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Estrogen utilization of IGF-1-R and EGF-R to signal in breast cancer cells

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Abstract

As breast cancer cells develop secondary resistance to estrogen deprivation therapy, they increase their utilization of non-genomic signaling pathways. Our prior work demonstrated that estradiol causes an association of ER α with Shc, Src and the IGF-1-R. In cells developing resistance to estrogen deprivation (surrogate for aromatase inhibition) and to the anti-estrogens tamoxifen, 4-OH-tamoxifen, and fulvestrant, an increased association of ER α with c-Src and the EGF-R occurs. At the same time, there is a translocation of ER α out of the nucleus and into the cytoplasm and cell membrane. Blockade of cSrc with the Src kinase inhibitor, PP-2 causes relocation of ER α into the nucleus. While these changes are not identical in response to each anti-estrogen, ER α binding to the EGF-R is increased in response to 4-OH-Tamoxifen when compared with tamoxifen. The changes in EGF-R interactions with ER α impart an enhanced sensitivity of tamoxifen resistant cells to the inhibitory properties of the specific EGF-R tyrosine kinase inhibitor, AG 1478. However, with long term exposure of tamoxifen-resistant cells to AG 1478, the cells begin to re-grow but can now be inhibited by the IGF-R tyrosine kinase inhibitor, AG 1024. These data suggest that the IGF-R system becomes the predominant signaling mechanism as an adaptive response to the EGF-R inhibitor. Taken together, this information suggests that both the EGF-R and IGF-R pathways can mediate ER α signaling.

To further examine the effects of fulvestrant on ER α function, we examined the acute effects of fulvestrant, on non-genomic functionality. Fulvestrant enhanced ER α association with the membrane IGF-1 receptor (IGF-1R). Using siRNA or expression vectors to knock-down or knock-in selective proteins, we further demonstrated that the ER α /IGF-1R association is Src-dependent. Fulvestrant rapidly induced IGF-1R and MAPK phosphorylation. The Src inhibitor PP2 and IGF-1R inhibitor AG1024 greatly blocked fulvestrant-induced ER α /IGF-1R interaction leading to a further depletion of total cellular ER α induced by fulvestrant and further enhanced fulvestrant-induced cell growth arrest. More dramatic was the translocation of ER α to the plasma membrane in combination with the IGF-1-R as shown by confocal microscopy. Taken in aggregate, these studies suggest that secondary resistance to hormonal therapy results in usage of both IGF-R and EGF-R for non-genomic signaling.

Introduction

The biological actions of estradiol (E2) are mediated both by genomic effects on transcription via nuclear ER and by non-genomic actions outside of the nuclear compartment. The non-genomic effects lead to the rapid activation of the MAP kinase and PI-3-kinase signaling pathways. Both the genomic and non-genomic actions of E2 play pivotal roles in E2-induced cancer cell proliferation and survival (4). MAPK and Akt are downstream signals resulting from the actions of the IGF-1-R (IGF-1-receptor) and EGF-R (EGF-receptor) (5;6). We and others have demonstrated that both IGF-1-R and EGF-R are actively involved in E2 biological actions by interacting with ER-mediated signaling pathways, leading to MAPK activation in MCF-7 breast cancer cells (7-9). A critical interplay among these receptors mediates the process of resistance to aromatase inhibitors and tamoxifen (10-11)

Recent studies documented that IGF-1-R-induced MAPK activation in COS-1 non-breast cancer cells is EGFR-dependent. This process involves the sequential events of activation of matrix metalloproteinases (MMPs), shedding of the extra-cellular domains of HB-EGF and HB-EGF binding and activation of EGFR tyrosine kinase (12). EGF-R has been considered a nodal point which converges many cytokine- and hormone-induced signals to lead to MAPK activation. In a variety of systems, ligands bind to their respective receptors and activate G protein coupled receptors (GPCR), resulting in increased activity of metalloproteinases 2 and 9. These enzymes in turn cleave HB-EGF from its tethering to protein structures causing release of HB-EGF and allowing HB-EGF to activate the EGFR. This pathway has been utilized by diverse stimuli, such as angiotensin I, thrombin, alpha and beta-adrenergic receptors, certain cholinergic receptors, human growth hormone and prolactin to signal downstream through the EGF-R (13-16). From these observations, Luttrell et al have described the EGFR as a nodal point of convergence of IGF-1-R signals on MAPK activation(15). Because both IGF-1R and EGFR are involved in the rapid E2 action, we postulated that the same mechanism might exist in E2-induced MAPK activation in which EGFR transactivation is required for IGF-1-induced MAPK activation.

Our recent studies examined the roles of the IGF-1-R and EGF-R in E2-induced rapid MAPK activation as well as their biological roles in mediating E2-induced cell growth and protection from cell death in MCF-7 cells (8,17-18). We showed that E2 rapidly stimulates the phosphorylation of both IGF-1R and EGFR. The selective inhibitors or knockdown of both receptors diminished E2-induced MAPK activation, but also blocked E2 mitogenic and anti-apoptotic effects (18). A series of experiments demonstrated that the EGFR acts in a linear sequence downstream of the IGF-1-R and that metalloproteinases 2 and 9 are involved in IGF-1R mediated EGFR activation. The non-genomic effects of estrogen appear to be enhanced in cells deprived of estrogen long term, a cellular model to examine the adaptive changes induced by aromatase inhibitors (19).

To assess the dynamic changes in the EGF-R and IGF-1-R pathways during development of hormone resistance, we examined these pathways during long term exposure to the anti-estrogens tamoxifen, 4-OH-tamoxifen and fulvestrant (19). We demonstrated that long term tamoxifen exposure results in the translocation of ER alpha out of the nucleus and in increased utilization of the EGF-R and IGF-1-R pathways (19). Unexpectedly, fulvestrant translocated ER alpha to the plasma membrane where it activated MAP kinase. This manuscript summarizes our published and unpublished data relating to these issues.

