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Physiology

Physiological coupling of growth factor and steroid receptor signaling pathways: Estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor

(cross-talk/ligand independent/transgenic/uterotropic)

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ABSTRACT **Past studies have shown that epidermal growth factor (EGF) is able to mimic the uterotropic effects of estrogen in the rodent. These studies have suggested a "cross-talk" model in which EGF receptor (EGF-R) signaling results in activation of nuclear estrogen receptor (ER) and its target genes in an estrogen-independent manner. Furthermore, *in vitro* studies have indicated the requirement for ER in this mechanism. To verify the requirement for ER in an *in vivo* system, EGF effects were studied in the uteri of ER knockout (ERKO) mice, which lack functional ER. The EGF-R levels, autophosphorylation, and c-*fos* induction were observed at equivalent levels in both genotypes indicating that removal of ER did not disrupt the EGF responses. Induction of DNA synthesis and the progesterone receptor gene in the uterus were measured after EGF treatment of both ERKO and wild-type animals. Wild-type mice showed increases of 4.3-fold in DNA synthesis, as well as an increase in PR mRNA after EGF treatment. However, these responses were absent in ERKO mice, confirming that the estrogen-like effects of EGF in the mouse uterus do indeed require the ER. These data conclusively demonstrate the coupling of EGF and ER signaling pathways in the rodent reproductive tract.**

Estradiol, epidermal growth factor (EGF), and insulin-like growth factors are known mitogens in the rodent reproductive tract (1–3). Estrogen has been shown to increase the uterine levels of both EGF and its receptor (EGF-R) (3–10), suggesting a link between the mitogenic effects of estrogens and growth factors. Furthermore, EGF has been shown to mimic the effects of estrogen in the mouse reproductive tract in terms of increased DNA synthesis and cornification of the vaginal epithelium (11), as well as increased phosphorylation and nuclear retention of the estrogen receptor (ER) (12). When estradiol is administered in conjunction with an EGF-specific antibody, a 60–70% reduction in the hormone-induced proliferation of the epithelium is observed in the mouse uterus and vagina (11). These data indicate a possible role for EGF as a mediator of estrogen action. Further evidence of EGF/estrogen cross-talk was provided by experiments showing that pre-treatment of mice with the pure anti-estrogen ICI 164,384 greatly diminished the uterine response to EGF (12). Because ICI 164,384 significantly reduces the level of uterine ER (13), these studies suggest the necessity for the ER in the mitogenic actions of EGF. This was supported by studies in Ishikawa cells, a human endometrial carcinoma cell line devoid of ER, in which an estrogen-responsive chloramphenicol acetyltransferase reporter gene could only be activated by EGF after cotransfection with an ER-expression plasmid (14).

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These studies have led to a model in which EGF plays a role in ER-mediated events in a ligand-independent manner. To gain further insight into the role of the ER in this cross-talk mechanism, EGF studies were carried out in the ER "knockout" (ERKO) mice. In the ERKO, both alleles of the ER gene have been disrupted via homologous recombination, resulting in a female reproductive tract that is nonresponsive to estrogen as described (15, 16). To address the concern that lack of ER might disrupt the expression and functioning of the EGF-R signaling pathway, EGF-R levels, its autophosphorylation, and the induction of c-*fos*, an EGF-regulated gene, were determined. Then ERKO animals, as well as wild-type controls, were treated with EGF, and induction of DNA synthesis and the progesterone receptor (PR) gene were measured to confirm whether ER is required for the estrogenic activities of EGF.

MATERIALS AND METHODS

Animals. All animals were handled according to the National Institutes of Health guidelines for the humane treatment of animals under an approved National Institute of Environmental Health Sciences animal studies protocol.

EGF Receptor Assays. Uteri and livers of intact wild-type and ERKO animals were homogenized in TEGM buffer (10 mM Tris/1.5 mM EDTA/10% glycerol/3 mM $MgCl_2$/3 mM EGTA/50 μg/ml each leupeptin, antipain, soybean trypsin inhibitor, and chymostatin). The homogenate was centrifuged at 16,000 × *g* for 15 min to remove nuclei, and the supernatant centrifuged at 45,000 rpm in a 70.1 Ti rotor (Beckman) for 50 min to pellet membranes. The membrane pellets were drained and then frozen at −80°C until needed. The [^{125}I]EGF-R binding assay was done as described (17).

