Up-regulation of PI3K/Akt Signaling by 17β-estradiol through Activation of Estrogen Receptor-α in Breast Cancer Cells

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Purpose: Estrogen stimulates cell proliferation in breast cancer, the biological effects of which are mediated through two intracellular receptors: estrogen receptor-alpha (ERα) and estrogen receptor-beta (ERβ). However, the actual role of ERs in the proliferative action of estrogen remains to be established. It was recently found that ER activates phosphatidylinositol-3-OH kinase (PI3K), via its binding with the p85 regulatory subunit of PI3K. Therefore, possible mechanisms may include ER-mediated phosphoinositide metabolism, with the subsequent formation of phosphatidylinositol-3,4,5-trisphosphate (PIP3), which is generated from phosphatidylinositol 4, 5-bisphosphate (PIP2) via PI3K activation. The present study has demonstrated that 17β-estradiol (E2) up-regulates PI3K in an ERα, but not an ERβ dependent manner, and also stimulates cell growth in breast cancer cells.

Methods: To study this phenomenon, we treated ER-positive MCF-7 cells and ERα-negative MDA-MB-231 cells with 10 nM E2.

Results: The treatment of MCF-7 cells with E2 resulted in a marked increase in the expression of PI3K (p85), which was paralleled by increases in the levels of phospho-Akt (Ser-473) and PIP3. These observations were also correlated with increased E2-induced cell proliferation activity. However, no effects of E2 on breast cancer cells were observed in the MDA-MB-231 cell line, indicating the pathway of E2-mediated up-regulation of PI3K/Akt is ERα-dependent.

Conclusion: These results suggest that estrogen activates PI3K/Akt signaling via an ERα-dependent mechanism in MCF-7 cells.

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Key Words: Estrogen receptor, PI3K, PIP, 17β-estradiol

INTRODUCTION
Proliferative activity of estrogen in normal cells has been considered as a stimulant for initiation and promotion of tumors in these organs. Studies have indicated that prolonged exposure of reproductive organs to estrogen can be a risk factor for

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breast and uterine cancers. In an animal model, breast cancer progression is significantly greater in the mice exposed to estrogen compared to unexposed mice. Anti-estrogens such as tamoxifen inhibit the growth of breast cancers. Furthermore, the endocrine therapy with tamoxifen is clinically the most commonly used treatment for patients with breast cancer. These observations suggest that estrogen plays a critical role in tumorigenesis of breast tumor.

Biological effects of estrogen are mediated through two distinct intracellular receptors, estrogen receptor α (ER) and ERβ. Although the estrogen/ER signaling pathway is not clearly established, a study has shown that ER can bind the p85 regulatory subunit of phosphatidylinositol-3-OH kinase (PI3K) in a ligand-dependent manner. It has been demonstrated that PI3K is necessary for the estrogen/ER signaling.

PI3K is one of key enzymes, which regulates phosphoinositol metabolism and responsible for generation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) via phosphorylation of D-3 position of the inositol ring of phosphatidylinositol 4,5-bisphosphate (PIP2). The activation of PI3K results in PIP3-mediated activation of serine-threonine kinase Akt by phosphorylation. The Akt protein in turn modulates the function of numerous substrates involved in the regulation of cell functions. PI3K/Akt signaling is involved in the regulation of cell functions including cell proliferation, cell survival, and cell cycle progression. However, PI3K/Akt signaling is not clear in estrogen/ER signaling pathway of breast cancer cells.

In this study we examined the effect of 17β-estradiol (E2) on PI3K/Akt signaling in human breast cancer cells. Two types of cell lines were used: the MCF-7 cell line a human breast epithelial cell, expresses ERα and ERβ, and MDA-MB-231 cell line, a human breast epithelial cell, expresses ERβ only. The results showed that E2 up-regulates PI3K/Akt signaling in an ERα-dependent manner, but not ERβ, and induces cell growth in breast cancer cells.