Materials and Methods

Reagents

Tissue culture supplies were obtained from Fisher Scientific (Pittsburgh, PA). IMEM with or without phenol red (zinc option Richter's modification) and fetal bovine serum (FBS) were products of Gibco Life Technologies (Rockville, MD). Fulvestrant was purchased from AstraZeneca, Inc. (Wilmington, DE) and dissolved in ethanol with a final concentration of ethanol in medium less than 0.01%. The original designation of ICI was ICI 182780. A commonly used designation for this has evolved as it often is referred to merely as ICI, a term used throughout this manuscript. Later the agent was named fulvestrant. At the present time, ICI 182780, ICI and fulvestrant are used synonymously. The smartpool of double-stranded siRNA of Src (Cat # M-003012-04) and siControl RNAs (D-001210-01-05) were from Dharmacon Tech (Lafayette, CO). All siRNAs were dissolved in RNAase-free buffer based on the manufacturer's protocol at a concentration of 20 μ M. Tyrphostin AG1024 and PP2 were purchased from Calbiochem (La Jolla, CA). The full-length c-Src wt expression vector (Src/p) was a gift from Dr. Fukami, (Kobe University, Japan) and has been previously reported (20). The following antibodies were obtained from Santa Cruz Biotechnology: monoclonal anti-IGF-1R β domain antibody (3B7), polyclonal anti-IGF-1R antibody (C-20), poly anti-ER α antibody (H184). The monoclonal anti-ER α antibody (Ab-10) was purchased from NeoMarkers (Fremont, CA). The HRP-conjugated monoclonal anti-phosphotyrosine antibody (4G10) was obtained from Upstate Biotech. Polyclonal anti-active MAPK antibody (9101) and anti-MAPK (9320) are from Cell Signaling.

Cell culture, siRNA and vector transfection

A detailed description of the tissue culture methods and siRNA transfection techniques has been reported previously (7,18). Briefly, MCF-7 cells growing in 5% FBS-IMEM were routinely maintained in a humidified 95% air, 5% CO₂ incubator at 37°C. 24 hours prior to treatment, the medium was changed into phenol red-free IMEM supplemented with 1% Dextran-coated charcoal-stripped FBS (DCC-FBS) for 24 hr. Cells were then treated with different agents as indicated and collected for assays. For transfection experiments with siRNA directed against Src, the medium for MCF-7 cells was first changed into antibiotic-free and serum-free DMEM without phenol red (Gibco Life Technologies) for two hours. Cells were then transfected with non-specific scrambled siRNA or siRNA against the selective proteins at 50 pmol/ml. The transfection reagent was DharmaEFCT (Dharmacon Tech, Lafayette, CO) and the procedure was carried out according to the manufacturer's instructions. For vector transfection, MCF-7 cells were transiently transfected with both 1 μ g of empty vector and Src expression vectors respectively. Transfection was performed with Effectene transfection reagent (Qiagen, Valencia, CA) according to company specifications. 48 hours after transfection, cells were treated and cell extracts were collected for Western blots. All transfections were conducted at least three times and the data are presented as mean $\pm$ SD.

Immunoprecipitation and immunoblotting

Immunoprecipitation (IP) and immunoblotting (IB) were carried out as reported previously (21). Briefly, cells were washed once with ice-cold phosphate-buffered saline containing 1 mM Na₂VO₄ and extracted with binding buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 5% Glycerol, 1% Triton X-100, 25 mM NaF, 2 mM Na₂VO₄, 10 μ g/ml of each aprotinin, leupeptin and pepstatin). Cell lysates were centrifuged at 14,000x g for 10 minutes at 4°C to pellet insoluble material. The protein concentration of the supernatant was determined using a DC protein assay kit based on the Lowry method (Bio-Rad). Equal amounts of proteins from cell extracts (0.5 mg) of each treatment were immunoprecipitated using one of the following antibodies: 1 μ g monoclonal anti-IGF-1R β domain antibody, and 1 μ g monoclonal ER α antibody. Incubations proceeded for 4 hrs at room temperature or overnight at 4°C in the

presence of 35 μ l of 50% slurry protein-G Sepharose beads (Gibco Life Technology, Rockville, MD). The beads were washed three times in cold binding buffer. For Western blotting, the proteins (either eluted from the beads or from the whole cell extracts) were analyzed on 7.5% SDS-polyacrylamide gels, and transferred to PVDF membranes. The PVDF membranes were probed with one of the following primary antibodies including: HRP-conjugated monoclonal anti-phosphotyrosine antibody (4G10, Upstate), polyclonal anti-IGF-1R antibody (C-20, Santa Cruz Biotechnology), polyclonal anti-ER α antibody, polyclonal anti-active MAPK antibody (9101, Cell Signaling) and MAPK (9320, Cell Signaling) antibodies. After washing the PVDF membrane, the immunoblots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hr (Donkey against rabbit IgG was from PIERCE or Sheep against mouse IgG was from Amersham) and further developed using the chemiluminescence detection system (PIERCE).

Confocal analysis of co-localization of ER and, IGF-1R on Cell Membrane and MAPK phosphorylation

Detailed methods regarding confocal microscopic studies were previously described (7,21). Briefly, the following antibodies were used: mouse anti-IGF-1R (Santa Cruz Biol), Rabbit anti-ER α (Santa Cruz Biol), rabbit anti-active MAPK (Cell Signaling Technologies), Alexa 633-goat anti-mouse and Alexa 488 goat anti-rabbit secondary antibodies. Alexa 546-conjugated phalloidin was included for membrane actin staining. Serum-starved MCF-7 cells were fixed in 4% (vol/vol) formaldehyde for 30 min on ice, permeabilized where indicated with ice-cold acetone for 2 min, and further blocked for 60 min with 10% (vol/vol) normal goat serum (NGS) in PBS. The secondary antibody incubations were 60 min, and the cover-slips were washed 4 times with PBS after each incubation. Cover-slips were mounted on glass slides with a Vectashield mounting solution with or without DAPI. The cells were imaged using a Zeiss confocal microscope 510 equipped with a META polychromatic multichannel detector.

