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Altered Glucocorticoid Binding and Action in Response to Epidermal Growth Factor in HBL100 Cells

Kanury V. S. Rao, Robert E. Williams, and C. Fred Fox

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ABSTRACT

Incubation of adherent human breast epithelial HBL100 cells with epidermal growth factor (EGF) decreased [3H]-dexamethasone binding by 35% with no effect on affinity. Maximal inhibition was obtained at 3 nM EGF and the 50% effective dose was 0.2 nM EGF. Decreased dexamethasone binding induced by 3 nM EGF was maximal by 5 min of treatment and, in the continuous presence of EGF, persisted at a constant level over 4 days. The action of EGF was antagonized by 12-O-tetradecanoylphorbol-13-acetate, which did not inhibit dexamethasone binding significantly, and by concanavalin A. In homogenates of EGF-treated cells, decreased dexamethasone binding was observed only in the cytosolic fraction. Saturation dexamethasone binding inhibited the growth rate of HBL100 cells by approximately 50%, but concurrent treatment with EGF overcame this inhibition. The effect of EGF on dexamethasone-inhibited cell growth also was antagonized by 12-O-tetradecanoylphorbol-13-acetate.

INTRODUCTION

Glucocorticoids elicit an array of physiological responses in target tissue and cultured cells. These effects include modulation of gene expression and regulation of cell growth (for a review see Refs. 1 and 2). The first step in glucocorticoid hormone action is a reversible, noncovalent, high affinity binding to its intracellular receptor. Subsequent to this, the receptor undergoes "activation," in which it acquires the capacity to interact with chromatin (1).

There is evidence for cross-talks between steroid hormones and growth factors, presumably via action of their respective receptors. Progestins have been reported to stimulate [125I]-EGF (3) and [125I]-insulin (4) binding in cultured human breast cancer (T47D) cells. Incubation of adherent cells with progestins for 10 h resulted in a 2-fold increase in EGF binding with no change in affinity for this binding (3). Glucocorticoids have also been reported to enhance EGF binding in G1-S phase synchronized HeLa S3 cells (5) and in quiescent human diploid foreskin fibroblasts (6). Dexamethasone-treated human fibroblasts (HF cells) exhibited a 50 to 100% increased ability to bind physiological concentrations of [125I]-EGF, with maximal enhancement after 24 h of dexamethasone treatment (6). This increase was specific for glucocorticoids and required protein synthesis. Dexamethasone treatment of HF cells also enhanced the mitogenic response of these cells to EGF by 50% (6). Increased insulin binding was observed when Swiss mouse 3T3-C2 cells were treated with glucocorticoids (7). Maximal stimulation was obtained 12 h after glucocorticoid administration (3-5-fold). Based on heavy isotope density shift experiments, it was suggested that this process involved decreased receptor degradation rather than fresh insulin receptor synthesis (7). Glucocorticoids have also been reported to either potentiate (8) or antagonize (9) insulin-stimulated macromolecular synthesis in different breast carcinoma cell lines and to inhibit growth of breast (9, 10), prostate (11), and smooth muscle (12) tumor cell lines. Attenuation of that growth inhibition has been observed upon growth factor addition (11-13).

Our interest in steroid receptors is derived from tests of these substrates for the kinase action of EGF receptor in cultured cells. Previous studies from our laboratory have shown that the purified chick oviduct progesterone receptor is a high affinity substrate for tyrosine phosphorylation by purified EGF (14, 15) and insulin (15) receptor kinases. More recently, we demonstrated that EGF treatment of 32P, steady state-labeled human breast epithelial HBL100 cells resulted in enhanced phosphorylation of subsequently immunoprecipitated glucocorticoid receptors (16). Increased phosphorylation in response to EGF was detected on both serine and tyrosine residues. To test for possible biological relevance of EGF-stimulated glucocorticoid receptor phosphorylation, we examined the effects of EGF on glucocorticoid binding and action in HBL100 cells, a nontumorigenic epithelial cell line derived from human milk (17). HBL100 cells are rich in glucocorticoid receptors but exhibit essentially no binding capacity for other steroids (18). In this report we describe studies showing that EGF decreases glucocorticoid binding and antagonizes its action in these cells.

MATERIALS AND METHODS

Materials. [1,2,4,6,7-3H]Dexamethasone (70 Ci/mmol) was obtained from Amersham Corporation (Arlington Heights, IL). Unlabeled dexamethasone, TPA, leupeptin, dithiothreitol, EDTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Company (St. Louis, MO). Unless otherwise stated, all other chemicals were of reagent grade.

