Effects of Epidermal Growth Factor (EGF) and Prolactin on EGF Receptor Cytoskeletal Association in Mammary Epithelial Cells (44149)

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Abstract. **Prolactin treatment of NMuMG mammary epithelial cells inhibits the ability of epidermal growth factor (EGF) to transduce a variety of signals, possibly by interfering with receptor tyrosine phosphorylation. However, the mechanism by which prolactin inhibits EGF receptor signaling is unclear. The objective of this study was to evaluate the effects of prolactin on the dynamics of EGF receptor degradation and resynthesis, and on the association of the receptor with the cytoskeleton. EGF decreased the EGF receptor content of NMuMG cells, and this decrease was unaffected by prolactin treatment. Subsequent to the decrease in EGF receptors, cells re-accumulated EGF receptors, and this re-accumulation was also unaffected by prolactin. In other studies, EGF induced a rapid association of EGF receptor with Triton X-100–insoluble (cytoskeletal) elements. The cytoskeletally associated receptors were more heavily tyrosine phosphorylated than soluble receptors in the absence of prolactin. In the presence of prolactin, similar amounts of EGF receptor associated with the cytoskeleton, but both cytoskeletal and soluble receptors exhibited decreased tyrosine phosphorylation. These studies indicate that the effects of prolactin on EGF receptor signaling are not likely to be due to altered receptor dynamics or cytoskeletal association but are more likely due to an alteration in receptor kinase activity.**

[P.S.E.B.M. 1997, Vol 215]

Epidermal growth factor (EGF) and related growth factors, such as transforming growth factor–α (TGFα), are produced in the mammary gland and appear to be developmentally regulated, probably under hormonal control (1–3). Both factors are mitogenic to mammary epithelium in culture (4–6) and increase development of the mammary ductal and alveolar system when administered *in vivo via* local release implants (7, 8).

Both EGF and TGFα appear to share a common receptor (9, 10) that acts as a tyrosine kinase. EGF receptors have been identified in the mammary gland (11) and are developmentally regulated (12). Activation of the EGF receptor by its ligand induces a variety of responses, including increased receptor tyrosine autophosphorylation (13, 14), increased association with SH2 domain containing proteins (15), increased activity of the *ras-raf*-MAPK pathway (16, 17), and increased tyrosine phosphorylation of a number of substrates, including phospholipase Cγ (18, 19). In addition to these signals, ligand binding to the receptor also appears to increase its association with the cytoskeleton, which may increase receptor signal transduction (20, 21).

Prolactin has long been known to have important roles in regulating mammary gland growth and differentiation (22). Recently, Fenton and Sheffield (23) observed that prolactin, at least under some conditions, is capable of decreasing EGF mitogenesis in mammary epithelial cells. The effect of prolactin appeared to be associated with decreased tyrosine phosphorylation of several EGF receptor kinase

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This work was supported by the University of Wisconsin College of Agricultural and Life Sciences, USDA Hatch Project WIS 3769, and NRICGP/USDA Grant 9202994.

Received July 17, 1996. [P.S.E.B.M. 1997, Vol 215]
Accepted April 13, 1997.

0037-9727/97/2154-0393$10.50/0

substrates and the EGF receptor itself (24). More recently, Johnson *et al.* (25) observed that prolactin blocked the ability of EGF to induce *ras* activity in mammary epithelium. This was associated with decreased Grb2 and Shc association with EGF receptor, and with decreased receptor tyrosine phosphorylation. However, the mechanism of these effects is unclear. Several possibilities exist, including that prolactin induces altered EGF receptor downregulation, recycling or cytoskeletal association. Therefore, the objective of this study was to determine the effects of prolactin on EGF-induced EGF receptor downregulation and cytoskeletal associations in mammary epithelium.

Materials and Methods

Cell Culture and Treatments. NMuMG cells (26) (ATCC, Rockville, MD) were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY), streptomycin (10 μg/ml), and penicillin (10 U/ml) (Sigma Chemical Co., St. Louis, MO). Cells were plated onto 60- or 100-mm^2 dishes (approximately $5 \times 10^5/cm^2$) and cultured for 24 hr. Quiescent cells were obtained by incubation in serum-free medium 18–24 hr prior to application of treatments. For hormone treatments, ovine prolactin (NIDDK) and murine EGF (Harlan Bioproducts, Indianapolis, IN) were used at indicated concentrations and for indicated times. All experiments were performed on cells between the passages of 15 to 21.

