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Prolactin Receptors in Explant Cultures of Carcinogen-induced Rat Mammary Tumors

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Abstract—*The turnover, down-regulation and role of intracellular organelles in the down-regulation of prolactin (PRL) receptors have been investigated in N-nitrosomethylurea (NMU)-induced rat mammary tumors cultured in short-term explants. Tumor explants are capable of maintaining PRL receptors for* 24–48 hr. *This maintenance reflects a dynamic phenomenon involving receptor synthesis, since addition of cycloheximide* (1 μg/ml) *in the culture medium results within* 12 hr *in a marked decline of PRL receptor levels. A down-regulation of total PRL receptors (measured after exposure of membranes to* 3M $MgCl_2$) *is observed in cultures containing concentrations of* 20 μg/ml *or greater of ovine PRL (oPRL). Lysosomotropic agents, such as chloroquine* (100 μM) *are ineffective in either increasing basal PRL receptor levels or in preventing the PRL-induced down-regulation in NMU-induced mammary tumor explants. Cytochalasin B* (20 μM), *without effect on basal PRL binding, prevents the down-regulation of PRL receptors, whereas colchicine* (10 μM) *results in a decline of PRL receptor levels both in the absence and in the presence of oPRL. The present data suggest a different pattern of PRL receptor regulation* in vitro *for tumors compared to normal rabbit mammary explants.*

INTRODUCTION

ONE OF the most important factors in the growth and development of both the normal mammary gland and mammary tumors is prolactin. Receptors for prolactin are abundant in rabbit mammary glands and can be maintained in organ culture. This *in vitro* approach has been utilized to study prolactin receptor regulation [1–3]. In addition to its ability to up-regulate the level of its own receptors [4–6], prolactin can induce a down-regulation in normal rabbit mammary gland [7]. In this tissue down-regulation involves an increased rate of lysosomal degradation of hormone–receptor complex, since lysomotropic agents block this down-regulation [3, 7] whereas cytoskeleton-disrupting drugs do not [7].

Prolactin binding has also been reported in dissociated mammary tumor cells [8], as in primary cultures of DMBA-induced tumor cells [9] as well as in NMU-induced mammary tumor explants [10]. In the present study the turnover, down-regulation and role of intracellular organelles in the down-regulation of PRL receptors have been investigated in carcinogen-induced rat mammary tumors.

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MATERIALS AND METHODS

Animals

NMU mammary tumors were induced in female Sprague–Dawley (CD, Charles River) rats (Canadian Breeding Farms, St. Constant, Quebec, Canada) being given intravenous caudal vein injections of NMU (5 mg/100 g body weight) at 50–55 days of age. The NMU (ICN Pharmaceuticals Life Sciences Group, Plainview, NY) was acidified with a few drops of 3% acetic acid and dissolved in distilled water. Injections were repeated 4 and 8 weeks after the initial injection. Tumors began to appear 2 months after the first intravenous injection. DMBA mammary tumors were induced by a single intragastric dose of 20 mg DMBA in 0.5 ml sesame oil at 50–55 days of age. Tumors began to appear after about 1.5 months.

In order to obtain lower circulating PRL levels and increase PRL receptor levels, animals were

given three injections of 0.5 mg CB-154 (Sandoz, Basle, Switzerland) every 12 hr over the 36-hr period prior to death.

Organ culture

Animals bearing carcinogen-induced mammary tumors were killed by cervical dislocation. Mammary tumors were immediately removed and placed in a sterile Petri dish. After removal of adipose tissue, approximately 600 mg of tumoral tissue was cut into small fragments of 1–2 mm^3 which were placed on stainless-steel grids (15 explants per grid). Twelve grids were placed in three 100 × 15 mm Petri dishes with Medium 199 (Flow Laboratories) that contained an excess of amino acids, bicarbonate (2.2 g/l), penicillin G (100,000 U/l) and streptomycin (50 mg/l). Medium 199 was also supplemented with insulin (5 μg/ml) and cortisol (500 ng/ml). The medium was added to the dishes in such a way that the explants were not immersed. All these operations were made under a laminar flow hood. The Petri dishes were placed in an incubator at 37°C under a controlled atmosphere of 5% CO_2–95% air. Explants were cultured up to 24 hr in the presence of ovine prolactin (NIH-P-S13, 30 IU/mg) at different concentrations (1–40 μg/ml). Explants were cultured for 12, 24 and 48 hr in the presence of cycloheximide (Sigma, 1 μg/ml). Other added inhibitors were: chloroquine (Sigma), 100 μM; NH_4Cl (BDH), 10 mM; cytochalasin B (Sigma), 20 μM; colchicine (Sigma), 10 μM. When cultures were prolonged up to 48 hr the medium was replaced after 24 hr. At the end of the culture the grids were placed on filter paper and the explants removed, weighed and frozen at -20°C until receptor study.

