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Role of Insulin-Like Growth Factor-I in Regulating Estrogen Receptor-α Gene Expression

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Abstract The role of insulin-like growth factor-I (IGF-I) in regulating estrogen receptor-α (ER-α) gene expression and activity was investigated in the human breast cancer cell line MCF-7. Treatment of cells with 40 ng/ml IGF-I resulted in a 60% decrease in ER-α protein concentration by 3 h, and the amount of ER-α remained suppressed for 24 h. A multiple-dose ligand-binding assay demonstrated that the decrease in ER-α protein corresponded to a similar decrease of 50% in estradiol-binding sites with no effect on the binding affinity of ER-α. The dissociation constant of the estradiol-ER-α complex in the absence of IGF-I ($K_d = 3 \times 10^{-10} \pm 0.5 \times 10^{-10}$ M) was similar to the dissociation constant in the presence of IGF-I ($K_d = 6 \times 10^{-10} \pm 0.3 \times 10^{-10}$ M). The decrease in ER-α protein concentration was paralleled by an 80% decrease in the steady-state amount of ER-α mRNA by 3 h. The IGF-I induced decrease in ER-α mRNA was due to the inhibition of ER-α gene transcription. When an 128-base pair ER-α-promoter-CAT construct was transfected into MCF-7 cells, treatment with IGF-I resulted in a 40% decrease in CAT activity. In contrast to the effects on ER-α, treatment with IGF-I induced two endogenous estrogen-regulated genes, progesterone receptor and pS2, by 4- and twofold, respectively. The pure antiestrogen ICI-164,384 blocked this induction, suggesting that ER-α mediates the effects of IGF-I. Transient co-transfections of wild-type ER-α and an estrogen response element-CAT reporter into COS-1 cells demonstrated that IGF-I increased reporter gene activity. This effect was also blocked by ICI 164,384. Protein kinase A and phosphatidylinositol 3-kinase inhibitors blocked the IGF-I effects on ER-α expression and activity, suggesting that these kinases may be involved in the cross-talk between the IGF-I and ER-α pathways. J. Cell. Biochem. 76:605–614, 2000.

Key words: ER-α IGF-I effects; gene expression

Steroid receptors are ligand-regulated transcription factors belonging to the superfamily of intracellular receptors, which also includes the retinoid, thyroid hormone, and vitamin D receptors, as well as many orphan receptors [Weigel, 1996]. Estrogens regulate the growth, differentiation, and function of diverse target tissues by binding to the estrogen receptor (ER). The ER aporeceptor is usually found in a complex with heat shock proteins before ligand-dependent activation. Estrogens bind and induce an allosteric conformational change in the receptor that causes elimination of the heat shock proteins and facilitates dimerization of the receptor. The receptor dimer then binds with high affinity to DNA, directing gene transcription [Tsai et al., 1994]. Growth factors such as epidermal growth factor (EGF), insulin-like growth factors, and transforming growth factor-α and their receptors are target genes of the ER [Westley et al., 1994].

Surprisingly, steroid receptors are also activated by extracellular signals from growth factors (EGF) [Ignar-Trowbridge et al., 1993; Zhang et al., 1994], insulin-like growth factors [Aronica et al., 1993; Ma et al., 1994; Newton et al., 1994], and heregulin [Pietras et al., 1995], neurotransmitters (dopamine), second messengers (cyclic adenosine monophosphate [cAMP]) [Aronica et al., 1993; Zhang et al., 1994; Denner et al., 1990], and mitogen-activated protein kinase (MAPK) [Bunone et al., 1996]. Activation of steroid receptors in the absence of hormones,

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referred to as steroid-independent activation, has been demonstrated for ER [Bunone et al., 1996; Ignar-Trowbridge et al., 1992; El-Tanani et al., 1997a], progesterone receptor [Mani et al., 1994; Mani, 1996], and androgen receptor [Culig, 1994; Nazareth et al., 1996], whereas the glucocorticoid and the mineralocorticoid receptors have proved refractory to agents that activate the other receptors. While evidence has accumulated for a cross-talk between the peptide and the steroid pathways, the mechanism of this cross-talk has not yet been elucidated.

Insulin-like growth factor-I (IGF-I) belongs to a family of growth factors involved in the regulation of normal and malignant cell growth, differentiation, and development. This 70-residue, single-chain polypeptide [Umayahava et al., 1994] is produced by the liver under the control of growth hormone. Besides its endocrine effects, IGF-I is produced in most organs and tissues and can function as an autocrine or paracrine growth stimulator [Yee, 1994]. Insulin-like growth factor-I exerts its actions through binding to IGF-I receptor I, which is a transmembrane protein with tyrosine kinase activity. In all extracellular fluids, binding of IGF-I to the IGF receptor is modulated by a group of soluble proteins, called IGF-binding proteins [Yee, 1994]. The binding of IGF-I to its receptor initiates a cascade of phosphorylations that activate cellular kinases and nuclear transcription factors.