Solubilized membranes were subjected to an *in vitro* autophosphorylation assay by incubation in the presence or absence of EGF (17). After stopping the reaction, equivalent amounts of total protein (25 μg per lane) were separated by SDS/PAGE (8% acrylamide), transferred to Immobilon membrane (Millipore), and the tyrosine-phosphorylated proteins were immunodetected using a monoclonal anti-phosphotyrosine antibody (anti-PTyr 3300, Sigma) and enhanced chemiluminescence as described (17). The position of the EGF-R band was determined based on the molecular weight markers as previously determined (17).

Abbreviations: EGF, epidermal growth factor; EGF-R, EGF receptor; ER, estrogen receptor; ERKO, ER knockout; PR, progesterone receptor; RPA, RNase protection assay; DMSO, dimethyl sulfoxide; AF, activation functions.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M29016 and M68915).

RNase Protection Assay (RPA). RPAs were used to measure the relative amounts of c-*fos* mRNA in both wild-type and ERKO mice. The animals were ovariectomized 10–14 days prior to subcutaneous injection of 30 μg EGF (receptor grade, Collaborative Research) in PBS. Some animals were pretreated with 1 mg/kg of the estrogen antagonist ICI 182,780 in dimethyl sulfoxide (DMSO) 30 min prior to EGF treatment. Control animals were treated with DMSO and PBS. Thirty minutes after EGF treatment, animals were killed and total RNA was prepared from uteri using Trizol (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. RPAs were carried out using the Ambion HybSpeed RPA kit (Ambion, Austin, TX), according to the manufacturer's protocol. All probes were prepared using the Ambion Maxiscript *In Vitro* Transcription kit (Ambion) and labeled with [^{32}P]-CTP (Amersham). The probe for c-*fos* mRNA was generated from the pTRI-c-*fos*/exon 4-mouse template plasmid (Ambion) and protected a 250-nt fragment of exon 4 sequences of the mouse c-*fos* transcript. The probe for normalization was generated from the pTRI–cyclophilin–mouse template plasmid (Ambion) and protects a 103-nt fragment of the mouse cyclophilin transcript. Final RPA reactions were separated on a 5% bis-acrylamide/8.3 M urea/1× TBE gel (100 mM Tris–Borate/0.2 mM EDTA, pH 8.3) (National Diagnostics). Markers were generated from the Ambion Century Marker Template Set and labeled by incorporation of [^{32}P]-CTP.

[^{3}H]Thymidine Incorporation Assay. EGF (mouse receptor grade) was obtained from Collaborative Research and made into pellets (5 μg per pellet) by Innovative Research of America. Animals were ovariectomized 10–14 days before treatment. Pellets were split into four sections, animals were anaesthetized, and one-quarter of an EGF or placebo pellet was inserted into the intraperitoneal cavity through a 13-gauge trochar needle. Animals were sacrificed 16–18 hr later, and the uterus was removed and divided into two portions. One uterine horn was frozen in liquid nitrogen for RNA preparation (see below), while the other was slit longitudinally to expose the luminal epithelium and used in the [^{3}H]thymidine incorporation assay as described (13).

Northern Blot Analysis. RNA prepared from the uterine horns from the EGF- or placebo-treated animals was analyzed by Northern blot analysis as described (16). The PR probe was an 1100-bp cDNA fragment encoding the DNA and ligand-binding regions of the mouse PR (bp 1667–2781). Equivalent RNA loading was confirmed by probing with a portion of the ribosomal protein L-7 (PL-7) cDNA (bp 371–639) as described (16).

