Role of estrogen receptor (ER) α in insulin-like growth factor (IGF)-I-induced responses in MCF-7 breast cancer cells

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Abstract

Insulin-like growth factor-I (IGF-I) is a mitogenic polypeptide that induces proliferation of MCF-7 breast cancer cells, and cotreatment with the phosphoinositide 3-kinase (PI3-K) inhibitor LY294002 and the antiestrogen ICI 182780 inhibits IGF-I-induced growth. The role of estrogen receptor α (ER α) in mediating responses induced by IGF-I was investigated in cells transfected with small inhibitory RNA for ER α (iER α). The results showed that IGF-I-dependent phosphorylation of Akt and mitogen-activated protein kinase, induction of G₁-S-phase progression and enhanced expression of cyclin D1 and cyclin E were dependent on ER α . Moreover, these same IGF-I-induced responses were also inhibited by the antiestrogen ICI 182780 and this was in contrast to a previous report suggesting that ICI 182780 did not affect IGF-I-dependent activation of PI3-K or induction of cyclin D1 expression. ICI 182780 exhibits antimitogenic activity and iER α inhibits G₁-S-phase progression and proliferation of MCF-7 cells treated with IGF-I, suggesting that the effects of the antiestrogen are primarily related to downregulation of ER α .

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Introduction

Insulin-like growth factors (IGFs) are mitogenic polypeptides that play an important role in growth, differentiation and survival of normal cells as well as proliferation of tumor cells (Dickson & Lippman 1995, Gullick *et al.* 1998, Grimberg & Cohen 2000, Singh 2000, Yee & Lee 2000). IGFs can act in an autocrine or paracrine fashion through interaction with the membrane-bound IGF receptor (IGFR). IGF-dependent signaling is regulated at multiple levels including their interaction with IGF-binding proteins, which dramatically modulate the activity and function of these mitogens (Grimberg & Cohen 2000). IGF signaling pathways are important for growth of multiple cancers, and elevated serum IGF-I levels are associated with an increased risk of prostate and colon cancers (Chan *et al.* 1998, Cohen 1998, Wolk *et al.* 1998, Ma *et al.* 1999).

The mitogenic activity of IGF-I and interactions between growth factor and estrogen receptor (ER) signaling has been investigated extensively in breast cancer cells, and estradiol (E₂) and IGF-I induce many of the same responses in these cell lines (Dickson & Lippman 1995, Smith 1998, Weigel & Zhang 1998, Kato *et al.* 2000, Yee & Lee 2000, Luconi *et al.* 2002, Segars & Driggers 2002). For example, IGF-I and E₂

induce proliferation of ER-positive breast cancer cell lines and several genes, including pS2, cathepsin D, cyclin D1, the progesterone receptor and IGF-IR, are also induced by E₂ and IGF-I (Cavaillès *et al.* 1989, Wakeling *et al.* 1989, Katzenellenbogen & Norman 1990, Chalbos *et al.* 1993, Ignar-Trowbridge *et al.* 1993, Westley & May 1994, Martin *et al.* 2000, Oesterreich *et al.* 2001, Varma & Conrad 2002). The estrogen-like activity of IGF-I, transforming growth factor α (TGF α) and epidermal growth factor (EGF) are due, in part, to growth factor-induced phosphorylation of ER which in turn activates ER-dependent genes in the absence of E₂ (Ali *et al.* 1993, Kato *et al.* 1995, 2000, Bunone *et al.* 1996, Ignar-Trowbridge *et al.* 1996, Joel *et al.* 1998, Smith 1998, Weigel & Zhang 1998, Luconi *et al.* 2002). E₂ also exhibits growth factor-like activity and the rapid non-genomic actions of estrogens (Watson *et al.* 1998, 2002, Levin 2002) depend, in part, on extranuclear ER and its association with several proteins involved in kinase signaling pathways. For example, ER α directly interacts with the SH2 region of Src, the p85 α regulatory subunit of phosphoinositide 3-kinase (PI3-K), caveolins, G α_1 (a G protein), Shc and IGF-IR (Kahlert *et al.* 2000, Migliaccio *et al.* 2000, Simoncini *et al.* 2000, Sun *et al.* 2001, Wyckoff *et al.* 2001, Razandi *et al.* 2002, Song *et al.* 2002). The functional significance of these

interactions may be cell-context-dependent. For example, antibodies that block IGF-IR signaling by IGF-I did not inhibit activation of the mitogen-activated protein kinase (MAPK) pathway by E_2 in MCF-7 cells (Duan *et al.* 2001).

Research in our laboratory has concentrated on the mechanism of IGF-I-ER α crosstalk in breast cancer cells (Wang *et al.* 2000, Castro-Rivera *et al.* 2001, Duan *et al.* 2001, Xie *et al.* 2001, Qin *et al.* 2002, Ngwenya & Safe 2003), and this report describes our research on the role of ER α in mediating IGF-I-induced responses in MCF-7 cells. Transfection of small interfering RNA (siRNA) for ER α (iER α) into MCF-7 cells resulted in decreased ER α protein, and this was accompanied by decreased phosphorylation of MAPK and decreased PI3-K-dependent phosphorylation of Akt. In addition, loss of ER α also resulted in decreased IGF-I-induced G₁-S-phase progression and expression of proteins associated with cell-cycle progression such as cyclin D1 and cyclin E. In contrast to a recent report (Varma & Conrad 2002), ICI 182780 not only inhibited MCF-7 cell proliferation but also decreased IGF-I-induced kinase signaling and cyclin D1 and cyclin E expression in MCF-7 cells, and this was correlated with downregulation of ER α . We also investigated the role of insulin receptor substrate-1 (IRS-1) in mediating IGF-I-induced activation of MAPK and PI3-K pathways using siRNA for IRS-1 (iIRS-1), which effectively knocks down IRS-1. The results show that IRS-1 is not required for kinase activation by IGF-I. These results clearly demonstrated that IGF-I-dependent mitogenic activity in breast cancer cells is dependent on ER α and this is consistent with the estrogen-like activity of IGF-I in breast cancer cells.