EGF Receptor Content. NMuMG cells were cultured and treated as described above. At various times after adding EGF, cells were rinsed with cold Hanks' balanced salt solution, lysed with SDS loading buffer, heated for 10 min in a steam bath, and proteins separated by 7.5% SDS-PAGE (27). Proteins were then transferred to PVDF membranes (Millipore, Bedford, MA) by electrophoretic blotting (28). Blots were blocked by overnight incubation at 4°C with PBS-T (50 m*M* sodium phosphate, pH 7.0, 150 m*M* NaCl, 3% Tween-20) containing 2% bovine serum albumin (BSA). Blots were then incubated with anti-EGF receptor antibody at a dilution of 1:1000 (UBI, Lake Placid, NY), rinsed six times with PBS-T containing 0.1% BSA, incubated with peroxidase conjugated secondary antibody (Sigma), and then rinsed with PBS-T containing 0.1% BSA. Proteins were then detected using Enhanced Chemiluminescence (DuPont NEN, Boston, MA).

Cytoskeletal Extractions. NMuMG cells were cultured and treated as described above. Cells were then lysed with lysis buffer (25 m*M* HEPES, pH 7.5, containing 1 m*M* PMSF, 1 m*M* $NaVO_4$, and 0.15% Triton X-100) on ice for 10 min and centrifuged at 14,000 *g* for 15 min. Pellets (cytoskeleton) were then resuspended in lysis buffer by sonication. In initial studies, 0.15% Triton X-100 was found to give maximum solubility of EGF receptors, and this concentration was used in subsequent studies. EGF receptor content was determined by Western analysis, as described above, and tyrosine phosphorylation determined by immunoprecipitation, as described below.

Tyrosine Phosphorylation of EGF Receptor. Soluble and cytoskeletal fractions of cells were suspended in lysis buffer containing 6 *M* urea. Agarose-conjugated anti-phosphotyrosine (Oncogene Science, Manhasset, NY) was added and tyrosine phosphorylated proteins immunoprecipitated essentially as previously described (24). Proteins were then separated by SDS-PAGE and analyzed for EGF receptor content by Western analysis as described above.

Statistical Analysis. Data were analyzed by two-way analysis of variance using the linear model $Y_{ij} = \mu + \beta_i + \tau_j + (\beta\tau)_{ij} + \epsilon_{ij}$. Where Y_{ij} represents the *jth* treatment in the *ith* replicate, β_i is the *ith* replicate, τ_j the *jth* treatment, $(\beta\tau)_{ij}$ the replicate × treatment interaction, and ϵ_{ij} the error, which is inseparable from replicate in this design. Since replicates were assumed random and treatments fixed, the replicate × treatment interaction was used as the error term for analysis. Experimental units were the cultures used in each replicate. Replicates indicated independent replication on the number of occasions indicated for each study. Means were compared using Dunnett's *t* test or planned comparisons (29). Unless otherwise stated, significance was $P < 0.05$.

Results

Initial studies (data not shown) indicated that prolactin did not alter the number of EGF receptors in NMuMG cells ($P > 0.05$). EGF induced a decline in EGF receptor number ($P < 0.05$) (Fig. 1). Significant downregulation was observed after 30 min of EGF treatment, and reached a minimum by 60 min. The presence of prolactin did not alter the magnitude and time course of this EGF-induced decline in EGF receptor content of NMuMG cells ($P > 0.05$). Constant total protein in each treatment group was verified by immunoblotting with anti–β-actin and found to be equivalent, thus verifying a true downregulation event.

Subsequently, the effect of prolactin on resynthesis of EGF receptors was evaluated. EGF receptors gradually reappeared after withdrawal of EGF from cells (Fig. 2). This resynthesis of receptors was not affected by the presence of prolactin in culture medium ($P > 0.05$). Thus, prolactin does not appear to alter the dynamics of EGF receptor downregulation or resynthesis in NMuMG cells.

