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Adaptive hypersensitivity following long-term estrogen deprivation: involvement of multiple signal pathways☆

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Abstract

Long-term estrogen deprivation causes hypersensitivity of MCF-7 cells to the mitogenic effect of estradiol (E_2) which is associated with activation of mitogen-activated protein kinase (MAPK). However, several lines of evidence indicate that MAPK activation is not the exclusive mechanism for E_2 hypersensitivity and multiple signal pathways might be involved. The current study explores the possible role of the PI3 kinase (PI3K) pathway in development of E_2 hypersensitivity. Basal PI3K activity in long-term estrogen deprived MCF-7 cells (LTED) was elevated as evidenced by increased phosphorylation of three downstream effectors, Akt, p70 S6 kinase, and eukaryotic initiation factor-4E binding protein (4E-BP1), which was blocked by the specific inhibitor of PI3K, LY294002. Dual blockade of both MAPK and PI3K completely reversed E_2 hypersensitivity of LTED cells. Enhancement in aromatase activity is another phenomenon accompanied with E_2 hypersensitivity. In aromatase over-expressing MCF-7 cells, aromatase activity was reduced by inhibitors of MAPK and PI3K suggesting the involvement of protein phosphorylation in the regulation of aromatase activity. Our data suggest that in addition to the MAP kinase pathway, activation of the PI3 kinase pathway is involved in E_2 hypersensitivity, which develops during adaptation of MCF-7 cells to the low estrogen environment.
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Keywords: MCF-7 cells; Long-term estrogen deprivation; MAP kinase; PI3 kinase; Aromatase

1. Introduction

Women with hormone-dependent breast cancer usually respond to surgical or medical endocrine therapies, such as oophorectomy and the antiestrogen tamoxifen. However, tumor regression in response to the primary endocrine therapy lasts only for 12–18 months. Among the patients with relapsing disease, about 50% experience secondary tumor regression upon administration of an aromatase inhibitor that further reduces levels of circulating estrogen. These clinical observations suggest that breast cancer cells that survive estrogen deprivation can adapt themselves and re-grow.

To investigate potential mechanisms for adaptation of breast cancer cells to estrogen deprivation, our laboratory and those of others have established in vitro model systems. These involve long-term cultivation of MCF-7 cells in estrogen deprived medium [1,2]. Using these model cells, we have demonstrated that after long-term estrogen deprivation, MCF-7 cells become more sensitive to the mitogenic effect of estradiol both in vitro and in vivo [1,3]. Enhanced sensitivity to estrogen may explain re-growth of breast cancer cells in a low estrogen environment and tumor regression in response to secondary endocrine therapy. Understanding the mechanisms responsible for this adaptation will lay the basis for improving endocrine therapy of hormone-dependent breast cancer.

It is well documented that both estrogen and growth factors are mitogenic to normal and malignant breast epithelial cells. Interactions between these two pathways occur in estrogen-targeted tissues including breast cancer. These can involve one of several mechanisms including (1) induction of expression of growth factors and growth factor receptors by estrogen [4–7]; (2) direct activation of the growth factor signaling pathways by estrogen through non-genomic mechanisms [8,9]; (3) activation of the estrogen receptor through its phosphorylation by growth factor-activated kinases [10–14]; and (4) synergistic interactions at the level of the cell cycle through regulation of cyclins, cyclin-dependent kinases, and inhibitors [15–20].

Our previous studies have shown that the MAP kinase pathway is activated in LTED cells and that this effect represents a factor responsible for rapid cellular proliferation in

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vitro [21]. Elevated MAPK activation has also been found in other breast cancer cell lines following deprivation of estrogen for a long period of time [22]. We further demonstrated that enhanced MAPK activation is associated with the hypersensitive phenotype of the LTED cells and we were able to increase E_2 sensitivity in wild type MCF-7 cells using exogenous TGFα to stimulate MAPK activation [3,23].

The present studies provide evidence that the PI3 kinase pathway, in addition to MAP kinase, is constitutively activated in LTED cells. These two pathways, working in conjunction, account for the enhanced sensitivity of LTED cells to the mitogenic effect of E_2. Our preliminary data also suggest the involvement of these two signaling pathways in regulation of aromatase activity.

2. Materials and methods

2.1. Materials

Estradiol was purchased from Steraloids (Newport, RI). ICI 182,780 and letrozole were kindly provided by AstraZeneca (Cheshire, England) and Novartis Pharma AG (Basel, Switzerland), respectively. [1β-^{3}H] androstenedione (specific activity: 25.9 Ci/mmol) was purchased from Perkin-Elmer Life Sciences, Inc. (Boston, MA). The sources of the other reagents were the following: TGF alpha (Collaborative Biomedical Products, Bedford, MA), PD98059 (Biomol Research Laboratories, Plymouth Meeting, PA); U0126 (Promega Corporation, Madison, WI), anti-active MAPK monoclonal antibodies (Sigma, St. Louis, MO); anti-total p44/p42 MAPK antibody (Zymed Laboratories, Inc., South San Francisco, CA); and anti-hemagglutinin (HA) protein (F7) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies against phospho-Akt (Ser473), total Akt, phospho-p70 S6 kinase (Thr389), total p70 S6 kinase, phospho-4E-BP1 (Ser65) and total 4E-BP1 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Secondary antibodies conjugated with horseradish peroxidase were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Cell culture medium (IMEM), fetal bovine serum, glutamine, and trypsin were purchased from Gibco (Gaithersburg, MD). All chemicals were obtained from Sigma (St. Louis, MO).