RESULTS

Estrogen up-regulates the EGF-R in the mouse uterus (3). Therefore, removal of the ER during development might alter the level of EGF-R, and it was necessary to determine the levels of EGF-R in the ERKO mouse before proceeding with the studies of EGF action. As shown in Table 1, EGF-R was present in membranes from the uteri and liver of ERKO animals, although at a level 35% and 15% lower, respectively, than the wild-type levels. The EGF-R also undergoes autophosphorylation in both tissues in response to ligand (Fig. 1), indicating that the EGF-R can be activated by EGF in the ERKO mouse.

Table 1. [^{125}I]EGF binding to membrane fractions

	Wild type	ERKO
Uterus	16.3×10^3	10.6×10^3
Liver	23.6×10^4	20.1×10^4

The [^{125}I]EGF receptor binding assay was done as described (17). Values are the average of triplicate determinations and are expressed as cpm bound per milligram of protein.

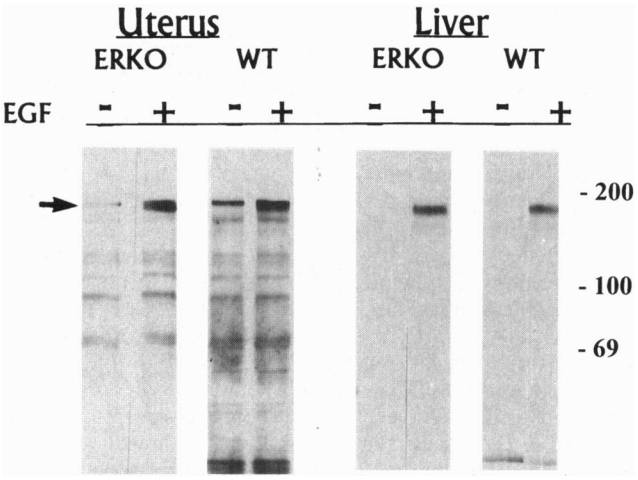

FIG. 1. EGF-stimulated autophosphorylation of the EGF-R in ERKO and wild-type mice. Solubilized membrane from uterus and liver was subjected to an *in vitro* autophosphorylation assay by incubation in the presence (+) or absence (−) of EGF (19). After stopping the reaction, equivalent amounts of total protein (25 μg per lane) were separated by SDS/PAGE. The arrow indicates the position of the EGF-R band. Migration of [^{14}C] molecular weight standards (Amersham), in kDa, is indicated at the right.

To determine if the level of EGF-R in the ERKO was sufficient to elicit a gene response, RNA was prepared from uteri of animals 30 min after subcutaneous injection of EGF, and c-*fos* levels were determined by RPA (Fig. 2). Levels of c-*fos* mRNA were induced 18-fold in the wild-type uterus and 13-fold in the ERKO, after normalizing to the level of cyclophilin mRNA. This response in both genotypes indicates that although the EGF-R level is lower in the ERKO (Table 1), the EGF-R signaling pathway is intact and able to mediate gene activation in ERKO mice. Because c-*fos* is also known to be an estrogen inducible gene (18), we wanted to determine whether the EGF induction might have an ER-mediated component via