The activity of the IGF-I receptor appears to play a critical role in the regulation of breast cancer cell growth [Yee, 1994; Lee et al., 1997]. The amount of IGF-I receptor is significantly higher in breast cancer than in normal breast tissue or benign tumors. In primary breast cancer, a correlation has been found between tumor size, the levels of the cellular substrate of the IGF-I receptor, insulin receptor substrate 1 (IRS-1), and recurrence of the disease [Rocha et al., 1995]. In the MCF-7 breast cancer cell line, the coexpression of either IRS-1 [Surmacz et al., 1995], IGF receptor I [Guvakova et al., 1997a], or IGF-II [Cullen et al., 1992] has been shown to reduce estrogen growth dependence [Guvakova et al., 1997b]. Inhibition of IGF receptor I signaling with anti-IGF receptor antibodies, antisense RNA to the IGF-I receptor, or antisense oligonucleotides to the insulin substrate-1 restricts breast cancer cell growth both in vitro and in vivo [Guvakova et al., 1997b]. A similar effect has been reported in breast tumor xenografts in vivo. Insulin-like growth factors dramatically increase cell proliferation in the presence of estrogen in MCF-7 cells [Stewart et al., 1990]. It has been postulated that the combined effects of estradiol and IGF-I may stimulate proliferation in normal mammary epithelium, increasing the risk of breast cancer. Antiestrogens have been shown to inhibit IGF receptor I-dependent growth through different mechanisms, such as downregulation of autocrine IGF secretion or modulation of IGF-binding protein expression [Winston et al., 1994]. In addition, antiestrogens decreased expression of IGF-I-binding sites and suppressed the activation of insulin receptor substrate-1-associated phosphatidylinositol 3-kinase in MCF-7 cells. Estrogens also regulate expression of IGF receptor-I and IGF-I messenger RNA (mRNA) in some ER-positive breast cancer cell lines, such as MCF-7 and T47D, as well as in the neuroblasoma cell line SK-ER3 [Ma et al., 1994], in uterine tissue [Westley et al., 1994; Sahlin et al., 1994], and in osteoblasts [Westley et al., 1994].

In contrast with the effects of estrogens on the IGF-I pathway, regulation of estrogen signaling by IGFs is less well defined. Early studies demonstrated that antiestrogens inhibited IGF-mediated proliferation [Chalbos et al., 1993]. It was suggested that inhibition was due to regulation of receptor-binding sites and tyrosine kinase activity by the antiestrogen. However, a number of later studies demonstrated that growth factors directly increased the transcriptional activity of ER, which was specifically inhibited by antiestrogens [Stancel et al., 1987; Ma et al., 1994; Newton et al., 1994; Westley et al., 1994; Clayton et al., 1997; Lee et al., 1997]. Although the mechanism by which growth factors activate the ER remains unclear, receptor phosphorylation is thought to play a role. Upon stimulation of uterine cells with IGF-I, the estrogen receptor is phosphorylated [Sahlin et al., 1994]; however, phosphorylation does not always correlate with transcriptional activity of the receptor [Lee et al., 1997].

This article reports work on the role of IGF-I in the regulation of ER-α gene expression and activity, which was studied in the ER-positive breast cancer cell line MCF-7. The effects of IGF-I on ER-α protein, mRNA, and transcription were studied. The effects of IGF-I on endogenous estrogen-regulated genes were measured

and the signal transduction pathway examined.

MATERIALS AND METHODS

Tissue Culture

Monolayer cultures of MCF-7 breast cancer cells were grown in improved minimal essential medium (IMEM) supplemented with 5% (vol/vol) fetal calf serum (FCS). When the cells were 80% confluent, the medium was replaced with phenol red-free IMEM containing 5% charcoal-treated calf serum (CCS). Calf serum was pretreated with sulfatase and dextran-coated charcoal to remove endogenous steroids. After 2 days under these conditions, the medium was changed to serum-free phenol red-free IMEM supplemented with 2 mg/ml fibronectin, 5 ng/ml glutamine, Hepes, 5 ml trace elements, and 5 ng/ml transferrin. The cells were treated with 10^{-9} M estradiol (Sigma Chemical Co., St. Louis, MO) or with 40 ng/ml IGF-I (Biosource, Camarillo, CA) for the appropriate times.

Plasmids

The probe for the ER-α, pOR-300, was constructed by subcloning a 300-base pair (bp) restriction fragment of pOR3 into the pGem4 polylinker regions using the restriction enzymes *Pst*I and *Eco*RI [Saceda et al., 1988]. The genomic clone corresponding to exon 1, Q7, is a 3-kilobase (kb) *Eco*RI-*Sal*I fragment subcloned into Bluescript $M13^+$ (Stratagene, La Jolla, CA) [Saceda et al., 1988]. The clone 36B4 was constructed by subcloning a 220-bp fragment of 36B4 into the *Pst*I restriction site of the pGem polylinker [Saceda et al., 1988]. In addition, the clones for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [Kastner et al., 1990], pS2, and progesterone receptor [Ignar-Trowbridge et al., 1992] were used as previously described.