METHODS

Materials. Antibodies against phosphatase and tensin homologue deleted on chromosome 10 (PTEN), ERα and ERβ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p85, p-Akt (Ser-473), and Akt were purchased from Cell Signaling (Beverly, MD, USA). Fetal calf serum (FBS) and charcoal-dextran treated FBS were obtained from Gibco BRL (Life Technologies, Grand Island, NY, USA), HBSS (Hanks balanced salt solution), 17β-estradiol, dimethyisulfoxide (DMEM), phenol red-free DMEM and β-actin antibody were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Cell Proliferation ELISA Kit was obtained from Roche (Lewes, UK).

Cell culture. Human breast cancer cell lines (MCF-7, MDA-MB-231) were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in DMEM medium containing 10% FBS, 2 mM glutamine, antibiotics (penicillin G 60 mg/L, streptomycin 100 mg/L, amphotericin B 50 μg/L) under a 5% CO2/95% air incubator. To elevate the estradiol effect, standard medium in which 10% FBS was replaced by 10% charcoal-dextran treated FBS.

Western blot analysis. Cells were seeded in 6-well plates at a density of 2×10^5 cells per well. Cells were preincubated for 1 h with 10% charcoal-dextran treated FBS, and then stimulated with E2. After washing with phosphate-buffered saline (PBS) and harvesting, cell pellets were lysed in ice-cold lysis buffer (20 mM Hepes pH 7.2, 1% Triton X-100, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, and 1 mg/ml aprotinin). After incubation for 30 min at 4 °C, cell debris was removed by centrifugation at 10,000 x g for 30 min, and supernatants were analyzed by sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoretic transfer from slab gel to nitrocellulose membranes and subsequent immunoblotting.
measured at 450 nm in an ELISA reader (Molecular devices corp. USA).

**Statistical analysis.** All experimental data are presented as means ± standard deviations (SD).

**RESULTS AND DISCUSSION**

**Expression profiling of ER responding to E2 in MCF-7 cells and MDA-MB-231 cells**

A previous study demonstrates that uterus show down-regulation of ERα expression following estrogen exposure of rodents. (16) To determine whether estrogen affects the expression of ER, MCF-7 and MDA-MB-231 cells were treated with E2 (10 μM), and the expression of ER proteins was determined by Western blotting. As shown in Fig 1A, treatment with E2 resulted in a decrease of ERα level in MCF-7 cells in a time-dependent manner. The ERα protein level at 72 h after E2 treatment was approximately 30% of control (p<0.002)(Fig 1B). The ERβ level was not changed. Consistent with the observations, the protein level of ERβ in MDA-MB-231 cells was not changed by E2 treatment (Fig 2A). Since this cell line does not express ERβ, there was no band corresponding to the Mr 65 kDa for ERα (Fig 2A). These results show that E2 exposure to breast cancer cells decreased in the level of ERα protein at only ERα-positive MCF-7 cells, indicating that the biologic effects of estrogen are mainly associated with ERα-dependent pathway in breast cancer cells.

**PI3K/Akt pathway responding to E2 in MCF-7 cells and MDA-MB-231 cells**

PI3K has been divided into three distinct classes (I, II and III). The predominant form of PI3K comprises p85, an adaptor/regulatory subunit of relative molecular mass 85,000 (Mr 85 kDa), and p110, a catalytic subunit of Mr 110 kDa. (17) PI3K promotes generation of PIP3, which binds to Akt. PIP3-bound Akt is translocated from cytoplasm to the plasma membrane, where it is activated through phosphorylation. To investigate ability of E2 to regulate PI3K/Akt pathway, MCF-7 or...
trol (p < 0.004) (Fig 7). However, these responses were not observed with MDA-MB-231 cells. These results suggest that F2 increases cell proliferation via ERα-dependent PI3K/Akt activity in MCF-7 cells.

CONCLUSION

We demonstrate that estrogen activates PI3K activity through ERα-dependent pathways, which paralleled an increase in phospho-Akt, PIP3 level, and cell proliferation in MCF-7 cells. These observations indicate that estrogen stimulates growth signal (PI3K/Akt) pathway through mediation of its specific receptor (ER α) in breast cancer cells. Thus, estrogen acts as a stimulant for initiation and promotion of tumors in breast tissues. Hence, we suggest that PI3K/Akt pathway may be a target for treatment of estrogen-associated diseases.

REFERENCES