Regulation of Epidermal Growth Factor Receptor by Estrogen*

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Administration of 17β-estradiol (E₂) to immature female rats produces a 3-fold increase in 125I-epidermal growth factor (EGF) binding to uterine membranes with no change in the affinity of membrane receptors for EGF. E₂ treatment also increases the EGF receptor visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after affinity labeling of uterine membranes and the EGF-stimulated receptor autophosphorylation activity. In addition, E₂ administration stimulates EGF-dependent tyrosine kinase activity in an assay system using exogenous angiotensin II as substrate. Following hormone treatment, EGF receptor levels increase between 6 and 12 h, remain elevated at 18 h, and decline between 24 and 36 h. This stimulation of EGF receptor levels by E₂ is specific, since the non-estrogenic hormones progesterone, dexamethasone, and dihydrotestosterone fail to elevate receptor levels. E₂-stimulated increases in EGF receptor levels are also blocked by cycloheximide and actinomycin D, suggesting that the observed effect represents de novo synthesis of the EGF receptor and may be mediated by a transcriptional mechanism. These results demonstrate that estrogen can regulate acutely the levels of EGF receptor in vivo and raise the possibility that events coupled to this receptor may play a role in estrogen-stimulated growth.

Epidermal growth factor is a polypeptide originally isolated from the submaxillary gland (1). Studies since that time have demonstrated that EGF has numerous effects, including the ability that events coupled to this receptor may play a role in physiological processes. EGF binding, affinity labeling, and EGF-stimulated kinase activity are regulated by estrogen stimulation. The results illustrated in Fig. 5 only, the steroids were administered in 0.1 ml of propylene glycol containing 2% acetone. This vehicle was used because of the decreased solubility of the non-estrogenic steroids used in aqueous solutions. For the experiments illustrated in Fig. 5 only, the same dose was then administered 6 h later; animals were killed at the indicated times. For the experiments illustrated in Fig. 5 only, the steroids were administered in 0.1 ml of propylene glycol containing 2% acetone. This vehicle was used because of the decreased solubility of the non-estrogenic steroids used in aqueous solutions. For the experiments illustrated in Fig. 5 only, the same dose was then administered 6 h later; animals were killed 12 h after the E₂ injection.

Membrane Preparation and Assays—All steps in the preparation of membranes were carried out at 0-4 °C. Uteri were pooled, minced, and homogenized with two 15-s bursts in a Polytron P-10 homogenizer in 15 volumes of homogenization buffer (20 mM PIPES, 0.25 mM sucrose, 1 mM EGTA, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.4). The homogenates were centrifuged at 800 X g for 15 min, and the supernatants were transferred to separate centrifuge tubes. The pellets were rehomogenized as above in the same volume of buffer and centrifuged at 800 X g. The 800 X g supernatants were combined and centrifuged at 105,000 X g for 60 min. The resulting pellets were suspended in homogenization buffer and centrifuged at 105,000 X g. The final pellets were resuspended in 20 mM PIPES, 0.15 M NaCl, pH 7.4 (2 ml/g of starting material), quickly frozen, and stored at -70 °C until use. For the estimation of protein content, an aliquot of the suspended membranes was diluted with an equal volume of 2 N NaOH, heated at 90 °C for 15 min, and then diluted with water prior to measurement of protein by the method of Lowry et al. (15). 5'-Nucleotide activity in diluted membrane preparations was measured as described by Aronson and Touster (15), except that the substrate concentration in the assay was doubled.

EGF binding, affinity labeling, and EGF-stimulated kinase activity were carried out as previously described (11, 13).

Assay of EGF-stimulated Kinase Activity with Exogenous Angiotensin II as Substrate—Frozen membranes prepared from 6-10 animals per group (see above) were thawed and extracted for 30 min at 0 °C with 1% Triton X-100 and 10% glycerol and centrifuged at 105,000 X g.