Materials and methods

Chemicals, cells, antibodies, oligonucleotides, and plasmids

MCF-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (DMEM/F-12) with and without Phenol Red, 100 $\times$ antibiotic/antimycotic solution, propidium iodide, E_2 and human recombinant IGF-I were purchased from Sigma (St Louis, MO, USA). Fetal bovine serum was purchased from Intergen (Purchase, NY, USA). [γ -³²P]ATP (300 Ci/mmol) was obtained from Perkin-Elmer Life Sciences. Poly(dI-dC) and T4 polynucleotide kinase were purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). Antibodies for lamin A/C, ER α , pAKT, and pERK proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for GAPDH was obtained from Ambion (Austin, TX, USA). Human ER α expression plasmid was provided by Dr Ming-Jer Tsai (Baylor College of

Medicine, Houston, TX, USA). Lysis buffer, luciferase reagent and RNase were obtained from Promega Corp. (Madison, WI, USA). PD98059 and LY294002 were purchased from Calbiochem (San Diego, CA, USA). ICI 182780 was provided by Dr Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; >98%) was prepared in this laboratory.

The pSRF construct containing five tandem serum-response factor (SRF) elements linked to a luciferase reporter gene was purchased from Stratagene (La Jolla, CA, USA), and the GAL4-ElkC plasmid was provided by Roger Treisman (Imperial Cancer Research Centre, London, UK). The pDRE₃-luc construct containing three tandem consensus dioxin-responsive elements (DREs) was prepared in this laboratory and the E_2 -responsive pC3-luc construct was provided by Dr Donald McDonnell (Duke University, Durham, NC, USA). DMSO was used as solvent for E_2 and the antiestrogens. Acetic acid (0.1 M) was used as solvent for IGF-I. The siRNAs for GL2, lamin A/C and ER α were prepared by Xeragon (Germantown, MD, USA), and the sequences of iRNA duplexes were indicated as follows: GL2, 5'-CGUACGCGGAAUACUUCGATT-3', 3'-TTGCAUGCGCCUUAUGAAGCU-5'; lamin A/C, 5'-CUGGACUUC CAGAAGAACATT-3', 3'-TTGACCUGAAGGUCUUCUUGU-5'; ER α , 5'-AGGCUCAUUC CAGCCACAGTT-3', 3'-TTUCCGAGUAAGGUCGGUGUC-5'. The siRNA for IRS-1 was comprised of four pooled siRNA duplexes (catalogue number M-003015) from Dharmacon (Lafayette, CO, USA).

Cell-proliferation assay

MCF-7 cells were seeded in DMEM/F-12 with 2.5% stripped fetal bovine serum and treated with different factors for 9 days. Cell numbers were determined using a Coulter Z1 counter, and results for each treatment are given as means $\pm$ S.D. from at least three different determinations for each treatment group. For the cell-proliferation study involving siRNA, cells were transfected with iER α or siRNA for GL2 (iGL2) and, after 24 h, treated with 10 nM IGF-I for 48 h. Cell numbers were then determined.

Transfection of MCF-7 cells

Cells were cultured in six-well plates in 2 ml DMEM/F-12 supplemented with 5% fetal bovine serum. After 16–20 h when cells were 30–50% confluent, siRNA duplexes and/or reporter gene constructs were transfected using Oligofectamine Reagent (Invitrogen, Carlsbad, CA, USA). For each well of a six-well plate, 0.2 μ mol iRNA duplex was transfected. Cells were harvested 36–44 h after transfection by

manual scraping in $1 \times$ lysis buffer (Promega). For whole-cell lysates, cells were frozen and thawed in liquid nitrogen, vortexed for 30 s, and centrifuged at 12 000 *g* for 1 min. Lysates were assayed for luciferase activity using luciferase assay reagent (Promega). β -Galactosidase activity was measured using Tropix Galacto-Light Plus assay system (Tropix, Bedford, MA, USA) in a Lumi-count microwell plate reader (Packard Instrument Co.).

Preparation of nuclear extracts

MCF-7 cells were seeded in a 100 mm tissue culture dish (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in DMEM/F-12 with 2.5% dextran/charcoal-stripped fetal bovine serum. After 24 h, cells were washed twice in PBS, scraped in 1 ml HEGD buffer (25 mM Hepes, 1.5 mM EDTA, 1 mM dithiothreitol and 10% (v/v) glycerol, pH 7.6) and homogenized. The cellular homogenate was centrifuged for 5 min at 1000 *g*. The supernatant was discarded and the pellet was suspended in 200 μ l HEGDK (HEGD with 0.5 M KCl) and incubated on ice for 15 min with frequent vortexing. Samples were centrifuged at 14 000 *g* for 1 min, and nuclear protein concentration in the supernatant was determined by Bradford assay (Bradford 1976). The supernatant was stored in small aliquots at -80°C for further use.