2.2. Cell culture

MCF-7 cells (kindly provided by Dr. R. Bruggemeier, Ohio State University, Columbus, OH) were grown in IMEM containing 5% fetal bovine serum (FBS). Long-term estrogen deprived cells (LTED) were routinely grown in phenol red free IMEM containing 5% charcoal–dextran stripped FBS (DCC-FBS). To reverse LTED cells to the wild type phenotype, LTED cells were cultured long-term in IMEM containing phenol red and 5% FBS.

2.3. Growth assay

Cells were plated in 6-well plates at the density of 60,000 cells/well in their culture media. Two days before treatment, the medium was replaced with phenol red- and serum-free IMEM. The cells were then treated in phenol red- and serum-free IMEM containing vehicle or treatment compound for 5 days with medium change on Day 3. The final concentration of vehicle (ethanol or DMSO) was 0.1–0.2%. At the end of treatment, cells were rinsed twice with saline. Nuclei were prepared by sequential addition of 1 ml HEPES−$MgCl_2$ solution (0.01 M HEPES and 1.5 mM $MgCl_2$) and 0.1 ml ZAP solution (0.13 M ethylhexadecyldimethylammonium bromide in 3% glacial acetic acid (v/v)), and counted using a Coulter counter.

2.4. Stable expression of constitutively active MAP kinase kinase (MEK-1) and aromatase in MCF-7 cells under the control of tetracycline

The CLONTECH Tetracycline-Off Gene Expression System was used to establish MCF-7 cell lines that stably express constitutively active MEK-1 or aromatase. MCF-7 cells were first transfected with pTET-off vector using the Qiagene Effectene Transfection kit. Transfected cells were selected in the medium containing G418 (600 μg/ml). Clones that survived G418 were screened for the expression of tetracycline-responsive transcriptional activator (tTA) by transient transfection with pTRE-luc vector and luciferase assay in the presence or absence of doxycycline. The cells that expressed high levels of luciferase in the absence of doxycycline which could be effectively inhibited by doxycycline in a dose-dependent fashion were used for the second stable transfection with either pTRE-MEK1 or pTRE-Arom vectors.

pTRE-MEK1^{218,222} vector was a gift from Dr. Michael Weber (University of Virginia, Charlottesville, VA). The aromatase cDNA was amplified by PCR using pHβ-arom plasmid as a template. The later was a gift of Dr. Shiuan Chen (City of Hope, CA). The PCR product was verified by sequence analysis before being subcloned into *Mlu I*/*Xba I* site of the pTRE2 vector. A pHyg vector was co-transfected with pTRE- MEK1^{218,222} or pTRE-Arom for hygromycin selection. Western analysis using anti-HA monoclonal antibody was used to detect expression of HA-tagged MEK-1. Expression of aromatase was determined by measurement of aromatase activity using the tritiated water release assay.

2.5. Immunoblotting

Cells grown in 60 mm dishes were washed with ice cold PBS. To each dish were added 0.5 ml lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1% Triton X 100, 1 mM β-glycerophosphate, 1 μg/ml leupeptin and aprotinin, 1 mM PMSF) or RIPA

buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM sodium orthovanadate, 1 mM PMSF, 20 μM leupeptin, and 0.15 μg/ml aprotinin). The dishes were incubated on ice for 5 min before collection. Cells were then pulse sonicated and centrifuged at 14,000 rpm for 10 min. Cell lysates were stored at −80 °C until analysis. Fifty microgram total protein was loaded and separated on 10% SDS polyacrylamide gel, and then transferred to a nitrocellulose membrane. The membrane was probed with primary antibodies for 2 h at room temperature. Secondary antibody conjugated with horseradish peroxidase (1:2000) was then applied. After reacting with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL), targeted protein bands were visualized by exposing the membrane to X-ray film. Specific protein bands were scanned and quantitated using a Molecular Dynamics scanner and ImageQuant program.

2.6. Aromatase assay (tritiated water release assay)

MCF-7$^{Tet\text{-}off\text{-}arom}$ cells grown in 6-well plates were rinsed with Hanks' solution and incubated at 37 °C with 1 ml serum-free medium containing approximately 1 μCi [1β^{3}H]androstenedione for 2 h. After incubation, the medium was transferred to a test tube. Two milliliters of chloroform were added to each tube to extract unconverted substrate and other steroids. An aliquot of 0.7 ml of the aqueous phase was then treated with equal amounts of 2.5% activated charcoal suspension to remove residual steroids. Tritiated water formed during aromatization of [1β^{3}H]androstenedione to estrogen was determined by measuring the radioactivity in the supernatant.

3. Results

3.1. Activation of the MAP kinase pathway and E_2 hypersensitivity

Long-term estrogen deprivation enhances the sensitivity of MCF-7 cells to the mitogenic effect of estradiol as shown by the reduced EC_{50} (concentration of estradiol which causes 50% stimulation of cell number) of estradiol with respect to cellular proliferation in the culture [1,23]. Our in vivo study also showed that LTED cell xenografts are hypersensitive, because they grew more rapidly than the wild-type MCF-7 cells in response to low doses of E_2 in nude mice [3].

We further demonstrated that the MAP kinase pathway in LTED cells is highly activated under basal conditions [3,21,23]. Activation of MAP kinase is tightly associated with the hypersensitive phenotype. We were able to induce E_2 hypersensitivity in wild type MCF-7 cells by co-administration of TGFα to activate MAP kinase [23]. As evidence that this effect involves MAP kinase and not some other action of TGFα, we demonstrated that PD 98059 could return the level of sensitivity back to that seen in the absence of TGFα.