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Materials

Animals—Immature Sprague-Dawley female rats (20-22 days of age, weight of 35-40 g) were obtained from Harlan-Sprague-Dawley (Houston) and ovariectomized 4-7 days prior to use. Animals received standard rat chow and water ad libitum. At the indicated times prior to death, animals received a subcutaneous injection of 17β-estradiol (40 μg/kg) administered in 0.5 ml of 20% saline, 5% ethanol; controls received the vehicle alone. Animals were killed by decapitation at the indicated times. For the experiments illustrated in Fig. 5 only, the steroids were administered in 0.1 ml of propylene glycol containing 2% acetone. This vehicle was used because of the decreased solubility of the non-estrogenic steroids used in aqueous solutions. For the indicated experiments, cycloheximide (4 mg/kg) and actinomycin D (8 mg/kg) were administered by intraperitoneal injection in a volume of 0.5 ml of saline. Actinomycin D was administered once at the same time as E₂, and the animals were killed 12 h later. Cycloheximide (4 mg/kg) was initially administered at the same time as E₂, and the dose was then administered 6 h later; animals were killed 12 h after the E₂ injection.

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for 60 min. The supernatant (0.5 ml containing 1-1.5 mg of protein) was mixed with an equal volume of packed wheat germ agglutinin-Sepharose (Pharmacia) and rotated end-over-end for 2 h at 0-4 °C. This mixture was poured into a small column, washed with 5 ml of 40 mM imidazole, pH 7.2, containing 0.5 M NaCl, 10% glycerol, and 0.05% Triton X-100. The column was eluted with 2 ml of the same buffer containing 0.3 M N-acetylglucosamine (16). The eluant was then concentrated by filtration using Centricon-30 filters (Amicon).

EGF-stimulated kinase activity was performed in a volume of 25 μl containing the final concentrations of the following: 20 mM imidazole, pH 7.2, 85% bovine serum albumin, 0.05% Triton X-100, 20 mM MgCl2, 10 mM p-nitrophenyl phosphate, 100 μM sodium vanadate, 2 mM dithiothreitol, and 100 μM [γ32P]ATP. Assays contained 2 mM angiotensin II (Sigma) as the substrate and were linear for 10 min at 25 °C. The source of kinase activity was 2-5 μg of the wheat germ agglutinin-purified material (see above) mixed with 15-20 μg of bovine serum albumin. This kinase preparation was preincubated with or without EGF (2 μg/ml) for 15 min at 25 °C and then added to initiate the reaction. Reactions were carried out for 10 min at 25 °C and stopped by the addition of trichloroacetic acid (to 5%). The source of kinase activity was 2-5 μg of the wheat germ agglutinin-purified material (see above) mixed with 15-20 μg of bovine serum albumin. This kinase preparation was preincubated with or without EGF (2 μg/ml) for 15 min at 25 °C and then added to initiate the reaction. After cooling on ice (10 min), the samples were centrifuged at 12,000 × g for 20 min, and incorporation was determined by spotting 20-μl aliquots of supernatant on phosphocellulose paper (17). Blank values (determined by adding the angiotensin II after the trichloroacetic acid) were subtracted for each assay, and EGF-stimulated activity was then determined for each preparation by subtracting the activity in the absence of the preincubation with EGF.

RESULTS

The results in Fig. 1 illustrate the effect of a single injection of estrogen on EGF binding in uterine membranes. Fig. 1 depicts results from three separate experiments performed on different days. Each bar represents the mean of triplicate measurements on a membrane fraction obtained by pooling uteri from 10 animals. Values are based on EGF binding/unit of membrane protein and represent specific binding assessed by incubations with 100-fold excess unlabeled EGF; nonspecific binding was typically 10-15% total binding. Similar results are obtained if EGF binding is expressed on the basis of 5′-nucleotidase activity in the membrane preparations.

The results illustrate that there is a sharp rise in EGF binding between 6 and 12 h after estrogen treatment; EGF binding remains elevated at 18 h and subsequently begins declining toward control levels. These results were obtained using 5 nM [125I]-EGF which, on the basis of previous work (11), is a saturating concentration of growth factor.

To further ensure that these observed changes represent an increase in the number of binding sites per se, Scatchard analysis (18) was performed with membrane preparations from control animals and animals receiving hormone treatment 18 h prior to death. These results (Fig. 2) illustrate that estradiol administration leads to an increase in the number of uterine EGF-binding sites without a major change in affinity. Kd values are 0.14 and 0.21 nM for control and estrogen-treated samples, respectively; Bmax values are 22 (control) and 66 (estrogen-treated) fmol of EGF bound per mg of membrane protein.