Cells and Cell Culture. HBL100 cells were obtained from W. L. McGuire (University of Texas Health Science Center, San Antonio, TX). The cells were maintained routinely in 55-cm² polystyrene Petri plates in McCoy's 5A (modified) culture medium (GIBCO, Chagrin Falls, OH) supplemented with l-glutamine (2 mM), 10% fetal bovine serum (GIBCO), and 10 μg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The cells were grown to near confluency and passed once a week with a change in the culture medium on the third day after passage.

For adherent cell binding studies, unless otherwise stated, cells were plated in 24-well polystyrene dishes (Corning Glass Works, Corning, NY). The medium was removed from the wells 24 h later and replaced with fresh medium containing 5% fetal bovine serum that had been stripped of steroids by treatment with dextran-coated charcoal (18). The cells were grown 4 days longer to about 0.7 × 10⁶ cells/cm² (60% confluency).

In binding experiments involving supernatant and particulate fractions of cell homogenates, HBL100 cells were grown in glass roller bottles. The roller bottles were gassed with CO₂ and the cells were cultured to approximately 80% confluence with a change in medium on the third day of culture.

Preparation of Cell Suspensions. HBL100 cell suspensions were obtained from cells grown in roller bottles. The cells were washed twice with 50 ml/roller bottle of Tris-buffered saline (150 mM NaCl and 10 mM Tris-Cl, pH 7.4). The cells were then incubated in 15 ml/roller...
bottle with Tris-EDTA [25 mm Tris-HCl, 1 mm NaCl, and 1 mm Na₂HPO₄ (pH 7.4 at 37°C)] at 37°C for 15 min to detach the cells. During this incubation period the roller bottles were rolled continuously at 4 rpm.

Preparation of High Speed Cytosol and Particulate Fractions following Homogenization. Suspended cells were collected by a 4000 × g centrifugation and washed twice with 5 cell pellet volumes of homogenization buffer [10 mm Tris-HCl, 1 mm disodium EDTA, 5 mm ethylene glycol bis(β-aminoethyl)ether)-N,N',N''-tetraacetic acid, 2 mm phenylmethylsulfonyl fluoride, 2 mm dithiothreitol, 40 μg/ml leupeptin, 10 mm sodium molybdate, and 10% (v/v) glycerol (pH 7.4)]. Cells were then homogenized in 2 pellet volumes of homogenization buffer using 50 strokes of a Dounce homogenizer in an ice bath. The cell homogenate was centrifuged at 40,000 × g and the supernatant fraction was collected. The particulate fraction was washed with 1 volume of the homogenization buffer and centrifuged at 40,000 × g. The supernatant fractions were combined and centrifuged at 6 × 10⁴ × g to obtain the high speed cytosol. The 40,000 × g pellet was suspended in 3 volumes of homogenization buffer to determine dexamethasone binding to the particulate-associated receptors.

Extraction of Particulate Fraction-associated Glucocorticoid Receptors at High Ionic Strength. Washed pellets were suspended in 2 volumes of homogenization buffer containing 0.5 M KCl. The suspension was incubated with gentle shaking on a rotating platform at 4°C for 60 min and then centrifuged at 40,000 × g. The supernatant fraction containing solubilized particulate-associated receptors was used for dexamethasone-binding experiments.

Dexamethasone Binding to Receptors Present in the Cytosol and Salt-extracted Particulate Fractions. Aliquots of 50 μl from the high speed cytosol or the particulate extract were incubated at 4°C for 16 h with 50 μM of homogenization buffer containing 0.2 to 100 nM [³H]dexamethasone. Three independent determinations were performed at each dexamethasone concentration and nonspecific binding was that occurring in the presence of 10 μM unlabeled dexamethasone. At the end of the incubations, bound radioactivity was determined by the dextrancoated charcoal assay (19). One part of dextran-coated charcoal suspension was added per sample and, after mixing by vortex action, the samples were incubated at 0°C for 5 min and centrifuged at 47,000 × g. The supernatant fraction was harvested using 50 strokes of a Dounce homogenizer in an ice bath. The cell homogenate was centrifuged at 40,000 × g and the supernatant fraction was collected. The particulate fraction was washed with 1 volume of the homogenization buffer and centrifuged at 40,000 × g. The supernatant fractions were combined and centrifuged at 6 × 10⁴ × g to obtain the high speed cytosol. The 40,000 × g pellet was suspended in 3 volumes of homogenization buffer to determine dexamethasone-binding experiments.

