

# Cross Talk between Peptide Growth Factor and Estrogen Receptor Signaling Systems

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Epidermal growth factor reproduces many of the effects of estrogen on the murine female reproductive tract and may partially mediate estrogen-induced growth and differentiation. The mechanism by which the actions of estrogens and epidermal growth factor (EGF) converge is unknown. The studies described herein were performed to investigate the possibility that some of the actions of EGF may be mediated through the estrogen receptor. A specific estrogen receptor (ER) antagonist inhibited estrogenlike effects of EGF in the mouse uterus, specifically induction of DNA synthesis and phosphatidylinositol turnover. In addition, EGF elicited enhanced nuclear localization of uterine ER and formation of a unique nuclear form of ER that is present after estrogen treatment. These *in vivo* observations indicated that EGF may elicit some of its actions by activation of nuclear ER. Thus, the effect of peptide growth factors on activation of a consensus estrogen response element was assessed in Ishikawa human endometrial adenocarcinoma cells, which contain negligible ER levels, and in BG-1 human ovarian adenocarcinoma cells, which contain abundant ER. EGF and TGF $\alpha$  induced transcriptional activation of a consensus estrogen response element (ERE) in an ER-dependent manner in both cell types. In addition, insulinlike growth factor I (IGF-I) was as potent as 17 $\beta$ -estradiol in BG-1 cells. Synergism between growth factors and estrogen was observed in both cell types, although synergism was not observed between the different classes of growth factors [i.e., transforming growth factor  $\alpha$  (TGF $\alpha$ ) and IGF-I] in BG-1 cells. The most potent activator of ERE-dependent transcription was a protein kinase C activator (TPA), which acted synergistically with 17 $\beta$ -estradiol. A protein kinase C inhibitor abolished the effect of TPA but not that of 17 $\beta$ -estradiol, IGF-I, or TGF $\alpha$ . A protein kinase A activator elicited ER-dependent activation of transcription and did not synergize with estrogen or growth factors. In conclusion, some physiologic actions of peptide growth factors are dependent on ER. Indeed, growth factors are capable of eliciting ER-dependent activation of an ERE. Both the protein kinase A and protein kinase C pathways can elicit ER-dependent transcriptional activation; however, it is unlikely that these pathways mediate the effects of peptide growth factors on the ER in BG-1 cells. — *Environ Health Perspect* 103(Suppl 7):35–38 (1995)

Key words: growth factors, EGF, TGF, IGF, estrogen, estrogen receptor

## Introduction

It has been proposed that polypeptide growth factors may act as autocrine or paracrine mediators of biological actions of estrogen (1,2). The observations that estrogen induces mRNA and protein for peptide growth factors [epidermal growth factor (EGF), transforming growth factor  $\alpha$  (TGF $\alpha$ ), and insulinlike growth factor I (IGF-I)] (3–7) and their receptors (8–11) in rodent uterus are consistent with this hypothesis and implicate a role for the

EGF receptor-signaling pathway in steroid hormone regulation of uterine biology. Our laboratory has previously demonstrated a convergence between the actions of EGF and estradiol in the mouse uterus. EGF reproduced the stimulatory effects of estrogen on uterine DNA synthesis, lactoferrin gene expression, and phosphatidylinositol lipid metabolism in ovariectomized mice (12,13). Furthermore, administration of EGF-neutralizing antibodies before estradiol blocks estrogen-induced uterine epithelial cell proliferation, which suggests that the presence of EGF may be necessary for estrogen-induced responses (12).

Presently, the mechanisms by which the actions of estrogens and growth factors converge are unknown. These studies address the intriguing possibility that some biological effects of peptide growth factors, which are extracellular ligands, may be mediated through a nuclear steroid hormone receptor, namely, the estrogen receptor. Several reports in the literature support this hypothesis. First, two reports by Power et al. (14,15) demonstrated the existence of cross talk between a membrane-associated extracellular receptor system and nuclear steroid receptors. In these studies,

dopamine elicited steroid receptor-dependent activation of steroid response element-regulated transcription in transfected CV1 cells. In addition, the ability of antiestrogens to suppress induction of progesterone receptor levels and DNA synthesis by peptide growth factors in MCF-7 cells (16–18) suggests a role for the estrogen receptor in the actions of extracellular mitogens.