Estrogen Receptor-α and Progesterone Receptor Protein Assays

For analysis of ER-α and progesterone receptor protein concentration, MCF-7 cells were cultured and treated as described above. The concentration of receptor protein was determined using an enzyme immunoassay kit from Abbott Laboratories (North Chicago, IL). To obtain total receptor protein, the cells were homogenized by sonication in a high salt buffer (10 mM Tris, 1.5 mM EDTA, 5 mM Na_2 Mo O_4, 0.4 M KCl, and 1 mM monothioglycerol with 2 mM leupeptin). The homogenate was incubated on ice for 30 min and centrifuged at 100,000*g* for 1 h at 4°C. Aliquots of the total extracts were then analyzed according to the manufacturer's instructions.

To measure the number of estrogen-binding sites and the dissociation constant (K_d) of the estradiol-ER-α complex, a whole-cell, multiple-dose ligand-binding assay was used [Stoica et al., 1997]. Cells were plated in 6-well plates, and at approximately 70% confluence they were treated with 0.4 ng/ml IGF-I for 24 h. Then the cells were incubated for 1 h with various concentrations of [^{3}H]estradiol. A 200-fold excess of diethylstilbestrol (DES) was used to distinguish between specific and nonspecific binding. The cells were washed and lysed. The protein content and radioactivity present in each sample was quantified and analyzed by the method of Scatchard.

Measurement of ER-α mRNA

Total cellular mRNA was extracted from MCF-7 cells by the RNazol method. The amounts of ER-α, 36B4, progesterone receptor, and GAPDH were determined by an RNase protection assay. Briefly, homogeneously ^{32}P-labeled antisense cRNA were synthesized in vitro from pOR-300, 36B4, pGAPDH using T7 polymerase and from progesterone receptor using SP6. Sixty micrograms of total RNA were hybridized for 12–16 h to the radiolabeled cRNA. After a 30 min digestion at 25°C with RNase A, ^{32}P-labeled cRNA probes protected by total RNA were separated by electrophoresis on 6% polyacrylamide gels. The bands were visualized by autoradiography and quantified using the phosphorimager. The amounts of ER-α mRNA and progesterone receptor mRNA were normalized to the internal controls 36B4 and GAPDH, respectively.

Transfection and CAT Assays

In the transfection assays, 10^6 MCF-7 cells were plated in 100 mm dishes and grown in IMEM supplemented with 10% CCS for 24 h before transfection. Calcium-phosphate DNA precipitates containing 5 μg ER-α promoter-CAT vector, 2 μg β-galactosidase vector, and 23 μg carrier DNA were prepared, and the cells were transfected by the method of Chen and Okayama [Chen et al., 1987]. At 18 h after transfection, cells were washed, and the medium was replaced with phenol red-free IMEM

supplemented with 10% CCS. After 24 h in estrogen-depleted medium 40 ng/ml IGF-I was added. Cell lysates were prepared 6 h after IGF-I treatment and analyzed for CAT activity by a standard protocol [Garcia-Morales et al., 1994]. The conversion of [^{14}C]chloramphenicol to its acetylated forms was determined by thin layer chromatography (TLC). The reaction products were analyzed by a phosphorimager (Molecular Dynamics). CAT activity was expressed as the percentage conversion of chloramphenicol to its acetylated forms and was normalized to the β-galactosidase activity. β-Galactosidase activity was determined as a measure of the transfection efficiency.

In a second series of transient transfection assay, COS-1 cells were maintained at 37°C in 5% CO_2 in IMEM phenol red-free medium with 10% CCS. COS-1 cells were plated at 3×10^6 cells per 150-mm plate. After 24 h, the plates were transfected by the calcium phosphate precipitation technique [Chen et al., 1987]. Cells were transfected with 3 ml of DNA precipitate containing 15 μg pRER expression vector encoding ER-α, 75 μg pb-CAT (S)MERE reporter construct, 2 μg β-galactosidase, and salmon sperm carrier DNA to a total of 90 μg of DNA. At 18 h later, cells were washed and IGF-I was added for 6 h. The cells were harvested and prepared for CAT assay as described above.

RESULTS

Effect of IGF-I on the Concentration of the Estrogen Receptor-α

To define the role of IGF-I in the expression of estrogen receptor-α in breast cancer, the effects of IGF-I on the concentration of the ER-α protein in MCF-7 cells was determined. MCF-7 cells were treated with 40 ng/ml IGF-I and the concentration of ER-α was measured using an enzyme immunoassay. The results presented in Figure 1 show that IGF-I treatment resulted in a decline in total receptor protein by about 60% at 3 h, and the amount of receptor remained suppressed for 24 h. Receptor protein declined from a concentration of approximately 457 fmol/mg protein in control cells to 183 fmol/mg protein in cells treated with IGF-I.