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RESULTS

Dexamethasone Binding in HBL100 Cells. Adherent HBL100 cells were incubated at 37°C with 0.25 to 75 nM [³H]dexamethasone to achieve equilibrium binding ("Materials and Methods"). Scatchard analysis of the data using the LIGAND program of Munson and Rodbard (20) revealed a single class of high affinity binding sites (Kᵣ 2 HM) with about 1 x 10⁹ sites/cell (not shown, but see Fig. 1).

In the course of our studies on progesterone receptor distribution in cultured breast carcinoma (T47D) cells we found that progesterin binding to adherent T47D cells revealed two classes of binding affinity (21). These two classes of binding sites were resolved by cell homogenization and subcellular fractionation to yield a soluble high affinity component and a particulate-associated low affinity component. While [³H]dexamethasone binding to HBL100 cells revealed only a single class of high affinity binding sites, a minor population of low affinity ligand binding sites might have escaped detection. We therefore examined dexamethasone binding in the resolved cytosolic, particulate, and salt-extracted particulate fractions from HBL100 cells ("Materials and Methods"). Both the cytosolic and particulate fractions displayed a single class of dexamethasone-binding sites with identical affinities (Table 1). The major portion of ligand binding activity was associated with the cytosolic fraction; about 20% was retained in the pellet fraction. While there was no evidence for a second, lower affinity component, high ionic strength extraction of the particulate fraction increased ligand binding activity by 2-fold with no change in affinity (Table 1).

In a separate experiment, no enhanced ligand binding activity

*J. C. Sarup, K. V. S. Rao, R. E. Williams, and C. F. Fox, submitted for publication.*
EGF ALTERATION OF GLUCOCORTICOID BINDING AND ACTION

Table 1  Scatchard analysis of specific dexamethasone binding to the cytosolic, particulate, and salt-extracted particulate fractions from HBL100 cell homogenates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dexamethasone bound (fmol/2 x 10^4 cells)</th>
<th>Kd (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>2560 ± 333</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Particulate fraction</td>
<td>656 ± 92</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Salt extract of particulate fraction</td>
<td>1320 ± 264</td>
<td>1.6 ± 0.3</td>
</tr>
</tbody>
</table>

Table 2  Influence of TPA on the EGF-induced decrease in dexamethasone binding

<table>
<thead>
<tr>
<th>Additions</th>
<th>Dexamethasone bound (10^-3 x sites/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>99 ± 5</td>
</tr>
<tr>
<td>EGF</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>TPA</td>
<td>84 ± 7</td>
</tr>
<tr>
<td>EGF + TPA</td>
<td>84 ± 11</td>
</tr>
</tbody>
</table>

Table 3  Influence of concanavalin A on the EGF-mediated decrease in specific dexamethasone binding

<table>
<thead>
<tr>
<th>Additions</th>
<th>Dexamethasone bound (10^-3 x sites/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>96 ± 9</td>
</tr>
<tr>
<td>EGF</td>
<td>62 ± 10</td>
</tr>
<tr>
<td>Concanaainin A</td>
<td>92 ± 10</td>
</tr>
<tr>
<td>EGF + concanavalin A</td>
<td>108 ± 8</td>
</tr>
</tbody>
</table>

was observed when the cytosolic fraction was treated at high ionic strength (not shown). High ionic strength is reported to inactivate ligand binding activity of the rat liver glucocorticoid receptor, but this inactivation can be overcome by including molybdate in the extraction buffer (22, as was the case in our experiment. Thus a significant population of dexamethasone-binding sites in the particulate fraction of HBL100 cells is not accessible to ligand. The increase in binding capacity noted for the salt extract of particulate fraction could be due either to hitherto inaccessible receptors becoming accessible on solubilization or by salt-induced activation of a cryptic population of particulate-associated receptors. This behavior does not appear to be a general characteristic of glucocorticoid receptors; extraction of receptors from the particulate fraction of cultured rat hepatoma cells at high ionic strength did not enhance glucocorticoid binding (23).

