The homogenization buffer was composed of 0.3 M sucrose, 25 mM Tris, 0.25 mM phenylmethylsulfonyl fluoride, 1.0 mM EGTA, 10 mM monothioglycolerol, and Trasylol (aprotinin) at

Abbreviations: PRL, prolactin; h, human; r, rat; LH, luteinizing hormone.

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100,000 kallikrein inactivator units per liter. The incubation buffer was composed of 0.1% bovine serum albumin in the above wash buffer. All of the chemicals used in buffers were purchased from Sigma.

**Normal Tissues.** Liver tissue of adult female 60-day-old Sprague–Dawley rats (Charles River Breeding Laboratories) were used to prepare the crude membranes of liver, which was used as a positive control. The liver membranes of immature, 21-day-old male rats were used as negative controls. Mammary tissue of adult female rats, rats at midpregnancy, and mammary and liver tissue of lactating (3-day postpartum) rats were used to prepare a preparation of prolactin receptors under normoprolactinemic and hyperprolactinemic conditions. Ventral and dorsolateral prostate tissues were obtained from 90-day-old adult male Sprague–Dawley rats.

**Tumor Tissues.** Mammary tumor membrane fractions were prepared from the MT/W9a adenocarcinoma implanted 120 days previously in Wistar/Furth female rats. This tumor was a gift from U. Kim (Roswell Park Memorial Institute, Buffalo, NY). Prostate cancer tissue was removed from Copenhagen × Fisher, male rats bearing the Dunning R-3327H prostate adenocarcinoma, 110 days after transplantation. Dunning tumors were provided by N. Altman (Department of Comparative Pathology, Miami University School of Medicine, Miami).

**Membrane-Receptor Preparation.** Tissues were removed and immediately placed in Petri dishes immersed in an ice bath. Tissues were cleaned of fat and/or connective tissues, cut into small slices, and homogenized in 4 times their volume of sucrose buffer with a Polytron homogenizer at a maximal speed (stroke five times for 10-sec durations at 30-sec intervals). The homogenate was centrifuged at 500 × g for 15 min at 4°C. The supernatant containing the crude membrane fractions was again centrifuged at 25,000 × g for 30 min at 2–4°C. The pellet was washed twice by resuspending in wash buffer and spinning. The final pellet was then used for the receptor binding studies and for determination of protein content by the method of Lowry et al. (25).

**Stoichiometric Iodination of PRL.** hPRL prepared from amniotic fluid (21–23), NIAADD-kPRL-I-7, or rPRL, NIAADD-rPRL-I-5, was used. The chloramine-T iodination method of Greenwood et al. (26) was modified to a mild stoichiometric procedure without the use of sodium metabisulfite. For iodination, 6 μg of the pure hormone in 15 μL of 0.1 M NaHCO3 (pH = 8.0) was placed in a 1.5-mL polypropylene conical vial, and 15 μL of 0.3 M phosphate buffer (pH = 7.6) was added. One and one-half microliters (1 Ci = 37 GBq) of a fresh batch of carrier-free Na125I (Amersham) was added. The iodination was performed at 0°C, and the reaction that lasted for 20 min was stopped by the addition of an excess of bovine serum albumin in 0.3 M phosphate buffer (pH = 7.6) as soon as the incorporation of one atom of 125I into one molecule of the hormone was completed. The separation of bovine serum albumin, the monomeric 125I-PRL, and free 125I was carried out by column chromatography at 4°C by using a 0.9 × 55 cm column of Sephadex G-100–120, previously precoated with 0.8 mL of 30% bovine serum albumin. The column was eluted with 0.1% bovine serum albumin/0.01 M phosphate buffer, pH 7.4/0.1% sodium azide, and 0.6-mL fractions were collected. Aliquots (10 μL) of each fraction were assayed on an Apex γ-scintillation spectrometer (Micromedic Systems, Huntsville, AL).