To determine whether the decrease in ER-α protein corresponded to a similar decrease in estradiol-binding sites, the binding capacity and affinity of ER-α were determined using a multiple-dose ligand-binding assay. Scatchard plots for control and IGF-I treated cells are presented in Figure 2. After treatment with 40 ng/ml IGF-I, a significant decrease in the estradiol-binding capacity was observed. The number of estrogen-binding sites decreased from 315 fmol/mg protein in control cells to 158 fmol/mg protein in treated cells, indicating a 50% decrease in ER-α-binding sites. These results were consistent with those obtained with the enzyme immunoassay. Scatchard analysis indicated that treatment with IGF-I did not alter the binding affinity of estradiol to the receptor ($K_d = 3 \pm 0.5 \times 10^{-10}$ M, n = 3, r = -0.89 in control cells compared with $K_d = 6 \pm 0.3 \times 10^{-10}$ M, n = 3, r = −0.79 in IGF-I-treated cells). These results suggest that although IGF-I decreased the number of receptor sites, it did not alter the affinity of the receptor for its ligand.

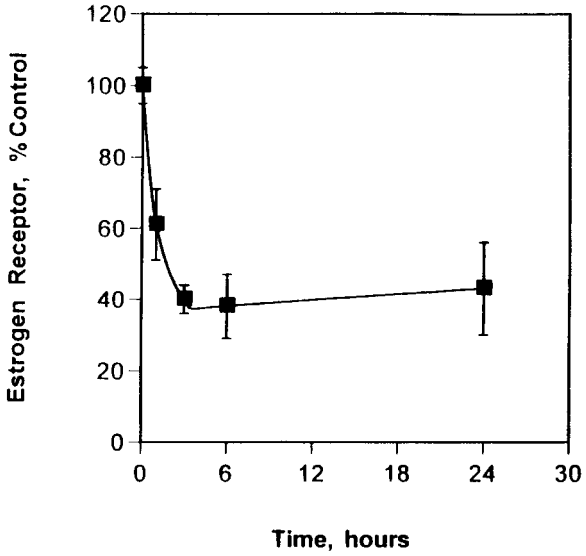

Fig. 1. Effect of insulin-like growth factor-I (IGF-I) on estrogen receptor-α (ER-α) protein concentration. MCF-7 cells were grown in 5% charcoal-treated calf serum (CCS). After 2 days, the medium was replaced with serum-free medium and the cells were treated with 40 ng/ml IGF-I for the times indicated. Total ER-α was determined with an enzyme immunoassay as described under Materials and Methods. Results were repeated 10 times and are presented as a percentage of control values ± SD.

Effect of IGF-I on the steady-state amount of the estrogen receptor-α mRNA. To determine whether the decline in estrogen receptor to a new steady-state amount was accompanied by a parallel decrease in ER-α mRNA, MCF-7 cells were treated with 40 ng/ml IGF-I and ER-α mRNA was measured using an RNase protection assay. The changes in ER-α mRNA were quantified by scanning densitometry, and the data are presented as the ratio of

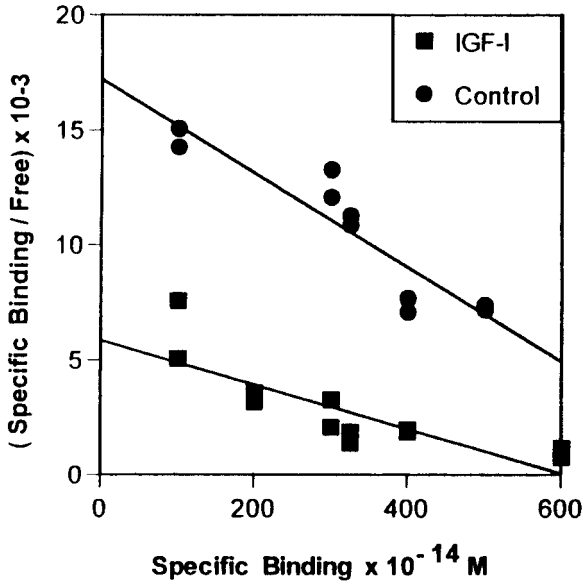

Fig. 2. Effect of insulin-like growth factor-I (IGF-I) on the binding of estradiol to the estrogen receptor-α (ER-α). MCF-7 cells were grown in 6-well plates and treated with increasing concentrations of [^{3}H]estradiol in the absence (B_T) or presence (B_N) of a 200-fold molar excess of diethylstilbestrol. Free radioactivity (F) was removed by washing the wells with medium supplemented with 1 mg/ml bovine serum albumin (BSA). The results were graphically represented according to the Scatchard equation. The specific binding, B_S, was calculated as the difference between the total binding, B_T, and the nonspecific binding, B_N. $B_{S'}$ is the binding capacity expressed in moles per liter (mol/L). From the slope of the plot and the abscissa to the origin, the dissociation constant of the complex (K_d) and the binding capacity (B_{max}) were determined, respectively. A representative assay is shown for control cells and IGF-I-treated cells. These experiments were repeated three times.