Assessment of cell growth and apoptosis

Both cell growth and apoptosis assays have been previously described (7,17-18). Briefly, cells were rinsed once with 0.9% saline and then lysed in ZAP buffer. The released nuclei were counted with a model Z1 Coulter counter (Coulter Corporation, Miami, FL). Cell apoptosis was measured by an ELISA method to test DNA fragmentation.

Statistical analysis

All reported values are the means $\pm$ SE. Statistical comparisons were determined with two-tailed Student's *t*-tests. Results were considered statistically significant if the *p* value was less than 0.05 (*p* < 0.05).

Results

Involvement of both IGF-1R and EGFR in E2-induced MAPK activation

As we reported previously, E2 increased the phosphorylation of both the IGF-1R and EGF-R and activated MAPK (18). We knocked down both receptors individually by selective siRNA expression and then treated cells with vehicle and E2 at 0.1 nM. As the end point, the degree of MAPK phosphorylation was measured at 15 min of E2 treatment. Expression of siRNA against either IGF-1R or EGFR or specific tyrosine kinase inhibitors against these molecules blocked E2-induced MAPK phosphorylation (18). We considered it important to fully characterize the components of this pathway by determining the levels of receptor present. Accordingly, we studied receptor expression in MCF-7 cells assessed by Western blot and real-time (RT) RCR methods. These results demonstrated that both the IGF-1R and EGFR mRNAs

are present but the IGF-R is about 2.4-fold higher than that of EGFR. In addition, both receptors are involved in E2-induced MAPK activation (18).

In order to focus on the upstream/downstream relationships of IGF-1R and EGFR in E2-induced MAPK activation in MCF-7 cells, we utilized a strategy to bypass the estrogen receptor and stimulate both receptors directly with their cognate ligands. IGF-1, but not EGF, increased IGF-1R phosphorylation in a dose-dependent manner (18). Unlike IGF-1-R, the phosphorylation status of EGF-R was increased not only by EGF-R, but also by IGF-1, indicating that IGF-1 is an upstream molecule which mediates EGFR tyrosine kinase activation. To further confirm the specificity of ligand-induced receptor activation, the IGF-1 and EGF-induced receptor activation status was studied in the presence of their selective inhibitors, AG1024, AG1478 or an inhibitor for matrix metalloproteinase 2 and 9 (MMP2/9 inhibitor). (18). IGF-1 increased its receptor phosphorylation, which was blocked by AG1024, but not by AG1478 and MMP2/9. EGF-induced EGFR phosphorylation was blocked only by AG1478. IGF-1 increased the phosphorylation status of EGFR, which was blocked by AG1024, AG1478 and MMP2/9 with the maximum effect seen in AG1478-treated cells, confirming the upstream role of IGF-R on the activation of EGFR in MCF-7 breast cancer cells.

To study the mechanistic role of IGF-1-R and EGF-R on MAPK activation on IGF-1 and EGF action, MAPK phosphorylation was studied in cells in which IGF-1R and EGFR were specifically knocked down with selective siRNA or blocked with specific inhibitors. The siRNAs and inhibitors against IGF-1R significantly blocked IGF-1-stimulated, but not EGF-stimulated, MAPK phosphorylation. These data indicate that the EGF-R is downstream of the IGF-R component (18).

Role of IGF-1R and EGFR on E2-induced cell growth and anti-apoptosis

After biochemical demonstration of the involvement of EGFR in IGF-1-induced MAPK activation, we wished to assess the biological consequences of both receptors on E2 action in MCF-7 cells. As biological endpoints, we examined both cell proliferation and apoptosis in response to E2 (18). For this purpose, the dual knockdown and inhibitor strategies were again employed: cells were transfected with the selective siRNA against either IGF-1-R or EGF-R and then cell numbers were counted at day 4 after treatment with E2 or vehicle (ethanol). As shown in figure 1 A, the cell numbers in non-specific siRNA transfected groups were 0.68×10^6 cells/well in vehicle-treated, 2.3×10^6 cells/well in E2-treated, 1.7×10^6 cells/well in IGF-1-treated and 1.2×10^6 cells/well in EGF-treated groups. Knockdown of either IGF-1-R or EGF-R significantly decreased the basal levels of cell number to 0.47×10^6 cells/well and 0.36×10^6 cells/well, respectively. Compared with vehicle treatment, knockdown of IGF-1R significantly blocked E2 or IGF-1, but not EGF, induced cell stimulation, suggesting that ligand-induced EGFR activation is a downstream event in E2 or IGF-1 action. At the same time, knockdown of EGF-R with siRNA significantly retarded the stimulatory effect induced by E2, IGF-1 and EGF, supporting that EGF-R is a common converging point mediating E2 and IGF-1-R action. Similar results were found with use of inhibitors (18). (Fig. 1B).

Because a decrease in cell number can result either from reduced growth or increased apoptosis, we further investigated whether blockade of IGF-1-R or EGF-R could reverse the effects of E2 or growth factors on protection against cell death. A statistically significant, approximately 2-fold increase of apoptosis was observed in both AG1024 and AG1478-treated groups when compared with the cells treated with vehicle (figure 1C) (18). Treatment of cells with E2, IGF-1 and EGF not only significantly decreased cell death rate compared with vehicle-treated control, but also in general protected cell apoptosis induced by both inhibitors (i.e. AG1024 and AG1478). Interestingly, AG1024 had little effect on EGF-induced cell growth, but significantly blocked EGF-induced cell death protection. Results using the si RNA strategy were similar.

Taken together the data indicate that both IGF-1R and EGFR play a role in the effect of E2 to protect against cell death and that IGF-1R might exert a stronger anti-apoptotic effect than on proliferation.

