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Coupling of dual signaling pathways: Epidermal growth factor action involves the estrogen receptor

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ABSTRACT **Epidermal growth factor (EGF) reproduces many of the effects of estrogen on the murine female reproductive tract and may partially mediate estrogen-induced growth and differentiation. This study was performed to investigate the mechanism by which EGF elicits estrogen-like actions in the whole animal. EGF was administered to adult ovariectomized mice by slow release pellets implanted under the kidney capsule. The induction of uterine DNA synthesis and phosphatidylinositol lipid turnover by EGF or administration of diethylstilbestrol (5 μg/kg), a potent estrogen, was attenuated by the estrogen receptor antagonist ICI 164,384. Furthermore, EGF mimicked the effects of estrogen on enhanced nuclear localization of the estrogen receptor and the formation of a unique form of the estrogen receptor found exclusively in the nucleus. These results suggest that EGF may induce effects similar to those of estrogen in the mouse uterus by an interaction between the EGF signaling pathway and the classical estrogen receptor. The demonstration of cross-talk between polypeptide growth factors and steroid hormone receptors may be of importance to our understanding of the regulation of normal growth and differentiation as well as the mechanisms of transmission of extracellular mitogen signals to the nucleus.**

It has been proposed that polypeptide growth factors may act as autocrine or paracrine mediators of estrogen-induced mitogenesis (1, 2). The observations that estrogen induces mRNA and protein for both epidermal growth factor (EGF) (3, 4) and its receptor (5–7) in rodent uterus are consistent with this hypothesis and implicate a role for the EGF receptor signaling pathway in steroid hormone regulation of uterine tract growth. Furthermore, exogenous EGF administration to adult ovariectomized mice mimics the effects of estrogen on proliferation and differentiation in the murine female reproductive tract (8). EGF-induced mitogenesis in this model is not affected by adrenalectomy or hypophysectomy, which indicates that adrenal or pituitary hormones do not mediate the effects of EGF. In addition, the fact that an EGF-specific antibody administered prior to estradiol partially blocks estrogen-induced uterine epithelial cell proliferation (8) suggests that production of EGF may be necessary for estrogen-induced responses.

Presently, the mechanism by which the actions of estrogens and EGF converge is unknown. This study addresses the intriguing possibility that some of the physiological actions of EGF, an extracellular ligand, may be mediated through a nuclear steroid hormone receptor, namely, the estrogen receptor (ER). Two recent reports by Power *et al.* (9, 10) support such a hypothesis. These studies demonstrated that dopamine, an extracellular ligand, was able to stimulate transcriptional enhancement by three members of the steroid receptor superfamily [the progesterone receptor (PR), ER, and COUP (chicken ovalbumin upstream promoter) transcriptional enhancer], which were transfected into CV1 monkey kidney cells. Furthermore, in MCF-7 human breast cancer cells, the ability of antiestrogens to suppress the induction of mitogenesis and elevation of PR levels (classical estrogen-inducible responses) by peptide growth factors (11, 12) suggests a role for ERs in the actions of extracellular mitogens.

In this investigation, we have examined the *in vivo* effect of a "pure" antiestrogen, ICI 164,384 (ICI) (13), on estrogen- and EGF-inducible responses in the mouse uterus. In addition to EGF-induced uterine DNA synthesis, the effect of EGF on phosphatidylinositol (PI) lipid metabolism was assessed. Activation of uterine PI lipid turnover is one of the earliest and most persistent effects of estrogen administration and this effect is mediated by the ER (14, 15). The metabolic products of PI lipids are mediators of signal transduction and cellular proliferation for many hormones and polypeptide growth factors, including EGF, which is thought to enhance metabolism of PI lipids by phosphorylation and activation of phospholipase Cγ (16, 17). This signaling pathway, therefore, is a potentially important point of convergence of the actions of EGF and estrogen in the uterus.

