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Requirement of Estrogen Receptor-α in Insulin-like Growth Factor-1 (IGF-1)-induced Uterine Responses and *in Vivo* Evidence for IGF-1/Estrogen Receptor Cross-talk*

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In the uterus insulin-like growth factor-1 (IGF-1) signaling can be initiated by estradiol acting through its nuclear receptor (estrogen receptor (ER)) to stimulate the local synthesis of IGF-1. Conversely, *in vitro* studies have demonstrated that estradiol-independent ER transcriptional activity can be induced by IGF-1 signaling, providing evidence for a cross-talk mechanism between IGF-1 and ER. To investigate whether ERα is required for uterine responses to IGF-1 *in vivo*, both wild-type (WT) and ERα knockout (αERKO) mice were administered IGF-1, and various uterine responses to IGF-1 were compared. In both WT and αERKO mice, IGF-1 treatment resulted in phosphorylation of uterine IGF-1 receptor (IGF-1R) and formation of an IGF-1R/insulin receptor substrate-1/phosphatidylinositol 3-kinase signaling complex. In addition, IGF-1 stimulated phosphorylation of uterine Akt and MAPK in both WT and αERKO mice. However, IGF-1 treatment stimulated BrdUrd incorporation and proliferating cell nuclear antigen expression in WT uteri only. To determine whether ERα can be activated *in vivo* by IGF-1 signaling, transgenic mice carrying a luciferase gene driven by two estrogen response elements (ERE-luciferase mice) were utilized. Treatment of ovariectomized ERE-luciferase mice with IGF-1 resulted in an increase in uterine luciferase activity that was attenuated in the presence of the ER antagonist ICI 182,780. Together these data demonstrate that 1) functional signaling proximal to IGF-1R is maintained in the αERKO mouse uterus, 2) ERα is necessary for IGF-1 induction of uterine nuclear proliferative responses, and 3) cross-talk between IGF-1R and ER signaling pathways exists *in vivo*.

Epithelial cells of the mammalian uterus undergo a wave of DNA synthesis followed by mitosis in response to 17β-estradiol (E_2),[1] which regulates the transcription of numerous target genes by binding to and activating the nuclear estrogen receptor (ER). Among the genes identified as targets for regulation by the E_2/ER complex in the uterus is that encoding insulin-like growth factor-1 (IGF-1). Studies have demonstrated that rodent uterine IGF-1 mRNA levels increase after exposure to E_2 (1, 2). Furthermore, presumably through increasing local production of IGF-1, E_2 has been shown to stimulate uterine IGF-1 receptor (IGF-1R) signaling as measured by tyrosine phosphorylation of IGF-1R and the formation of a signaling complex composed of IGF-1R, insulin receptor substrate-1 (IRS-1), and p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) (3, 4). These studies suggested that IGF-1 signaling is involved in E_2-induced uterine growth, and in support of this mechanism, other studies have shown that, like E_2, IGF-1 can induce DNA synthesis in cells of the rodent uterus (5). A more recent study further demonstrated a role for IGF-1 in E_2-induced uterine proliferation by demonstrating that IGF-1 is required for E_2-induced uterine epithelial cell mitosis (6). In that study, DNA synthesis occurred in IGF-1 knockout (IGF-1KO) mouse uteri in response to E_2. However, there was a significant decrease in the E_2-stimulated mitotic index in the uterine epithelium, indicating that IGF-1 is necessary for the cells to progress properly through mitosis in response to E_2. Finally, a recent study by this laboratory has confirmed that increased synthesis of uterine IGF-1 mRNA and stimulation of uterine IGF-1R signaling by E_2 are ERα-dependent events (7). Collectively, these studies have illustrated that E_2, by acting through ERα, stimulates IGF-1/IGF-1R signaling and increases uterine epithelial cell mitosis in the mouse uterus.

Conversely related *in vitro* studies have described another level of integration between the ER and IGF-1 signaling pathways by demonstrating that ER-mediated responses can be induced or increased by IGF-1. An initial study showed that, in the absence of E_2, IGF-1 can stimulate the phosphorylation of ER and induce the expression of an estrogen-responsive, ERE-containing reporter construct, an effect that was blocked by antiestrogen (8). Subsequent studies confirmed these findings and further showed that IGF-1 activation of the ERE-reporter construct occurred only in the presence of ER, demonstrating that IGF-1 was indeed inducing reporter gene transcription through activation of ER (9). More recently, the mechanism through which IGF-1 activates ER was investigated, and it was

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[1] The abbreviations used are: E_2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; αERKO, ERα knockout; WT, wild type; IGF-1, insulin-like growth factor-1; IGF-1R, IGF-1 receptor; IRS-1, insulin receptor substrate-1; PI 3-kinase, phosphoinositol 3-kinase; BrdUrd, bromodeoxyuridine; PCNA, proliferating cell nuclear antigen; MAPK, mitogen-activated protein kinase.

determined that IGF-1 activation of ER is mediated, at least in part, through a mechanism involving Akt (10). Thus, a model develops in which it appears that E_2, acting through its nuclear receptor, initiates responses that include the induction of IGF-1. The IGF-1, in turn, interacts with its cognate membrane receptor to initiate a signaling cascade that involves phosphorylation-dependent kinases, such as Akt. The membrane receptor-mediated IGF-1 signaling pathway further appears to be linked to the ERα pathway via a cross-talk mechanism whereby activation of the IGF-1R can stimulate "ligand-independent" ERα-mediated responses.