Membrane preparation

We used the procedure derived from the original method of Shiu *et al.* [11], as adapted to tissue explants by Djiane *et al.* [2]: explants are homogenized in 6 vol. (w/v) of 0.25 M sucrose using a Polytron PT-10 homogenizer at a setting of 5 for 2 or 3 periods of 10 sec with an interval of 10 sec for cooling. The homogenate was then centrifuged (12,000 *g*, 15 min) and the resulting pellet was resuspended in 25 mM Tris–HCl, pH 7.4, and 10 mM $MgCl_2$ with a Teflon and glass homogenizer. An aliquot was taken to determine protein content [12]. Membrane fractions were stored at -20°C until further use.

Dissociation of endogenously bound prolactin

Since a large proportion of prolactin still remains bound to the receptors at the end of the membrane preparation, it was necessary to remove endogenously bound prolactin in order to determine total prolactin receptor levels [13]. To 1.5 mg of mammary tumor membrane protein 0.5 ml of 3 M $MgCl_2$ was added, the tubes were vortexed and after 3 min 3 ml of assay buffer was added to each tube. Tubes were centrifuged at 2300 *g* for 15 min and the resulting supernatant was aspirated. The pellets were resuspended in 0.5 ml of assay buffer and binding studies were carried out.

Binding assay

It has been shown previously that [^{125}I]-hGH binds with the same affinity and specificity as [^{125}I]-oPRL to membrane fractions prepared from prolactin-responsive tissues [14]. We have used [^{125}I]-hGH for prolactin binding since labelled samples of this hormone have proved more stable than labelled prolactin. A modification of the method of Hunter and Greenwood [15] using low concentrations of chloramine T (500 ng) and 5 μg hGH with an incubation for 3 min at 23°C was employed to iodinate hGH [13]. The iodinated hormone was purified on a column of Sephadex G-75 (0.9 × 100 cm) and the tubes of radioactivity comprising the protein peak were diluted in assay buffer (25 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$ and 0.1% bovine serum albumin). Specific activity, as calculated by isotope recovery, ranged between 75 and 95 μCi/μg. The integrity of the iodinated hGH was verified after iodination using a laboratory-standard receptor preparation. At the concentration of mammary gland receptor used (300 μg) at least 40% of the iodinated hGH was specifically bound before the tracer was considered acceptable.

Carcinogen-induced mammary tumor membranes (300 μg protein) were incubated with approximately 100,000 counts/min [^{125}I]-hGH in the presence and absence of an excess (1 μg) of unlabeled oPRL. Specific binding was calculated as the difference between the counts/min bound in the absence and presence of excess unlabelled hormone and was expressed as a percentage of the total radioactivity added to the tube.

Each assay was performed with at least duplicate determinations. Receptor assays were calculated using a program developed in this laboratory. Calculations were performed using a Hewlett-Packard 9845B desk-top calculator. Statistical significance was determined using Student's *t* test or Duncan's multiple range test [16]. All data presented except for Figs 2 and 3 are means ± S.E.M.

RESULTS

In the absence of ovine prolactin, both NMU- and DMBA-induced mammary tumors are capable of maintaining PRL receptors in explant

culture. As illustrated in Fig. 1, more than 80% of specific binding measured in control tissue (before culture) can be measured after 24 hr culture in medium containing insulin and cortisol.

Free receptors when measured over a 48-hr period tend to fluctuate slightly. For NMU tumors (Fig. 2) there is a rapid decline at 12 hr followed by a return towards 0-hr values. For DMBA tumors (Fig. 3) binding increases slightly at 12 hr, with a return to values measured prior to culture by 24 and 48 hr. Addition of cycloheximide (1 μg/ml) causes a rapid decline of