Development of anti-estrogen resistance

Our group has reported an increased association of ER α with c-Src as well as with the EGF-R in cells developing resistance to estrogen deprivation (as a surrogate for aromatase inhibition), tamoxifen (19). At the same time, we demonstrated translocation of ER α out of the nucleus and into the cytoplasm and cell membrane (Fig 2). Blockade of cSrc with the Src kinase inhibitor, PP-2 caused relocation of ER α into the nucleus. The changes in EGF-R interactions with ER α impart an enhanced sensitivity of tamoxifen-resistant cells to the inhibitory properties of the specific EGF-R tyrosine kinase inhibitor, AG 1478 (19) (figure 3). However, with long term exposure of tamoxifen-resistant cells to AG 1478, the cells begin to re-grow but could be inhibited by the IGF-R tyrosine kinase inhibitor, AG 1024 (unpublished data). This suggests that the IGF-R system becomes the predominant signaling mechanism as an adaptive response to the EGF-R inhibitor.

Fulvestrant down-regulates total ER α protein in MCF-7 cells

Further studies evaluated the effects of another anti-estrogen, fulvestrant. Initial studies examined its acute effects in MCF-7 cells. As shown in Fig.4 (upper panel), both fulvestrant at 1 nM and 17 β -estradiol (E2) at 0.1 nM strongly decreased total cellular ER α level when compared with the vehicle-treated control. On the other hand, treatment of cells with tamoxifen at 100 nM, IGF-1 at 20 ng/ml and EGF at 20 ng/ml all had little effect on ER α down-regulation. We next determined whether fulvestrant-induced ER α downregulation is dose-dependent. MCF-7 cells were exposed to fulvestrant at different doses as indicated for 3 hrs. The result shows that fulvestrant greatly down-regulated ER α level in a dose-dependent manner (Fig. 4, lower panel). The maximum effect of fulvestrant-induced ER α depletion is seen at the concentration of both 1 and 100 nM.

Fulvestrant increases ER α interaction with the IGF-1-R

We had previously demonstrated that 0.1 nM E2 rapidly increased extra-nuclear ER α association with IGF-1R in human breast cancer MCF-7 cells but fulvestrant at 1 nM blocked this effect(7). During the process, we found that fulvestrant at higher doses (i.e 100nM) also increased the ER α interaction with IGF-1R, even if it down-regulated total ER α protein. In order to characterize how fulvestrant specifically regulates *extra-nuclear* ER α , we tested the effect of fulvestrant on the interaction of ER α with IGF-1R biochemically and morphologically. To study ER α interaction with IGF-1R, MCF-7 cells were treated for 10 min with vehicle, different doses of fulvestrant and 0.1 nM E2 as positive control. The ER α and IGF-1-R interaction was determined by immunoprecipitation of IGF-1R and detection of co-immunoprecipitated ER α on Western blot. Fig. 5A shows that there is a basal level of ER α /IGF-1R interaction and E2 at 0.1 nM increased the protein complex formation. Fulvestrant at 100 nM, but not at doses of 0.01 and 1 nM, strongly induced the interaction of ER α with IGF-1R (Fig. 5A, upper panel). The IGF-1R and ER α interaction was further confirmed using the reverse order of antibodies (Fig. A, lower panel). We next tested the effects of fulvestrant on the time course of ER α /IGF-1-R interactions. Fulvestrant at 100 nM rapidly increased ER α interactions with IGF-1-R as early as 5 min with maximal peak at 15-30 minutes (Fig. 5B, upper panel). The protein complex between ER α and IGF-1R persisted for 48 hrs after fulvestrant treatment (Fig. 5B, lower panel). Together, these results suggest that fulvestrant, in addition to its effect on total ER α down-regulation, stimulates ER α interaction with IGF-1R in the plasma membrane.

To further confirm the extra-nuclear ER α interaction with IGF-1R, a confocal microscopy method was employed. Cells were stained under after fixation under non-permeable and permeable conditions (see methods for specific details on the permeabilized and non-permeabilized conditions). As shown in Fig. 5C, under non-permeabilized conditions in which the nuclear ER α is not stained, the expression of the extra-nuclear ER α in green is low. The IGF-1R is shown in red and mainly expressed along the cell membrane with little expression in the cytosol. The merged image shows little overlapping of IGF-1R and ER α on the cell membrane due to the low expression of the extra-nuclear ER α . Fig. 5D shows that fulvestrant at 100 nM did not change IGF-1R expression (red), but strongly induced the ER α staining (green) both in the cytosol and on the membrane, indicating that fulvestrant treatment induced ER α shuttling from the nucleus to the extra-nuclear space. The arrows in the merged image illustrate the ER α co-localization with IGF-1R on the cell membrane as the yellow color development due to the overlapping of IGF-1R (red) and ER α (green). Together, the confocal images support our view that ER α interacts and co-localizes with IGF-1R in the membrane under the fulvestrant treatment.

With non-permeabilizing staining conditions, the extra-nuclear ER α level, including that associated with IGF-1R, greatly increased in response to fulvestrant treatment. Next we used permeabilized conditions to examine the redistribution of ER α in response to 100 nM fulvestrant (Fig. 5E). In vehicle-treated cells, ER α green staining is mainly localized in the nucleus with dense staining of nucleoli. There is also faint, scattered ER α staining in the cytosol. Fulvestrant treatment not only decreased the dense staining of ER α in the nucleus but also strongly increased the cytosol and membrane ER α staining, suggesting that fulvestrant shuttles ER α from the nucleus to the cytosol and plasma membrane.

Role of Src and IGF-1R in fulvestrant-induced ER α /IGF-1R interaction

We next studied the mechanism of ER α /IGF-1R interaction induced by fulvestrant. We postulated that fulvestrant at higher doses might act as an estrogen agonist on extra-nuclear events. Accordingly, we tested several selective signaling pathway inhibitors on fulvestrant-induced ER α /IGF-1R interactions. Fig. 6A shows that fulvestrant greatly increased ER α and IGF-1R interaction at 5 min. The c-Src inhibitor PP2 or IGF-1R tyrosine kinase inhibitor AG1024, abrogated the fulvestrant-induced the ER α /IGF-1R interaction, indicating that both Src and IGF-1R activation are required for fulvestrant-induced ER α /IGF-1R interaction. Blockade of EGF-R with AG1478 or MAPK with PD98059 had little effect on the fulvestrant-induced ER α interaction with IGF-1R.