## Interaction of EGF with Uterine Estrogen Receptors *in Vivo*

The first set of experiments involved *in vivo* administration of EGF via slow-release, cholesterol-based pellets containing 1.25  $\mu$ g mouse EGF per quarter pellet implanted under the kidney capsule of adult ovariectomized mice anesthetized with methoxyflurane (13). The characterization of this procedure is detailed in Nelson et al. (12). Both the pellets and the serum of the mice were devoid of estrogen, and the mitogenic effect induced by EGF administration in the uterus was not affected by adrenalectomy or hypophysectomy. These pellets can also be placed in the peritoneal cavity without attenuation of effect (unpublished observations). Placebo

This paper was presented at the Symposium on Estrogens in the Environment, III: Global Health Implications held 9–11 January 1994 in Washington, DC. Manuscript received: March 15, 1995; manuscript accepted: April 4, 1995.

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Abbreviations used: EGF, epidermal growth factor; TGF $\alpha$ , transforming growth factor  $\alpha$ ; IGF-I, insulinlike growth factor I; ER, estrogen receptor; ERE, estrogen response element; CAT, chloramphenicol acetyltransferase; TPA, phorbol 12-myristate 13-acetate; DES, diethylstilbestrol; vitA2 ERE, vitellogenin A2 consensus/estrogen response element; PKC, protein kinase C; PKA, protein kinase A; 8-Br cAMP, 8-bromoadenosine-3'5'-cyclic monophosphate.

pellets were implanted in control animals. Mice receiving ip injections of diethylstilbestrol (DES) or saline were sham operated. Administration of EGF (1.25  $\mu\text{g}$ ) or DES (5  $\mu\text{g}/\text{kg}$ ) resulted in similar inductions of both DNA synthesis (10-fold at 16 hr posttreatment) and phosphatidylinositol turnover (2-fold) versus respective controls. DNA synthesis and phosphatidylinositol metabolism were assessed as described (13,19). The effects of both EGF and DES were inhibited by pretreatment with 1 mg/kg of the pure antiestrogen ICI 164,384 (provided by Alan Wakeling, ICI Pharmaceuticals, Mereside, United Kingdom) (20).

The ability of an ER antagonist to block the cellular actions of EGF in the whole animal prompted an investigation of the effects of EGF on biochemical characteristics of the estrogen receptor (ER) (13). Estrogen treatment rapidly enhances ER affinity for chromatin so that the ER is retained in the nuclear fraction after tissue homogenization. This phenomenon is quantitated by a  $^3\text{H}$ -estradiol-binding assay (21,22). Significant augmentation of nuclear ER levels was observed 1.5 hr after EGF pellet implantation or DES treatment. Enhanced nuclear retention of ER after EGF treatment was corroborated by immunodetection with H222 ER monoclonal antibody (a gift from Chris Nolan, Abbott Laboratories, Abbott Park, IL), which demonstrated that there was a larger quantity of protein in the ER-specific bands in the uterine nuclear fractions after EGF or DES treatment. The interaction of the ER with the estrogen response element (ERE) is a prerequisite for transcriptional activation. This property of ER found in uterine nuclear extracts from EGF-treated mice was compared to that found after DES treatment using a gel retardation assay (23). In this assay, nuclear proteins were salt extracted from uterine homogenates and concentrated by ammonium sulfate precipitation. The nuclear extracts were incubated in the presence or absence of H222 ER antibody overnight followed by incubation with a  $^{32}\text{P}$ -labeled vitellogenin A2 consensus ERE. The resulting complexes were separated on a 5.625% polyacrylamide gel. The band-shifting pattern observed after EGF treatment was very similar to that seen after DES administration. The two specific ER-ERE complexes present after EGF or DES treatment were supershifted by H222 antibody, which demonstrated that immunoreactive ER was present in these complexes. These results suggest that the nuclear ER from EGF-treated mice interacts with an ERE in a

manner similar to that observed after estrogen treatment. It has previously been demonstrated that estrogen treatment induces heterogenous nuclear ER forms of 65- and 66.5-kDa in uterine nuclear extracts (22) and that the 66.5-kDa form is hyperphosphorylated ER (24). Similar to the effect of estrogen, EGF treatment elicited the formation of the unique nuclear 66.5-kDa form of ER as assessed by immunodetection with H222 antibody. This form was not observed in the uterine cytosolic fractions from EGF- or DES-treated mice.