Western immunoblot analysis

An aliquot of whole-cell lysate containing 30 μ g protein was diluted with loading buffer, boiled and loaded on a 10% SDS/polyacrylamide gel. Samples were electrophoresed at 150–180 V for 3–4 h, and separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) in buffer containing 48 mM Tris/HCl, 29 mM glycine and 0.025% SDS. Proteins were detected by incubation with polyclonal primary antibodies for lamin A/C, ER α -H184, GAPDH, p-AKT1/2/3-Ser473 and p-ERK (1:1000 dilution) followed by blotting with horseradish peroxidase-conjugated anti-rabbit (for ER α and p-AKT), anti-goat (for lamin A/C) or anti-mouse (for GAPDH and p-ERK) secondary antibody (1:5000 dilution). Blots were then exposed to chemiluminescent substrate (PerkinElmer Life Sciences) and placed in Kodak X-Omat AR autoradiography film. Band intensities were determined by a scanning laser densitometer (Sharp Electronics Corp., Mahwah, NJ, USA) using Zero-D Scanalytics software (Scanalytics Corp., Billerica, MA, USA).

FACS analysis

Cells were transfected with iER α or iGL2. After 20–24 h cells were treated with 0.1 M acetic acid or 10 nM IGF

(acetic acid as a vehicle) for 18–20 h in serum-free medium. Cells were then trypsinized, and $\sim 2 \times 10^6$ cells were centrifuged and resuspended in 1 ml staining solution (50 μ g/ml propidium iodide (PI), 4 mM sodium citrate, 30 units/ml RNase and 0.1% Triton X-100, pH 7.8). Cells were then incubated at 37°C for 10 min, and prior to FACS analysis, NaCl was added to give a final concentration of 0.15 M. Cells were analyzed on a FACS Calibur flow cytometer (BD PharMingen) using CellQuest acquisition software (BD PharMingen). PI fluorescence was collected through a 585/542 nm bandpass filter, and list mode data were acquired on a minimum of 12 000 single cells defined by a dot plot of PI width versus PI area. Data analysis was performed in ModFit LT (Verity Software House, Topsham, ME, USA) using PI width versus PI area to exclude cell aggregates. FlowJo (Treestar, Palo Alto, CA, USA) was used to generate plots shown in the figures.

Gel electrophoretic mobility shift assay (EMSA)

The probe containing a consensus estrogen-responsive element (ERE; 5'-GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT-3') was synthesized, annealed and ^{32}P -labeled at the 5'-end using T4 polynucleotide kinase (Roche) and [γ - ^{32}P]ATP. The binding reactions were performed on ice. For each lane, an appropriate amount of HEGDK buffer was added to 5 μ g MCF-7 cell nuclear extracts to bring the total volume to 5 μ l. HEGD buffer (15 μ l) was added to dilute the salt concentration and 1 μ g poly(dI-dC) was used to block the non-specific binding. After incubation for 5 min, 0.01 pmol radio-labeled probe was added and incubated for 10 min. ER antibody (sc-7202; Santa Cruz Biotechnology) was then added to the mixture and incubated for another 5 min if applicable. The mixture was resolved on 5% non-denaturing PAGE and protein-DNA complexes were visualized using Storm Imager system (Molecular Dynamics, Sunnyvale, CA, USA).

Immunostaining

MCF-7 cells were seeded in DMEM/F-12 with 2.5% dextran/charcoal-stripped fetal bovine serum in two-well Lab-Tek chamber slides (Nalge Nunc International Corp., Naperville, IL, USA). In experiments involving siRNAs, after 24 h cells were transfected with siRNA for lamin A/C (iLMN) or iER α with Oligofectamine according to manufacturer's recommendation and incubated for 48 h before methanol fixation. Otherwise, cells were directly treated and fixed. Cells were fixed in methanol at -20°C for 10 min. Slides were air-dried, washed in PBS/0.3% Tween-20 for 10 min, and blocked with 1:20 goat serum in antibody dilution buffer (1% BSA, PBS, 0.3% Tween-20 and 31% glycerol with

Figure 1 IGF-I-induced proliferation of MCF-7 cells and interactions with LY294002 (LY; A), ICI 182780 (ICI; B), and PD98059 (PD; C). MCF-7 cells were seeded in six-well plates and treated with 10 nM IGF-I and/or 1, 5, or 15 μM of the different chemicals for 9 days, and cell numbers were determined as described in the Materials and methods. Results from the experiments are given as means ± S.D. from at least three different determinations for each treatment group. IGF-I significantly ($P < 0.05$) increased growth (compared with solvent control) and only ICI 182780 and LY294002 significantly decreased IGF-I-induced proliferation.

0.5 M Na_2CO_3 , pH 9.5) for 1 h in a humidified chamber. ER antibody (sc-7202) in antibody dilution buffer (1:100 dilution) was added and incubated in a humidified chamber at 4 °C for overnight. Slides were washed three times, every 20 min in PBS/0.3% Tween-20, and then probed with FITC-conjugated anti-rabbit IgG antibody (sc-2012; Santa Cruz Biotechnology) in antibody dilution buffer (1:1000 dilution) for 2 h. Slides were then washed for 30 min (three times) in PBS/0.3% Tween-20 and then in deionized water for 15 min and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Immunostaining was observed with a Zeiss Axioplan2 microscope fitted with a Hamamatsu-C5810 chilled 3 CCD color camera.