Recently, the ERE-luciferase mouse was described as an innovative model system for profiling the *in vivo* dynamics of ER activity (11). This mouse has been engineered to carry a luciferase transgene that is driven by two EREs from the vitellogenin promoter upstream of a minimal thymidine kinase promoter. As a result, the luciferase gene is expressed only in response to transcriptionally active ER. In an effort to further understand the cross-talk between the IGF-1/IGF-1R and E_2/ERα signaling pathways, the current study was designed to evaluate the *in vivo* role of ERα in IGF-1-mediated uterine responses. By utilizing both the ERα knockout (αERKO) and ERE-luciferase mouse models, we establish the requirement for ERα in IGF-1-induced uterine nuclear responses and provide *in vivo* molecular evidence demonstrating the existence of cross-talk between the uterine IGF-1 and ER signaling pathways.

EXPERIMENTAL PROCEDURES

Materials—17β-Estradiol was purchased from Steraloids (Newport, RI). Long R3 IGF-1, a biologically active IGF-1 derivative with low affinity for IGF-1-binding proteins, was obtained from Diagnostic Systems Laboratories (Webster, TX). The following antibodies were purchased: IGF-1R (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phosphotyrosine (PY20, ICN Biomedicals, Inc., Aurora, OH); anti-IRS-1 and anti-p85 (Upstate Biotechnology, Lake Placid, NY); Akt, phospho Akt, p42/44 MAPK, and phospho p42/44 MAPK (Cell Signaling Technology, Beverly, MA); PCNA 19A2 (Beckman Coulter, Hialeah, FL), biotinylated anti-mouse IgM (Vector Laboratories, Inc., Burlingame, CA); BrdUrd (Accurate Chemical and Scientific Corp., Westbury, NY).

Animals—All animals were handled in accordance with an approved NIEHS, National Institutes of Health animal study protocol. Adult female WT, αERKO, and ERE-luciferase mice were ovariectomized, held for 10–14 days to clear endogenous ovarian hormones, and then used in studies. Animals were either injected intraperitoneally with 20 μg/kg estradiol (E_2) in saline, or a mini-osmotic pump (Alza Corporation, Palo Alto, CA; 3 days, 1 μl/h) containing 100 μl of 2.4 μg/μl-long R3 IGF-1 dissolved in 0.1 M acetic acid was implanted in the peritoneal cavity. Control animals were injected with saline. For BrdUrd incorporation and PCNA immunohistochemistry, mice were injected with BrdUrd (1 mg/mouse) 16 h after E_2 or IGF-1 treatments, and uteri were collected 1 h later. A segment of each uterine horn was frozen for Western analysis, and the rest of the uterine tissue, as well as a piece of the intestine, was fixed in 10% cold formalin and processed for BrdUrd or PCNA staining.

For studies utilizing ERE-luciferase mice, ovariectomized mice were injected intraperitoneally with either 50 μg/kg E_2, vehicle (vegetable oil), or two injections of 200-μg-long R3 IGF-1 in 200 μl vegetable oil, with the second injection of IGF-1 given 3 h after the first injection. Uteri were collected 6 h after injection with E_2 or vehicle or 3 h following the second IGF-1 injection.

IGF-1R Immunoprecipitation—IGF-1 signaling components were analyzed by immunoprecipitation and Western blot procedures as previously described (7). In brief, frozen samples of uterine horns were homogenized at 4 °C in 200 μl of solubilization buffer (20 mM HEPES, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100) containing protease and phosphatase inhibitors (20 μg/ml aprotinin, 20 μg/ml leupeptin, 4 μg/ml a-phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 20 mM NaF, 0.05 mM Na_2MoO_4). Homogenates were normalized for protein concentration (BCA Protein Assay, Pierce), and equivalent amounts of protein were pre-cleared by incubation with protein A-Sepharose and then subjected to immunoprecipitation with 5 μg of anti-mouse IGF-1R polyclonal antibody with an equal volume of 2× immunoprecipitation buffer (100 mM Tris, pH 8.5, 300 mM NaCl, 10 mM EDTA, 1% Triton X-100) for 1 h at 4 °C. Antigen-antibody complexes were captured with protein A-Sepharose for 3 h and subjected to three sequential washes (Wash 1: 0.5% Triton X-100, 1 mM EDTA, 500 mM NaCl in 50 mM Tris, pH 8.5; Wash 2: 0.5% Triton X-100, 1 mM EDTA, 150 mM NaCl in 50 mM Tris, pH 8.5; Wash 3: 0.1% Triton X-100 in 10 mM Tris, pH 8.5). Precipitated antigen was eluted from the protein A-Sepharose by resuspending it the pellets in Laemmli sample buffer and boiling for 5 min.

Immunoblots—Immunoprecipitated proteins or uterine extracts were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked in either Tris-buffered saline with 5% bovine serum albumin for detection of IGF-1R, phosphotyrosine, Akt, phospho-Akt, p42/44 MAPK, phospho-p42/44 MAPK, and p85, or phosphate-buffered saline with 3% nonfat dry milk for detection of IRS-1. Membranes with IGF-1R immunoprecipitates were probed with anti-phosphotyrosine, anti-IRS-1, anti-p85, or anti-IGF-1R antibodies. Membranes with total uterine proteins were probed with anti-Akt, anti-phospho-Akt, anti-p42/44 MAPK, or anti-phospho p42/44 MAPK antibodies. Enhanced Chemiluminescence (ECL, Amersham Pharmacia Biosciences) was used for detection of immunoreactive proteins according to the manufacturer's specifications.