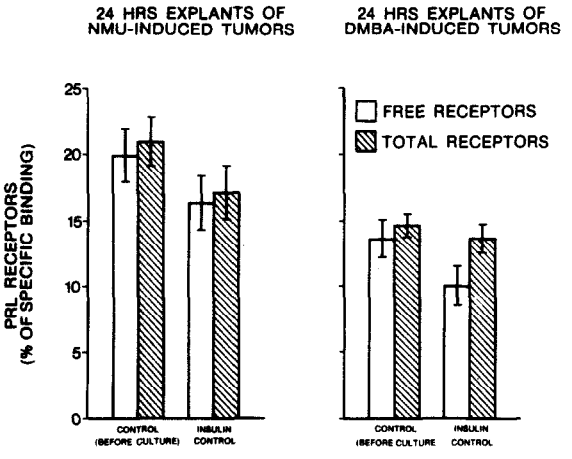

Fig. 1. Free and total PRL receptor levels in 24-hr explants of carcinogen-induced rat mammary tumors. Organ cultures of NMU- (left panel) and DMBA-induced (right panel) mammary tumors have been carried out in medium 199 supplemented with insulin (5 μg/ml) and cortisol (500 ng/ml). Values are means ± S.E.M. of 5 explant cultures.

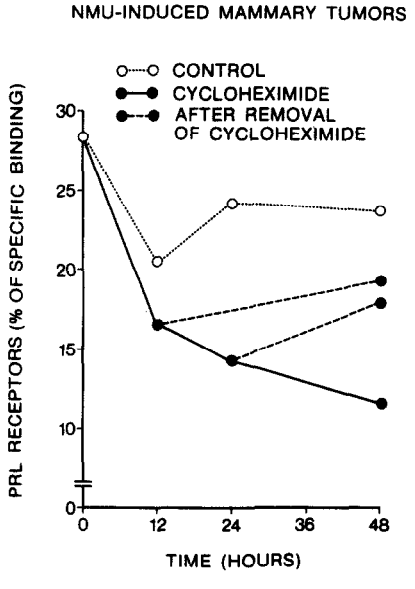

Fig. 2. Effect of cycloheximide (1 μg/ml) on free PRL receptors in NMU-induced mammary carcinoma. Mammary tumor explants were cultured in medium 199 containing insulin (5 μg/ml) and cortisol (500 ng/ml) in the absence and presence of cycloheximide after which the medium was changed at 12 or 24 hr and cycloheximide was removed.

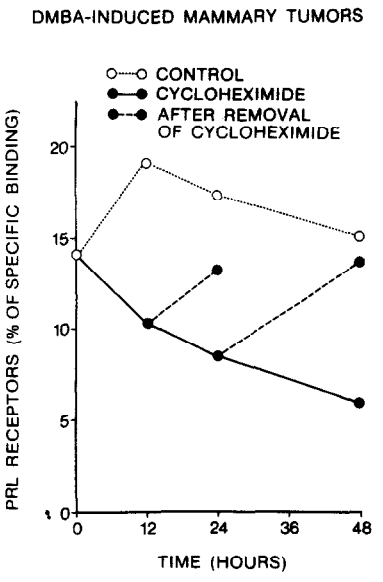

Fig. 3. Effect of cycloheximide (1 μg/ml) on free PRL receptors in DMBA-induced mammary carcinoma (see legend to Fig. 2).

binding during the first 12 hr and this inhibition remains present for the next 48 hr. This concentration of cycloheximide reduces protein synthesis, measured by the incorporation of [^{3}H]-amino acids into total protein, by more than 90%. Figures 2 and 3 demonstrate this as well as the reversibility of the effect of cycloheximide in both NMU- (Fig. 2) and especially in DMBA-induced (Fig. 3) mammary tumor explants. Removal of cycloheximide results in a return of prolactin binding towards control levels 12–36 hr later.

Free and total receptors (after membrane preparation exposure to 3M $MgCl_2$) were measured in explants cultured for 24 hr in the absence and presence of different concentrations of ovine prolactin. As shown in Fig. 4, at concentrations greater than 1 μg/ml a dose-dependent decline of free PRL receptors can be observed which is maximal (63%) at 40 μg/ml. Total receptors are also markedly reduced (39% decline at 20 μg/ml, $P < 0.05$), demonstrating that prolactin can induce a down-regulation of its own receptor in NMU-induced rat mammary tumors. A similar effect (down-regulation ranging between 38 and 50%) with high concentrations of oPRL can also be observed in DMBA-induced mammary tumors (data not shown).