We report here that EGF- and diethylstilbestrol (DES)-induced uterine DNA synthesis and PI lipid metabolism were significantly attenuated by an ER antagonist. Furthermore, the observation that EGF and DES modified the biochemical characteristics of the nuclear ER in the uterus similarly suggests that an interaction between the EGF signaling pathway and ER may occur in normal tissues *in vivo*.

MATERIALS AND METHODS

Animals. Adult female CD-1 mice were ovariectomized between 7 and 8 weeks of age and were treated 2 weeks after surgery. The mice were anesthetized with methoxyflurane before surgery. Slow-release pellets containing 5 μg of EGF (receptor grade from Collaborative Research) were split into quarters, which were implanted under the kidney capsule. Placebo pellets consisted of the same matrix with no growth factor added. Detailed procedures and characterization of the effects of the EGF pellets in the female reproductive tract are described in ref. 8. Sham operations were performed immediately prior to intraperitoneal injection of DES (5 μg/kg) or vehicle. In the antagonist experiments, either ICI 164,384 (1 mg/kg) or dimethyl sulfoxide vehicle was injected i.p. (in 100 μl) 30 min prior to surgery, and mice were killed 16 h after pellet implantation or steroid treatment. Mice were killed 1.5 h after agonist treatment in biochemical studies of the ER.

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Abbreviations: EGF, epidermal growth factor; ER, estrogen receptor; PR, progesterone receptor; PI, phosphatidylinositol; DES, diethylstilbestrol; ERE, estrogen responsive element.

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PI Lipid Metabolism. This technique is described in detail in ref. 15. Briefly, uteri were removed, slit longitudinally to expose the endometrial surface, and labeled with *myo*-[^{3}H]inositol (0.4 mCi/ml; 1 Ci = 37 GBq) in Dulbecco's modified Eagle's medium/F12 medium for 1.5 h. The lipids were extracted from homogenized tissue and the total radioactivity and phosphate in the lipid fraction were determined. Data are expressed as dpm per nmol of phosphate.

DNA Synthesis. DNA synthesis was measured by incorporation of [^{3}H]thymidine into nuclei 16 h after EGF or DES administration as described (8). For biochemical analysis, tissue was homogenized in TEGM buffer [10 mM Tris·HCl/1.5 mM EDTA/10% (vol/vol) glycerol/3 mM $MgCl_2$, pH 7.6] and the nuclear pellet was resuspended in TEGM buffer containing 2.2 M NaCl. The amount of trichloroacetic acid-precipitable radioactivity per μg of DNA was assessed as described (15). Treatment of tissue for histological examination was performed as described (8). Uterine tissue was fixed in Bouin's fixation solution and embedded in paraffin for sectioning. The number of labeled and unlabeled luminal epithelial cells along the basement membrane was quantitated in sections that had been dipped in Kodak photographic emulsion (Eastman Kodak), developed after 2 weeks exposure, and stained with hematoxylin and eosin. Quantitation was performed on six to eight uteri per group. Data are presented as the mean of the percentage of labeled nuclei per unit basement membrane.

Biochemical Analyses of the ER. The [^{3}H]estradiol binding assay was performed as described (18) with modifications (19). Uterine tissue was homogenized in ice-cold TEGM buffer and the nuclear pellet was washed twice in this buffer. [^{3}H]Estradiol binding was measured in both the nuclear and cytosolic fractions. Data are expressed as fmol of ER bound per 100 μg of DNA in the sample.

SDS/PAGE of nuclear and cytosol fractions was performed as described in ref. 20 with a 3% acrylamide/bisacrylamide stacking gel and a 10% separating gel with Acrylaide crosslinker (FMC, Rockland, ME). Proteins were blotted to nitrocellulose and ER immunodetection was performed as described (21) using the ER monoclonal antibody H-222.