Immunohistochemistry—Immunohistochemical staining of PCNA was performed as described previously (7). The primary antibody used was anti-mouse PCNA 19A2 (Beckman Coulter), and the secondary antibody used was biotinylated anti-mouse IgM (Vector Laboratories). ExtrAvidin peroxidase (Sigma) was added and detected with the NovaRed detection system (Vector Laboratories) according to the manufacturer's instructions. BrdUrd was detected using the BrdUrd staining kit from Oncogene (Boston, MA) according to the manufacturer's instructions. Immunohistochemistry for luciferase was performed as described previously (11). The primary antibody used was a polyclonal anti-luciferase antibody (Sigma, 1:1800 dilution in phosphate-buffered saline with 10% goat serum and 0.3% Tween 20) for 16 h; an anti-rabbit secondary antibody (1:200 dilution in phosphate-buffered saline with 1% goat serum and 0.3% Tween 20) was also used. Antibody-antigen detection was performed with the ABC kit (Vector Laboratories) according to manufacturer's specifications. Immunostaining was visualized by exposure to 3,3′-diaminobenzidine.

Luciferase Assays—Assays for uterine luciferase activity were performed as described previously (11). Briefly, uterine extracts were prepared by homogenization in 500 μl of 100 mM KPO_4 lysis buffer (pH 7.8) containing 1 mM dithiothreitol, 4 mM EGTA, 4 mM EDTA, 0.7 mM phenylmethylsulfonyl fluoride followed by three cycles of freezing-thawing and 30 min of microcentrifugation at maximum speed. Supernatants containing luciferase were collected, and the protein concentration was determined by Bradford's assay. Luciferase enzymatic activity in tissue extracts was measured by a commercially available kit (Luciferase Assay System, Promega, Madison, WI) according to the manufacturer's instructions. The light intensity was measured with a luminometer (Lumat LB 9501/16, Berthold) over 10 s and was expressed initially as relative light units over 10 s/μg proteins.

Statistical Analysis—Luciferase activities were analyzed by analysis-of-variance followed by *post hoc* analyses with the Scheffé test.

RESULTS

Analysis of Estrogen- and IGF-1-stimulated Uterine DNA Synthesis and PCNA Expression—One indication of estrogen activity is the wave of DNA synthesis and mitosis that occurs in the uterine epithelium following administration of hormone. [^{3}H]thymidine incorporation has been shown to increase to a maximal level at ~16 h following E_2 treatment of ovariectomized mice (12). Previous studies indicated that this estrogen-dependent response is negligible in the αERKO mouse (13). To determine whether IGF-1 can initiate a similar DNA synthesis response in the αERKO uterus as well as to identify the specific uterine cell types that respond to IGF-1, immunohistochemical analysis of BrdUrd incorporation was performed. As expected, E_2 treatment of WT animals resulted in extensive BrdUrd incorporation in the luminal and glandular epithelium (Fig. 1*A*) 16 h following hormone treatment. Long R3 IGF-1 infusion also resulted in positive BrdUrd immunoreactivity in epithelial cells. However, the response was decreased compared with that of the E_2 treatment group (Fig. 1*A*). This confirms that IGF-1 signaling initiates an estrogen-like DNA synthesis response in

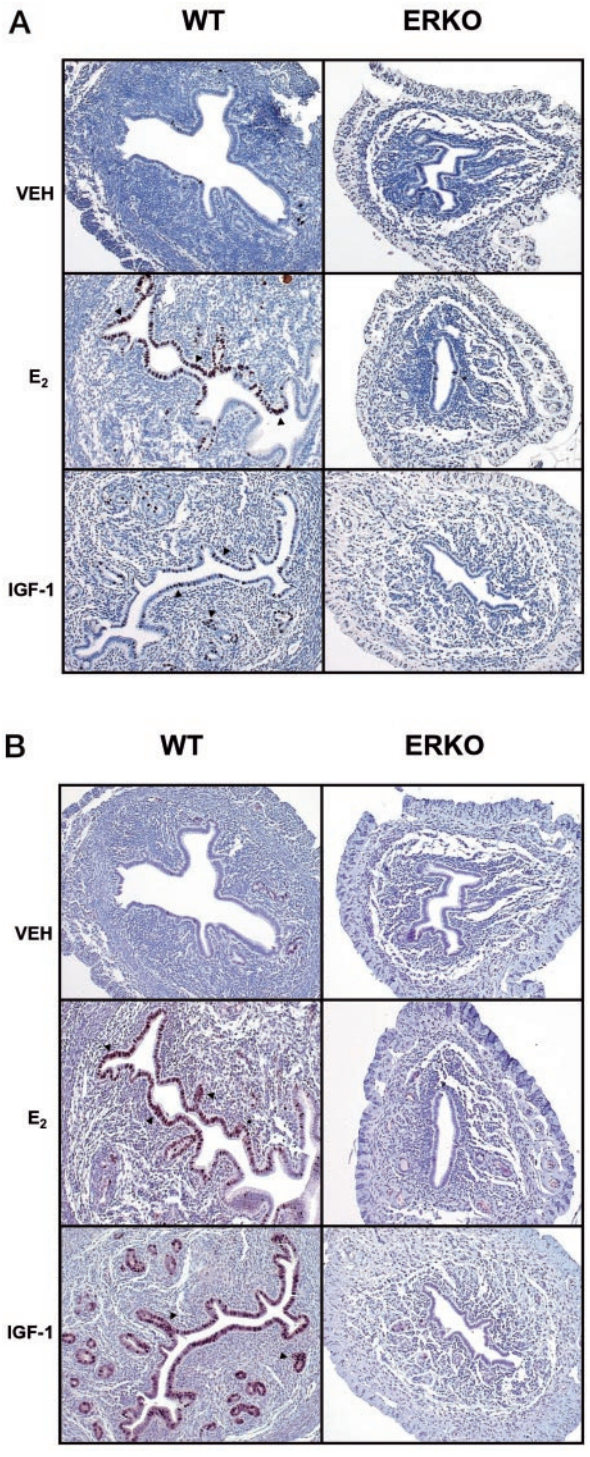

FIG. 1. **Uterine BrdUrd and PCNA immunohistochemistry after E2 or IGF-1 treatment.** 16 h after treatment with vehicle (*VEH*), E_2, or IGF-1, tissues were prepared as described under "Experimental Procedures" and stained for BrdUrd (*A*, *dark brown/black* staining) or PCNA (*B*, *dark red* staining). In each case, the *left panels* represent WT uteri, and the *right panels* represent αERKO uteri. The *arrows* point to immunoreactive epithelial cells. Each treatment group contained three or four mice, and the panels shown are representative of two separate experiments. αERKO uteri were photographed at a higher magnification (40×) than the WT uteri (20×) to better present the absence of immunoreactivity in the αERKO uteri.