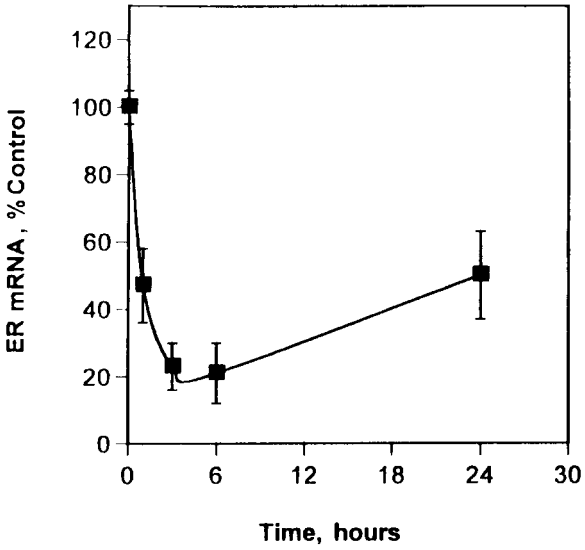

Fig. 3. Effect of insulin-like growth factor-I (IGF-I) on the steady-state amount of estrogen receptor-α (ER-α) mRNA. MCF-7 cells were grown as described in Fig. 1 and treated with 40 ng/ml IGF-I. Total RNA was isolated using Rnazol, and 60 μg was analyzed using an RNase protection assay as described under Materials and Methods. Results are presented as percentage of control values. Data represent the mean of six experiments ± SD.

the integrated ER-α signal to the integrated 36B4 signal. The results are shown in Figure 3. Insulin-like growth factor-I treatment of MCF-7 cells resulted in a maximum suppression of ER-α mRNA to approximately 20% of the control value by 3 h, with a small, but statistically nonsignificant, recovery in the amount of mRNA at 24 h.

Effect of IGF-I on Estrogen Receptor-α Gene Transcription

The IGF-I-mediated decrease in the amount of ER-α mRNA may be due to either a transcriptional or a posttranscriptional mechanism. To rule out a posttranscriptional process, the effect of IGF-I on the stability and nuclear transport of ER-α mRNA was measured. In these studies, IGF-I did not alter ER-α mRNA transport between the nucleus and cytoplasm and had no effect on ER-α mRNA stability (data not shown). These data strongly suggested a transcriptional mechanism. To determine whether IGF-I inhibited transcription of the ER-α-gene, 128 bp of the proximal promoter from nucleotides −128 to +1 were linked to the CAT reporter gene and transfected into MCF-7 cells. The transfected cells were treated with 40 ng/ml IGF-I for 6 h. Cells were harvested and assayed for CAT activity. To control for transfection efficiency, β-galactosidase activity was measured. The results are presented in Figure 4 as percentage of control values. IGF-I treatment resulted in an approximately 40% decrease in CAT activity, suggesting that the IGF-I induced decrease in ER-α mRNA was due to an inhibition of transcription of the ER-α gene.

Signal Transduction Pathways in the Regulation of Estrogen Receptor-α

To gain insight into the signal transduction pathways involved in the regulation of ER-α expression by IGF-I, several inhibitors of protein kinases were employed. The inhibitor KT5720 was used to block protein kinase A [Fishman, 1997]. Wortmannin (KY 12420), a fungal metabolite, was employed to inhibit phosphatidylinositol 3-kinase [Vlakos, 1994], and ultimately protein kinase B [Jones, 1995]. The

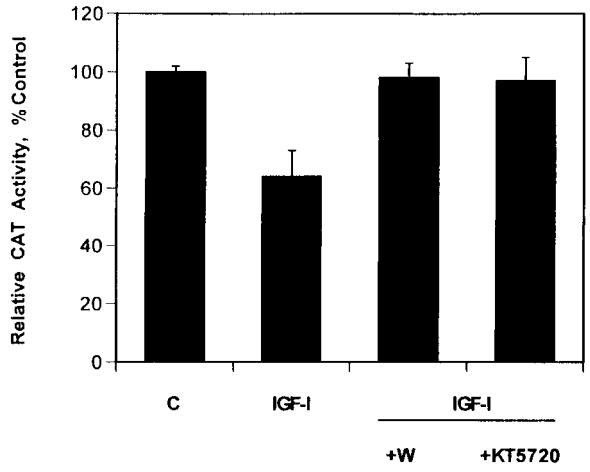

Fig. 4. Effect of insulin-like growth factor-I (IGF-I) on the estrogen receptor-α promoter. MCF-7 cells were transiently transfected with the estrogen receptor-α (ER-α) promoter-chloramphenicol acetyl transferase (CAT) vector, pER 128-CAT, which contains 128 bp of the proximal promoter, linked to the CAT reporter gene. After transfection, cells were treated with 40 ng/ml IGF-I for 6 h in the presence or absence of 100 nM wortmannin or 5×10^{-7} M of KT7520. Cells were harvested and assayed for CAT activity as described under Materials and Methods. The results are expressed as a percentage of control. The experiment was performed in triplicate and repeated five times ± SD.