Statistical analysis

Statistical significance was determined by analysis of variance and Scheffe's test, and the levels of probability are noted. The results are expressed as means ± S.D. from at least three separate (replicate) experiments for each treatment group.

Results

Mitogenic activity of IGF-I

The mitogenic activity of IGF-I was initially investigated in MCF-7 cells and the results in (Fig. 1) show that treatment with 10 nM IGF significantly induced proliferation of these cells. Treatment of MCF-7 cells with 5 μM LY294009, 15 μM PD98059, and 1 μM ICI 182780 also significantly inhibited MCF-7 cell growth compared with solvent (DMSO)-treated controls. How-

ever, in cells cotreated with IGF-I plus these inhibitors, growth factor-induced proliferation of MCF-7 cells was inhibited by LY294002 and ICI 182780 but not PD98059. These results are consistent with previous reports on the effects of LY294002 and ICI 182780 on IGF-I induced growth (Wakeling *et al.* 1989, Dufourny *et al.* 1997, Oesterreich *et al.* 2001, Varma & Conrad 2002) and suggest a role for PI3-K and ERα in mediating the mitogenic activity of IGF-I.

IGF-I-induced responses are dependent on ERα: effects of iERα

The role of ERα in mediating IGF-I-induced proliferation and gene activation was further investigated in MCF-7 cells using RNA interference with iERα. Whole-cell lysates from MCF-7 cells transfected with iERα or iLMN (non-specific control) and untransfected cells were analyzed by western blot analysis (Fig. 2A). iERα specifically induced downregulation of ERα but not LMN (non-specific) protein, whereas iLMN decreased iLMN but not ERα protein. Over several studies, transfected iERα decreased ERα protein by >50% in whole-cell lysates and this is consistent with the high transfection efficiencies observed in MCF-7 cells. iERα-mediated decreases in nuclear ERα were confirmed in a gel mobility shift assay with nuclear extracts from MCF-7 cells and [^{32}P]ERE (Fig. 2B). The intensity of the specifically bound retarded ERα-ERE band (Fig. 2B, lane 3), was decreased in cells transfected with iERα (Fig. 2B, lane 4) or after incubation with excess unlabeled ERE (Fig. 2B, lane 2). Extracts from cells transfected with iLMN did not affect retarded band intensity (Fig. 2B, lane 5) and ERα antibodies supershifted the specifically bound band (Fig. 2B, lane 1).

Figure 2 RNA interference with iERα. (A) Effects of iERα on ERα protein expression in MCF-7 cells. Cells were transfected with iERα and iLMN (as control siRNA), and whole-cell extracts were analyzed for ERα, lamin A/C (LMN) and GAPDH proteins by Western blot analysis as described in the Materials and methods section. Results are expressed means ± S.D. from three replicate determinations for each treatment group, and a significant ($P < 0.05$) decrease in ERα (compared with solvent control or iLMN) protein levels was observed (**). CTL, control. (B) Gel mobility shift assay. MCF-7 cells were treated with solvent, iERα, or iLMN, and binding of nuclear extracts to [³²P]ERE was determined in gel mobility shift assays as described in the Materials and methods section. Specifically bound bands and supershifted complexes are indicated by arrows. (C) Immunocytochemistry. MCF-7 cells were transfected with iERα or iLMN, and stained with ERα or lamin A/C antibodies. Immunofluorescence was determined as described in the Materials and methods section.

iERα-mediated decreases in ERα protein is also evident in MCF-7 cells immunostained for ERα (Fig. 2C). Compared with control (untreated) cells or cells transfected with iLMN, ERα protein was significantly decreased in MCF-7 cells transfected with iERα.

The specificity of the RNA interference assay was further investigated in MCF-7 cells transfected with an E₂-responsive pC3-luc construct which contains the human complement C3 gene promoter insert. E₂-induced luciferase activity in cells transfected with pC3-luc and iLMN, whereas hormone-induced activity was decreased in cells transfected with iGL2 (which targets bacterial luciferase) or iERα (Fig. 3A). As a negative control, we show that iERα does not affect

TCDD-induced transactivation in MCF-7 cells transfected with an aryl hydrocarbon-responsive construct (pDRE₃-luc; Fig. 3B). These results are consistent with previous studies showing that IGF-I induced E₂-responsive genes through ligand-independent activation of nuclear ERα (Stoica *et al.* 2000, Wang *et al.* 2000, Xie *et al.* 2001). IGF-I also activates MAPK- and PI3-K-dependent pathways including phosphorylation-dependent activation of Elk-1 and SRF, which are serum-response element (SRE)-dependent genes (Duan *et al.* 2001, 2002). Compared with the solvent control, using acetic acid, IGF-I induced transactivation in MCF-7 cells transfected with iLMN, a GAL4-Elk-1 expression plasmid, and a pGAL4 reporter construct

Figure 3 Effects of iER on transactivation in MCF-7 cells transfected with several ligand-activated constructs. Cells were transfected with iLMN, iGL2, or iER α , and the E₂-responsive pC3-Luc (A), aryl hydrocarbon-responsive pDRE₃-Luc (B), MAPK-regulated Gal4-Elk-1/Gal4-Luc (C), and a PI3-K-regulated pSRF-Luc (D) constructs. Cells were treated with DMSO, 10 nM E₂, 10 nM TCDD, or 10 nM IGF-I, and luciferase activity was determined as described in the Materials and methods section. Results summarized in A–D are means $\pm$ s.d. from three replicate determinations for each treatment group, and significantly ($P < 0.05$) increased activities (compared with solvent control) are indicated by * and significant inhibition by siRNAs is indicated by **. CTL, control; HAC, acetic acid vehicle.