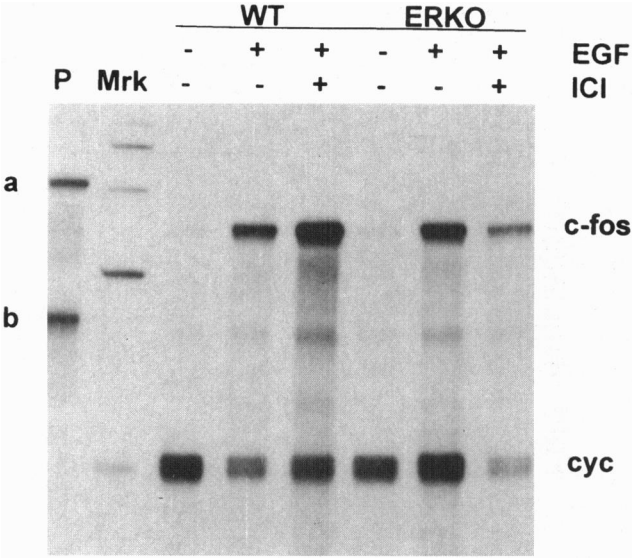

FIG. 2. Uterine c-*fos* mRNA is induced by EGF in uteri of wild-type and ERKO mice. RPA was used to measure the relative amounts of c-*fos* mRNA from mice treated with PBS vehicle, EGF for 30 min, or pretreated with ICI prior to EGF treatment. Positions of c-*fos* (250 nt) and cyclophilin (cyc, 103 nt) are indicated. Lane P shows the positions of the riboprobes for c-*fos* (a) and cyclophilin (b) before digestion. Molecular weight markers (Ambion) (Mrk) are at 500, 400, 300, 200, and 100 nt.

the proposed cross-talk mechanism. The antiestrogen ICI 182,780, however, did not diminish the induction of c-*fos* by EGF in wild-type animals, indicating that ER is not involved in EGF induction of c-*fos* at this early time point of 30 min. The increased EGF induction after ICI pre-treatment is most likely due to the DMSO vehicle, since DMSO pre-treatment caused a similar enhancement of EGF activity (data not shown), possibly due to increased EGF-R autophosphorylation following DMSO treatment (19).

The ERKO uterus has previously been shown to lack a growth response following estradiol treatment as measured by a uterotropic bioassay and [^{3}H]thymidine incorporation (15, 16). To determine if EGF would initiate DNA synthesis in the ERKO uterus, ovariectomized ERKO and wild-type animals were implanted with EGF releasing pellets, and DNA synthesis measured using relative [^{3}H]thymidine incorporation. After 16–18 hr of EGF treatment, wild-type uteri showed a 4.3-fold increase in [^{3}H]thymidine incorporation over placebo-treated animals, whereas the ERKO mice showed no increase in DNA synthesis compared with the placebo control (Fig. 3).

The PR gene is also estrogen regulated in the rodent uterus (20, 21); however, it is not induced by estrogen in the ERKO (16). To determine whether PR mRNA could be induced by EGF, RNA was prepared from uterine tissue after *in vivo* treatments with EGF pellets with or without pre-treatment with the pure anti-estrogen ICI 182,780. Northern blot analysis (Fig. 4) revealed that PR message increases after EGF treatment in the wild-type animals, but not in the ERKO mice. In addition, the anti-estrogen ICI 182,780 is able to block the EGF induction of the PR in the wild type, and has no effect on the ERKO.

DISCUSSION

This study, in combination with our previous studies and those of others (11, 12, 14, 22), have led us to propose a model of "cross-talk" or interaction between the EGF and estrogen signaling pathways such that EGF-R activation can result in activation of ER target genes in the absence of estrogen (Fig. 5). In this model, the EGF-R and ER pathways can initiate distinct and separable signals that modulate responses of their respective target genes. However, it is also possible to couple the two pathways when, as depicted by the cross-hatched arrow in Fig. 5, the EGF-R signal activates the ER-mediated response of estrogen targets. The ERKO mouse is the definitive model to demonstrate the role of ER in this mechanism, since removal of the ER should disable the cross-talk. Indeed, both DNA synthesis and PR mRNA, which are estrogen responsive, could be induced by EGF in wild-type mice but not in ERKO mice. Induction of c-*fos*, which required only the EGF-R

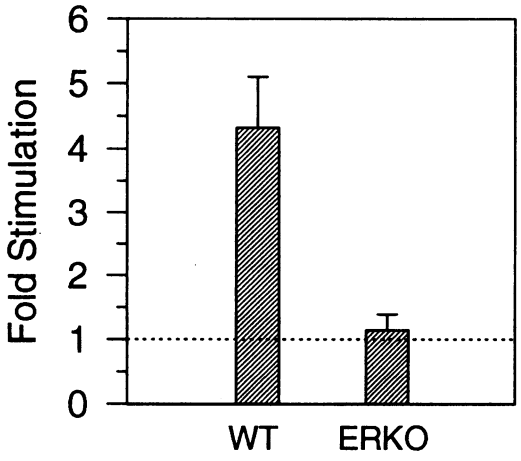

FIG. 3. EGF does not increase DNA synthesis in the ERKO uterus. Relative [^{3}H]thymidine incorporation into uteri of wild-type or ERKO mice was measured following insertion of a placebo pellet or an EGF-containing pellet. [^{3}H]Thymidine incorporation was calculated as dpm/μg of DNA and is plotted as -fold increase after EGF treatment compared with placebo control. Values are the means of two independent experiments and the bars show the ranges of values.