We recognized that TGFα activates multiple signaling pathways involved in cell proliferation and that more direct evidence was required to confirm the role of the MAPK pathway in development of E_2 hypersensitivity. We decided to directly interfere with MAP kinase activation and to see if we can alter E_2 sensitivity. Two approaches were employed: over-expression of constitutively active MAP kinase kinase (MEK-1) in wild type MCF-7 cells and specific blockade of MAP kinase activation with the MEK inhibitor, U0126, in LTED cells.

Wild type MCF-7 cells were transfected with pTRE-MEK1^{218,222}, a vector that expresses constitutively active MEK-1 [24] under the control of tetracycline response elements. The resultant stable line, MCF-7$^{Tet\text{-}Off\text{-}MEK}$, expressed high levels of HA-tagged MEK-1 in the absence of doxycycline as shown on the Western blot probed with anti-HA antibody (upper panel, Fig. 1). Accordingly, ERK-1/2 MAP kinase was highly activated without doxycycline (middle panel, Fig. 1). Treatment of MCF-7$^{Tet\text{-}Off\text{-}MEK}$ cells with doxycycline for 4 days dose-dependently inhibited MEK-1 expression and MAP kinase activation. Activation of endogenous ERK1/2 was not affected by doxycycline.

Over-expression of MEK1 accelerates cellular proliferation regardless of serum concentration in the culture medium (data not shown). We then tested whether MAP

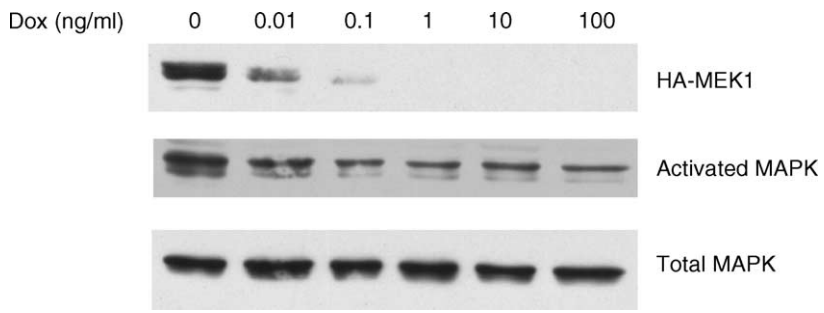

Fig. 1. Expression of constitutively active MEK1 and activation of MAP kinase in MCF-7$^{Tet\text{-}Off\text{-}MEK}$ cells. MCF-7$^{Tet\text{-}Off\text{-}MEK}$ cells were cultured in 60 mm dishes in the absence or presence of doxycycline at indicated concentrations for 4 days. Cell lysates of 50 μg total protein were subjected to Western analysis with primary antibodies against hemagglutinin (upper panel), phosphorylated ERK1/2 (middle panel), and total ERK1 (lower panel).

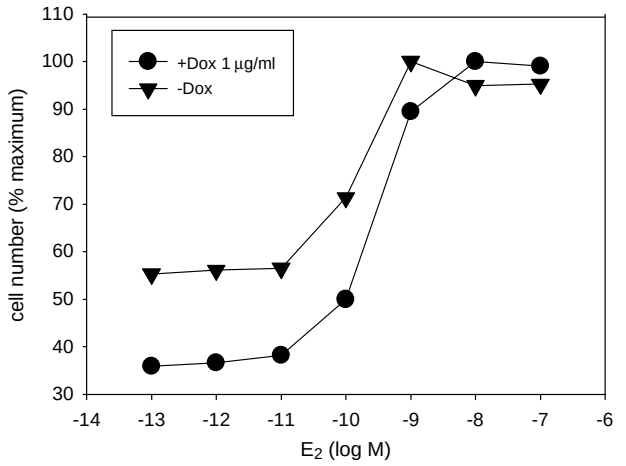

Fig. 2. Growth of MCF-7$^{Tet\text{-}Off\text{-}MEK}$ cells in response to estradiol. Sixty thousand MCF-7$^{Tet\text{-}Off\text{-}MEK}$ cells kept in cultures with or without doxycycline (1 μg/ml) were plated into each well of 6-well plates in their culture medium. Two days later, the cells were treated with various concentrations of estradiol in the presence of ICI 182,780 (10^{-9} M) in phenol red-free medium DMEM containing 1% DCC-FBS. Cell number was counted in 5 days.

kinase activation as a result of MEK1 over-expression could enhance E_2 sensitivity. The growth assay was carried out in serum-free medium containing various concentrations of estradiol with or without doxycycline (1 μg/ml). The responses of MCF-7$^{Tet\text{-}Off\text{-}MEK}$ cells to estradiol for proliferation at different status of MEK-1 expression were compared. As shown in Fig. 2, the E_2 dose–response growth curve in the absence of doxycycline (high expression of MEK1) was shifted to the left indicating that the sensitivity of MCF-7$^{Tet\text{-}Off\text{-}MEK}$ cells to estradiol was increased. However, the extent of the shift was smaller than that induced by TGFα [23]. These data suggest that activation of the MAP kinase pathway is partially responsible for E_2 hypersensitivity.

To further verify the role of the MAP kinase pathway in development of E_2 hypersensitivity, we carried out growth assays using LTED cells to determine whether blockade of MAP kinase activation with the specific MEK inhibitor, U0126, could reduce E_2 sensitivity. The concentration of U0126 used in this experiment has been demonstrated to completely inhibit MAP kinase activation (data not shown). In the presence of U0126, the E_2 dose–response curve was shifted to the right (Fig. 3). The experiment was repeated several times with consistent results. The average EC_{50} of E_2 in the presence of U0126 was reduced by 0.3 logs, indicating that MAP kinase plays a role in hypersensitivity. The difference was statistically significant ($P < 0.05$). Again, blockade of MAP kinase activation partially reduced E_2 sensitivity. These data, together with those of MCF-7$^{Tet\text{-}Off\text{-}MEK}$ cells, suggest that in addition to the MAP kinase pathway other pathways may also be involved in E_2 sensitivity.