Figure 5 illustrates the lack of effect of chloroquine (100 μM, a dose which presumably inhibits lysosomal degradation) on PRL receptor levels in explants of NMU-induced mammary tumors. Chloroquine is also ineffective in preventing PRL-induced down-regulation. Similar results have been also observed with tumor explant cultures in the presence of 10 μM NH_4Cl,

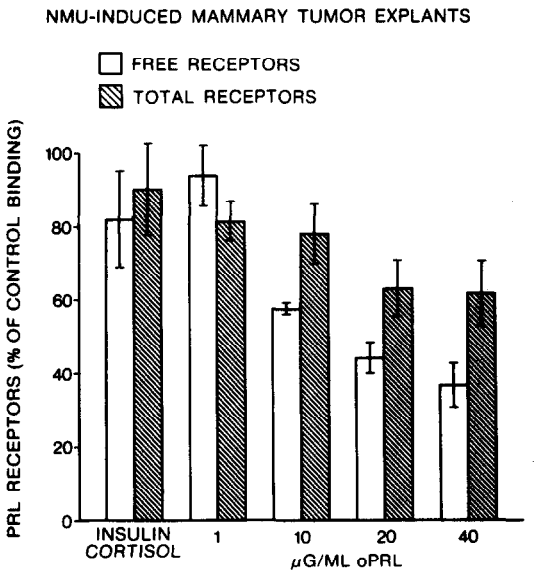

Fig. 4. The effect of increasing concentrations of oPRL (1–40 μg/ml) on free and total PRL receptor levels in organ cultures of NMU-induced mammary tumors. Explants were cultured for 24 hr. Values are means ± S.E.M. of 10 explant cultures and are represented as a % of control binding in uncultured tissue, which was 21.0 ± 1.6 and 24.4 ± 1.8% for free and total receptors respectively.

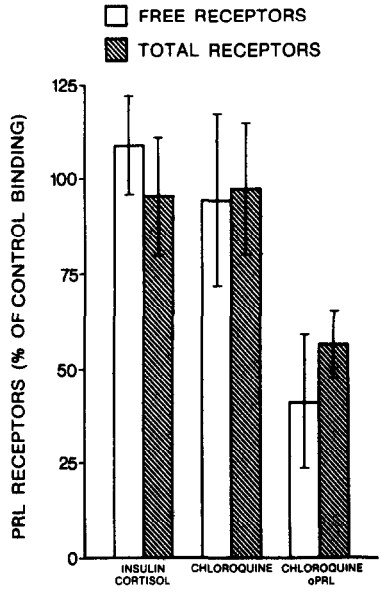

Fig. 5. Free and total PRL receptor levels in 24-hr explants of NMU-induced mammary carcinoma in medium 199 supplemented with insulin (5 μg/ml) and cortisol (500 ng/ml) in the presence of chloroquine (100 μM) or a combination of chloroquine and oPRL (20 μg/ml). Values are means ± S.E.M. of 5 explant cultures represented as % of control binding in uncultured tissue, which were 16.1 ± 2.4 and 17.3 ± 3.1% for free and total receptors respectively.

another agent capable of inhibiting lysosomal activities (data not shown).

Cytoskeleton-disrupting drugs have been also studied, as shown in Figs 6 and 7. Cytochalasin B (20 μM), a microfilament-disrupting agent, does not alter basal PRL receptor levels in explants of

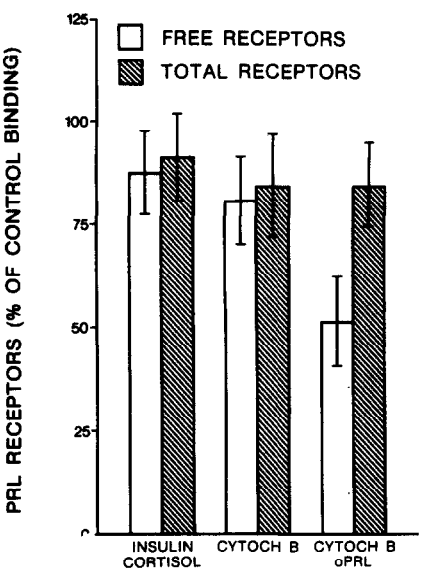

Fig. 6. Free and total PRL receptor levels in 24-hr explants of NMU-induced mammary carcinoma in medium 199 supplemented with insulin (5 μg/ml) and cortisol (500 ng/ml) in the presence of cytochalasin B (20 μM) or a combination of cytochalasin B and oPRL (20 μ/ml). Values are means ± S.E.M. of 5 explants cultures and represented as a % of control binding in uncultured tissue which were 13.0 ± 1.8 and 14.7 ± 1.2% for free and total receptors respectively.