Four peaks were routinely obtained. The first peak, representing iodinated bovine serum albumin, was regarded as the void volume of the column. The second, a small “shoulder” representing a damaged PRL aggregate, was then eluted. A third (major) peak of monomeric 125I-hPRL was obtained at Kav = 0.42 ± 0.01. The last peak of free iodide (Na125I) was regarded as the Kav = 1 of the column. The main fractions of the monomeric hPRL were pooled. These fractions were found to be 96–99% free of nonprotein iodine and had high specific activity between 170 and 186 μCi/μg as calculated by the isotope recovery. The pooled and labeled hormone solution was divided into 1-ml portions and stored at −70°C until used. The 125I-hPRL was found to be stable for at least 3 wk after iodination. Normally, even at 21 days, the deiodination did not exceed the 10% value, so that the ligand could be used without repurification. The high specific activity of the 125I-hPRL permitted the use of high (≈200,000 cpm), contained in a relatively small amount (1.0 ng) of PRL-hPRL, that was sufficient for saturation analysis for each assay point. Before use, the frozen, labeled hormone was thawed, checked for the percentage of free iodine, and then diluted with the assay buffer to contain 1.0 ng of 125I-hPRL per 0.1 ml.

**Freeing of Receptors from Bound Endogenous PRL.** A certain portion of the PRL receptors may be occupied by the endogenous circulating PRL. Since the binding of PRL to its receptors is relatively stable, most of these receptors remain bound after the homogenization and membrane fractionation procedure (27). Therefore, these “latent” receptors are undetectable unless the bound PRL is removed. To dissociate the bound PRL, the membrane preparation was exposed to a high concentration of MgCl2 as described by Kelly et al. (28). For each volume of membrane solution, 6 volumes of ice-cold 3 M MgCl2 was added. After 3 min at 0°C, the solution was diluted with 4 times its volume of ice-cold wash buffer and then was centrifuged at 2–4°C at 25,000 × g for 30 min. The pellet was resuspended in wash buffer to prepare the membrane solution for incubation. This procedure resulted in a loss of about 50–61% in the measurable protein content of the membrane preparation.

**Assay of PRL Receptors.** Binding experiments were performed in 12 × 75 mm polypropylene tubes containing 1.0 ng of 125I-PRL (about 200,000 cpm) with increasing amounts (1–3000 ng) of unlabeled PRL. Fifty micrograms of protein, as determined by the Lowry method (25), from the tissue membrane preparation in 0.1 ml of wash buffer was added, and the final volume was adjusted to 0.5 ml with the incubation buffer. The incubation was performed at 20°C for 16 hr, after which 150-μl aliquots in triplicate were layered over 200 μL of ice-cold incubation buffer in 400-μL polyethylene microcentrifuge tubes placed in an ice bath. These tubes were centrifuged at 10,000 × g for 3 min in a Beckman Microfuge B microcentrifuge at 22–24°C. The supernatant was aspirated, and the pellet was washed with 300 μL of ice-cold incubation buffer. The samples then were centrifuged for 1 min. The supernatant was aspirated, the tip of the tube containing the pellet was cut off and transferred into a 12 × 75 mm polystyrene tube, and the radioactivity was determined in a γ-scintillation counter. All of the results were calculated from the Scatchard plot (29), based on displacement curves obtained from the binding of 125I-hPRL with tissue membranes in the presence of increasing amounts of unlabeled PRL. Evaluation of the presence of one or more classes of receptor populations and the determination of the amount and the affinity of receptors were performed by using Rodbard’s computerized program adjusted for an IBM personal computer (30).

**RESULTS**

**Labeled Ligands: 125I-hPRL vs. 125I-rPRL.** The specific binding characteristics of labeled hPRL and labeled rPRL to PRL binding sites in the ventral prostate of normal rats are depicted in Fig. 1. The binding in other rat tissues was similar. When hPRL was used as a ligand, two substantially different classes of receptors were revealed. The first class of receptors showed high affinity (KD1 = 3.46 × 10−10 M) and medium
capacity \( (K_d = 1.26 \ \text{nmol/mg}) \), and the other class had lower affinity \( (K_d = 1.93 \times 10^{-8} \ \text{M}) \) and higher capacity \( (r_2 = 86.4 \ \text{nmol/mg}) \) (Fig. 1 Upper). However, when labeled rPRL was used, only one class of receptors with high affinity \( (K_d = 2.90 \times 10^{-10} \ \text{M}) \) but low capacity \( (r = 0.546 \ \text{nmol/mg}) \) was measured (Fig. 1 Lower).