Effect of EGF on Dexamethasone Binding. Adherent HBL100 cells grown to about 60% confluency were incubated with 0.01 to 20 nM 125I-EGF at 4°C for 4 h to characterize EGF binding. Scatchard analysis of the resulting data revealed 41,000 ± 4,000 (SE) EGF-binding sites per cell. Over 95% were low affinity sites (Kd 8 nm), and the remainder were high affinity sites (Kd 0.1 nm) (not shown).

Incubation of adherent cells with 10 nM [3H]dexamethasone and 0 to 5 nM EGF caused a dose-dependent decrease in dexamethasone binding (Fig. 2). Maximal inhibition was achieved at 3 nM EGF with a 50% effective dose of about 0.2 nM EGF. This suggests that the high affinity component of EGF receptors (Kd 0.1 nm) may facilitate the process resulting in inhibition.

To test this, experiments were conducted in which the tumor promoter TPA was also added to the incubation medium along with EGF. TPA is known to inhibit formation of the high affinity EGF binding component (24, 25), apparently by stimulating protein kinase C-mediated phosphorylation of EGF receptor on Thr 654 (26). TPA inhibited dexamethasone binding slightly (~15%), but simultaneous incubation with EGF and TPA produced no additional inhibition (Table 2). On the other hand, EGF induced a 36% decrease in dexamethasone binding in this experiment in the absence of TPA.

Wakshull and Wharton (27) have reported that concanavalin A prevents endocytosis of the EGF-EGF receptor complex by mouse fibroblasts (27). The effect of concanavalin A on EGF-induced inhibition of dexamethasone binding was studied under conditions used in that study. HBL100 cells were first incubated with 3 nM EGF at 4°C for 4 h and then with 200 μg/ml concanavalin A for 20 min at 4°C. The cells were then warmed to 37°C and incubated with 10 nM [3H]dexamethasone for 60 min. EGF had no effect on dexamethasone binding in concanavalin A-treated cells but inhibited dexamethasone binding by 36% in cells not treated with concanavalin A (Table 3). Concanavalin A alone had no significant effect on dexamethasone binding. In agreement with the findings of Wakshull and Wharton (27), 125I-EGF uptake and degradation by HBL100 cells were inhibited by greater than 50% under the conditions of Table 3 (not shown).

Failure of EGF to Alter the Binding Affinity of Occupied Receptors. To determine if EGF inhibits dexamethasone binding by altering glucocorticoid receptor affinity as opposed to inactivating ligand binding activity, adherent cells were incubated at 37°C to achieve equilibrium binding with 0.3 to 25 nM [3H]dexamethasone in either the presence or the absence of 3 nM EGF. Scatchard analysis revealed 33% lowered dexamethasone binding in response to EGF but no significant change in dexamethasone binding affinity (Fig. 1). In four separate experiments, the Ka for dexamethasone binding was 2.3 ± 1.4 for untreated cells and 2.1 ± 1.2 for EGF treated cells, and the average decrease in receptor number due to EGF was 36 ± 2%.

Inhibitory Effect of EGF on Dexamethasone Binding is Detected only in the Cytosolic Fraction of Cell Homogenates. To
determine if EGF decreased dexamethasone binding in all subcellular fractions, dexamethasone binding was studied in the cytosolic, particulate, and salt extract of the particulate fractions of EGF-treated and untreated cells (Table 4). Significantly decreased dexamethasone binding was observed only in the cytosolic fraction in response to EGF.

Rapid Initiation and Persistence of Decreased Dexamethasone Binding by EGF. The inhibitory effect of EGF on [3H]dexamethasone binding was studied as a function of time at 37°C. Cells were incubated throughout the course of the experiment with 10 nM [3H]dexamethasone in the presence or absence of 3 nM EGF. The ratio of dexamethasone binding in untreated versus EGF treated cells is presented in Fig. 3. Inhibition of dexamethasone binding was evident as early as 2 min of EGF treatment and was maximal within 5 min (Fig. 3A). This decrease in dexamethasone binding persisted over 4 days (Fig. 3B).