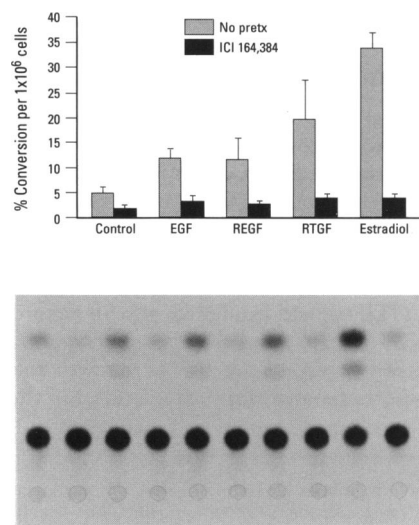
In summary, some estrogenlike effects of EGF in the uterus are dependent on the estrogen receptor. Furthermore, EGF elicits biochemical modifications of the uterine estrogen receptor that are similar to those of estrogen. Thus, cross talk may indeed exist between EGF and ER-signaling systems. This evidence suggests a role for the ER in mediating physiological actions of EGF in the reproductive tract in the absence of estradiol.

### Peptide Growth Factor Regulation of Estrogen Receptor-dependent Activation of an Estrogen Response Element

The following studies were performed to assess whether peptide growth factors could activate transcription from a consensus estrogen response element in an ER-dependent manner. To accomplish this goal, we first used Ishikawa human endometrial adenocarcinoma cells, which contain negligible levels of ER, in transfection experiments (25). Ishikawa cells were plated on 6-well plates and grown in DMEM/F-12 media containing 5% fetal bovine serum. At 50% confluency, the cells were transfected with 2  $\mu\text{g}$  of pCAT sv40 promoter reporter vector (Promega, Madison, WI) containing a consensus vitellogenin A2 ERE sequence (2  $\mu\text{g}/\text{well}$ ) and 0.15  $\mu\text{g}$  of pSG5 expression vector (Stratagene Cloning Systems, Inc., LaJolla, CA) containing the full-length cDNA for mouse ER (gift from Malcolm Parker) using transfectam (Promega). The cells were maintained in serum-free medium from the time of transfection until harvest. Cells were treated with 100 ng/ml of human recombinant EGF or TGF $\alpha$  or with 17 $\beta$ -estradiol for 18 hr before harvest. Cells were frozen and thawed to obtain supernatants in which chloramphenicol acetyltransferase (CAT) enzyme activity was assayed, as previously described (25).

Neither 17 $\beta$ -estradiol nor the peptide growth factors elicited a change in expression of the reporter gene (CAT) in the

absence of ER transfection or in the presence of the control reporter vector (without ERE). However, in Ishikawa cells transfected with both vitA2 ERE pCAT and the ER expression vector, both EGF and TGF $\alpha$  as well as 17 $\beta$ -estradiol elicited transcription from the ERE. The activation of transcription by these agents was completely antagonized by the pure antiestrogen ICI 164,384 ( $1 \times 10^{-7}$  M). These data are shown in Figure 1. EGF in this figure refers to the mouse receptor-grade EGF used in the original pellet studies. It is clear from these data that the ER is required for the peptide growth factors to elicit transcription from the ERE. In addition, the effects of EGF and TGF $\alpha$  were inhibited approximately 70% by a mixture of human EGF receptor-neutralizing antibodies (Upstate Biotechnology Inc., Waltham, MA) (26,27), which demonstrated that the growth factors are dependent on the extracellular receptor for activity in this model. Neither basal transcription nor the effect of estradiol was inhibited by the antibodies. Interestingly, the combination of 17 $\beta$ -estradiol ( $1 \times 10^{-10}$ ) with either EGF or TGF $\alpha$  elicited ERE-dependent transcription of larger magnitude than the sum of their separate effects.