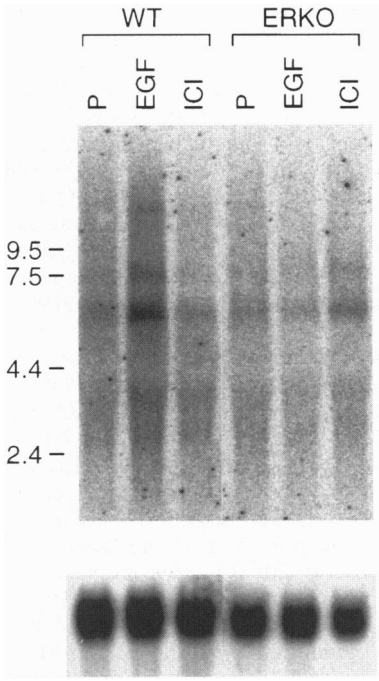

FIG. 4. PR mRNA is increased by EGF treatment of wild-type but not ERKO mice. Twenty micrograms of total RNA isolated from uteri was analyzed by Northern blot for PR mRNA (*Top*) as described. RNA samples were from wild-type (WT) or ERKO animals containing either placebo pellets (P), EGF pellets, or were injected with 1 mg/kg ICI 182,780 (ICI) 30 min prior to insertion of EGF pellets. The bottom panel shows the same blot reprobed with PL-7, a ribosomal protein used to confirm equal loading of samples.

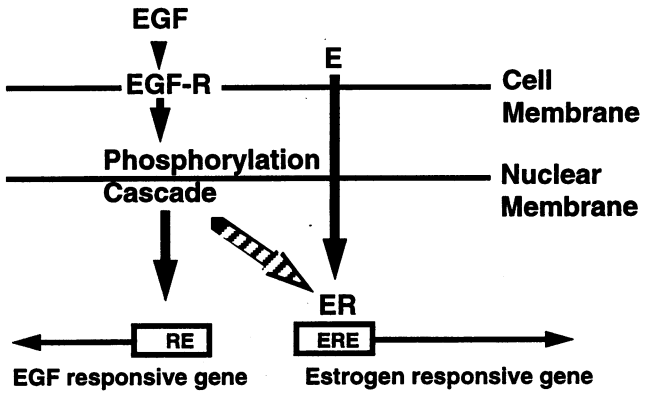

FIG. 5. Model of cross-talk between EGF-R/ER signalling pathways. The model depicts the separate EGF and estrogen inducible pathways. EGF binds to its membrane-bound receptor, which initiates a cascade of phosphorylation modulating the activities of cellular kinases and nuclear transcription factors (depicted as the phosphorylation cascade in the model). The activation of transcription factors results in regulation of responsive genes. In contrast, estrogen (E) diffuses to the nucleus where it binds and activates the nuclear ER thereby modulating transcription of estrogen-responsive genes. Cross-talk between the pathways (depicted by the cross-hatched arrow) occurs when EGF initiates the phosphorylation cascade, which activates the ER, presumably via ER phosphorylation, regardless of the presence of estrogen.

pathway, occurred in both genotypes. Because EGFR activates several pathways and the precise pathway involved in EGF induction via the ER has not been determined, it is only possible to definitely conclude that the c-*fos* induction mechanism is intact. However, it appears that ER-mediated responses, including EGF-R initiated cross-talk with ER, but not processes dependent only on the EGF-R signaling pathway, are disrupted in the ERKO.