3.2. The PI3 kinase pathway is activated in LTED cells

Activation of growth factor receptors on the surface of the cell membrane results in recruitment of Ras to the membrane and the turn on of several downstream signaling pathways [25] including the PI3 kinase pathway [26]. Since the PI3 kinase pathway plays a critical role in cell proliferation and survival, we decided to examine whether this pathway is also activated in LTED cells and whether it contributed to hypersensitivity to estradiol.

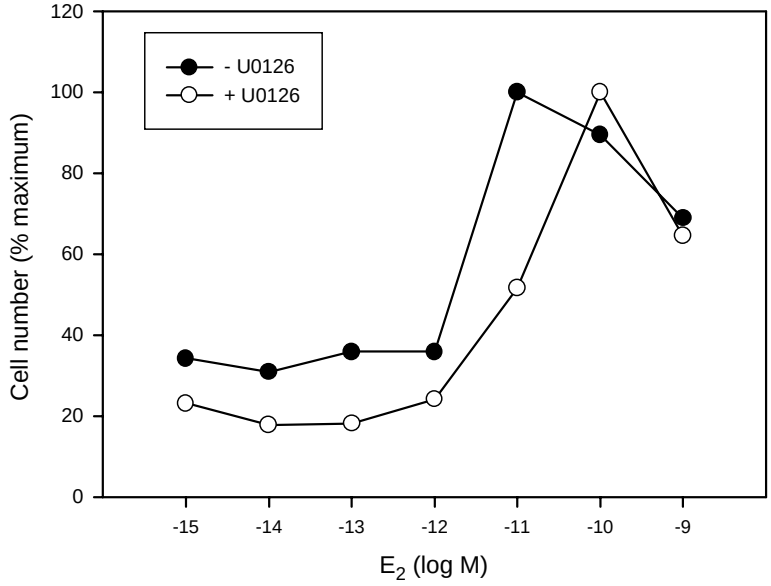

Fig. 3. Effect of U0126 on growth of LTED cells in response to estradiol. Sixty thousand LTED cells were plated into each well of 6-well plates in phenol red-free IMEM containing 5% DCC-FBS. Two days later, the cells were shifted to serum-free IMEM and cultured for another 2 days before treatment with various concentrations of estradiol plus ICI 182,780 (10^{-9} M) with or without U0126 (1 μM). Cell number was counted in 5 days.

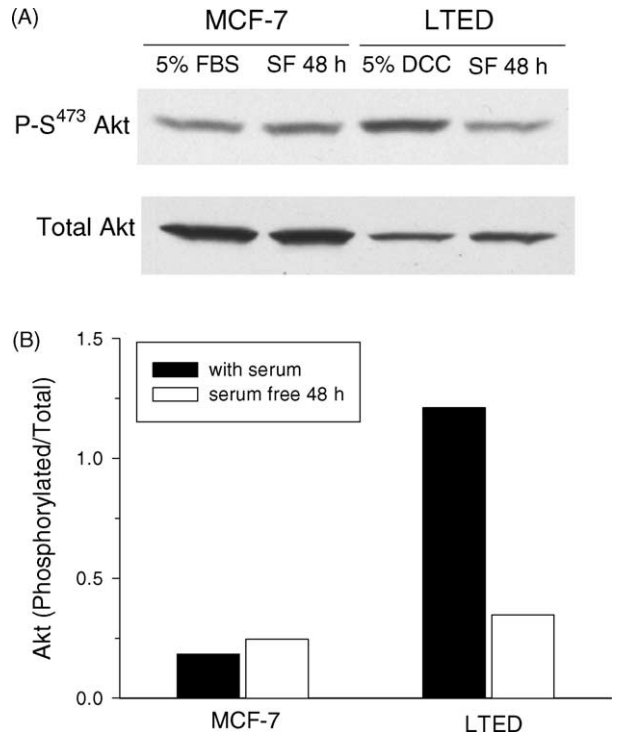

Fig. 4. Phosphorylation of Akt in MCF-7 and LTED cells. MCF-7 and LTED cells were grown in 60 mm dishes in their culture medium or in serum free medium for 48 h before lysis. (A) Western blot with anti-phospho-Akt (Ser^{473}) or total Akt antibodies; (B) densitometry quantitation.

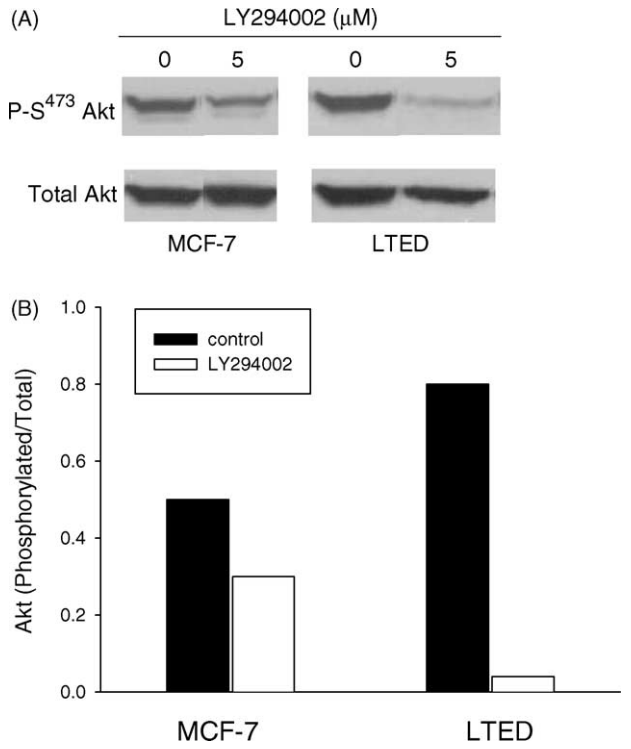

Fig. 5. Effect of LY294002 on Akt phosphorylation in MCF-7 and LTED cells. MCF-7 and LTED cells, grown in 60 mm dishes in serum containing media (5% FBS for MCF-7 and 5% DCC-FBS for LTED), were treated with 5 μM LY294002 for 24 h before lysis. (A) Western blot with anti-phospho-Akt (Ser^{473}) or total Akt antibodies; (B) densitometry quantitation.