The gel-retardation assay was performed as described (22). Briefly, uteri were homogenized in TGM buffer (10 mM Tris·HCl/10% glycerol/3 mM $MgCl_2$, pH 7.6) containing protease inhibitors (23). Uterine nuclear proteins were extracted in 0.4 M KCl on ice for 1 h. Nuclear extracts were concentrated by precipitation with ammonium sulfate at 40% saturation for 1 h. Proteins were resuspended in TGM buffer with protease inhibitors and were incubated in the presence or absence of H-222 overnight at 4°C before adding ^{32}P-labeled vitellogenin A2 estrogen responsive element (ERE).

Statistics. A two-way analysis of variance was used to analyze the effects of ICI 164,384 on EGF- and DES-induced DNA synthesis and PI lipid metabolism. The *F* values for the interaction between treatments are shown in the figure legends. A significant interaction as determined by two-way analysis of variance was interpreted as inhibition of the effects of either EGF or DES by the ER antagonist. Student's unpaired *t* test was used to analyze [^{3}H]estradiol binding assay results.

RESULTS

Data in Figs. 1*B* and 2*B* demonstrate that administration of DES to sham-operated ovariectomized mice enhanced both DNA synthesis and PI lipid turnover, respectively, and these effects, as expected, were significantly attenuated by ICI 164,384. Interestingly, the stimulatory effect of EGF on both of these responses was also blocked by pretreatment with ICI 164,384 (Figs. 1*A* and 2*A*).

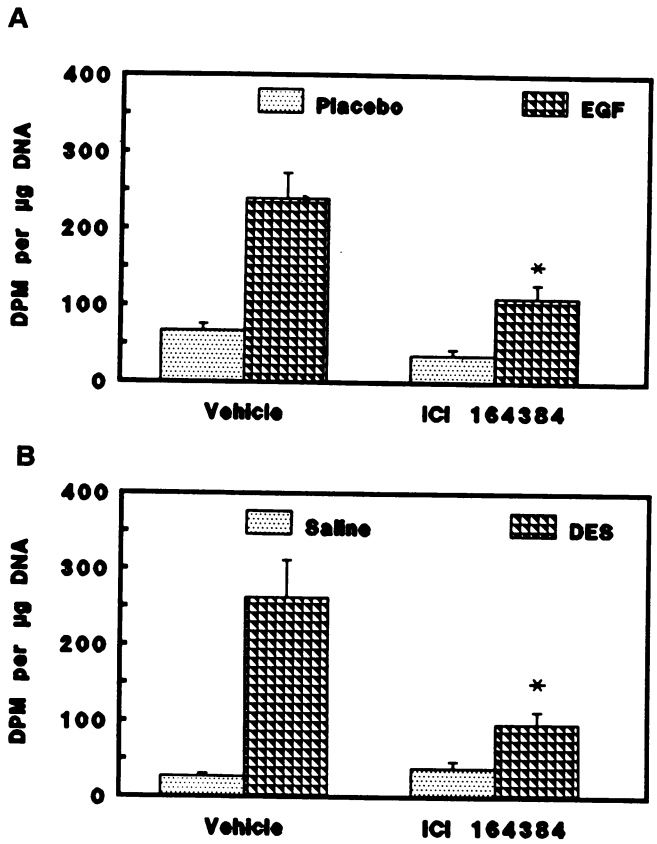

FIG. 1. Effect of ICI 164,384 on uterine DNA synthesis induced by EGF or DES. Ovariectomized adult mice were pretreated for 30 min with dimethyl sulfoxide vehicle or ICI 164,384 (1 mg/kg) in dimethyl sulfoxide. (*A*) Mice received placebo or an EGF pellet. (*B*) Sham-operated mice received vehicle or DES (5 μg/kg) i.p. Data are the pool of two experiments and are expressed as the mean ± SEM. The asterisk indicates that the effects of EGF and DES were significantly attenuated by a 30-min pretreatment with ICI 164,384 (N = 11–16). (*A*) $F_{1,52}$ = 8.2. (*B*) $F_{1,49}$ = 11.7.