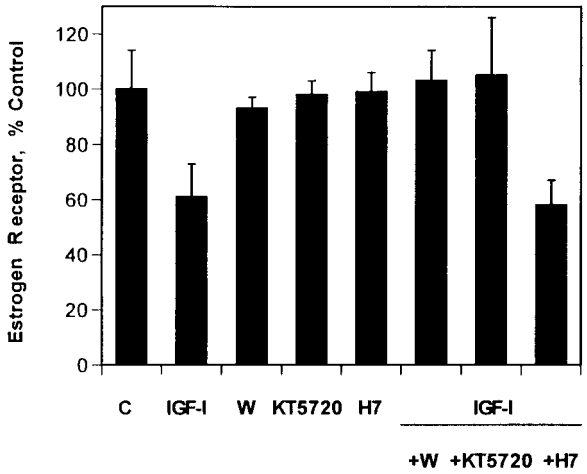

Fig. 5. Effect of protein kinase inhibitors on the insulin-like growth factor-I (IGF-I) regulation of estrogen receptor-α (ER-α). MCF-7 cells were grown as described in Fig. 1 and treated with either 10^{-9} M estradiol or 40 ng/ml IGF-I in the presence or absence of 5×10^{-7} M KT7520, H7, or 100 nM wortmannin. The concentration of ER-α was measured by the enzyme immunoassay as described under Materials and Methods. The results are the mean of four experiments ± SD and are expressed as a percentage of control values.

most potent and selective synthetic inhibitor, H-7, was selected to block protein kinase C [Ingles, 1997]. In this study, MCF-7 cells were treated for 6 h with 5×10^{-7} M of KT5720, 100 nM wortmannin, or 5×10^{-7} M H7 in the presence or absence of 40 ng/ml IGF-I. ER-α protein concentration was determined using the enzyme immunoassay. The results are presented in Figure 5. While the protein kinase inhibitors had no effect on the expression of ER-α, KT5720 and wortmannin blocked the IGF-I induced decrease in ER-α protein by approximately 90%. Similar results were observed at the level of ER-α mRNA (data not shown). In addition, KT7520 and wortmannin blocked the effect of IGF-I on the ER-α promoter (Fig. 4), suggesting that the effect of IGF-I on ER-α gene expression is mediated by protein kinase A and phosphatidylinositol 3-kinase.

Effect of IGF-I on the Activity of ER-α

To test the effect of IGF-I on the transcriptional activity of ER-α, the ability of IGF-I to induce estrogen-regulated genes, progesterone receptor and pS2, was determined. The amounts of progesterone receptor mRNA and pS2 mRNA were measured by an RNase protection assay and the results are presented in Figures 6 and 7, respectively. Estradiol induced a fourfold increase in progesterone receptor mRNA, whereas IGF-I increased the steady-state amount of progesterone receptor mRNA by threefold. When estradiol and IGF-I were added simultaneously, there was an additive effect (sevenfold induction) (Fig. 6). Similar results were obtained with pS2 mRNA. Estradiol induced a threefold increase in pS2 mRNA. A twofold induction was obtained after IGF-I treatment and an approximately fivefold induction was observed when estradiol and IGF-I were added simultaneously (Fig. 7). In both cases, the IGF-I induced effect was blocked by the antiestrogen ICI 164,384, suggesting that the IGF-I effect is mediated by ER-α.

The effect of IGF-I on the ER-α activity was also investigated in transient co-transfection assays employing ER-α and an estrogen response element-CAT reporter construct. The ER-α expression vector and the reporter construct were co-transfected into COS-1 cells treated with either estradiol or IGF-I in the presence or absence of ICI 164,384, and CAT activity was determined by thin layer chromatography. The results from several independent experiments are summarized in Figure 8. Estradiol increased CAT activity fourfold, IGF-I produced an approximately twofold increase in CAT activity, while estradiol plus IGF-I produced an approximately fivefold increase in CAT activity. The increase in CAT activity was also