Table 4  EGF decreases specific dexamethasone binding in the cytosolic fraction of cell homogenates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dexamethasone bound (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Cytosol</td>
<td>580 ± 81</td>
</tr>
<tr>
<td>Particulate fraction</td>
<td>114 ± 46</td>
</tr>
<tr>
<td>Salt extract of particulate fraction</td>
<td>500 ± 75</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of EGF on specific dexamethasone binding as a function of time. A, effect over 60 min of EGF treatment. Cells in 24-well dishes were incubated at 37°C for the times shown in 0.5 ml medium containing 10 nM [3H]dexamethasone either with or without 3 nM EGF. To determine nonspecific binding 1 μM nonradioactive dexamethasone binding was also added to some wells. Cells were processed at the times indicated to determine specifically bound dexamethasone ("Materials and Methods"). B, effect of long term incubation with EGF. HBL100 cells were plated in 24-well dishes at a density of 3 × 10⁴ cells/cm². At 24 h (day 0) the medium was replaced with fresh medium containing [3H]dexamethasone either with or without EGF as described for A. The cells were incubated at 37°C for the times shown with a change in medium at day 2 (arrow). At the times indicated cells were processed to determine specifically bound dexamethasone ("Materials and Methods"). Cell counts were determined with a hemocytometer and dexamethasone bound was normalized for cell number.

Antagonism of Inhibitory Action of Dexamethasone on Growth of HBL100 Cells by EGF. Dexamethasone inhibits thymidine incorporation in several human breast cancer cell lines, and the extent of this inhibition correlates well with their glucocorticoid-binding capacity. For example, 100 nM dexamethasone inhibited the growth of MCF-7 cells by over 50% (10). Glucocorticoids have been shown to inhibit proliferation of several cultured cell lines by arresting them either in the Go or the G1 phase of the cell cycle. Growth factor addition can overcome this inhibition (11-13). Attenuation of glucocorticoid-induced inhibition of cell growth has been observed in prostate-derived R3327H-G8-A1 tumor cells with EGF (11) and in a smooth muscle tumor cell line, DD7, MF-2, with platelet-derived growth factor (12, 13).

Dexamethasone also inhibited the growth of HBL100 cells (Fig. 4). Maximal inhibition was achieved at 100 nM dexamethasone with a 50% effective dose of less than 5 nM (Fig. 4), in close agreement with the observed dissociation constant for dexamethasone binding (2 nM). Dexamethasone-inhibited inhibition of HBL100 cell proliferation was blocked by 3 nM EGF (Fig. 5), although EGF alone had no significant stimulatory action on HBL100 cell growth (Fig. 5). Our results are thus consistent with observations of others (11-13).

Table 5  Insensitivity of occupied glucocorticoid receptors to short term EGF action

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Dexamethasone preincubation</th>
<th>Control</th>
<th>+EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−</td>
<td>112 ± 13</td>
<td>69 ± 5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>87 ± 13</td>
<td>85 ± 15</td>
</tr>
<tr>
<td>2</td>
<td>−</td>
<td>91 ± 3</td>
<td>70 ± 5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>76 ± 6</td>
<td>81 ± 11</td>
</tr>
</tbody>
</table>
while the magnitude of the effect of EGF on dexamethasone binding is not substantial, nevertheless this interaction could be important in regulating at least the short term effects of glucocorticoids. Amplified responses to small changes in stimuli are common regulatory events in biological systems (44) and the condition has been referred to as a "threshold" phenomenon.

The mechanism by which EGF acts to attenuate HBL100 cell growth inhibition occurring in response to dexamethasone is not obvious from our study. It is unlikely that EGF acts to uncouple glucocorticoid growth-inhibitory action by decreasing glucocorticoid binding. This would require that EGF (a) act to decrease glucocorticoid binding nearly completely in a subpopulation of cells which is unique in its growth inhibition sensitivity to glucocorticoids or (b) induce preferential phosphorylation of a unique subpopulation of glucocorticoid receptors which are responsible for the growth-inhibitory action. While we consider these possibilities extremely unlikely, we are pre-
paring to test for differences in responses to glucocorticoids or EGF in subclones of the HBL100 cell line.

The action of EGF to attenuate glucocorticoid-induced inhibition of HBL100 cell growth reinforces the observations of Smith et al. (11) and Syms et al. (12) with cultured R3327H-G8-A1 and DDT,MF-2 cells. Glucocorticoid-induced growth inhibition of R3327H-G8-A1 and DDT,MF-2 cell growth can be reversed nearly completely by EGF in the former case and by platelet-derived growth factor in the latter (11, 12). This indicates that growth factors can act through pathways independent of and dominant over those through which glucocorticoids act to inhibit cell growth. This underscores the value of determining EGF receptor levels (and responsiveness) and possible autocrine or paracrine EGF or transforming growth factor-α secretion (45) in tumors in assessing the prognosis of glucocorticoids as tumor growth inhibitors.

REFERENCES