**Figure 1.** Effect of ICI 164,384 on CAT activity induced by EGF, TGF $\alpha$ , or estradiol in Ishikawa cells transfected with vit A2 ERE and mouse ER. Cell culture and transfection were performed as described in the text. Transfected cells were pretreated for 30 min with  $1 \times 10^{-7}$  M ICI 164,384 before addition of 100 ng/ml mouse receptor grade EGF, human recombinant EGF (REGF), human recombinant TGF $\alpha$  (RTGF), or  $1 \times 10^{-8}$  M 17 $\beta$ -estradiol. No pretx refers to the groups that did not receive ICI 164,384 pretreatment. The order of samples in the representative autoradiogram is the same as that in the graph. Data are expressed as the mean  $\pm$  SEM and are a pool of three to six samples from four separate experiments.

The results in the Ishikawa cells supported our cross-talk hypothesis derived from the *in vivo* experiments. However, we wished to corroborate the *in vitro* data in a cell line that contained endogenous ER as opposed to receptors replaced by transfection. This type of model is also optimal for experiments in which the signal-transduction pathways that may mediate the growth factor interaction with the ER can be elucidated. We discovered that the BG-1 human ovarian adenocarcinoma cell line (28) contains abundant ER as assessed by immunodetection with H222 antibody. TGF $\alpha$  and IGF-I at 1, 10, or 100 ng/ml stimulated proliferation of BG-1 cells over a 3-day period in serum-free medium in a dose-dependent manner. At the highest dose, an increase of approximately 200 to 250% was observed. 17 $\beta$ -estradiol ( $1 \times 10^{-10}$  M) only stimulated proliferation approximately 20%; however, in combination with IGF-I, a greater than additive effect was observed, which suggests an interaction between IGF-I and estrogen. Since the BG-1 cells responded to both TGF $\alpha$  and IGF-I, we then performed experiments to determine if the growth factors could regulate endogenous ER with respect to enhanced transcriptional activation. We performed transfection experiments as described above except that the mouse ER expression vector was not included and the cells were starved in 0.5% fetal bovine serum/DMEM-F12 media for 24 hr before transfection. EGF, TGF $\alpha$ , and IGF-I (100 ng/ml) induced CAT activity in BG-1 cells transfected with the vitA2 ERE-CAT vector. IGF-I had activity similar to estradiol ( $1 \times 10^{-10}$  M) eliciting a 10- to 12-fold induction, whereas EGF and TGF $\alpha$  elevated CAT levels approximately 4-fold.

One potential explanation for this effect is an elevation of ER levels induced by growth factor treatment. However, under conditions described for the transfection assays, TGF $\alpha$  induced a gradual decrease in receptor to approximately 50% control at 18 hr posttreatment as assessed by immunodetection and quantitation of [ $^{125}$ I]-secondary antibody bound to the blot with an image analysis system from Molecular Dynamics (Sunnyvale, CA). As expected, estradiol treatment produced the same effect on ER levels. Increased proteolytic degradation of the mouse uterine ER after estradiol treatment has been previously reported (29). Thus, the effect of the peptide growth factors was not dependent on enhanced ER levels. To rule out the possibility that the strong sv40 promoter

could be responsible for the growth factor activity, we performed transfection experiments using a reporter vector that contained a minimal promoter, namely the adenovirus E1b TATA box (30) upstream of a vitellogenin A2 promoter fragment containing a consensus ERE (a gift from John Cidlowski, University of North Carolina). Both TGF $\alpha$  and IGF-I activated transcription using this reporter vector. Transcriptional activation induced by TGF $\alpha$ , IGF-I, and 17 $\beta$ -estradiol with either the sv40 or E1b minimal promoter was completely inhibited with ICI 164,384 ( $1 \times 10^{-7}$  M). Thus transcriptional activation in the BG-1 model is also dependent on the presence of the estrogen receptor.