In order to further substantiate this increase in EGF-binding sites, we performed affinity labeling studies and measurements of EGF-stimulated kinase activity. These results are shown in Figs. 3 and 4, respectively. For affinity labeling studies, membranes were incubated with [125I]-EGF, briefly washed by centrifugation to remove unbound [125I]-EGF, and cross-linked with disuccinimidyl suberate. The cross-linked membranes were then extracted and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis; labeled material was observed by autoradiography.

As seen in Fig. 3, major (Mr = 170,000) and minor (Mr = 150,000) species of cross-linked [125I]-EGF are present, and hormone treatment produces a substantial increase in both species. It is important to note that binding in both samples is abolished by the presence of excess unlabeled EGF in the initial incubation.

EGF also stimulates the phosphorylation of a species of similar Mr, in extracts from control and hormone-treated membranes (Fig. 4); it is clearly seen that hormone treatment
Regulation of EGF Receptor by Estrogen

UTERINE EGF - R
AFFINITY LABELLING

TB NB - 170 -

TIME AFTER ESTRADIOL
( Hours )

FIG. 3. Affinity labeling of EGF receptor in uterine membranes of control and \( E_2 \)-treated animals. Groups of animals (10 per group) were treated with \( E_2 \) (40 \( \mu \)g/kg) or the vehicle alone 18 h prior to death. Uteri in each group were pooled, membranes were prepared, and affinity labeling was performed as described under "Experimental Procedures." TB, membranes were incubated with 3 \( nM \) \(^{125}\)I-EGF prior to cross-linking with disuccinimidyl suberate; NB, membranes were incubated with 3 \( nM \) \(^{125}\)I-EGF plus a 100-fold excess of unlabeled EGF prior to cross-linking. EGF-R, EGF receptor.

also leads to a substantial increase in the level of this species, which represents receptor autophosphorylation. We have previously shown (11) that this autophosphorylation occurs largely at tyrosine residues.

Since the EGF receptor may function by phosphorylation of cellular substrates, tyrosine kinase activity was also measured with angiotensin II as an exogenous substrate (19). For these studies, uterine membranes were prepared from animals receiving estrogen treatment 18 h prior to death and from vehicle-treated control animals. The EGF receptor was solubilized by detergent treatment and partially purified by passage over a wheat germ agglutinin column. The EGF-stimulated kinase activity was then determined by adding angiotensin II as an exogenous substrate. The results of these studies are illustrated in Table I.

As seen in Table I, estrogen treatment causes a 2-3-fold increase in activity. This is in agreement with results from the previous binding (Figs. 1 and 2), affinity labeling (Fig. 3), and autophosphorylation data (Fig. 4).

In order to examine the hormonal specificity of the estrogen-induced increase in EGF receptor level, we tested the effects of other non-estrogenic steroids. As seen in Fig. 5, neither dexamethasone (600 \( \mu \)g/kg), nor dihydrotestosterone (400 \( \mu \)g/kg), nor progesterone (4 \( mg/kg \)) treatment produced the increase in EGF receptor content caused by estradiol (40 \( \mu \)g/kg) administration.

As an initial approach to elucidating the mechanism by

TABLE I
Stimulation of uterine EGF-stimulated kinase activity by estradiol

For each experiment, groups of 8-10 rats were treated with estradiol (40 \( \mu \)g/kg) or the vehicle alone (control) 18 h prior to death. Uteri in each group were pooled, and membranes were isolated. Membrane proteins were extracted and purified, and EGF-stimulated kinase activity was measured with angiotensin II as substrate.

| Experiment | Treatment | Kinase activity* | \( E_2 / \text{control} \) |
|------------|-----------|------------------|----------------------|
| No. 1      | Control   | 9.9              | 3.1                  |
|            | \( E_2 \)  | 30.3             |                      |
| No. 2      | Control   | 9.2              | 2.2                  |
|            | \( E_2 \)  | 20.3             |                      |
| No. 3      | Control   | 7.5              | 2.5                  |
|            | \( E_2 \)  | 18.4             |                      |