the uterine epithelium and that the response to this particular method of long R3 IGF-1 treatment is less extensive than the response to E_2. Uteri from αERKO mice revealed no BrdUrd staining at 16 h following exposure to E_2 or long R3 IGF-1 (Fig. 1*A*), indicating that DNA synthesis is not induced by either E_2 or IGF-1 pathways in the absence of ERα.

Adjacent tissue sections from WT and αERKO uteri were also stained with antibody for PCNA as a marker for cell proliferation. As with the BrdUrd staining, both E_2 and long R3 IGF-1 induced an increase in PCNA immunoreactivity in the WT uterus, whereas neither E_2 nor long R3 IGF-1 treatment resulted in increased PCNA staining in the αERKO uterus (Fig. 1*B*). These results suggest that ERα is an integral component in the IGF-1-stimulated uterine epithelial proliferative response. When WT mice were given long R3 IGF-1 together with E_2, the response did not appear to be increased compared with E_2 alone (data not shown), suggesting that both of these stimuli exert their effects on DNA synthesis and PCNA expression through a common pathway.

The DNA synthesis observed in the wild-type uterus following IGF-1 infusion is less robust than that observed in response to E_2, suggesting that ERα may not have full transcriptional activity in the absence of estrogen ligand. Although this finding is in agreement with *in vitro* data showing that ERE-driven reporter and endogenous genes are not as responsive to IGF-1 as to E_2 (9, 34), it should also be considered that the peak time for DNA synthesis after IGF-1 exposure may be decreased compared with the peak time for DNA synthesis following exposure to E_2.

Proximal IGF-1R Signaling Is Preserved in the αERKO Uterus—To eliminate the possibility that dysfunctional IGF-1R signaling was the basis for the lack of IGF-1-induced DNA synthesis and PCNA expression in the αERKO uterus, ovariectomized mice were treated with E_2 or infused with long R3 IGF-1, and uterine extracts were isolated and analyzed for IGF-1-stimulated responses. Because E_2-stimulated IGF-1 production is an induced response that occurs for only a fixed amount of time (14), phosphorylation of IGF-1R can only be measured from ~3–6 h after exposure to E_2 (3). Therefore, for this set of experiments extracts from E_2-treated animals were prepared 6 h after injection with E_2, whereas IGF-1-treated uteri were prepared 16 h after continuous infusion of long R3 IGF-1. Following long R3 IGF-1 treatment and in agreement with previously published data (7), uterine IGF-1R was tyrosine-phosphorylated in αERKO mice, confirming that the delivery of long R3 IGF-1 was effective and that the IGF-1R in the αERKO uterus was responsive to IGF-1 (Fig. 2*A*). Also as previously described, treatment with long R3 IGF-1 increased the formation of an IGF-1R signaling complex comprising IGF-1R, IRS-1, and p85 ((Refs. 3 and 4, Fig. 2*B*). Furthermore, after long R3 IGF-1 treatment, the IRS-1 associated with IGF-1R was tyrosine-phosphorylated (Fig. 2*A*). None of these responses occurred in the αERKO uterus after treatment with E_2 (Fig. 2, *A* and *B*). In wild-type mice IGF-1R activation and recruitment of IRS-1 and p85 occur in response to either E_2 or long R3 IGF-1 infusion (data not shown; Ref. 7), confirming that in this mouse model either E_2 or IGF can induce IGF-1R activation.

The p85 component associated with the IGF-1R complex is the regulatory subunit of PI 3-kinase, and the PI 3-kinase substrate Akt is phosphorylated and activated in response to IGF-1 by this pathway (10, 15). To further characterize the downstream IGF-1R signaling events in the αERKO, uterine Akt phosphorylation was monitored after treatment with E_2 or long R3 IGF-1. As shown in Fig. 3*A*, E_2 treatment increased phosphorylation of Akt on Ser 473 in the WT but not the αERKO uterus, whereas IGF-1 treatment stimulated uterine Akt phosphorylation in both genotypes. This indicates that activation of the PI 3-kinase pathway by IGF-1 can occur in the αERKO but that E_2-initiated Akt activation requires the presence of functional ERα. Taken together these data (Figs. 2 and