Triton X-100, at a concentration of 0.15%, was able to solubilize most of the EGF receptor in NMuMG cells. EGF increased the association of EGF receptors with Triton X-100 insoluble material from about 5%–6% in controls to about 20% in EGF-treated cells. However, prolactin had no effect on the Triton X-100 solubility of EGF receptor. Similarly, when cells were pretreated with prolactin, the ability of EGF to induce cytoskeletal association of the EGF receptor was unaltered (Fig. 3).

While prolactin did not alter EGF receptor association with the cytoskeleton, the tyrosine phosphorylation of EGF

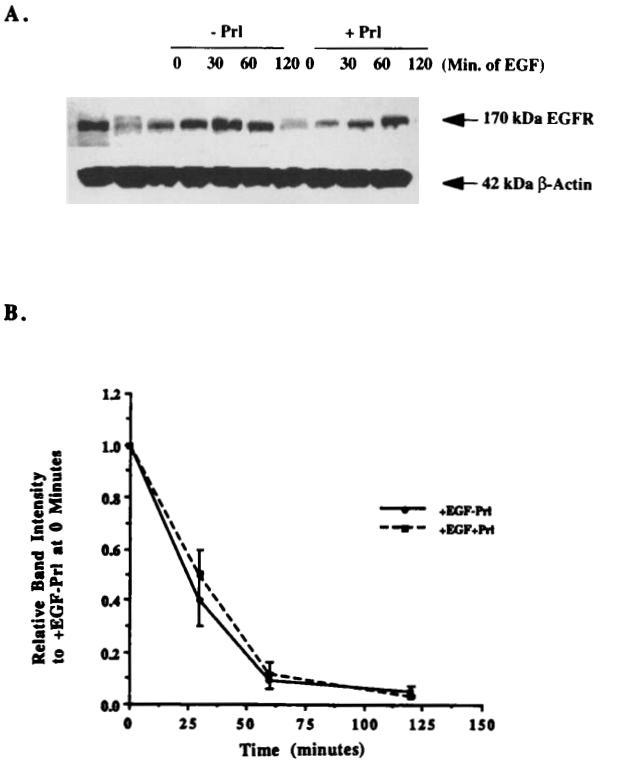

Figure 1. Influence of EGF and prolactin on EGF receptor loss following addition of EGF to cells. (A) Representative immunoblot. (B) Densitometric analysis of 1A (top). Cells were treated with 0 or 100 ng/ml prolactin (Prl) in the presence of 100 ng/ml EGF for indicated times. EGF receptor (top) or β-actin (bottom) content was then determined as described in Materials and Methods. Values represent mean ± pooled SEM of $n = 3$ observations.

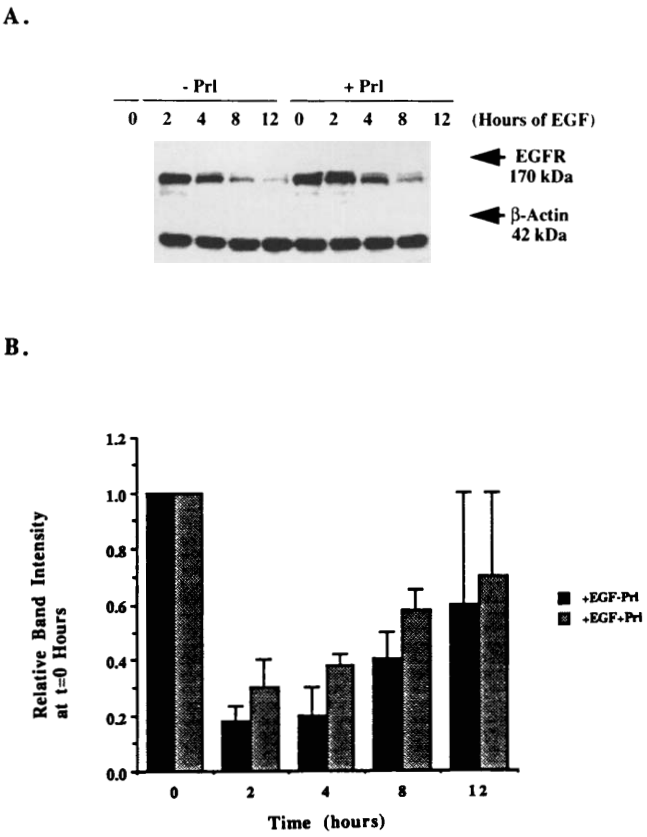

Figure 2. Effect of prolactin on reappearance of EGF receptors after EGF-induced downregulation. (A) Representative immunoblot. (B) Densitometric analysis of 1A (top). Cells were treated with 0 or 100 ng/ml prolactin (Prl) and 100 ng/ml EGF for 0–12 hr. After the indicated treatment times, EGF receptor (top) or β-actin (bottom) content was determined as described in Materials and Methods. Mean ± pooled SEM of $n = 4$ observations.