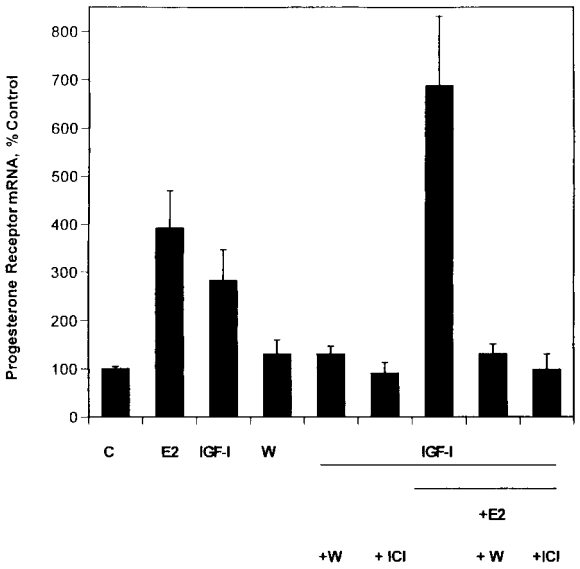

Fig. 6. Effect of insulin-like growth factor-I (IGF-I) on expression of the progesterone receptor. MCF-7 cells were grown as described in Fig. 1 and treated with 10^{-9} M estradiol, 40 ng/ml IGF, or estradiol plus IGF-I in the presence or absence of 100 nM wortmannin or 5×10^{-7} M ICI for 6 h. The concentration of progesterone receptor was determined by the enzyme immunoassay as described under Materials and Methods. The results represent the mean value of five experiments ± SD and are presented as a percentage of control values.

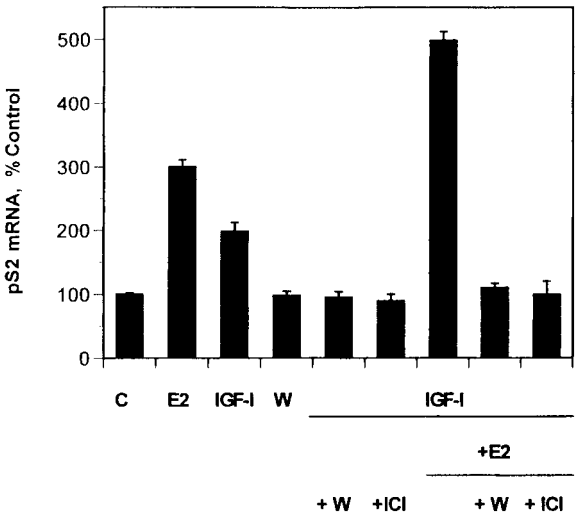

Fig. 7. Effect of insulin-like growth factor-I (IGF-I) on the steady-state concentration of pS2 mRNA. MCF-7 cells were grown and treated as described in Fig. 5. Total RNA was isolated using Rnazol, and 60μg was analyzed by an RNase protection assay as described under Materials and Methods. The results represent the mean value of four experiments and are presented as a percentage of control values.

blocked by ICI 164,384. The inhibitor wortmannin was also employed to identify the pathway involved in the regulation of ER-α activity (Figs. 6–8). Similar to the results observed on the

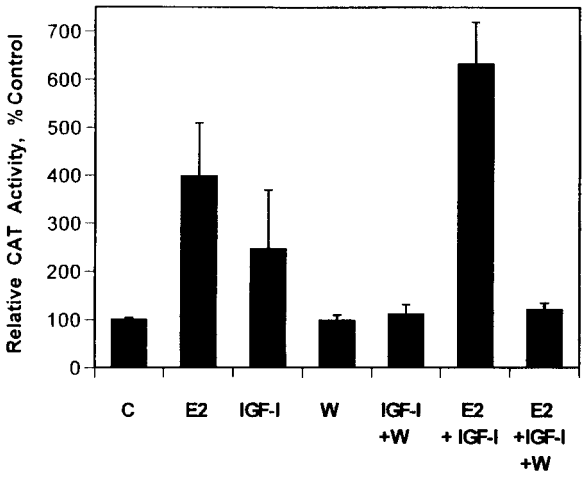

Fig. 8. Effect of insulin-like growth factor-I (IGF-I) on estrogen receptor-α activity in COS-1 cells. COS-1 cells were transiently co-transfected with a wild-type ER-α expression vector and an estrogen response element-CAT reporter construct using the calcium phosphate method as described under Materials and Methods. Transfected cells were treated with 10^{-9} M estradiol, 40 ng/ml IGF-I, or IGF-I plus estradiol in the presence or absence of 100 nM wortmannin for 6 h. CAT activity was measured as described under Materials and Methods. The results were normalized to the β-galactosidase activity. The data represent the mean value of four experiments and are expressed as a percentage of the CAT activity in untreated cells.

expression of ER-α, wortmannin blocked the effect of IGF-I on ER-α activity, suggesting that the pathway that regulates ER-α expression also regulates ER-α activity.