Data in Fig. 1 depict a pool of two experiments in which the effects of EGF and DES were diminished ≈56 and ≈75%, respectively, by the ER antagonist ICI 164,384. Histological analysis of DNA synthesis in the luminal epithelium from one of these experiments is presented in Table 1. These data corroborate the biochemical analysis inasmuch as the enhancement of [^{3}H]thymidine incorporation into the nuclei of luminal epithelium by DES and EGF was significantly inhibited by antagonist pretreatment.

The metabolism of PI lipids was assessed by *myo*-[^{3}H]inositol incorporation into the phospholipid fraction. We have shown (15) that a component of the increased incorporation is dependent on increased turnover of PI rather than simply an increase in the absolute mass of uterine lipid. Data in Fig. 2 depict one representative experiment in which the effects of EGF and DES were diminished by 51 and 88%, respectively. Pooled data from two experiments expressed as the percentage of the mean ± SD of control data are as follows: EGF (184.3 ± 29.4%) versus ICI 164,384 plus EGF [146.8 ± 22.3%; $P < 0.001$; n = 13–16 (t test)]; DES (192.9 ± 28.7%) versus ICI 164,384 plus DES (135.9 ± 32.0%; $P < 0.001$; n = 12–16).

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 ER. Estrogen treatment rapidly enhances ER affinity for chromatin so that the ER is retained in the nuclear fraction after tissue homogenization. This phenomenon can be quantitated by a [^{3}H]estradiol binding assay (18, 19). As shown in Fig. 3,

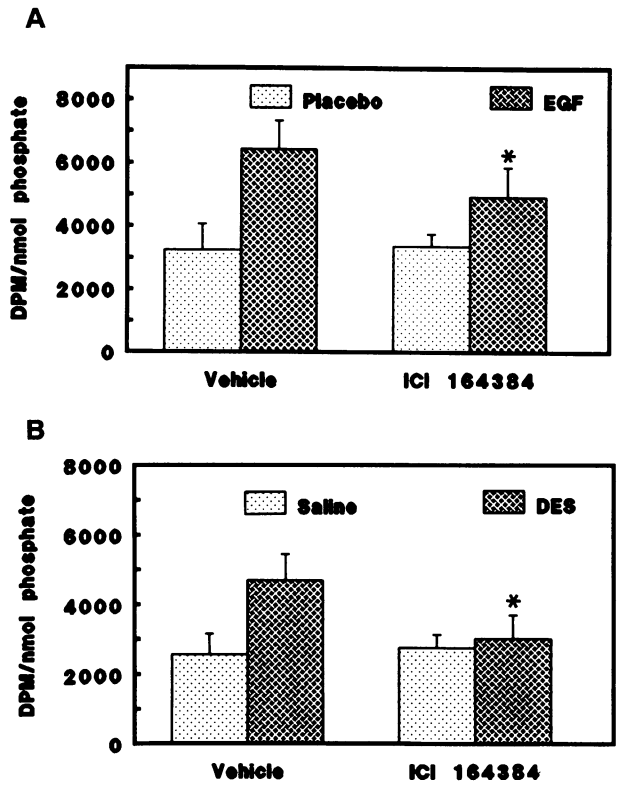

FIG. 2. Effect of ICI 164,384 on uterine PI lipid metabolism induced by EGF and DES. Mice were treated as described in Fig. 1. Data are expressed as the mean ± SD. The asterisk indicates that the effects of EGF and DES were significantly attenuated by pretreatment with ICI 164,384 (N = 6–9). (A) $F_{1,26}$ = 7.2. (B) $F_{1,25}$ = 16.7.

significant augmentation of nuclear ER levels was observed 1.5 h after EGF pellet implantation or DES treatment. Enhanced nuclear retention of ER after EGF treatment was further corroborated by the immunodetection of greater levels of ER protein, which migrated at ≈65 kDa in uterine nuclear extracts as detected with the ER monoclonal antibody H-222 (Fig. 4*A*). The protein that migrated at ≈54 kDa is a proteolytic fragment of the ER (23). Since a corresponding decrease in cytosolic ERs was observed by both receptor binding and Western blot, an actual redistribution of receptors probably occurs rather than simply a change in the amount of ER (data not shown).