(Fig. 3C; containing five tandem GAL4-response elements), or an SRF-luc construct (Fig. 3D; containing three tandem SRF motifs). In contrast, activation of both constructs was significantly inhibited by transfection with iGL2 or iER α demonstrating that ER α plays a role in IGF-I-induced transactivation in MCF-7 cells.

We further investigated the role of ER α in mediating IGF-I-induced gene expression by investigating phosphorylation of Akt or MAPK (Fig. 4A and B). Figure 4A shows that, compared with untransfected cells or cells transfected with iLMN (a control; Fig. 4A, lanes 1 and 2), iER α significantly decreased ER α protein levels in the presence or absence of IGF-I (Fig. 4A, lanes 3 and 6). IGF-I treatment did not affect levels of ER α protein but induced Akt phosphorylation (Fig. 4A, lanes 4–6); however, in cells cotransfected with iER α there was a significant decrease in IGF-I-induced Akt phosphoryl-

ation. In a separate experiment using a comparable approach, it was also apparent that iER α also decreased MAPK phosphorylation (Fig. 4B), and this was consistent with decreased Elk-1 and SRF activation by IGF-I in cells transfected with iER α (Fig. 3C and D). These results suggest that ER α is required for activation of MAPK/PI3-K-dependent kinases by IGF-I.

IGF-I-induced signaling in the mouse uterus (Klotz *et al.* 2002) suggested that activation of PI3-K required IRS-I, whereas activation of MAPK was IRS-I-dependent. The results in Fig. 4C show that transfection of iIRS-I in MCF-7 cells decreases IRS-I protein expression but does not significantly decrease phosphorylation of Akt or MAPK. These results suggest that IRS-I is not required for IGF-I-induced activation of PI3-K or MAPK in MCF-7 cells, and differs from the activation pathway in the mouse uterus (Klotz *et al.* 2002).

Figure 4 Effects of iER α and iIRS-1 on IGF-I-dependent kinase activation. Effects of iER α on phosphorylation of Akt (A) and MAPK (B). MCF-7 cells were transfected with siRNAs as described in the Materials and methods section, and phosphoproteins were determined by Western blot analysis of whole-cell lysates. The intensity of the different bands were determined by densitometry and plotted as means $\pm$ S.D. from three replicates in each treatment group. Significant ($P < 0.05$) increases in band intensity (compared with solvent control) are indicated by * and significant inhibition by siRNAs is indicated by **. (C) Effects of iIRS-1 on phosphorylation of Akt and MAPK. The experiments were carried essentially as described in (A) and (B) except the phosphorylation levels were determined in DMSO (D; solvent control) and IGF-I-treated cells after treatment for 10 or 30 min. CTL, control.

Figure 5 Effects of iER α on IGF-I-induced cell-cycle progression and proliferation in MCF-7 cells. (A) FACS analysis. Serum-starved MCF-7 cells were treated with DMSO or 10 nM IGF-I alone or cotransfected with iGL2 and iER α , and the percentage of distribution of cells in G₁/G₀, S, and G₂/M were determined by FACS analysis as described in the Materials and methods section. The results of three separate FACS experiments showed that after transfection with iER α there was a $13.0 \pm 1.44\%$ increase in G₀/G₁ and a $9.27 \pm 3.47\%$ decrease in S-phase distribution of the cells. Results are expressed as means $\pm$ S.E. (B) Cell proliferation. MCF-7 cells were transfected with iER α or iGL2 and, after 24 h, cells were treated with 10 nM IGF-I for 48 h, and cell numbers were determined as described in the Materials and methods section. Results are expressed as means $\pm$ S.E. for three replicate determinations for each treatment group, and a significant ($P < 0.05$) decrease in cell number was observed in cells transfected with iER α compared with cells transfected with iGL2.

Previous studies (Wakeling *et al.* 1989, Dufourny *et al.* 1997, Oesterreich *et al.* 2001, Varma & Conrad 2002) and results in Fig. 1 suggest that IGF-I-induced growth is dependent on activation of the PI3-K pathway, and therefore the effects of iER α on cell-cycle progression were investigated. The distribution of MCF-7 cells in G₀/G₁, S, and G₂/M phases was 82.4, 9.5, and 8.1%, respectively (Fig. 5A). Similar results (83.6, 9.9, and 6.5%) were observed in cells transfected with iGL2, a non-specific inhibitor of bacterial luciferase. In cells

transfected with iER α , there was a further increase in the percentage of cells in G₀/G₁ phase (86.5%) and a decrease of those in S phase (5.8%), suggesting that ER α contributes, in part, to the G₀/G₁-S-phase progression of untreated (control) MCF-7 cells. Treatment of MCF-7 cells with 10 nM IGF-I for 18–20 h significantly decreased (25–28%) the percentage of cells in G₀/G₁ and a comparable increase in the percentage of cells in S phase was observed. However, in MCF-7 cells transfected with iER α , IGF-I-induced G₁-S-phase