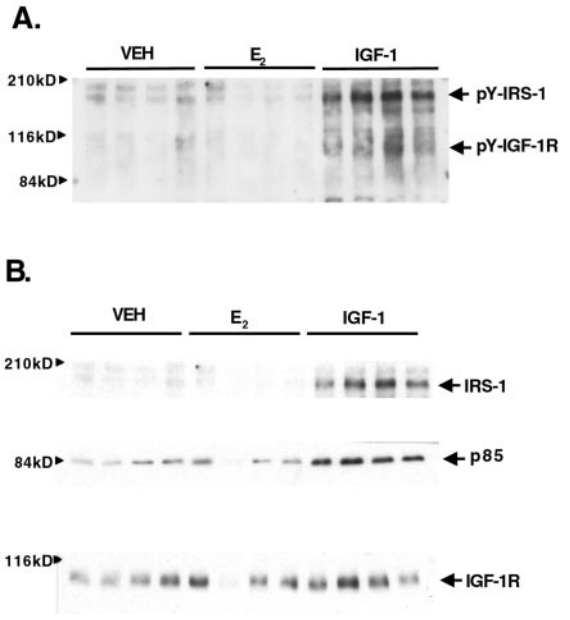

FIG. 2. **Proximal uterine IGF-1R signaling is functional in the αERKO mouse uterus.** Six h after treatment with vehicle (*VEH*) or E_2 or 16 h after treatment with long R3 IGF-1, uterine homogenates were prepared, and IGF-1R was immunoprecipitated as described under "Experimental Procedures" and analyzed by immunoblotting for phosphotyrosine (*pY*), IRS-1, p85, and IGF-1R. Each *lane* contains a sample from a single uterus and is representative of uteri from at least 3 separate experiments where a treatment group consisted of no less than 3 animals. *A*, IGF-1 R immunoprecipitates immunoblotted and probed with anti-pY antibody. *B*, IGF-1R immunoprecipitates were immunoblotted and probed with antibodies to IRS-1, p85, or IGF-1R proteins.

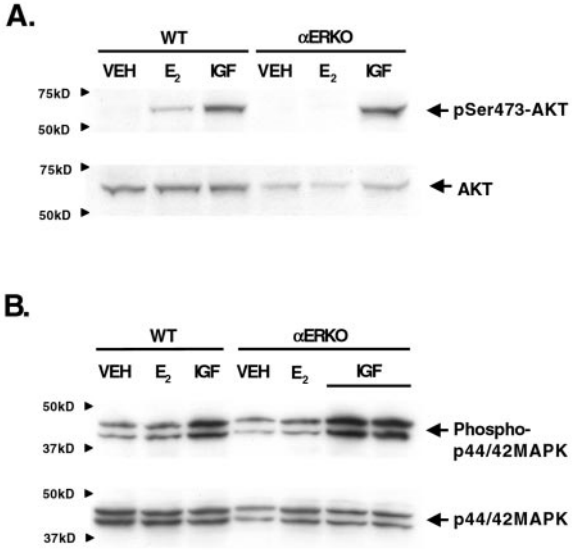

FIG. 3. **Activation of uterine Akt by E_2 or IGF-1.** Six h after treatment with vehicle (*VEH*) or E_2 or 16 h after treatment with IGF-1 (*IGF*), uterine extracts were analyzed by immunoblotting using antibodies to Akt or phosphoserine 473 Akt (*pSer473-Akt*) (*A*) or to p44/p42 MAPK or phosphorylated p44/p42 MAPK (*B*). Each *lane* represents a single animal and no less than 3 animals/treatment group.

3*A*) demonstrate that the αERKO uterus is fully responsive to IGF-1 with respect to activation of signaling events proximal to IGF-1R.

Mitogen-activated protein kinase (MAPK) has been shown to be activated by E_2 *in vitro* (16, 17), and activation of MAPK by IGF-1 has also been demonstrated *in vitro* in hormone-responsive cells (18). In addition, phosphorylation of ER by MAPK has been reported (19, 20). In WT uteri IGF-1 increased the phosphorylation of uterine p44/42 MAPK (Fig. 3*B*). In the αERKO uteri IGF-1 also increased p44/42 MAPK phosphorylation, indicating that ERα is not required for MAPK activation by IGF-1 in the uterus. A basal level of p44/42 MAPK phosphorylation was observed in the control groups of both genotypes (Fig. 3*B*). Phosphorylation of MAPK by E_2 above basal levels did not occur in the majority of the WT samples; however, the results varied, possibly because of a suboptimal sampling time for this response after E_2 treatment, and further investigation is necessary to determine the effects of E_2 on MAPK activity *in vivo*. MAPK was not activated above basal levels by E_2 in any of the αERKO uterine samples.

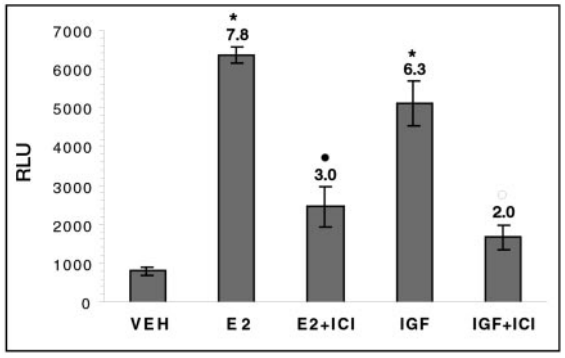

FIG. 4. **IGF-1-induced activation of uterine ERE-luciferase activity.** Six h after treatment with vehicle (*VEH*), E_2, IGF-1 (*IGF*), E_2 + ICI 182,780 (*ICI*), or IGF + ICI, tissue extracts were prepared and assayed for luciferase activity as described under "Experimental Procedures." Each *bar* is the average of at least 6 individual animals. *Error bars* represent standard deviation. *, $p < 0.01$ compared with vehicle; ●, $p < 0.01$ compared with E_2; ○, $p < 0.01$ compared with IGF-1. The *numbers* given *above* each *bar* indicate the average fold-induction above vehicle.