To examine the activation of the PI3 kinase pathway in LTED cells, specific antibodies against phosphorylated forms of three downstream effectors were used for Western analysis. We first looked at Akt phosphorylation at serine 473 (Fig. 4). The basal level of phosphorylation of Akt was low in wild type MCF-7 cells and appeared not to be affected by serum starvation. In marked contrast, the level of Ser^{473}-phosphorylated Akt was six-fold higher in LTED cells and significantly reduced upon serum withdrawal. A specific inhibitor of PI3 kinase, LY294002, blocked Akt phosphorylation in both cell types with more dramatic inhibition in LTED cells (Fig. 5).

We next examined the level of activation of p70 S6 kinase (p70 S6K), a downstream effector of the PI3 kinase pathway that drives translation of a set of mRNAs encoding primarily ribosomal proteins and components of the translational apparatus [27]. The level of phosphorylation of p70 S6K at threonine 389 (a marker of activation) was more than three-fold higher in LTED cells than in the wild type MCF-7 cells (Fig. 6). LY294002 at the concentration of 5 μM almost completely abolished p70 S6K phosphorylation in LTED cells (Fig. 7).

The third molecule we examined was 4E-BP1. When existing in its hypophosphorylated state, this protein binds to eukaryotic initiation factor-4E (eIF4E) and inhibits its function. Once phosphorylated, 4E-BP1 dissociates from eIF4E and protein translation occurs. Phosphorylation of 4E-BP1 at serine 65 was significantly enhanced in LTED cells in comparison with wild type MCF-7 cells (Fig. 8). There are multiple serine residues in 4E-BP1 that can be phosphorylated. The levels of phosphorylation of 4E-BP1 at other sites were also elevated in LTED cells as reflected by different migration rates of this protein in the Western blot probed with the antibody against total 4E-BP1 (Fig. 8A). LY294002 (5 μM) significantly reduced phosphorylation at Ser^{65} and other serine sites in LTED cells (Fig. 8B).

The above results with three separate phosphorylated proteins are consistent and indicate that the PI3 kinase pathway is activated in LTED cells under basal conditions. Based upon these observations, we postulated that activation of both the MAP kinase and PI3 kinase pathways was required for the development of full E_2 hypersensitivity. To test this hypothesis, we performed estradiol dose-response growth assays in LTED cells in the presence or absence of a MAP kinase inhibitor in combination with a PI3 kinase inhibitor. With inhibition of MAP kinase alone, the rightward shift was only 0.3 logs (as shown in Fig. 3). Blockade of the PI3 kinase pathway in combination with MAP kinase caused a further right-shift of approximately 2 logs (Fig. 9). These data suggest that activation of both the MAP kinase and PI3 kinase pathways is involved during adaptation of MCF-7 cells to the low estrogen environment and regrowth.

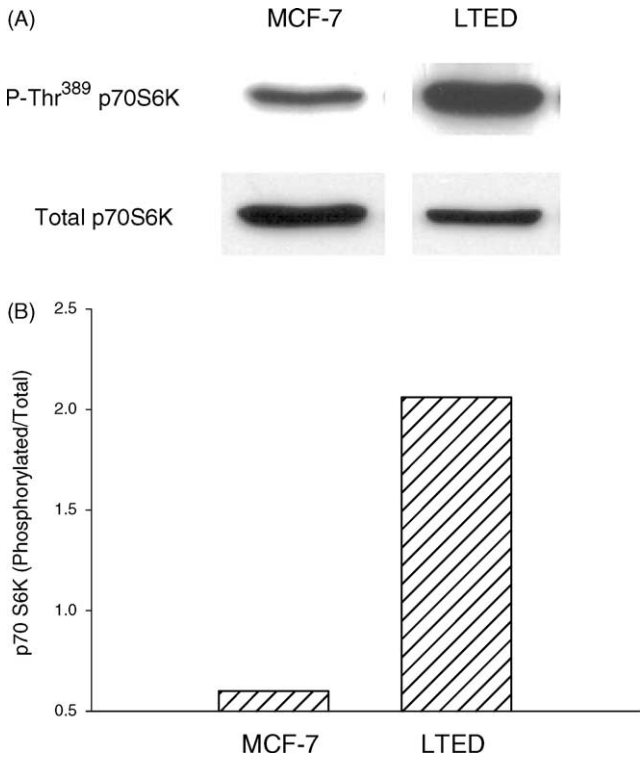

Fig. 6. Phosphorylation of p70S6 kinase (p70 S6k) in MCF-7 and LTED cells. MCF-7 and LTED cells were cultured in the medium containing 5% FBS or 5% DCC-FBS until sub-confluence. Cell lysates were then prepared for Western analysis. (A) Western blot with anti-phospho-p70 S6k (Thr^{389}) or total p70 S6k antibodies; (B) densitometry quantitation.