receptor induced by EGF was substantially altered by prolactin. EGF induced phosphorylation of EGF receptors in both soluble and cytoskeletal fractions (Fig. 4). However, the cytoskeletal fractions represented a small percentage of the EGF receptors, and autophosphorylation of receptors in the cytoskeleton was substantially greater than in the soluble fraction. Prolactin significantly inhibited receptor phosphorylation in both soluble and cytoskeletal fractions.

Discussion

Previously, a number of investigators have reported that EGF decreases EGF receptor content of mammalian cells (30–33), including mammary gland (34). Thus, the EGF-induced receptor disappearance in the present study is not surprising. However, other authors have noted that the EGF-induced receptor internalization appears to require tyrosine phosphorylation of the receptor (14, 35). In the present study, although prolactin inhibited receptor autophosphorylation, it had no measurable effect on EGF-induced receptor downregulation.

Previous studies using A431 cells and rat fibroblasts overexpressing EGF receptors have clearly shown that a small percentage of EGF receptors are closely associated with the cytoskeleton in resting cells (20, 21). Thus, the finding of a cytoskeletal association of EGF receptors in

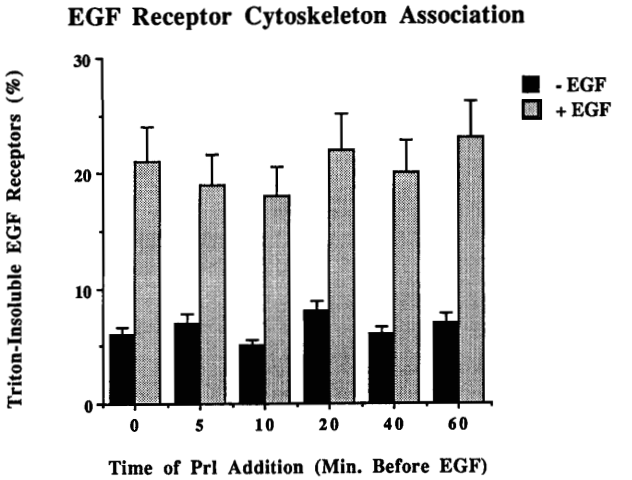

Figure 3. Effect of prolactin and EGF on amount of EGF receptor associated with cytoskeleton. Cells were treated with 0 or 100 ng/ml prolactin, then with 100 ng/ml EGF for 15 min. EGF receptor distribution between Triton X-100–soluble and insoluble (cytoskeletal) EGF receptors, was determined as described in Materials and Methods. Mean ± pooled SEM of four observations.