IGF-1 Stimulates ERα Transactivation in Vivo—The data described in the preceding sections demonstrate that IGF-1 alone can not reproduce E_2-induced effects and indicate that ERα plays an active role in IGF-1-induced proliferative responses in the uterus rather than simply a permissive role through induction of IGF-1 synthesis. To determine whether long R3 IGF-1 can activate ER *in vivo*, ovariectomized adult ERE-luciferase mice were treated with E_2 or long R3 IGF-1. Based on the initial data described for these mice (11), uteri were removed and assayed for luciferase activity 6 h following injection of E_2. Additionally, a pilot study demonstrated that 3 h after injection of IGF-1 in oil, uterine IGF-1R was tyrosine-phosphorylated; 6 h after injection uterine IGF-1R was still tyrosine-phosphorylated, although to a lesser extent (data not shown). Therefore, giving two successive injections of long R3 IGF-1, 3 h apart, was chosen as the course for administering IGF-1 for these experiments. Treatment with E_2 stimulated approximately an 8-fold increase in the amount of uterine luciferase activity compared with vehicle-treated controls. Treatment with long R3 IGF-1 increased the amount of luciferase activity by ~6-fold above vehicle-treated controls (Fig. 4). To verify that in this model system the IGF-1 was indeed inducing luciferase expression through an ER-dependent mechanism, mice were simultaneously treated with long R3 IGF-1 and the ER antagonist ICI 182,780. Cotreatment with ICI 182,780 abrogated the induction of luciferase activity that was observed in response to both E_2 and long R3 IGF-1 (Fig. 4). As a control for both E2 and IGF-1 activity, livers from treated mice were also sampled and assayed for luciferase activity. Both E2 and IGF-1 stimulated luciferase activity in liver, and ICI inhibited this stimulation (data not shown).

It is noteworthy that the IGF-1-induced activation of ER-mediated transcription measured in the ERE-luciferase mouse

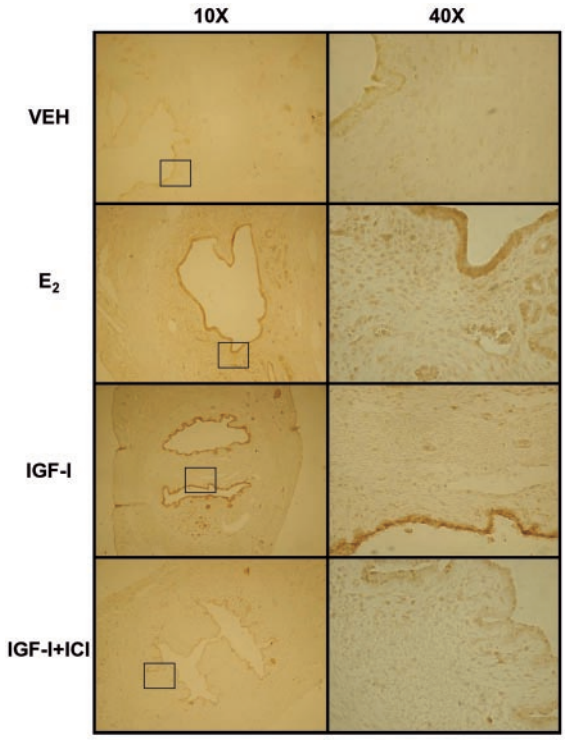

FIG. 5. **Immunolocalization of E_2 and IGF-1-induced luciferase expression.** Six h after treatment with vehicle (*VEH*), E_2, IGF-1, or IGF-1 + ICI 182,780 (*IGF-1 + ICI*), uterine sections were prepared, and immunolocalization of luciferase was performed as described under "Experimental Procedures." Each section is representative of at least 4 animals/treatment group.

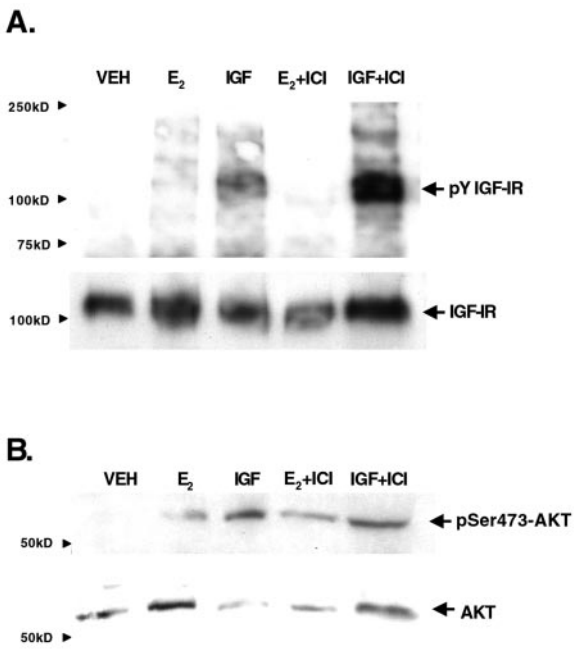

FIG. 6. **Activation of IGF-1R signaling in ERE-luciferase mice.** Six h after treatment with vehicle (*VEH*), E_2, IGF-1, E_2 + ICI 182,780 (*ICI*), or IGF-1 + ICI, uterine homogenates were prepared and IGF-1R immunoprecipitations were performed as described under "Experimental Procedures" and analyzed by immunoblotting for phosphotyrosine (*pY*) and IGF-1R. Additionally, tissue extract was immunoblotted and probed with antibodies for Akt and phosphoserine 473 Akt (*pSer473-Akt*). Each *lane* is a sample from an individual animal and is representative of at least 4 animals/treatment.

was comparable with that observed in response to E_2, unlike the stimulation of DNA synthesis, which may suggest that in this case ER was fully activated. However, it should be recognized that treatment of ovariectomized mice with E_2 or IGF-1 is a form of pharmacological manipulation, and both experiments administered IGF-1 in separate ways and at different concentrations. In addition, the luciferase response measures a single reporter gene that has been engineered for maximal induction by ER and is not comparable with the mitotic response, which is the result of modulation of many necessary components and might have varying sensitivity to the growth factor activated ER. Therefore, differences between local concentrations of E_2 and IGF-1 that may arise from endogenous *versus* exogenous hormone sources, methods of administration, and differences in the measured response preclude any firm conclusions being drawn with respect to the potency of E_2 compared with IGF-1 in the activation of ER and downstream events.