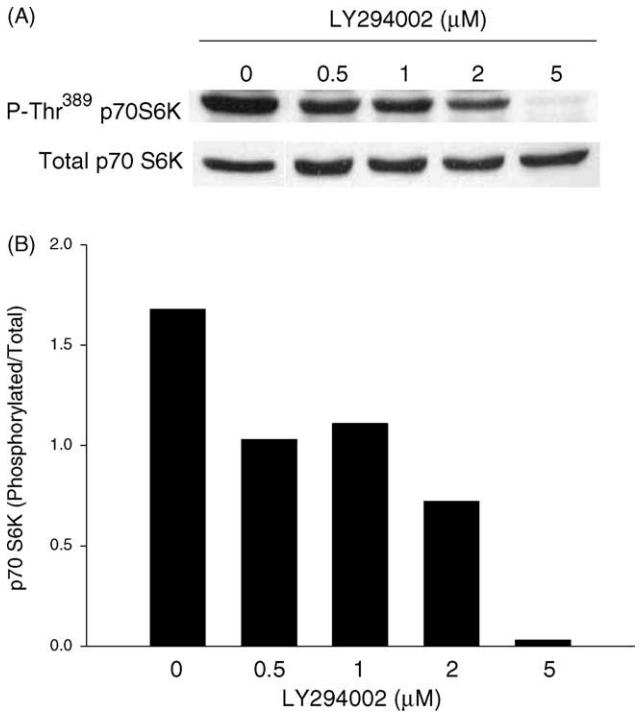

Fig. 7. Effect of LY294002 on p70 S6k phosphorylation in LTED cells. LTED cells grown in IMEM with 5% DCC-FBS were treated with LY294002 at indicated concentrations for 24 h. Cell lysates were then prepared for Western analysis. (A) Western blot with anti-phospho-p70 S6k (Thr^{389}) or total p70 S6k antibodies; (B) densitometry quantitation.

3.3. Blockade of the MAP kinase and the PI3 kinase pathway reduces aromatase activity

We have shown that long-term estrogen deprivation enhances the sensitivity of MCF-7 breast cancer cells to the mitogenic effect of estradiol, which is associated with activation of the MAP kinase and PI3 kinase pathways. We also found that aromatase activity in LTED cells was increased [28]. However, it was not clear whether there is a connection between these two phenomena.

To study the possible role of these kinases in regulation of aromatase activity, we established a stable line of MCF-7, MCF-7$^{\text{Tet-Off-Arom}}$, that expresses aromatase under the control of tetracycline. Aromatase expression in MCF-7$^{\text{Tet-Off-Arom}}$ cells was validated by measurement of aromatase activity using the tritiated water release assay. In the absence of doxycycline, aromatase activity in MCF-7$^{\text{Tet-Off-Arom}}$ cells was about 500 fmol/mg/h. Doxycycline dose-dependently reduced aromatase activity indicating an inhibition of aromatase expression. Aromatase activity could be significantly inhibited by letrozole, the specific aromatase inhibitor (Fig. 10). Pretreatment of the cells with U0126 (10 μM) or LY294002 (5 μM) for 4 days reduced aromatase activity by 60 and 30%, respectively (Fig. 11). The inhibitory effect of these two compounds

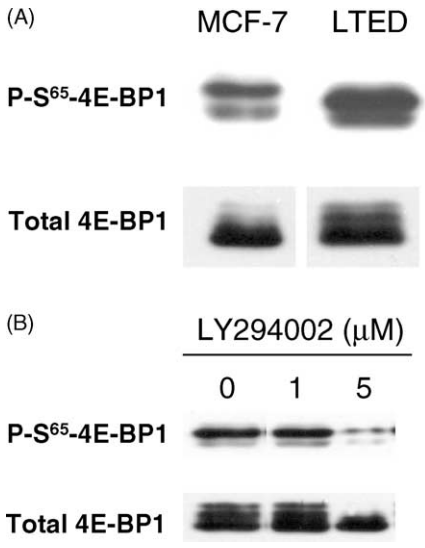

Fig. 8. Phosphorylation of 4E-BP1 in MCF-7 and LTED cells. MCF-7 and LTED cells were cultured in the medium containing 5% FBS or 5% DCC-FBS until sub-confluence. Cell lysates were then prepared for Western analysis. (A) Western blot with anti-phospho-4E-BP1 (Ser^{65}) or total 4E-BP1 antibodies; (B) Western analysis of lysates prepared from LTED cells treated with LY924002 for 24 h.

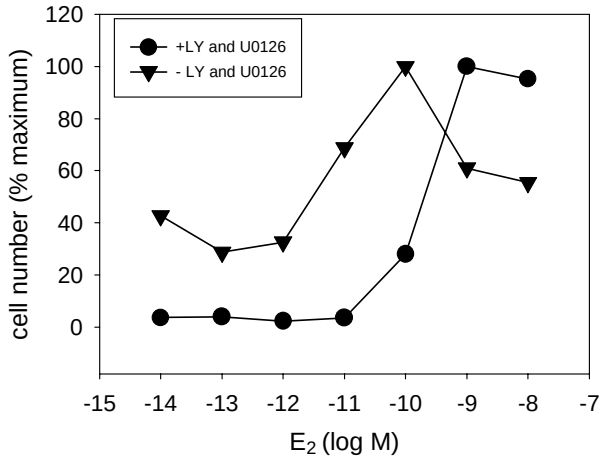

Fig. 9. Effect of U0126 and LY294002 on growth of LTED cells in response to estradiol. Fifty thousand LTED cells were plated into each well of 6-well plates in phenol red-free IMEM containing 5% DCC-FBS. Two days later, the cells were shifted to serum-free IMEM and cultured for another two days before treatment with various concentrations of estradiol plus ICI 182,780 (2×10^{-9} M) with or without U0126 (1 μM) and LY294002 (2 μM). Cell number was counted in 5 days.