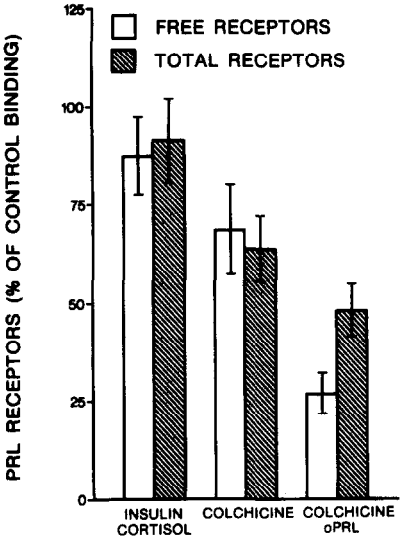

Fig. 7. Free and total receptor levels in 24-hr explants of NMU-induced mammary carcinoma in medium 199 supplemented with insulin (5 μg/ml) and cortisol (500 ng/ml) in the presence of colchicine (10 μM) or a combination of colchicine and oPRL (20 μg/ml). Values are means ± S.E.M. of 5 explants cultures. Controls are identical to those described in legend to Fig. 6.

NMU-induced tumors, whereas a non-significant 25% decline in both free and total PRL binding is observed in the presence of 10 μM colchicine. The effects of the same agents on PRL-induced down-regulation are also depicted after a 4-hr preincubation of cytochalasin B with the explants (Fig. 6); addition of oPRL (20 μg/ml) is not

capable of inducing a decline in total receptor levels. Colchicine does not prevent the PRL-induced down-regulation (Fig. 7), in spite of a greater occupation of free receptors than observed with cytochalasin B.

DISCUSSION

These results confirm that both NMU and DMBA-induced mammary tumors maintain PRL receptors in short-term (24-hr) explant cultures [10, 17]. As in normal mammary gland [1], this reflects a dynamic phenomenon since cycloheximide, a translational inhibitor of protein synthesis, results (within 12 hr) in a marked decline of PRL receptor levels in carcinogen-induced mammary tumors. This inhibition is reversible, especially as is demonstrated for DMBA tumors (Fig. 3), by changing the culture medium with one which does not contain the inhibitor. In normal rabbit mammary gland PRL binding has been shown to be reduced by 60% within 6 hr [3] and to return to control levels 18–24 hr after removal of the cycloheximide, indicating a rapid turnover.

It has been previously shown that in NMU-induced tumors the ability of PRL to down-regulate its own receptors increased with the tumor age [10]. Nevertheless, it has been possible in the present study to observe, regardless of tumor age, a dose-dependent occupation of free PRL binding sites which was maximal in the presence of 40 μg/ml oPRL. A decline in total PRL receptor levels could be observed only at concentrations of 20 μg/ml or greater, representing non-physiological hormone levels. Costlow and Hample [18] in DMBA-induced mammary tumor cells in culture have reported that down-regulation only occurs at non-physiologic hormone levels. In fact, free receptors in rat mammary tumors do not decline to the same extent as those in rabbit mammary tissue [1, 19], suggesting some differences in the accessability of prolactin receptors. In normal rabbit mammary gland a PRL-induced down-regulation can be observed in the presence of 100 ng/ml oPRL or less [19]. Down-regulation in this experimental model is the reflection of an equilibrium between synthesis and degradation of PRL receptors. Indeed, chloroquine increased PRL receptor levels and prevented down-regulation [7]. In contrast to what has been observed in rabbit mammary explants, lysomotropic agents such as chloroquine or ammonium chloride are ineffective in either increasing basal PRL receptor levels or in preventing the PRL-induced down-regulation of PRl receptor. This lack of effect of lysomotropic agents has been also reported in DMBA-induced mammary tumor cells [18]. These data imply differences in the level of lysosomal degradation of PRL receptors between normal vs cancerous tissue or, alternatively, this may represent species differences.

Cytochalasin B, which interferes with the functions of microfilaments, appeared capable of inhibiting the down-regulation of total PRL receptors. This is surprising for this drug is inactive in all cases where hormone-receptor degradation has thus far been analyzed [20, 21]. The lack of a decline in total receptors can be partially explained by the reduced occupation of free prolactin binding sites. Microfilaments or a cytochalasin B-binding component may be partially involved in the binding of PRL to its receptors. Colchicine, which prevents microtubule polymerization, appeared to result in a loss of PRL receptor levels both in the absence and presence of oPRL. This fact supports the hypothesis of an action of colchicine at a step beyond hormone binding, since no effect in down-regulation is observed whereas the response of mammary cells is inhibited [22–24].

These data show that in carcinogen-induced rat mammary tumors there is a different pattern of prolactin receptor regulation *in vitro* compared to normal rabbit mammary explants. Whether this difference is linked to species or to the pathological tissue condition remains to be determined.

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