To further confirm the above observations, we then employed a dual strategy by knock-down or over-expression of Src and IGF-1R in MCF-7 breast cancer cells. As shown in Fig. 6B, transfection of cells with siRNA against Src greatly knocked down Src expression (third row, upper panel). Compared with fulvestrant-induced ER α /IGF-1R interaction in non-specific, scrambled siRNA-transfected cells, knock-down of Src blocked ER α /IGF-1R association induced by fulvestrant (Fig. 6B, upper panel). This observation provides strong evidence that Src is a mediator of the ER α /IGF-1R interactions. The role of Src in protein complex formation was further tested by over-expression of wt Src in MCF-7 cells. The lower panel of Fig. 6B shows that fulvestrant increased ER α /IGF-1R interactions in empty-vector transfected cells. Over-expression of wild type Src not only increased the basal level of protein interaction but also further potentiated the fulvestrant-induced association of both proteins.

We next studied the role of IGF-1R on protein complex formation by knock-down of IGF-1R with selective siRNA in MCF-7 cells. As shown in Fig. 6C, fulvestrant increased ER α /IGF-1R interactions in non-specific, scrambled siRNA-treated controls. Expression of selective siRNA against IGF-1R greatly knocked down the protein (third row, lower panel), which blocked the fulvestrant-induced ER α interaction with IGF-1R. Our data demonstrate that IGF-1R is an

important molecule to recruit ER α in response to fulvestrant treatment. Together, our findings demonstrate that IGF-1-R, as a membrane receptor, provides a docking site for ER α membrane association. Src plays an important role linking ER α to IGF-1R.

After demonstrating the role of Src and IGF-1R in recruiting ER α , we were interested to know the functions of Src and IGF-1R in fulvestrant-induced total ER α degradation. To pursue this purpose, we investigated the effects of fulvestrant on cellular ER α degradation in the presence of a series of selective pathway inhibitors. As shown in the upper panel of Fig. 6D, fulvestrant alone greatly decreased cellular ER α level compared with the vehicle-treated control. MG132, an inhibitor of ubiquitin proteinases, was used as a positive control for blockade of fulvestrant-induced cellular ER α degradation. Blockade of Src with PP2 did not alter the basal ER α expression level. When combined with fulvestrant, it potentiated the fulvestrant effect on the decrease of ER α level, suggesting that ER α association with IGF-1R protected itself from protein degradation. Next, we tested the combination of fulvestrant with AG1024, AG1478 or PD98059 on the total ER α expression. The lower panel of Fig. 6D shows that fulvestrant decreased ER α level compared with the control. AG1024, but not AG1478 or PD98059, further potentiated the fulvestrant effect on ER α degradation. Together, our data support the findings that the association of ER α with IGF-1R, appears to prevent degradation by a ubiquitin proteinase system.

Fulvestrant induced phosphorylation status of IGF-1R and MAPK

PP2 and AG1024 are selective tyrosine kinase inhibitors of Src and IGF-1R which have been known to be upstream molecules in MAPK activation cascade. In our experiments, both inhibitors blocked fulvestrant-induced ER α /IGF-1R interaction. We postulated that fulvestrant at 100 nM might induce the activation of IGF-1R and MAPK. To test this hypothesis, fulvestrant-induced activation of the IGF-1R and MAPK was examined by measuring their phosphorylation status in MCF-7 cells. Fig. 7A shows that fulvestrant time-dependently increased IGF-1R phosphorylation with an optimal time at 5 min. IGF-1, used as a positive control, strongly increased IGF-1R phosphorylation. To test MAPK activation, we measured MAPK phosphorylation induced by doses of fulvestrant. As shown in Fig. 7B, fulvestrant at lower doses, i.e. 0.01 and 1 nM, had little effect on MAPK phosphorylation, but 100 nM fulvestrant greatly increased MAPK phosphorylation when compared with a vehicle-treated control. We then tested the time course of fulvestrant on MAPK activation and showed that fulvestrant activated MAPK as early as 10 min (Fig. 7C). Since Src and IGF-1R are both upstream molecules on MAPK activation and both are involved in fulvestrant-induced ER α /IGF-1R association, we hypothesized that Src and IGF-1R might be upstream molecules with respect to fulvestrant-induced MAPK activation. Accordingly, cells were pre-treated with PP2 and AG1024, then challenged with 100 nM fulvestrant. Fig. 7D shows that fulvestrant at 100 nM greatly increased the phosphorylation status of MAPK compared with the control. Both PP2 and AG1024 attenuated this effect with the strongest inhibitory effect seen in PP2-treated cells.

To further confirm our Western blot results on fulvestrant-induced MAPK activation, we analyzed MAPK phosphorylation using the confocal microscope method. Fig. 7E shows that the phosphorylated MAPK is stained in green, cell membrane actin in red and the nucleus in blue. In vehicle-treated cells, the phosphorylated MAPK staining is very low with only a few green spots around the cytosol (**a and d**). Fulvestrant at 100 nM increased the green staining in both cytosol and nucleus indicating an increase of the phosphorylated MAPK (**e and h**). As a positive control, IGF-1 at 10 ng/ml strongly enhanced the green staining of the phosphorylated MAPK (**i and l**).

Extra-nuclear action of fulvestrant is dissociated from its genomic action

Since fulvestrant at a 100nM dose acts estrogenically in non-genomic settings of MCF-7 breast cancer cells, we examined the effect of the same dose on estrogen-regulated genomic responses. Accordingly, we investigated the genomic action of fulvestrant on the estrogen-responsive endpoints, ERE transcription and E2F1. In this experiment, we used estrogen as a positive control. As shown in Fig. 8A, fulvestrant dose-dependently decreased both ERE and E2F1 activities in MCF-7 cells. Unlike fulvestrant, estrogen dose-dependently increased ERE and E2F1 promoter activities. These observations suggest that fulvestrant, even at higher doses, continues to exert anti-estrogenic effects on transcriptional events in MCF-7 breast cancer cells.