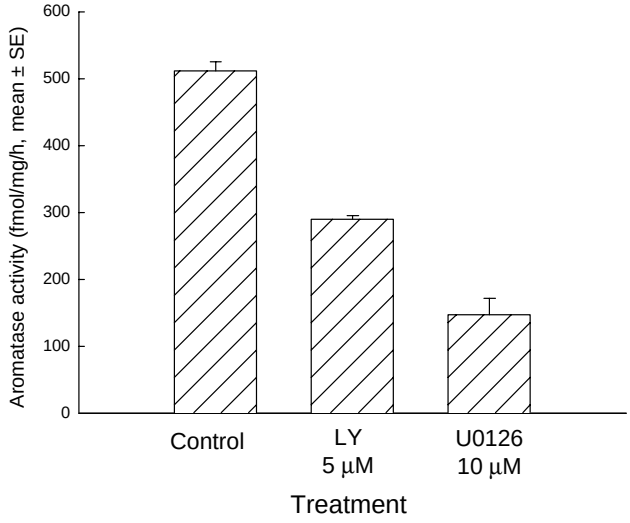

Fig. 11. Effect of U0126 and LY294002 on aromatase activity in MCF-7$^{Tet\text{-}Off\text{-}Arom}$ cells. MCF-7$^{Tet\text{-}Off\text{-}Arom}$ cells grown in 6-well plates were treated with U0126 (10 μM) or LY294002 (5 μM) for 4 days. Aromatase activity was measured by tritiated water release assay in the absence of above compounds. Each bar represents the average activity of duplicate wells.

was observed in the experiment with 2 h treatment and no direct inhibition on aromatase activity was seen when tested using placental microsomes (data not shown). These data suggest that blockade of activation of MAP kinase or PI3 kinase leads to inhibition of aromatase activity presumably through dephosphorylation of the enzyme. More detailed studies of the possible mechanisms now need to be conducted.

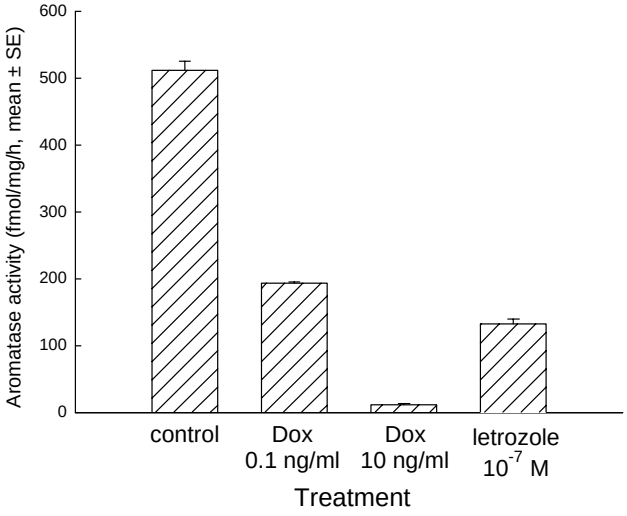

Fig. 10. Aromatase activity in MCF-7$^{Tet\text{-}Off\text{-}Arom}$ cells. MCF-7$^{Tet\text{-}Off\text{-}Arom}$ cells grown in 6-well plates were treated with doxycycline (0.1 or 10 ng/ml) or letrozole (10^{-7} M) for 4 days. Aromatase activity was measured by tritiated water release assay in the absence of above compounds. Each bar represents the average activity of duplicate wells.

4. Discussion

Long-term estrogen deprivation results in adaptation of breast cancer cells and allows growth in a low estrogen environment as shown by enhanced sensitivity to the mitogenic effect of estradiol in vitro and in vivo. Enhanced E_2 sensitivity is associated with elevated activation of MAP kinase in LTED cells. We were able to alter the sensitivity of wild type MCF-7 cells to estradiol by stimulation of MAP kinase activation. While the MAP kinase pathway is necessary for the development of E_2 hypersensitivity, it seems not to be the only mechanism involved. This was evidenced by the fact that expression of constitutively active MEK-1 in wild type MCF-7 cells only slightly increased their sensitivity to E_2. Further, blockade of MAP kinase activation in LTED cells reduced but did not completely reverse E_2 hypersensitivity. We found that the PI3 kinase pathway was also up-regulated in LTED cells. We further demonstrated that the PI3 kinase pathway was another component required for E_2 hypersensitivity. Blockade of both pathways with specific inhibitors resulted in complete reversion of the hypersensitive phenotype of LTED cells.

The MAP kinase pathway is an amplification system that links extracellular mitogenic signals to cell proliferation. Activation of this pathway has been associated with aggressive progression of breast cancer and poor prognosis [29]. Independent studies from our laboratory and those of others have shown that activation of MAP kinase in hormone-dependent breast cancer cells could result from long-term deprivation of estrogen in the culture medium [21,22]. We then explored whether enhanced activation of MAP kinase was responsible for the hypersensitive phenotype of LTED cells.