The incorporation of BrdUrd and elevated expression of PCNA in response to IGF-1 were observed only in the uterine epithelium (Fig. 1). To identify which cells within the uterus were responding to long R3 IGF-1 by inducing ER/ERE-driven luciferase expression, immunohistochemical localization of luciferase was performed. In agreement with the stimulation of DNA synthesis and expression of PCNA, long R3 IGF-1 induced expression of luciferase in the epithelial cells of the uterus (Fig. 5).

To confirm that the long R3 IGF-1 treatment was activating signaling components of the IGF-1 pathway and, importantly, that ICI was not interfering with that activation, separate sections of the same uteri used in the above experiment were examined as described earlier. As expected, both E_2 and IGF-1 stimulated IGF-1R and Akt phosphorylation (Fig. 6, *A* and *B*), confirming that IGF-1 signaling proximal to the IGF-1R was activated. At this 6-h time point, only residual IGF-1R phosphorylation in response to E_2 could be observed. In the presence of ICI 182,780 IGF-1 also induced the activation of IGF-1R and AKT (Fig. 6, *A* and *B*), demonstrating that inhibition of IGF-1-stimulated luciferase activity by ICI 182,780 was mediated through ER antagonism and was not a result of interference between ICI 182,780 and proximal IGF-1 signaling events. ICI inhibited E_2-induced activation of IGF-1R, presumably through inhibition of IGF-1 gene expression. Interestingly, ICI did not inhibit E_2-stimulated Akt activity, indicating that E_2 may activate Akt through more than one mechanism, at least one of which may be IGF-1-independent and perhaps another of which is not affected by ICI inhibition of ER. The apparent increase in IGF-1R phosphorylation in the uteri of mice cotreated with IGF-1 and ICI, compared with IGF-1 alone, is most likely because of increased immunoprecipitation of IGF-1R and interanimal variation in the particular sample presented here (Fig. 6 *A, lower panel*).

DISCUSSION

Growth factors such as EGF and IGF-1 are believed to be an integral component in the growth response of the rodent uterus to E_2. Expression of EGF and IGF-1 as well as IGF-1R signaling can be induced by E2 (21–24), suggesting that these growth factors may play a fundamental role in mediating the uterine response to E_2. Studies utilizing the IGF-1 knockout mouse model have shown that although DNA synthesis occurs in the uterine epithelium in response to E_2, there is a significant decrease in mitotic index after E_2 treatment, indicating that estrogen must induce IGF-1 expression for a full uterine mitotic response to occur (6). It is unknown, however, whether IGF-1 replacement would rescue normal mitosis in this model. A transgenic mouse that overexpresses IGFBP-1, resulting in lower serum IGF-1 levels, and possibly lower levels of locally produced IGF-1 in the uterus, was also shown to have an impaired uterine response to E_2 (26). Both the thymidine in-

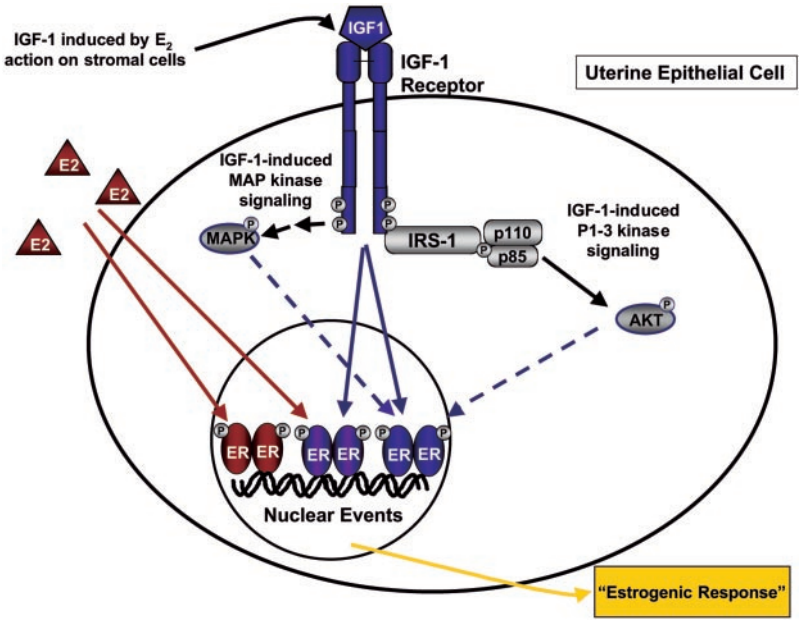

FIG. 7. **Schematic diagram of proposed model for IGF1/ER cross-talk in the uterus.** E_2 can activate ER directly through the classical E_2/ER binding mechanism (*solid red line arrow* and *red oval*). Alternatively, these data, along with previously reported *in vitro* data, demonstrate that IGF-1 can also activate ER (*solid blue line arrow* and *blue oval*), possibly through a mechanism involving PI 3-kinase/Akt and/or MAPK (*broken line arrow*). Either mechanism of ER activation appears to be sufficient to elicit a DNA synthesis response and expression of the proliferative markers (PCNA). Activation of ER appears to be the crucial molecular event for this response as in the αERKO uterus IGF-1-induced DNA synthesis does not occur in the absence of ER. It remains to be determined whether ER activation by either pathway alone is sufficient for a full estrogenic response or whether concomitant activation of ER by both pathways (*purple oval*) is necessary.

corporation and uterine weight increases that occur in response to E_2 were blunted in IGFBP-1-overexpressing mice compared with nontransgenic mice. Although both of these studies underscore the importance of IGF-1 for a complete uterine response to E_2, they provide no information about the specific role of IGF-1 in mediating the uterotropic effects of E_2.