Since interaction of the ER with the ERE is a prerequisite for transcriptional activation, the nuclear ER from EGF-treated mice was tested for this ability to determine if the

Table 1. [^{3}H]Thymidine-labeled nuclei in uterine luminal epithelium

Treatment	% labeled nuclei per unit basement membrane
Vehicle/placebo	7.8 ± 2.2
Vehicle/EGF	75.3 ± 1.7
ICI 164,384/placebo	7.0 ± 4.3
ICI 164,384/EGF	17.9 ± 3.7*
Vehicle/saline	4.4 ± 0.72
Vehicle/DES	71.4 ± 3.0
ICI 164,384/saline	5.0 ± 1.2
ICI 164,384/DES	18.6 ± 6.8*

Asterisks indicate that the effects of EGF and DES are significantly attenuated by pretreatment with ICI 164,384 as assessed by a two-way analysis of variance ($P < 0.01$). F values for the interaction of EGF and DES with ICI 164,384 are $F_{1,21}$ = 80.0 and $F_{1,22}$ = 44.4, respectively (N = 6–8 mice). Data are expressed as the mean ± SEM.

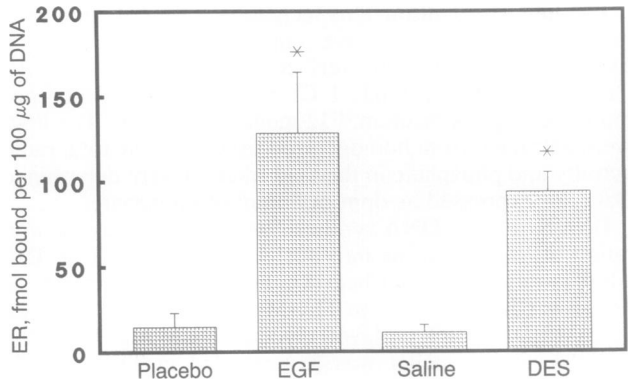

FIG. 3. [^{3}H]Estradiol binding in uterine nuclear fractions after EGF or DES treatment. Ovariectomized mice were treated with vehicle or DES (5 μg/kg) or received placebo or EGF pellet implants. Mice were killed 1.5 h after pellet implantation or injection and the uteri were frozen on dry ice and stored at −100°C until the [^{3}H]estradiol binding assay was performed (19). Data are from one representative experiment of three experiments and are expressed as fmol of ER bound per 100 μg of DNA. The asterisk indicates treatment group different from control group [$P < 0.01$ (Student's unpaired t test)]. Each bar represents the mean ± SD of three samples each consisting of a pool of three uteri.

increased level of ER in the nucleus consisted of ERs that could interact with the ERE in the same manner as after estrogen treatment. The interaction of the ER from nuclear extracts of uterine tissue from DES- and EGF-treated mice