Figure 6 Effects of iER α on IGF-I-induced cell-cycle proteins. MCF-7 cells were transfected with iLMN (as a control siRNA) or iER and treated with either (10 nM) IGF-I or solvent vehicle for 24 h. The expression of cyclin D1 (CD1) and cyclin E (CE) proteins were determined by Western blot analysis as described in the Materials and methods section, and intensities were determined and plotted as means $\pm$ S.D. from three replicate for each treatment group. Significant ($P < 0.05$) increases in band intensities (compared with solvent control) are indicated by * and significant inhibition by siRNA is indicated by **.

progression was partially reversed, demonstrating a role for ER α in mediating IGF-I-dependent effects on this specific phase of the cell cycle. In addition, ER α knockdown in MCF-7 cells decreased IGF-I-induced cell proliferation (Fig. 5B). It is possible that some component of IGF-I-induced G₁-S-phase progression and cell proliferation may be ER α -dependent.

The potential role of cell-cycle-regulatory proteins associated with ER α -dependent actions of IGF-I were further investigated by western blot analysis (Fig. 6). Untreated (Fig. 6, lanes 1–3) and IGF-I-treated (Fig. 6, lanes 4–6) cells were untransfected (Fig. 6, lanes 1 and 4) or transfected with iLMN (Fig. 6, lanes 2 and 4) or iER α (Fig. 6, lanes 3 and 6), and whole-cell lysates were analyzed by western blot analysis. The patterns of ER α expression were similar to those illustrated in Figs 4A and B, and the various treatments did not affect levels of cdk2 or cdk4 or phospho-Rb proteins, and levels of IGF-I receptor were also unchanged (data not shown). In contrast, IGF-I induced cyclin D1 and cyclin E

protein levels and cotransfection with iER α decreased induction of both proteins. The results indicate that ER α plays a role in endogenous expression of both cyclin D1 and cyclin E since levels of these proteins were significantly decreased in untreated cells transfected with iER α . These data suggest that induction of cyclin D1 and cyclin E by IGF-I is an important response associated with G₁-S-phase progression. Previous studies also show that cyclin D1 and cyclin E were induced by IGF-I in MCF-7 cells (Dufourny *et al.* 1997, Dupont *et al.* 2000) and this is consistent with the induction of G₁-S-phase progression (Fig. 5) which is also dependent, in part, on expression of ER α .

Effects of ICI 182780 on IGF-I-induced responses

A previous study reported that although ICI 182780 inhibited IGF-I-induced growth of MCF-7 cells (Varma & Conrad 2002), the antiestrogen did not completely

Figure 7 Inhibition of IGF-I-induced responses by ICI 182780. (A) Activation of kinases (short term). Cells were treated with 10 nM IGF-I alone or in combination with 1 μ M ICI 182780 for 10 min, and whole-cell lysates were analyzed by Western blot analysis for Akt, p-Akt, Erk-1, and pErk-1 as described in the Materials and methods section. (B) Time-dependent inhibition on IGF-I-induced kinases. MCF-7 cells were pretreated with ICI 182780 for different periods of time and then treated with IGF-I for 10 min, and whole-cell lysates were analyzed by Western blot analysis as described in the Materials and methods section. Band intensities were determined by densitometry as described in the Materials and methods section. Each sample was determined in triplicate, and the data shown are from a representative experiment, and expressed in the bar graphs as means $\pm$ s.d. CTL, control. (C) Immunocytochemical analysis of ER α . MCF-7 cells were untreated (DMSO; D) or treated with ICI 182780 (ICI) for 2 h, and stained with ER α antibody or IgG. Immunofluorescence was determined as described in the Materials and methods section. DAPI (4,6-diamidino-2-phenylindole) staining was shown to demonstrate nuclear staining.

block IGF-I signaling including phosphorylation of Akt and IRS-I or induction of cyclin D1. These results are surprising since ICI 182780 induces proteasome-dependent downregulation of ER α in breast cancer cells (Stenoien *et al.* 2001, Wijayaratne & McDonnell 2001, Fan *et al.* 2003, Wormke *et al.* 2003). Based on the results obtained in the siRNA knockdown studies with iER α (Figs 2–6), we further investigated the effects of ICI 182780 on IGF-I-induced pathways in MCF-7 cells.

Results of initial studies showed that cotreatment of MCF-7 cells with 1 μ M ICI 182780 and 10 nM IGF-I (8 min) only slightly decreased MAPK and Akt phosphorylation (Fig. 7A). Since ICI 182780 induces a time-dependent decrease in levels of ER α (Fan *et al.* 2003, Stenoien *et al.* 2001, Wijayaratne & McDonnell 2001, Wormke *et al.* 2003), we therefore determined the effects of 1 μ M ICI 182780 on IGF-I-induced phosphorylation for up to 4 h after treatment with the

Figure 8 Inhibition of IGF-I-induced cell-cycle proteins by ICI 182780. MCF-7 cells were treated with 10 nM IGF-I and/or 1 μ M ICI for 24 h, and whole-cell lysates were analyzed by Western blot analysis as described in the Materials and methods section. The experiment was carried out in triplicate and results from densitometric analysis are plotted as means $\pm$ S.D. from three replicates for each treatment group. Significant ($P < 0.05$) increases in band intensity (compared with solvent control) are indicated by * and significant inhibition by ICI 182,780 is indicated by **.