Next, we investigated the effects of the 100 nM dose of fulvestrant on cell growth arrest in the presence or absence of PP2 and AG1024 in MCF-7 cells. As shown in Fig. 8B, the cell number in vehicle-treated group is 0.81×10^6 cells/well, which represents 100% of the control. Fulvestrant at 100 nM significantly inhibited cell growth to 71% of the control levels. The result indicates the extra-nuclear effect of a higher dose of fulvestrant had little effect on cell growth regulation. PP2 and AG1024 alone inhibited cell numbers to 87% and 82% of that in the vehicle-treated group respectively. However, 100 nM fulvestrant, when in combination with PP2 and AG1024, further lowered the fulvestrant alone-induced cell growth arrest to 61% and 58% of the control, which is statistically significant when compared with fulvestrant-treated alone ($p < 0.01$). The observation demonstrates that further down-regulation of ER in the presence of PP2 and AG1024 potentiates the cell growth arrest induced by fulvestrant alone. To confirm our findings, we further tested knock-down of IGF-1-R with siRNA on fulvestrant-induced cell growth arrest (Fig. 8C). Our data confirmed that down-regulated IGF-1R expression potentiated fulvestrant-antiestrogenic action.

Discussion

Estradiol (E2), mediated by its ER alpha and beta receptors, regulates gene transcription and also exerts extra-nuclear effects on the activation of many signaling molecules (22-23). Our laboratory and those of others demonstrated that ER α can co-opt classical membrane growth factor receptor signaling pathways involving IGF-1-R and EGF-R (7;18,24). These alternative signaling pathways are up-regulated in a model of long term estradiol deprivation which is used as a cell culture model to mimic the effects of aromatase inhibitor therapy (25). Our studies reviewed here tested the hypothesis that E2 can utilize a linear signaling pathway which involves the sequential interactions of ER α , IGF-1-R, matrix metalloproteinases and EGF-R to activate MAPK phosphorylation. Observations supporting this hypothesis involve two separate experimental strategies: examination of E2-initiated effects and dissection of the sequential linear effects of ligands for IGF-1R and EGFR. Regarding E2 effects, we found that (i) E2 rapidly induced IGF-1-R and EGF-R phosphorylation and MAPK activation; (ii) blockade or knockdown of either IGF-1-R or EGF-R abrogated E2-induced MAPK phosphorylation; (iii) knockdown or blockade of either IGF-1-R or EGF-R counteracted the stimulation of cell growth and protection against cell death induced by E2. The second strategy, involving use of IGF-1-R and EGF-R ligands to dissect out the linear, sequential nature of events, provided the following additional evidence in support of our hypothesis: (i) MMP2 and MMP9 are involved in IGF-1-R action upstream of EGF-R activation; (ii) inhibition of IGF-1 action by the IGF-1-R inhibitor AG1024 or by siRNA knockdown of IGF-1-R or abrogation of MMP2/9 with an inhibitor blocks EGFR activation and biologic effects on cell proliferation and apoptosis (18).

The working hypothesis for our studies is that long term treatment with hormonal therapy causes breast cancer cells to adapt by up-regulating various signaling pathways. Our strategic approach is to examine cells acutely exposed to anti-estrogens and then sequentially during long term treatment. We have previously reported these findings for tamoxifen and 4-OH-

tamoxifen (19). In examining fulvestrant, we found surprising results during acute exposure to this agent. Fulvestrant is generally considered a pure anti-estrogen which blocks both the genomic and non-genomic functions of estrogen (26). Contrary to this concept, our data show that fulvestrant at 100 nM initiates rapid non-genomic events similar to those induced by estrogen. Summarizing our findings, we demonstrated that: i) fulvestrant from 0.01 nM to 100 nM down-regulates total cellular ER α within 3 hrs but at 100 nM rapidly induces a strong ER α /IGF-1R complex formation beginning at 5 minutes and lasting for 48 hours ii) confocal microscopy shows that fulvestrant administration is associated with export of ER α from the nucleus into the cytosol and physical interaction with the IGF-1-R on the cell membrane iii) both the Src inhibitor PP2 and the IGF-1-R inhibitor AG1024 interrupt formation of the ER α /IGF-1R complex and lead to an enhanced fulvestrant-induced ER α downregulation and cell growth inhibition than fulvestrant treatment alone; iv) Src and IGF-1-R knock-down or knock-in strategies further confirmed that Src and IGF-1R are actively involved in fulvestrant estrogenic action in non-genomic settings, including a rapid increase of IGF-1R and MAPK phosphorylation. Taken together, these data suggest that fulvestrant acted as an estrogen agonist on extra-nuclear functions but blocked genomic effects on transcription and cell proliferation in the studies described.

Our hypothesis to explain these findings relates to the fact that effects can differ depending on sub-cellular compartmental localization. The data show that ICI down-regulates ER α in unfractionated extracts from MCF-7 cells (Figure 4) but at the same time, there is an increased association of ER α with IGF-1-R by western blot. IGF-1R resides predominantly in the cell membrane. Our hypothesis is the ICI causes proteolytic down-regulation of ER α in the cytoplasm and nucleus but when ER α is translocated to the plasma membrane, it is protected from degradation. There it is stimulated by ICI to bind to IGF-1-R. While there is an overall decrease in ER α in the cell, the fraction in the cell membrane is increased. While this hypothesis fits with all of our data, we do not have direct proof based on ER α cell fractionations in these studies.

It is surprising that ICI exerts estrogenic properties in the plasma membrane and anti-estrogenic effects in the nucleus. However, if one examines the structure of ICI it represents estradiol with a long side chain. ICI blocks transcription in the nucleus by two mechanisms, down-regulation of ER α and altered binding to the various co-activators. If down-regulation of ER α does not occur in the plasma membrane and there are no co-activators there., ICI could easily bind to ER α in the membrane, activate cSrc and allow ER α to bind to the IGF-1-R. While consistent with data in the literature, our explanation requires direct proof in additional studies.