The αERKO mouse provides an appropriate model for the expansion of parallel studies of the converse role of ERα in growth factor action to an *in vivo* system. Previous studies from our laboratory demonstrated that treatment with EGF alone stimulated an increase in [^{3}H]thymidine incorporation in the wild-type but not in the αERKO mouse uterus, providing *in vivo* evidence that ERα is necessary for growth factor-induced uterine DNA synthesis (32). In the present study we demonstrate that both IGF-1 and E_2 are capable of eliciting proliferative responses in the uterus. Furthermore, the data establish that ERα is required for a proper response to either stimulus and that the IGF-1 and ERα pathways have separate yet interacting functions.

In cell culture models with defined and controlled components, both EGF and IGF-1 have been shown to stimulate ERα-mediated responses independent of E_2 (8, 9, 27–31). These studies described the ability of growth factors to activate ER(α), allowing for the hypothesis that one role of the growth factors in the normal physiologic response of the uterus to E_2 may be the amplification of ER signaling whereby signaling through growth factor membrane receptors may result in further or more persistent activation of ERα. The recent generation of the ERE-luciferase mouse model has provided a unique tool for examining the physiological activity of estrogen receptors *in vivo*. By using this model system in the present study, we are able to report the novel observation that, in agreement with the previously described *in vitro* studies, IGF-1 can indeed activate ER-mediated transcription of a target gene *in vivo*. This transgenic mouse should provide a wealth of information regarding the role of growth factor signaling pathways in ligand-independent activation of ER as well as provide a model system for the dissection of the molecular mechanisms involved the activation of ER by growth factors.

The identification of Akt as an integral component in the mechanism of ERα activation by IGF-1 is described in a recent report demonstrating that Akt is activated by IGF-1 and is required for stimulation of ERα functions in MCF-7 cells and COS-1 cells (10). An additional study described the role of PI 3-kinase in ligand-independent activation of ERα, providing data suggesting that PI 3-kinase activates ERα through both Akt-dependent and -independent mechanisms (33). The failure of DNA synthesis to occur in the αERKO uterus even after full activation of proximal IGF-1R signaling by IGF-1, including Akt activation, suggests that the requirement of ERα for the mechanism to progress may be as a target for Akt, as the recent *in vitro* study indicates (10). The data herein do not demonstrate activation of MAPK by E_2; however, the time after E_2 exposure at which MAPK activation was measured may not be optimal. Previous *in vitro* studies demonstrated that activation of MAPK by E_2 occurred in a rapid fashion and returned to basal levels within 1 h after exposure (16, 17). Based on the results reported here that demonstrate the increased activation of uterine MAPK in response to IGF-1 in both the wild-type and αERKO uterus, a requirement for the MAPK signaling pathway in the activation of ER by IGF-1 has not been ruled out.

Estrogen is a potent mitogen in the mouse uterus, and previous studies with the αERKO have shown that ERα is essential for hormone-initiated proliferation (7, 13). In the present study, it is apparent that the role of ERα in this mechanism is not merely to induce growth factors such as IGF-1, which then lead to proliferative responses. IGF-1 infusion does not restore uterine responses in the absence of ERα even in the presence of a functional IGF-1 signaling cascade, underscoring the importance of ERα as an active mediator of IGF-1 and possibly other growth factor proliferative actions in the uterus. Furthermore, we provide novel evidence that IGF-1/ER cross-talk occurs *in vivo* by demonstrating that IGF-1 alone can activate ER-medi-

ated transcription in the mouse uterus. Based on the data presented, we hypothesize that uterine DNA synthesis can be initiated by activation of ERα either directly by binding E_2 or indirectly by IGF-1R pathway-mediated activation of ERα (Fig. 7). When E_2 does not induce IGF-1 ligand, as in the IGF-1 knockout, ERα can still be activated directly by E_2 and DNA synthesis occurs. In the αERKO not only is IGF-1 induction in response to E_2 disrupted but also ERα is not present as a target of either E_2 binding or IGF-1R signaling; therefore, neither molecule can induce DNA synthesis. These observations, combined with those reported here in the ERE-luciferase mouse, demonstrating *in vivo* activation of ER by IGF-1, illustrate the role of ERα as a target for both E_2 and IGF-1 and suggest that ERα activation by either mechanism is essential for a uterine proliferative response to occur.

With respect to the relevance of these findings to normal *in vivo* physiology, it is important to consider the role of IGF-1 in disease. In light of the present findings demonstrating that IGF-1 can activate ER transactivation functions in the absence of E_2, it may be worthwhile to consider that IGF-1 and perhaps other growth factors may affect endocrine-related cancers, even in the absence of steroid hormones, by circumventing the need for the hormone to elicit a proliferative response in target cells. It will, therefore, be both interesting and important to compare the gene expression profiles elicited by IGF-1 and E_2 to determine whether IGF-1 induces unique genes or is simply one mediator of E_2-stimulated gene expression. Additionally, a comparison of IGF-1 activation of genes regulated by ER through EREs to those regulated by ER through AP-1 sites will be informative with respect to the role of IGF-1, and potentially other growth factors, in the activation of ER-mediated gene expression. All three model systems mentioned here, the IGF-1KO, the αERKO, and the ERE-luciferase mice, can be outstanding tools for comparisons that will help broaden our understanding of the role(s) of IGF-1 in E_2-induced responses in target tissues in the context of both normal and neoplastic conditions.

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Requirement of Estrogen Receptor-α in Insulin-like Growth Factor-1 (IGF-1)-induced Uterine Responses and *in Vivo* Evidence for IGF-1/Estrogen Receptor Cross-talk

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