It was found that enhanced MAP kinase activation was tightly associated with E_2 hypersensitivity. Reversion of the hypersensitive phenotype by re-exposure of LTED cells to estradiol reduced the level of activated MAP kinase [23]. This relationship between the activation of MAP kinase and E_2 hypersensitivity led to our hypothesis that activation of MAP kinase causes hypersensitivity. To test this hypothesis, we decided to alter the levels of activated MAP kinase and examine whether this would change E_2 sensitivity. We treated wild type MCF-7 cells with TGFα to activate MAP kinase and examined their responses to estradiol. Although the data from this proof of principle experiment were supportive [23], more direct evidence was required to draw a conclusion on the causal relationship between the activation of MAP kinase and the hypersensitive growth phenotype exhibited in LTED cells. We first induced stable expression of constitutively active MEK-1 in wild type MCF-7 cells, which resulted in enhanced MAP kinase. In these cells, we did find enhanced E_2 sensitivity. However, the extent of left-shift of the E_2 dose–response curve was not as significant as that induced by TGFα. We then used U0126 to block MAP kinase activation in LTED cells. This approach reduced but not completely reversed E_2 sensitivity of LTED cells. These results suggested that the MAP kinase pathway is involved in but is not an exclusive cause of E_2 sensitivity in LTED cells.

The PI3 kinase pathway is another important pathway conveying mitogenic signals upon activation of growth factor receptors. Once activated, PI3 kinase activates downstream kinases through sequential phosphorylations. Several lines of evidence indicated the requirement of multiple signaling pathways in mediating growth factor dependent mitogenesis. For example, coordination between the MAP kinase and the PI3 kinase pathways is required for arrested fibroblasts to progress into the cell cycle upon stimulation with growth factors [30] or from Ras activation [31]. We postulated that activation of the PI3 kinase pathway was also important for LTED cells to develop E_2 hypersensitivity.

To test our hypothesis, we initially examined whether the PI3 kinase pathway is activated in LTED cells by exploring phosphorylation of three downstream effectors of PI3 kinase. One effector is Akt, an immediate serine/threonine kinase downstream of PI3K, which is involved in regulation of cell proliferation and survival. The other two effectors, p70S6 kinase and 4E-BP1, are more distal and have been reported to be important regulators of protein translation [32]. We found consistent elevation in phosphorylation of all three effectors of PI3 kinase in LTED cells compared to the wild type MCF-7. LY294002, the specific PI3 kinase inhibitor, effectively reduced phosphorylation of Akt, p70S6 kinase and 4E-BP1. These data provide compelling evidence that the PI3 kinase pathway is upregulated in LTED cells. The key evidence for the involvement of PI3 kinase activation in E_2 hypersensitivity came from the growth assay of LTED cells treated with U0126 and LY294002. Dual blockade of MAP kinase and PI3 kinase pathways completely reversed the hypersensitive phenotype of LTED cells as shown by a 2-log right shift of E_2 dose–response growth curve compared with that in the absence of these inhibitors.

In the past decade, clinical and experimental studies have revealed the importance of growth factor signaling pathways in mediating the failure of endocrine therapy of hormone-dependent breast cancer. The major emphasis has been on HER2/neu, a member of the EGF receptor family. The data from these studies have convincingly shown a significant inverse relationship between the expression of the EGF receptor or HER2 and responsiveness to endocrine therapy in breast cancer [33–36]. More recent studies have reported the ability of antibodies against HER2 to increase the inhibitory effect of tamoxifen or the pure antiestrogen ICI 182,780 on the proliferation of breast cancer cells with or without HER2 overexpression [37,38]. In addition, combined treatment with Iressa, an EGFR tyrosine kinase inhibitor, delayed the development of TAM resistance [39]. Our findings provide a more precise mechanism responsible for adaptation of breast cancer cells to the low estrogen environment and regrowth. It is predictable that the combination of antiestrogens with agents that inhibit signal transduction of growth factor pathways will delay the development of E_2 hypersensitivity and will prolong the duration of disease free survival from endocrine therapy of hormone dependent breast cancer.

Enhancement in aromatase activity is another phenomenon accompanied with E_2 hypersensitivity following long-term estrogen deprivation [28]. We observed an inverse correlation between tissue estradiol concentrations and aromatase activity under several circumstances: in cultured breast cancer cells, or in aromatase-transfected MCF-7 xenografts in nude mice, and in human breast cancer specimens [28]. However, the mechanisms regulating these changes are unknown. Aromatase is regulated at the transcriptional level through the alternative use of tissue specific promoters. There are a few studies regarding post-translational regulation of aromatase activity but these observations have been controversial [40,41]. Upregulation of the MAP kinase and the PI3 kinase pathways and aromatase activity in LTED cells implicate the possibility that aromatase activity might be regulated through phosphorylation by these kinases.

To avoid confounding results from transcriptional regulation of growth factor on aromatase expression, MCF-7 cells stably expressing aromatase under the control of tetracycline were utilized in our experiments. The expression system uses a minimal CMV promoter. Aromatase cDNA is expressed only in the absence of tetracycline. When the cells were treated with inhibitors of MEK and PI3 kinase, aromatase activity was significantly reduced (Fig. 11). The inhibitory effect of U0126 and LY294002 is unlikely due to down-regulation of aromatase at the transcriptional level because it occurred as early as two hours after treatment. There was no direct inhibition of these two kinase inhibitors on aromatase activity when applied to placental

microsomes. These data, though preliminary, suggest that aromatase activity can be regulated through phosphorylation. Further studies are required to reveal the mechanism.

In summary, long-term estrogen deprivation results in activation of the MAP kinase and the PI3 kinase pathways. These two pathways contribute equally to the hypersensitive phenotype of LTED cells with respect to the mitogenic effects of estradiol. The results from our studies using an in vitro model system suggest that responsiveness of hormone-dependent breast cancer cells can be managed by interfering with growth factor pathways. The combination of inhibitors of growth factor pathways with antiestrogens or aromatase inhibitors may provide additional benefit for patients with hormone-dependent breast cancer in the future.

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