DISCUSSION

While it is clear that estrogen affects insulin-like growth factor action by altering the expression of several members of the IGF signal pathway, this article describes the regulation of ER-α gene expression and activity by IGF-I. The relationship between the steady-state amounts of ER-α protein, receptor mRNA, and promoter activity were examined in the ER-positive breast cancer cell line, MCF-7. The results presented herein demonstrate that treatment with IGF-I resulted in an approximately 60% decrease in the steady-state amount of ER-α protein with no alteration in the binding affinity of estradiol similar to the down-regulation observed in rat uterus [Sahlin et al., 1994]. This study further demonstrates that the decline in ER-α protein accompanied a parallel decrease (approximately 80%) in the amount of receptor mRNA. Transient transfections using an ER-α promoter-CAT construct also showed an approximately 60% decrease in CAT activity after IGF-I treatment, suggesting that a poten-

tial negative IGF-I response element is present within the proximal promoter of the ER-α gene. IGF-I response regions elements have been identified in the chicken δ-1-crystalline gene [Alemany et al., 1990, 1992] and the rat elastin gene [Wolfe et al., 1993; Jensen et al., 1995]. These regions are GC-rich domains that bind the ubiquitous transcription factor Sp1 [Bunone et al., 1996; Yee, 1994]. A 30-bp GC-rich IGF response element was also identified in the porcine P-450 cholesterol side-chain cleavage gene, P-450 11A [Urban et al., 1994]. This P-450 11A IGF response element stimulates gene expression in MCF-7 cells [Urban et al., 1996]. In addition to positive IGF-I response elements, negative insulin response elements have been identified in the genes of the IGF-binding protein-1 [Suwanickul et al., 1993], α-amylase [Johnson et al., 1993], phosphoenolpyruvate carboxykinase (PEPCK) [O'Brien et al., 1990], and glucagon [Philippe, 1991]. Each of these negative insulin response elements contains an AT-rich region with four consecutive thymidine residues. Alignment of the sequences of the negative response elements provides a consensus sequence, CTTTG. The ER-α promoter contains a similar sequence between -59 and -54. The role of this putative negative response element in mediating the effects of IGF-I on ER-α gene expression is unknown but is currently under investigation in our laboratory.

Steroid-independent activation of the ER-α by IGF-I has been demonstrated in MCF-7 cells [Katzenellenbogen et al., 1990; El-Tanani et al., 1997b; Lee et al., 1997; Chalbos et al., 1993] and in transient transfection assays in pituitary tumor cells [Newton et al., 1994], neuroblastoma cells [Ma et al., 1994], human ovarian adenocarcinoma cells [Stancel et al., 1987], Chinese hamster ovary cells [Ignar-Trowbridge et al., 1996], and MCF-7 cells [Pakdel et al., 1993]. However, ER-α activation by IGF-I is not a universal phenomenon. In transiently co-transfected HeLa cells, IGF-I failed to activate the estrogen receptor [Jensen, 1996]. In this study, the ability of IGF-I to activate ER-α was demonstrated in MCF-7 and COS-1 cells. In MCF-7 cells, IGF-I induced several endogenous estrogen-regulated genes. The induction of progesterone receptor and pS2 was additive with estradiol and was blocked by the pure antiestrogen ICI 164,384. Additionally, IGF-I increased CAT activity from an estrogen response element-CAT reporter plasmid either transiently transfected into MCF-7 cells or co-transfected with a wild-type human ER-α expression vector into COS-1 cells. In both cases, the pure antiestrogen ICI 164,384 blocked the effect of IGF-I, providing additional evidence for the cross-talk between the IGF-I and ER-α pathways.

Cell proliferation and differentiation in response to IGF-I is mediated principally by two signaling pathways: the Ras-Raf-MAPK pathway and the phosphatidylinositol 3-kinase and p70/S6 kinase pathway. The pathway involved in mediating these responses depends on the cell [Coolican et al., 1997; Petley et al., 1999]. In myoblasts [Coolican et al., 1997], adipocytes [Valverde et al., 1997], and 3T3 fibroblasts [Teruel et al., 1996], IGF-I clearly employs the Ras-Raf-MAPK pathway for signaling cell proliferation, whereas in MCF-7 cells [Dufourny et al., 1997], brain capillary cells [Kanda et al., 1997], and human fibroblasts [Takahashi et al., 1997], the proliferative response to IGF-I is mediated by phosphatidylinositol 3-kinase and p70/S6 kinase. Alternatively, IGF-I-induced differentiation in myoblasts [Coolican et al., 1997] and adipocytes [Kaliman et al., 1998] is mediated by the phosphatidylinositol 3-kinase pathway, and in neuroblastoma cells [Valverde et al., 1997], differentiation is mediated by the MAP kinase pathway. In this report, we demonstrate that regulation of ER-α expression and activity by IGF-I was blocked by wortmannin and KT7520, providing evidence that phosphatidylinositol 3-kinase and protein kinase A are involved in mediating the IGF-I response. These data suggest that the pathway that mediates the proliferative response to IGF-I also regulates ER-α expression. However, further studies are necessary to more precisely define the cross-talk between ER-α and IGF-I.

The data obtained in this study demonstrate that IGF-I decreases ER-α gene expression while increasing the activity of the receptor. The decrease in the ER-α gene transcription may be mediated by a putative negative response element in the proximal promoter of the gene. The effects of the growth factor on ER-α expression and activity require protein kinase A and phosphatidylinositol 3-kinase, suggesting a link between the proliferative pathway of IGF-I and ER-α.

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