antiestrogen (Fig. 7B). IGF-I (10 min) alone induced phosphorylation of Akt and MAPK. After cotreatment with ICI 182780 for 20 min, there was a significant decrease of ER α protein, and phosphorylation of Akt and MAPK was also decreased compared to treatment with IGF-I alone. Quantitation of ER α protein levels and kinase phosphorylation in cells cotreated with IGF-I plus ICI 182780 showed a time-dependent decrease in these parameters and decreased Akt phosphorylation was the most pronounced response at the 20-min time point. Decreased ER α protein in MCF-7 cells treated with 1 μ M ICI 182780 for 2 h was also confirmed by immunocytochemical analysis (Fig. 7C) and the results were similar to those observed in MCF-7 cells transfected with iER α (Fig. 2C). Moreover, ICI 182780 also inhibited IGF-I-induced transactivation in cells transfected with PI3-K- and MAPK-dependent constructs (data not shown) as observed in MCF-7 cells transfected with iER α (Fig. 3C and D).

The effects of ICI 182780 on induction of cyclins D1, A and E by IGF-I were also investigated in MCF-7 cells treated with 10 nM IGF-I (24 h), 1 μ M ICI 182,780

(24 h), or their combination (Fig. 8). ICI 182780 alone decreased levels of ER α protein but did not significantly affect cyclins D1, A and E, and this was in contrast to the decrease in cyclin D1 and A protein levels in cells transfected with iER α (Fig. 6). This suggests that other activities of ICI 182780 may prevent degradation of these proteins. IGF-I alone induced cyclin D1, A and E proteins; however, in the combined treatment (ICI 182780 plus IGF-I), the antiestrogen significantly inhibited induction of the cyclins by IGF-I. ER α protein was not affected by IGF-I but decreased after combined treatment with ICI 182780. Thus, degradation of ER α by iER α or ICI 182780 is linked to inhibition of IGF-I signaling in MCF- cells, indicating that extranuclear ER α plays an essential role in IGF-I-induced cell proliferation, cell-cycle progression, MAPK and PI3-K activation and gene expression.

Discussion

Growth factors play an important role in mammary tumor growth and induce breast cancer cell proliferation

through both endocrine and paracrine pathways. IGF-I and other polypeptide growth factors interact with their cognate membrane-bound receptors, which have tyrosine kinase activities, and these receptor–ligand interactions initiate activation of kinase cascades and their downstream nuclear genomic targets. Although growth factors induce mitogenic responses and gene expression in cells/tissues that do not express the ER, in E₂-responsive cells/tissues growth factor-induced responses are tightly coupled with ER expression. For example, in ER α -knockout (ERKO) mice, the effects of EGF and IGF-I are attenuated or suppressed (Curtis *et al.* 1996, Klotz *et al.* 2002). EGF induces uterine EGF receptor autophosphorylation and c-Fos expression in ERKO mice, whereas induction of uterine progesterone receptor mRNA and DNA synthesis by EGF were not observed (Curtis *et al.* 1996, Klotz *et al.* 2002). IGF-I induces uterine DNA synthesis in wild-type but not ERKO mice; in contrast, IGF-I receptor-dependent activation of PI3-K and MAPK is induced by IGF-I in both wild-type and ERKO mice, suggesting that kinase activation in the absence of ER α is insufficient for uterine proliferation (Klotz *et al.* 2002). IGF-I induces proliferation of MCF-7 cells (Fig. 1) and this response is inhibited by antiestrogens. Moreover, IGF-I does not induce growth of an ER α -negative MCF-7 cell subline; however, re-expression of ER α in these cells restores IGF-I responsiveness (Oesterreich *et al.* 2001).

One level of IGF–ER α crosstalk involves ligand-independent activation of E₂-responsive genes through selective phosphorylation of ER α (Ali *et al.* 1993, Kato *et al.* 1995, Bunone *et al.* 1996, Ignar-Trowbridge *et al.* 1996, Joel *et al.* 1998). This pathway involves genomic or nuclear pathways of ER α action in which growth factor-dependent phosphorylation of the receptor is sufficient for induction of genes that require nuclear ER α –DNA (promoter) or ER α –protein–DNA interactions (Cavailles *et al.* 1989, Wakeling *et al.* 1989, Katzenellenbogen & Norman 1990, Chalbos *et al.* 1993, Ignar-Trowbridge *et al.* 1993, Westley & May 1994, Wang *et al.* 2000, Xie *et al.* 2001). IGF-I-induced responses may also involve extranuclear ER α which associates with several key proteins involved in kinase pathways, including the IGF-IR, p85 α (PI3-K regulatory subunit), G proteins, Src, and Shc (Kahlert *et al.* 2000, Migliaccio *et al.* 2000, Simoncini *et al.* 2000, Sun *et al.* 2001, Wyckoff *et al.* 2001, Razandi *et al.* 2002, Song *et al.* 2002). Inhibition of IGF-I-induced cell proliferation by ICI 182780 and LY294002 (Fig. 1) implies that both ER α and PI3-K are necessary for this response. The role of ER α in mediating IGF-I-induced gene expression, kinase activation, and cell-cycle progression was extensively investigated using iER α to efficiently knockdown ER α protein (Fig. 2). This approach could then be used to demonstrate that ER α was required for IGF-I-dependent activation of MAPK and PI3-K