Similar to the results obtained in the Ishikawa cells, both TGF $\alpha$  and IGF-I in combination with estradiol induced CAT-activity levels that were greater than the sum of their separate activity. However, an interaction between IGF-I and TGF $\alpha$  treatments was not observed. One explanation for the observed synergism is that the growth factor-signaling mechanism induces ER conformational changes that are different from and are cooperative with those induced by ligand binding. Synergism between peptide growth factors and steroids has been reported previously. IGF-I and estrogen synergistically induced progesterone receptor levels in MCF-7 breast cancer cells (17). Synergism between progesterone and EGF in T47D mammary tumor cells transfected with a progesterone response element has also been reported (31). In addition, the interaction between dopamine and the ER in HeLa cells is very similar to that of EGF and ER (32). Dopamine alone stimulated transcription in cells transfected with an ERE and an estrogen receptor expression vector in an ER-dependent manner. The combination of submaximal amounts of dopamine and 17 $\beta$ -estradiol induced CAT activity more than either agonist by itself.

The next set of experiments began to address the elucidation of signaling pathways involved in the interaction between peptide growth factors and the estrogen receptor. In BG-1 cells transfected with the vitA2-sv40-ERE reporter construct, treatment with the protein kinase C activator phorbol 12-myristate 13-acetate (TPA, 100 nM) resulted in a 40-fold increase in transcription whereas 17 $\beta$ -estradiol ( $1 \times 10^{-10}$  M) elicited only 50% of that level of activity. However, the combination of estradiol and TPA induced a 180-fold increase in CAT activity. Synergism was not observed in combination treatments

with TPA and either IGF-I or TGF $\alpha$ . ICI 164,384 inhibited the effect of TPA, demonstrating a dependence on the ER for the enhancement of CAT activity. Bisindolylmaleimide (1  $\mu$ M), an antagonist of PKC, completely inhibited the effect of TPA but had no effect on 17 $\beta$ -estradiol or the peptide growth factor effects. Thus, the protein kinase C pathway does not mediate the effects of 17 $\beta$ -estradiol or peptide growth factors on the ER in BG-1 cells. The TPA experiments were repeated using the E1b minimal promoter construct with very similar results. Finally, an activator of protein kinase A, 8 bromoadenosine 3'5' cyclic monophosphate (8 Br cAMP,  $1 \times 10^{-4}$  M) elicited only a 3.5-fold increase in CAT activity while TGF $\alpha$ , IGF-I, and 17 $\beta$ -estradiol induced 7.5-, 14.5-, and 11-fold increases in CAT activity. Additivity was observed in combinations of peptide growth factors or 17 $\beta$ -estradiol with 8 Br cAMP; the effect of 8 Br cAMP was blocked by ICI 164,384. Given the weak activation observed at a high dose of 8 Br cAMP, it seems unlikely that the protein kinase A pathway mediates the effects of the peptide growth factors or 17 $\beta$ -estradiol.

Aronica and Katzenellenbogen (33) have recently shown that 17 $\beta$ -estradiol, IGF-I, and protein kinase A activators similarly enhance transcription (8- to 10-fold) from a minimal promoter—the ERE reporter construct transfected into ER-positive rat primary uterine cells. Transcriptional activation by these agonists was inhibited by ICI 164,384 or H8, a protein kinase A inhibitor. They concluded from these results that, in their system, the effects of IGF-I and 17 $\beta$ -estradiol are mediated through the activity of PKA. Similar to our findings in BG-1 cells, they did not observe synergism with the PKA agonist. Aronica and Katzenellenbogen (34) also reported that in ER-positive MCF-7 cells and ER-negative CHO cells they observed very weak transcriptional activation when these cells were treated with either a protein kinase A or C activator. In MCF-7 cells, the combination of 17 $\beta$ -estradiol with a protein kinase A or C activator elicited synergistic activation of transcription. However, only the protein kinase A interaction with 17 $\beta$ -estradiol is synergistic in CHO cells. The results of the Katzenellenbogen laboratory taken together with our observations point to cell-specific second-messenger regulation of the effects of extracellular ligands on nuclear hormone receptor transcriptional activation.

The presence of cross talk between peptide-growth factor receptors and the estrogen receptor suggests that interactions

between growth factors and steroid receptors may modulate hormonal activity influencing normal and aberrant function in mammalian cells. The problems inher-

ent in inappropriate exposure to estrogens through environmental sources may extend to chemicals that could activate peptide-growth factor signaling systems. Thus,

environmental agents that do not appear to be estrogens from their chemical structures may act as estrogens through intracellular signaling to the nuclear estrogen receptor.

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