Moreover, labeled rPRL exhibited a similar low binding capacity with all other types of rat tissue tested. Hence, it was decided to use only labeled hPRL in subsequent studies.

Free and Total Receptors in Various Rat Tissues. To check whether the circulating PRL levels can affect the number of measurable binding sites, the PRL receptors in the rat liver and mammary glands were determined (i) during early lactation when serum PRL is the highest (300–700 ng/ml), (ii) in midpregnancy when circulating PRL is fairly high (20–100 ng/ml), and (iii) at diestrous when serum PRL levels are relatively low (3–15 ng/ml). No free PRL receptors were detected in the mammary glands or in the liver of the postpartum lactating rats. However, when in vitro pretreatment with 3 M MgCl\(_2\) was applied to these membranes lacking free receptors, it was possible to detect two classes of receptors in the liver tissue and one class in the mammary glands. In the liver membranes, after the process of desaturation, two types of "hidden" receptors, as depicted in Fig. 2, were revealed: the first had a high affinity and low capacity \( (K_d = 4.54 \times 10^{-10} \ \text{M}; r = 0.354 \ \text{nmol/mg}) \), and the second had a lower affinity and higher capacity \( (K_d = 7.82 \times 10^{-9} \ \text{M}; r = 3.1 \ \text{nmol/mg}) \). In the mammary glands and lactating rats, one type of specific binding site with a relatively low affinity and high capacity \( (K_d = 1.77 \times 10^{-8} \ \text{M}; r = 10.6 \ \text{nmol/mg}) \) was detected. In the rat mammary gland, a high amount of one class of free receptors was found at diestrous and a small amount in midpregnancy. Fig. 3 illustrates PRL receptors that were found in mammary glands of rats at midpregnancy. Altogether, in mammary tissue we detected only one class of PRL receptors with moderate affinity, ranging between \( K_d = 1.77 \times 10^{-8} \) to \( 6.41 \times 10^{-9} \) M but in a relatively high amount ranging from \( r = 1.75 \) to 10.6 nmol/mg. In contrast to the mammary glands, two classes of PRL receptors were found in both prostate and liver tissues (Figs. 1 Upper and 2). The total amount of PRL receptors in these two organs considerably exceeded the levels found in the mammary glands.

Changes in PRL Receptors in Malignant Neoplasms. Changes in the type of PRL receptors were investigated in MT/WT9a mammary adenocarcinoma and in Dunning R3327H prostate cancer. In both of these tumors, there was a decrease in the amount of PRL receptors as compared to the respective normal tissue and, in the case of the prostate, there was a complete loss of one class of PRL receptors. While in
the normal prostate two types of receptors were found (Fig. 1 Upper), in the Dunning prostate tumor there was only one class of receptors. In some specimens of prostate tumors, only the class of PRL receptors with a high affinity \( (K_d = 4.6 \times 10^{-10} \text{ M}) \) was found (Fig. 4 Upper Left), and in other specimens only the receptors with a lower affinity \( (K_d = 8.8 \times 10^{-9} \text{ M}) \) were detected (Fig. 4 Upper Right). In the mammary tumor, low levels of PRL receptors \( (r = 0.027 \text{ pmol/mg}) \) with a very high affinity \( (K_d = 6.8 \times 10^{-14} \text{ M}) \) were detected (Fig. 4 Lower).