signaling pathways (Fig. 4) and subsequent transactivation of SRE- and SRF-dependent promoter–reporter constructs (Fig. 3). These results are in contrast to the effects of IGF-I in the uterus of wild-type and ERKO mice where both Akt and MAPK are phosphorylated after treatment with IGF-I (Klotz *et al.* 2002). This report (Klotz *et al.* 2002) also suggested that IRS-I may be important for IGF-I-induced activation of PI3-K, and another study demonstrated that re-expression of ER α in ER-negative MCF-7 cells not only restored IGF-I responsiveness but was accompanied by increased expression of IRS-I and IGF-IR (Ignar-Trowbridge *et al.* 1996). However, using iIRS-I to knockdown the protein (Fig. 4C) it was apparent that induction of MAPK and Akt phosphorylation by IGF-I were unaffected, whereas knockdown of ER α clearly decreased kinase-dependent phosphorylation (Fig. 4A and B). Thus, although IRS-I is an important signaling molecule activated by IGF-I in MCF-7 cells (Nolan *et al.* 1997, Jackson *et al.* 1998, Lee *et al.* 1999), our results suggest that IRS-I does not directly regulate IGF-I-dependent activation of MAPK or PI3-K.

The critical role of ER α in mediating the mitogenic activity of IGF-I in MCF-7 cells was also confirmed in ER α -knockdown experiments, which demonstrated that IGF-I-induced G₁–S-phase progression and cell proliferation (Fig. 5) and cyclin D1 and E protein expression were dependent on ER α (Fig. 6). While these results were consistent with previous reports on the mitogenic activity of IGF-I (Dufourny *et al.* 1997, Dupont *et al.* 2000), these data are in contrast to a study showing that ICI 182780 inhibited IGF-dependent growth of MCF-7 cells but not IGF-I-dependent kinase activation (Varma & Conrad 2002). For example, ICI 182780 did not inhibit IRS-I, MAPK, or Akt phosphorylation or cyclin D1 induction in MCF-7 cells treated with 10 nM IGF-I (Varma & Conrad 2002). These results are surprising since ICI 182780 induces proteasome-dependent down-regulation of ER α in MCF-7 cells (Wijayaratne & McDonnell 2001, Fan *et al.* 2003, Wormke *et al.* 2003) and RNA-interference studies (Figs 2–6) clearly demonstrate an essential role for ER α in IGF-I signaling. Moreover, Lee and coworkers (1999) also reported that ICI 182780 inhibited IGF-I-induced phosphorylation of MAPK, IRS-I, and IGF-IR. We therefore further investigated the time-dependent effects of ICI 182780 on IGF-I-induced activation of kinases, and cyclins (Figs 7 and 8). Preliminary studies showed that short-term cotreatment of MCF-7 cells with IGF-I plus ICI 182780 did not decrease IGF-I-induced phosphorylation of MAPK or PI3-K (Fig. 7A) as previously reported (Varma & Conrad 2002). However, longer-term studies with ICI 182780 showed that the inhibitory effects of ICI 182780 were time-dependent. The results clearly show that ICI 182780 inhibits IGF-I-induced cyclin D1, E, and A protein expression (Fig. 8), and this was

paralleled by inhibition of IGF-I induced phosphorylation of Akt and MAPK (Fig. 7B) and IRS-I (data not shown). These data, coupled with ICI 182780-induced degradation of ER α (with or without IGF-I; Fig. 7B and C), complement the results of RNA-interference studies with iER α showing that IGF-I action in MCF-7 cells is dependent on crosstalk with extranuclear ER α (Figs 2–6). Although ICI 182780 did not inhibit some IGF-I-induced cell-cycle proteins or Akt/MAPK phosphorylation in the study by Varma and Conrad (2002), they reported that proliferation of MCF-7 cells induced by IGF-I was inhibited by ICI and this corresponded to results of this study (Fig. 1). It was also shown that although ICI 182780 did not affect IGF-I-induced cyclin D1 protein, the antiestrogen inhibited growth factor-dependent upregulation of both cyclin D1 mRNA and cyclin A protein levels (Varma & Conrad 2002), and the latter response was also observed in this study (Fig. 8). The remaining differences between our results and the previous study could also be due to variations in MCF-7 cell passage and origin, serum lot, and the different concentrations of ICI 182780 (1 μ M (this study) versus 100 nM (Varma & Conrad 2002)).

In summary, results from this study demonstrate the important role of ER α in mediating the mitogenic activity of IGF-I in MCF-7 cells and demonstrate that there were clear differences between the mechanisms of IGF-I-induced signaling in breast cancer cells and in the mouse uterus (Klotz *et al.* 2002). Both E $_2$ and IGF-I induce many comparable responses in MCF-7 and other breast cancer cell lines, and the activities of both mitogens are ER-dependent. However, there are also mechanistic differences in the actions of E $_2$ and IGF-I. For example, IGF-I-induced activation of an SRE (PI3-K/MAPK) was inhibited by the H1356 polypeptide, which is an IGF-I receptor antagonist, whereas E $_2$ -induced activation of this promoter was unaffected (Duan *et al.* 2001, 2002). A recent study showed that E $_2$ -induced formation of an ER α -Shc-IGF-IR complex in MCF-7 cells, whereas treatment with IGF-I did not recruit ER α to this complex (Song *et al.* 2004). Current studies are focused on further delineating the mechanisms of IGF-I/E $_2$ -induced responses in breast cancer cells and determining the differential role of ER α and its interactions with extranuclear factors.

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