Clinically, fulvestrant is given at 250 mg by intramuscular injection, which results in plasma concentrations from 14-19 nM (27-28). In our study, fulvestrant at 100 nM induces a strong interaction between ER α and IGF-1-R and rapidly increases IGF-1-R and MAPK phosphorylation. These data suggest that fulvestrant might initiate similar actions in the cells of breast cancer patients. We postulate that the fulvestrant-induced responses shown have the following potential: i) prevention of ER α molecule degradation outside of the nucleus; ii) preservation of extracellular ER α as a functional molecule which might be related to later development of endocrine-resistance. Further studies in tumor samples will be required to confirm these possibilities.

Fulvestrant has been previously shown to disrupt ER α nucleocytoplasmic shuttling and cause receptor cytoplasmic localization (29). In agreement with these studies, we observed that ER α relocated to the cytoplasmic compartment of breast cancer cells in response to fulvestrant treatment. However, our new findings demonstrated that fulvestrant at higher doses also increased extra-nuclear ER α interaction with the membrane IGF-1-R, and increased IGF-1-R and MAPK phosphorylation. Although the mechanism underlying these rapid actions remains

elusive, it appears that under higher concentrations, fulvestrant, like estrogen, may act directly at the cell membrane and exert agonist effects on many membrane-initiated events. Others have provided data suggesting the non-genomic estrogenic actions of fulvestrant. In MCF-7 cells, the McDonnell group reported that fulvestrant alone, like estrogen, rapidly increased a detectable calcium oscillation (30). We postulate that higher doses of fulvestrant, like estrogen, may initiate a similar pathway recruiting ER α on the cell membrane by binding to IGF-1-R. Our key finding is that fulvestrant at higher doses appears to be an estrogen agonist on non-genomic end-points. A component of fulvestrant resistance might then be the interplay with membrane growth factor receptors such as IGF-1R.

Mounting evidence suggests that the crosstalk between non-genomic ER α action and growth factor receptor pathways is one mechanism in the development of endocrine resistance (31-32). Our data regarding cells exposed long term to estrogen, a biologic condition analogous to that induced by aromatase inhibitors, support this concept (25). These cells exhibit up-regulation of the MAP kinase and PI-3-kinase pathways and specific inhibitors of them block cellular proliferation. Long term exposure to tamoxifen and 4-OH-tamoxifen is associated with translocation of ER α out of the nucleus and increased dependence on the EGF-R and IGF-R pathways (19). The data of Osborne and Schiff et al further demonstrate the sequential adaptation process during development of hormonal resistance (32). Taken together, these data suggest that breast cancer cells are highly plastic and can utilize multiple pathways to overcome the inhibitory effects of various anti-hormonal agents.

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Fig. 1A

Fig. 1B

Fig. 1C

Figure 1.

Evidence of the involvement of IGF-1R and EGFR in E2-induced cell growth and cell death protection. **A:** Cells were transiently transfected with smart pool of siRNA of siControl or siRNA against IGF-1R and EGFR using the siPORT lipid transfection reagent. At day 2, cells were treated with either vehicle, 0.1 nM E2, 20 ng/ml IGF-1 or 20 ng/ml EGF. Cell numbers were counted after four-days of treatment. * $p < 0.05$ compare E2 or growth factor-treated, siRNA expressed cells with vehicle, E2 or growth factor-treated alone. **B & C.** MCF-7 cells were pretreated with either 1 μ M AG1024 or 1 μ M AG1478 for 30 min and then treated with vehicle, 0.1 nM E2, 20 ng/ml IGF-1 or 20 ng/ml EGF. Cell numbers were counted as above and cell apoptosis was assayed with ELISA method as described in "Materials and Methods". All experiments were repeated at least three times and one representative is shown. * $p < 0.05$ compare the combination of inhibitors and E2 or growth factor-treated cells with vehicle, E2 or growth factor-treated alone.

Figure 2.

Altered localization of ER alpha in TAM-R cells. Cultured on coverslips for 2 d, cells were fixed and incubated with anti-ER antibody overnight at 4 degrees C. After a thorough wash with PBS, the cells were incubated with Alexa Fluo488-labeled secondary antibody (green) for 1 h. Actin was stained with Alexa Fluor 546 labeled phalloidin (red). Confocal images were captured under Zeiss LSM 510 confocal microscope (40 power objective)

Fig 3 **Cell growth** **Apoptosis**

Figure 3.

Enhanced sensitivity to growth inhibition by AG1478 in TAM-R cells. **A.** Growth responses to AG 1478. TAM-R and control cells were plated in six-well plates at a density of 60,000 per well in IMEM containing 5% FBS. The cells were then treated with different concentrations of EGFR inhibitor AG1478 for 5 d, and cell number was counted. Points represent the average of four independent experiments; bars, SE. **B.** apoptotic response to AG1478. Cells were plated into 12-well plates. A parallel set of plates were used for cell counting. Two days later, the cells were treated with AG1478 (5 Amol/L) for 48 h before ELISA for apoptosis.

Figure 4.

Fulvestrant (ICI) down-regulated total cellular ER α level in MCF-7 cells. MCF-7 breast cancer cells were treated with 100 nM ICI, 0.1 nM 17 β -estradiol (E2), 100 nM Tamoxifen (Tam), 10 ng/ml IGF-1 and 10 ng/ml EGF (upper panel) or different doses of ICI (lower panel) for 3 hrs. The expression levels of ER α and actin were assayed by Western blot using anti-ER α and anti-actin antibodies.

Fig. 5A

Fig. 5B

Fig. 5C

Fig. 5D

Figure 5.