**DISCUSSION**

The results of this study demonstrate that the micromethod for measurement of PRL receptors described here is highly sensitive and can be used to detect these receptors in normal tissues in various physiological conditions and malignant neoplasms. An interesting finding is that labeled hPRL binds much better to rat tissues than does iodinated rPRL. In many cases using \(^{125}\text{I}-\text{rPRL}, we failed to measure PRL receptors, but when we used \(^{125}\text{I}-\text{hPRL}, we found high levels of these receptors. The reasons for this are not fully understood, and several explanations are possible. One possibility is that the purity of the rPRL was inadequate, although the highest grade of rPRL (NIADDK-rPRL-1-5) available was used. Another possibility is that the rPRL molecule is much more sensitive to the iodination procedure than is hPRL, although iodination was carried out under extremely mild conditions. After the iodination, the rPRL molecule could change its conformation and subsequently lose much of its binding capacity, even though it kept its full immunological activity.

Similar phenomena are known to occur for other rat hormones. For instance, human chorionic gonadotropin (HCG) or human luteinizing hormone (LH) bind to rat Leydig cells better than does iodinated rat LH (personal communication from K. Catt). Thus, hPRL, iodinated under the mild conditions described in the present study, is superior to labeled rPRL for binding experiments with various rat tissues. An important finding of this study is that, under certain conditions of hyperprolactinemia, PRL receptors can be totally occupied and therefore undetectable. This is probably due to the “irreversible” nature of PRL binding to its receptors (27) and to the high circulating PRL levels. Thus, in the postpartum lactating state, despite the presence of very high levels of PRL receptors in the mammary glands and in the liver, these receptors were not detectable. In the liver tissue, these occupied receptors were found to be abundant and composed of two classes (Fig. 2). These receptors are “hidden” and undetectable unless an in vitro dissociation procedure is applied. This important finding must be taken into account when receptor levels are being measured in order to assess hormone-dependency of the tumor. In order to eliminate the false/negative results, the amount of total receptors, and not only free receptors, must be measured in the tumor tissue. The results obtained on tumors indicate that the PRL receptors in the MT/W9a mammary adenocarcinoma are of a very high affinity. This suggests that they might bind circulating PRL and hence are affected by it, even when the plasma levels of PRL are relatively low. The detection and measurement of these PRL receptors in the cancerous tissues is also necessary for predicting responsiveness to hormonal therapy. The method reported can also be used for
the measurement of PRL receptors in the pellet fraction remaining from human breast cancer specimens that had undergone processing for estrogen receptors. After measurement of >300 biopsy specimens, we found significant levels of PRL receptors in 42% of U.S. and Israeli women with breast cancer (M.B.-D., T.K., J. L. Wittliiff, S. Biran, and A.V.S., unpublished data).

It is surprising that mammary tissue, which is the principal target for PRL, was found to possess only one type of PRL receptor with a moderate capacity. On the other hand, our data showed that organs such as the liver and the prostate had two classes of PRL receptors with relatively high affinities and altogether had a higher quantity of PRL receptors than did the breast. The significance of this finding and the role that PRL may play in pathogenesis [such as tumor promoter in rat liver (31)] or in the normal physiology of these organs remains to be established. Several actions have been attributed to PRL (32) that have not yet been fully investigated.

If the PRL receptors are present in significant amounts in a cancerous tissue, then the way to neutralize a possible stimulatory effect of PRL in the process of tumorogenesis would be to eliminate it from circulation. This might be achieved by treatment with PRL-suppressing agents (28) that can drastically lower the levels of circulating PRL. A future approach to suppress PRL secretion might consist of the use of somatostatin analogs (33, 34) or hypothalamic PRL release-inhibiting factor, which is possibly related to gonadotropin-releasing hormone-associated peptide (35). Treatments with these polypeptides could be combined with long-term treatment of LH-releasing hormone (LH-RH) agonists (36) that were found to be effective in estrogen-dependent human breast cancer when given either alone (37–39) or in combination with other regimens (40). Some LH-RH agonists and somatostatin analogs can be administered once-a-month by using the delayed-release microcapsules (41). In addition to suppressing gonadal activity, the LH-RH agonists may prevent PRL hypersecretion induced by haloperidol (42) and exhibit direct effects on human breast cancer cells growing in vitro (43). This effect may be exerted by an action on specific binding sites that are detected in breast carcinoma (38).

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