*Activity is given as pmol of \(^{32}\)P incorporated per min/mg of protein.
which estrogen increases uterine EGF receptor levels, we tested the effects of cycloheximide and actinomycin D on the induction process. For these studies, animals were treated with estradiol and inhibitors 12 h prior to death, the earliest time we observed a consistent increase in EGF receptor levels (Fig. 1). This time was chosen (rather than the 18-h treatment used in most previous experiments) to minimize the in vivo exposure of the test animals to these toxic inhibitors. The results of these studies are illustrated in Fig. 6. Neither inhibitor had any effect on the levels of EGF receptor in vehicle-treated control animals. Cycloheximide completely abolished the increase in uterine EGF levels following estrogen administration, and actinomycin D substantially decreased the effect of the hormone.

**DISCUSSION**

In a previous study, we demonstrated that uterine membranes contain EGF receptors with properties similar to EGF receptors from other sources (11). These include a high affinity for EGF (K_d = 0.4 nM), specificity, saturability, molecular weight of 170,000-150,000, and an EGF-stimulated kinase activity which phosphorylates both tyrosine and serine residues on the receptor itself. Based on studies in other systems, it is likely that the minor species of M_r = 150,000 is a proteolytic fragment of the major M_r = 170,000 species (7, 9, 20). At present, we do not know the distribution of EGF receptors in the various uterine cell types in the rat.

In this study, we have shown that 17β-estradiol elevates uterine EGF receptor levels approximately 3-fold within 12 h. This increase in EGF receptor levels assessed by 125I-EGF binding (Figs. 1 and 2) is also accompanied by increases in EGF receptor levels visualized by affinity labeling (Fig. 3), EGF-stimulated receptor autophosphorylation (Fig. 4), and EGF-stimulated tyrosine kinase activity measured with an-
Uterine EGF receptor levels increase between 6 and 12 h after estrogen administration. This increase in receptor levels precedes increases in uterine DNA synthesis, which begin approximately 18 h after estrogen treatment and are maximal approximately 24 h after hormone administration (25–27). It is especially interesting to note that several studies have shown that events occurring between 6 and 12 h after hormone administration play a critical role in triggering true uterine growth and DNA synthesis (28–30). It is thus possible that this observed increase in EGF receptor levels plays a role in the regulation of uterine DNA synthesis. Studies in progress are aimed at investigating this hypothesis.

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REFERENCES

1. Cohen, S. (1962) J. Biol. Chem. 237, 1555–1562
2. Carpenter, G., and Cohen, S. (1979) Annu. Rev. Biochem. 48, 193–216
3. Hollenberg, M. D. (1979) Vitam. Horm. 37, 89–109
4. Carpenter, G., and Cohen, S. (1978) in Biochemical Actions of Hormones (Litwack, G., ed) Vol. 5, pp. 203–247, Academic Press, New York
5. O’Keefe, E., Hollenberg, M. D., and Cuatrecasas, P. (1974) Arch. Biochem. Biophys. 164, 518–526
6. Cohen, S., Carpenter, G., and King, L., Jr. (1980) J. Biol. Chem. 255, 4834–4842
7. Linsley, P. S., and Fox, C. F. (1980) J. Supramol. Struct. 14, 461–471
8. Buhrow, S. A., Cohen, S., and Staros, J. V. (1982) J. Biol. Chem. 257, 4019–4022
9. Cohen, S., Ushiro, H., Stoscheck, C., and Chinkers, M. (1982) J. Biol. Chem. 257, 1523–1531
10. Rubin, R. A., and Earp, H. S. (1983) J. Biol. Chem. 258, 5177–5182
11. Mukku, V. R., and Stancel, G. M. (1985) Endocrinology, in press
12. Gonzalez, F., Lakshmanan, J., Farr, A. L., and Randall, R. J. (1981) J. Biol. Chem. 256, 265–275
13. Aronson, N. N., Jr., and Touster, O. (1974) Methods Enzymol. 31, 90–102
14. Piko, L. J., Kuenzel, E. A., and Krebs, E. G. (1984) J. Biol. Chem. 259, 6543–6547
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