Fulvestrant increased ER α /IGF-1R association in MCF-7 cells. **A:** Doses of fulvestrant on ER α association with IGF-1R. MCF-7 cells were treated with vehicle, 0.1 nM E2 as a positive control and different doses of 100 nM ICI for 15 min. The IGF-1R was immunoprecipitated and the co-immunoprecipitated ER α was detected on Western blot (upper panel). The reverse order of antibodies was used to confirm the ER α /IGF-1R interaction (lower panel). Non-specific IgG was used in the immunoprecipitation step as negative control. **B** Time course of fulvestrant on ER α association with IGF-1R. MCF-7 cells were treated with ICI at 100 nM for different times as indicated. Then ER α /IGF-1R association was detected as described in “Materials and Methods”. **C and D:** Confocal microscope study of fulvestrant-induced ER α and IGF-1R co-localization on the cell membrane in non-permeabilized condition. Cells were starved in 1% DCC medium for 24 hr and then treated with either vehicle (C) or ICI at 100 nM (D) for 15 min. Treated cells were fixed in 4% formaldehyde and stained with mouse monoclonal anti-IGF-1R antibody (a), rabbit polyclonal anti-ER α antibody (b), or nuclear staining with DAPI (c). The merged images are shown in d. Scale bar = 20 μ m. **E:** Confocal microscopic study of fulvestrant-induced ER α exportation from the nucleus to the plasma membrane under permeabilized conditions. Cells under E2-deprived conditions were treated with vehicle or ICI at 100 nM for 15 min. Treated cells were fixed in 4% formaldehyde, permeabilized with cold acetone, and stained with rabbit polyclonal anti-ER α antibody (green) and phalloidin to stain membrane filamentous actin (red). Scale bar = 10 μ m.

Fig. 6A

Fig. 6B

Fig. 6C

Fig. 6D **Figure 6.**

Role of Src and IGF-1R in fulvestrant-induced ERα/IGF-1R interaction. **A:** Inhibitors of Src and IGF-1R tyrosine kinase blocked fulvestrant-induced ERα/IGF-1R interaction. Cells were pre-treated 30 min with 5 μM PP2, 1 μM AG1024, 1 μM AG1478 or 20 μM PD98059 (PD). Cells were then challenged with ICI at 100 nM for 15 min. The interaction of ERα and IGF-1R was assayed as described in Materials and Methods. **B:** Src expression altered fulvestrant-induced ERα/IGF-1R interaction. Cells were transiently transfected with non-specific scramble siRNA (siCont) or siRNA against Src (upper panel) or expression vector with or without insert of wild type Src (lower panel) for 3 days. Then cells were challenged with vehicle or ICI at 100 nM for 15 min. The interaction of ERα and IGF-1R was assayed as described in Materials

and Methods. The Western blots (WB) in both upper and lower panels show either Src knockdown or over-expression in the cells. The MCF-7 cell extract loaded on Western blot to monitor protein migrations was used as a positive control (pc). **C**: Knock-down of IGF-1R abolished fulvestrant-induced ER α and IGF-1R association. Cells were transiently transfected with non-specific scramble siRNA (siCont) or siRNA against IGF-1R for 3 days, then cells were challenged with vehicle or ICI at 100 nM for 15 min. The interaction of ER α and IGF-1R was assayed as described in Materials and Methods. **D**: PP2 and AG0124 further potentiate the fulvestrant effect on cellular ER α degradation. Cells were pretreated with the selective inhibitors as indicated for 30 min and then challenged with vehicle or ICI at 100 nM for 3 hrs. The total ER α level was assayed by Western blot. The blot was also probed with actin to normalize protein loadings.

Fig. 7A & 7B

Fig. 7C & 4D

Figure 7.

Fulvestrant at 100 nM activates both IGF-1R and MAPK in MCF-7 cells. **A:** Fulvestrant induced IGF-1R phosphorylation. Cells were treated with either vehicle, 10 ng/ml IGF-1 as positive control or 100 nM ICI for the time indicated and the IGF-1R phosphorylation was assessed by immunoprecipitation of IGF-1R and detection of its phosphorylation status using anti-pY antibody 4G10. **B and C** Fulvestrant-induced MAPK phosphorylation. Cells were treated with vehicle or fulvestrant at the doses indicated for 15 min (**B**) or 100 nM fulvestrant for the time indicated (**C**). The phosphorylation status and total protein loading of MAPK were assayed using specific anti-active and anti-MAPK antibodies. **D:** PP2 and AG1024 blocked fulvestrant-induced MAPK phosphorylation. Cells were pre-treated with 5 μ M PP2 or 1 μ M AG1024, and then challenged with vehicle or 100 nM fulvestrant for 15 min. The phosphorylation status and total MAPK protein loading were assayed using specific anti-active and anti-MAPK antibodies. **E:** Confocal microscopic study of fulvestrant-induced MAPK phosphorylation. Cells were starved in 1% DCC medium for 24 hr and treated with either vehicle (upper row), ICI at 100 nM (middle row) or IGF-1R at 10 ng/ml (bottom row) for 15 min. Treated cells were fixed in 4% formaldehyde, permeabilized with cold acetone, and stained with rabbit polyclonal anti-phosphor MAPK antibody (**a**, **e**, **i**), phalloidin to stain membrane filamentous actin (**b**, **f**, **j**) or nuclear staining with DAPI (**c**, **g**, **k**). The merged images are shown in **d**, **h**, **l**. Scale bar = 20 μ m.

Fig. 8A

Fig. 8B

Fig. 8C **Figure 8.**

Biological effect of fulvestrant on reporter gene expression and MCF-7 cell growth. **A:** Fulvestrant exerts anti-estrogenic activities on genomic endpoints. MCF-7 cells were transiently transfected with the luciferase reporter genes driven by either ERE (left panel) or E2F1 (right panel) promoters for 2 days. Then cells were treated with vehicle, different doses of E2 and ICI as indicated for 9 hrs. The luciferase activities were measured using a luminometer. **B and C:** Blockade or knock-down of Src or IGF-1R potentiates fulvestrant anti-estrogenic activity. Cells were pretreated with 5 μ M PP2 or 1 μ M AG1024 for 30 min (**B**) or transiently transfected with empty or IGF-1R expression vectors for 2 days (**C**). Then cells were challenged with vehicle or ICI at 100 nM for 5 days. The cell number was counted. Data are mean $\pm$ SEM (n=6). * $p < 0.05$ compare with either vehicle- or fulvestrant-treated alone.