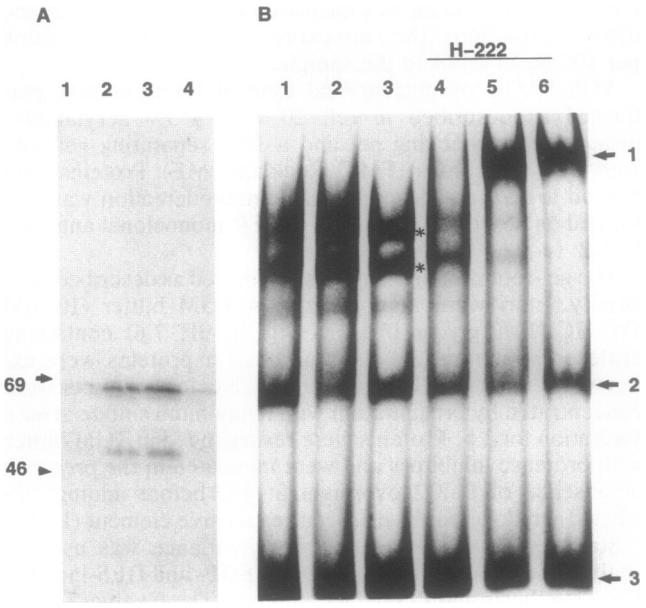

FIG. 4. Effect of EGF on nuclear ER immunolocalization and binding to the vitellogenin A2 ERE. Mice were treated as described in Fig. 3. (A) SDS/PAGE of uterine nuclear fractions and ER immunodetection. Each lane contained 150 μg of protein. Lanes: 1, vehicle; 2, DES; 3, EGF pellet; 4, placebo pellet. Positions of ^{14}C-labeled molecular mass markers (in kDa) are denoted by arrows to the left. (B) Uterine nuclear extracts were assayed for ER binding to the vitellogenin A2 ERE by using a gel-retardation assay as described (22). Uterine nuclear proteins were incubated in the presence or absence of anti-ER monoclonal antibody H-222 and were then incubated with a ^{32}P-labeled vitellogenin A2 ERE. The complexes were separated on a 5.625% polyacrylamide gel. Each lane contained 25 μg of protein. Lanes: 1, placebo pellet; 2, EGF pellet; 3, estradiol control; 4–6, same order as lanes 1–3 except extracts were incubated with H-222. Asterisks indicate specific ER–ERE complexes. Arrows: 1, H-222–ER–ERE complexes; 2, nonspecific band; 3, free ^{32}P-labeled vitellogenin A2 ERE.

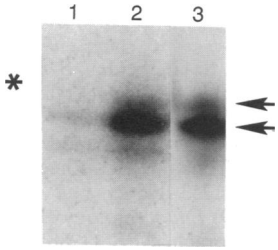

FIG. 5. Heterogenous nuclear ER forms induced by EGF treatment. Animals and tissue were treated as in Fig. 3. Nuclear proteins were extracted from uterus, precipitated with ammonium sulfate, and then in preparation for the gel-retardation assay, were subjected to SDS/PAGE on a 32-cm Tall Boy gel (Hoeffer Scientific) and transferred to nitrocellulose, and the ER was detected with anti-ER monoclonal antibody H-222. Lanes: 1, placebo pellet; 2, EGF pellet; 3, DES. The two ER bands that form the doublet are marked by arrows. The lowest molecular mass band observed in both placebo and EGF samples is believed to be a proteolytic fragment of the ER (23). The asterisk indicates the position of the 69-kDa molecular mass marker.

with the ERE was assessed using a vitellogenin A2 ERE in a gel-retardation assay. The results shown in Fig. 4*B* demonstrate that the band-shifting pattern observed after EGF treatment was very similar to that seen after estradiol administration. The two specific ER–ERE complexes (lanes 2 and 3, marked with asterisks) present after EGF or estradiol treatment were further shifted by addition of H-222 antibody (lanes 5 and 6, arrow 1), which demonstrated that immunoreactive ER was present in these complexes. A similar amount of ER and band-shifting intensity in nuclear extracts was observed after EGF treatment when compared with sham-operated DES-treated mice (data not shown). The results suggest that the nuclear ER from EGF-treated animals interacts with the DNA in a manner similar to that observed after estrogen treatment.

This laboratory has demonstrated (19) that *in vivo* estrogen treatment induces heterogenous nuclear forms of the 65- and 66.5-kDa ER in uterine nuclear extracts. This type of phenomenon has also been observed with chicken oviduct PR after progesterone treatment (24). Phosphorylation of both ER forms is stimulated by estrogen treatment but this effect is more prominent for the higher molecular weight form found exclusively in the nuclear fraction (20). Similar to the effect of estrogen, EGF treatment induced the appearance of the 66.5-kDa nuclear form of the ER (Fig. 5, lane 2). A typical uterine nuclear extract from DES-treated mice is shown in Fig. 5, lane 3. Doublet formation is not detected in the cytosol after DES or EGF treatment (refs. 19 and 20 and data not shown).