Sumida and Pasqualini showed induction of PR protein by EGF in the absence of estrogen (22), indicating that an estrogen responsive gene can be activated by EGF. The inhibition of this response by an anti-estrogen indicated a role for ER in mediating this response (22). In this study we were able to show an EGF-induced increase in PR mRNA *in vivo*. In addition, the mRNA induction required ER because EGF did not induce the PR in ERKO mice or in wild-type mice that had been treated with the anti-estrogen ICI 182,380.

The mechanism of this EGF-R-mediated ER activation is unknown, but *in vitro* studies using a truncated ER illustrated the importance of amino terminal sequences for activation by EGF (14). The ER mediates transcriptional activities by using two transcription activation functions or AFs (2, 23–25). AF-1 is a ligand-independent activation function in the N terminus and AF-2 is a ligand-dependent activation function in the ligand-binding domain. An ER deletion mutant lacking the hormone-binding domain was activated by EGF, whereas a deletion mutant lacking the AF-1 was not (14). Phosphorylation in the AF-1 region of the ER is a likely mechanism of EGF-mediated activity, because EGF-R activation results in phosphorylation and consequent modulation of the activities of several cellular kinases (26–28), and subsequent phosphorylation and activation of nuclear transcription factors (26–28). Serines 122, 156, 158, and 298 of the mouse ER are known to be phosphorylated in COS-1 cells (29). Serines 104, 106, and 167 of the human ER have been identified as phosphorylation sites as well (30–32). Mutation of Ser-118 to alanine, which is located in AF-1 of the human ER, eliminated phosphorylation of this serine and resulted in reduction of transcriptional activity by 40–75% in HeLa and COS-1 cells (30), indicating that phosphorylation of Ser-118 may mediate transcriptional activity of ER and therefore could be a target for EGF-R activated kinase. Furthermore, recent *in vitro* data show that Ser-118 of the human ER can be phosphorylated by the mitogen-activating protein kinase pathway (33), resulting in increased estrogen-dependent activity in transfected COS-1 cells. Estrogen-independent activation of the human ER by EGF via the mitogen-activating protein kinase pathway and phosphorylation of Ser-118 has been shown in HeLa and SK-Br-3 cells (34). It will be interesting to determine if serine 122 of the mouse ER, which is analogous to Ser-118 in the human ER, is required for EGF activity, and whether mitogen-activating protein kinase-mediated phosphorylation results in mouse ER transcriptional activity. Alternatively, other serines in the AF-1 region or other kinases may be required. Perhaps phosphorylation of substrates other than ER, such as steroid receptor coactivators or nuclear transcription factors, are required for ligand-independent activity.

The importance of estrogen-independent ER action to normal physiology is unclear. The levels of EGF and EGF-R are both increased by estradiol (3), which indicates EGF-R may play a role at a time when estrogen is already present. Perhaps during proestrous, when estrogen is low, EGF is able to mediate and maintain a constitutive level of estrogen-responsive gene expression. In addition, the EGF may play a greater role during fetal development or postnatally before estrogen levels increase.

Alternatively, EGF-R-mediated ER activation may synergize with the estrogen hormone signal by increasing the activity of the ER both in terms of the level of response of genes and broadening the spectrum of activated genes. Studies *in vitro* have suggested that AF-1 and AF-2 activities are promoter specific (35), and an increase in AF-1 activity as a result of EGF-R activation may allow for an enhanced response by genes requiring AF-1 activity.

The ERKO mouse has provided the first definitive link between growth factor pathways and ER *in vivo*. However, other membrane receptor pathways have also been shown to activate nuclear steroid receptors. Insulin and IGF-1 both increase growth and induce an estrogen-responsive chloramphenicol acetyltransferase reporter in human neuroblastoma cells that contain ER (36). Membrane permeable agents that increase cellular phosphorylation, such as 8-bromo-cAMP and okadaic acid, also enhance progesterone-dependent gene transcription (37). Dopamine, a neurotransmitter, has been shown to induce ligand-independent activation of several nuclear receptors including the chicken PR (38, 39) and the human ER (40). Together with the activation of mouse ER by EGF, these results confirm the existence of conserved cross-talk mechanisms between membrane receptors and nuclear receptors.

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