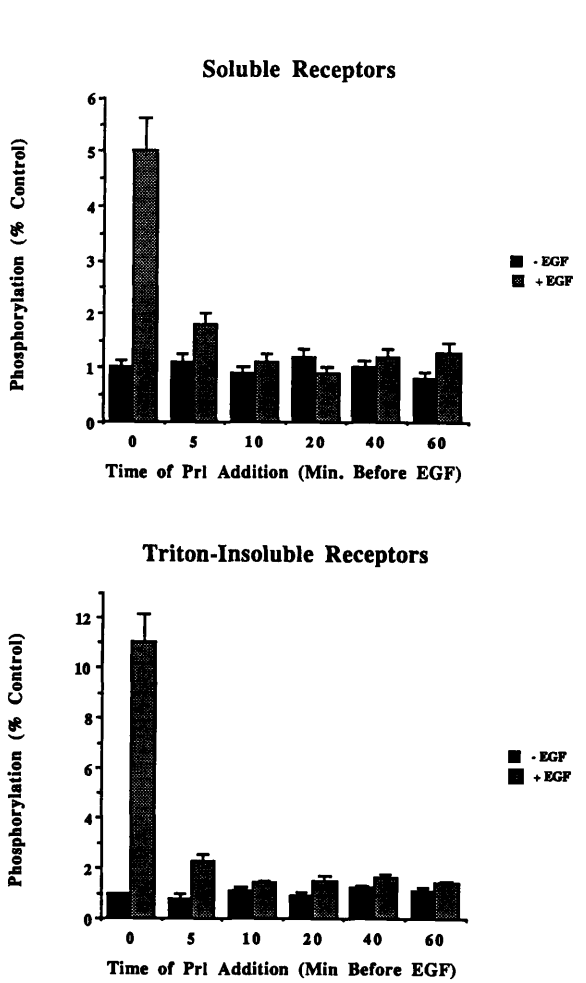

Figure 4. Effect of prolactin and EGF on tyrosine phosphorylation of EGF receptor in NMuMG cells. Cells were treated with 0 or 100 ng/ml prolactin, then with 100 ng/ml EGF for 15 min. Cells were then extracted with 0.15% Triton X-100 and EGF receptors in soluble (A) and cytoskeletal (Triton X-100–insoluble) (B) fractions immunoprecipitated. Tyrosine phosphorylation of the immunoprecipitated receptors was then determined by Western blot analysis as described in Materials and Methods. Mean ± pooled SEM of four observations.

NMuMG cells was not unexpected. Furthermore, receptor-cytoskeleton associations appeared to increase upon EGF stimulation of NMuMG cells, as occurs in other cells as well (21). The significance of the association of EGF receptors with the cytoskeleton is not entirely clear. Gronowski and Bertics (20, 21) observed that cytoskeletally associated EGF receptors had a greater kinase activity than soluble receptors, measured by autophosphorylation and phosphorylation of exogenous substrates. The present study also indicates that cytoskeletally associated EGF receptors exhibit a greater ligand-induced receptor tyrosine phosphorylation than soluble receptors. Furthermore, the results indicate that prolactin substantially reduces the kinase activity of both soluble and insoluble receptors. However, although the tyrosine phosphorylation of the receptor was reduced by prolactin, the Triton X-100 solubility of the receptor was unaffected. Thus, altered mass of receptor associated with the cytoskeleton does not explain the prolactin inhibition of EGF receptor function. However, these results do not address the possibility that a qualitative difference in cytoskeletal interactions exists in prolactin-treated cells.

Other studies (23–25) have reported that prolactin appears to decrease EGF receptor signaling in NMuMG cells, although the mechanism of this phenomenon is unclear. The major effect of prolactin on EGF receptor observed in this study was a substantial decrease in receptor phosphorylation, which may indicate a change in EGF-induced receptor auto-kinase activity. This reduction was evident in both soluble and cytoskeletally associated receptors.

The mechanism by which prolactin might desensitize the EGF receptor remains unclear. The EGF receptor is known to be phosphorylated by a variety of kinases, including protein kinase C (18, 36–38), and at least some of these phosphorylation events appear to regulate receptor function. Although the mechanisms of prolactin action are poorly understood, activation of protein kinase C is one proposed action (39–42). Also, although the prolactin receptor has been cloned and does not contain any consensus kinase-like sequences (43), the receptor does associate with several kinases, including JAK2, $p59^{Fyn}$, a member of the *src* family, and *raf* (44–47). Thus, the potential exists for prolactin to significantly modify EGF signaling pathways at a number of levels. The present data suggests that prolactin modification of EGF signaling occurs early in the signaling process.

Interestingly, uterine fibroblasts treated with prolactin show a similar decrease in activity of a growth factor extracted from uterine tumors (48). The exact identity of the growth factor is unclear, but it does not appear to be EGF. Prolactin does, however, appear to inhibit growth factor–induced phosphatidylinositol kinase activity (49). In addition, a similar decrease in EGF-stimulated mitogenesis in preadipocytes has been reported for growth hormone (50), although the magnitude of the decline was less than that reported for prolactin in mammary epithelium (23). In addition, the growth hormone antagonism of EGF action appears to occur by a different mechanism that does not involve disruption in receptor function, as was seen in our studies.

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