DISCUSSION

It is widely accepted that the members of the steroid hormone receptor superfamily are transcriptional enhancers when activated by their cognate steroid ligand (25). However, the evidence presented in this report suggests a role for the ER in mediating the physiologic actions of EGF in the murine female reproductive tract in the absence of estrogen, which raises the possibility of interactions between steroid receptors and polypeptide growth factor signaling pathways.

The mechanism of inhibition of steroid action elicited by steroid receptor antagonists is not completely clear and differs among the various agents. It has been suggested that some act as partial agonists or competitive antagonists or may promote or inhibit conformational changes of the receptor (26). For ICI 164,384, in the mouse uterus, the actual amount of ER decreases and the ER is not detectable for at least 16 h after antagonist administration in mouse uterus (27). This antagonist also causes a 60% decrease in ER level in MCF-7 breast cancer cells (28). Thus, the mechanism of antagonism of the biological effects of both DES and EGF in this study may be explained by the presence of diminished amounts of available ER. The responses to EGF evaluated in this study were inhibited by at least 50%. Although the blockade of PI lipid turnover induced by EGF was significantly less than that of DES, it is quite plausible that EGF elicits the remainder of its effect on PI lipid metabolism through other mechanisms or transcription factors. The data presented here demonstrate that the ER transcription factor is necessary for at least a portion of the biological responses to EGF in the uterus.

It is intriguing that the biochemical attributes of the ER after estrogen or EGF treatment are similar. It is thought that the ER binds more tightly to chromatin after *in vivo* steroid treatment and that a more highly phosphorylated form of ER is produced that is found only in the nuclear fraction (20). We have shown that EGF administered to the whole animal mimics the effects of estrogen on these biochemical characteristics. The ER seems to behave as if it is occupied by ligand, but to our knowledge serum estradiol is undetectable after EGF treatment of ovariectomized mice (8). Furthermore, EGF-induced uterine DNA synthesis occurred in mice that were hypophysectomized, adrenalectomized, and ovariectomized (8). In addition, the aforementioned cell culture results obtained in the absence of steroids that have been reported by several other groups (9–12) are suggestive of interactions between extracellular ligands and steroid receptors and support our *in vivo* observations. It remains to be proven, however, whether EGF can actually induce transcriptional activation through an ERE in the absence of estrogen. The studies by Power *et al.* (9, 10) that demonstrated that dopamine could activate the ER, PR, and COUP transcription factor in transfected CV1 cells support such a possibility. PR activation by dopamine, but not progesterone, was dependent on a specific serine residue in the PR that is phosphorylated after treatment with dopamine, an activator of adenylate cyclase. In addition, in the absence of progesterone, the PR could activate transcription after cells were treated with agents that enhance phosphorylation (presumably of the PR or other proteins involved in transcription) (29). These observations support a role for phosphorylation in the interaction of the extracellular ligand and the nuclear receptor. Certainly, EGF induces phosphorylation of various second messengers, thereby activating their signaling cascades (14, 15, 30, 31). One hypothesis to explain the results presented here is that EGF activates its receptor tyrosine kinase, which, in turn, influences key signaling intermediates that interact with the ER in the nucleus.

In summary, these results demonstrate that the action of a polypeptide growth factor may modulate a classical steroid hormone receptor, which adds another dimension to the autocrine loop model. Estrogens may upregulate autocrine or paracrine polypeptide growth factor signaling pathways whose effects, in turn, may be partially mediated by the ER itself. Our results in the whole animal suggest that cross-talk between growth factors and steroid hormone receptors may be relevant to the regulation of normal growth and differentiation in the female reproductive tract. Thus, the earlier hypothesis that steroid receptor transcriptional enhancers are activated only in the presence of steroid ligand may not be totally valid. Finally, the mechanism suggested in this report may be of global importance in understanding the physiological mechanisms of extracellular mitogen signaling interactions with transcription factors in the nucleus.

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