Akt Activation by Estrogen in Estrogen Receptor-negative Breast Cancer Cells

Eing-Mei Tsai, Shao-Chun Wang, Jau-Nan Lee, and Mien-Chie Hung

Abstract

It has been a common belief that estrogen regulates cellular responses through binding to its receptor, the estrogen receptor (ER). In the nucleus, estrogen modulates the expression of estrogen-responsive genes through the action of the ER at the transcriptional level. In the cytoplasm, the ER-dependent signaling pathway has been shown to be involved in the activation of Akt and the downstream molecules. It is not clear, however, whether estrogen can modulate cytoplasmic signaling in an ER-independent manner. Human breast cancer cell lines without detectable ERs such as MDA-MB-435 and MDA-MB-231 were treated in estrogen-depleted medium followed by a brief treatment with estrogen. The activation of Akt was evaluated with a phosphoserine antibody. Our results showed that estrogen stimulated Akt activation, as indicated by phosphorylation at Ser473 of the oncoprotein, in ER-negative breast cancer cells. Activation of Akt by estrogen in these cells was time and dose dependent and could be blocked by inhibitors of phosphatidylinositol 3'-kinase and Src kinase but not by estrogen antagonists. Our results provide evidence as well as the mechanism of the receptor-independent function of estrogen, in which the antiapoptotic factor Akt is activated.

Introduction

The steroid hormone 17β-estradiol (estrogen), together with its cognate receptor, plays a critical role in both physiological and pathological processes, and it is required for sex differentiation and establishment and maintenance of the reproductive cycle, as well as the metabolism of fat and bone tissues. One of the key signaling pathways downstream of estrogen is the PI3K pathway, which activates antiapoptotic Akt signaling. Akt, also known as protein kinase B or PKB, contains an NH2-terminal pleckstrin homology domain. The pleckstrin homology domain binds to the phosphorylated lipids generated by the activity of PI3K and translocates Akt to the cell membrane. Akt is then further activated by the membrane-associated kinase PDK1, which phosphorylates Akt at Thr308. Akt activation can also be achieved by phosphorylation at Ser473, although the in vivo kinase(s) catalyzing the phosphorylation is not clear. Akt activation leads to signals that either stimulate antiapoptotic cellular responses or block apoptotic functions of the cell.

The involvement of estrogen signaling in Akt activation is highlighted by the recent discovery that the cytoplasmic ER binds to PI3K in an estrogen-dependent manner, resulting in activation of Akt, which in turn activates endothelial nitric-oxide synthase (4). Activation of this pathway by estrogen protects cardiovascular muscle cells after ischemia and reperfusion. In addition, the estrogen/ER-PI3K-Akt pathway has been shown to be a neuroprotective factor against toxic insults for neuronal cells (5). Estrogen treatment reduced the neurotoxicity induced by glutamate, and the protective effect of estrogen was blocked by coadministration of a PI3K inhibitor (LY 294002). In cancer cells, estrogen has been shown to exhibit growth-promoting properties in breast epithelial cells, and abnormal estrogen exposure is associated with an increased risk of breast cancer. Consistently, it has been reported that estrogen mediated Akt activation in breast cancer cells with endogenous ER (6).

Despite the documented ER-dependent signaling mechanisms described above, evidence of a receptor-independent function of estrogen or its analogues is emerging. For instance, estrogen activates the mitogen-activated protein kinase cascade in brain cells derived from mice lacking ER-α expression (7). In this report, we describe a novel mechanism of estrogen-mediated Akt activation independent from known ERs in human breast cancer cells.

Materials and Methods

Cell Lines and Reagents. Human breast cancer cell lines MCF-7, MDA-MB-435, and MDA-MB-231 were grown in DMEM/F-12 supplemented with 10% FBS. The PI3K inhibitors LY 294002 and wortmannin were purchased from Calbiochem (San Diego, CA). Src kinase inhibitor PP2 [4-amino-5-(4-chlorophenyl)-7-[(tub3|yrazolo[3,4-d]pyrimidine] and the inactive control compound PP3 [4-amino-7-phenylpyrazolo[3,4-d]pyrimidine] were from Sigma Chemical Co. (St. Louis, MO). The ER antagonist ICI 182,780 was purchased from Toarcis (Ballwin, MO). Antibodies against Akt and the phosphorylated Akt at Ser473 were purchased from New England Biolabs (Beverly, MA).

Activation of Akt. Cells (1 × 10⁶) were plated in 100-mm plates in phenol red-free DMEM/F-12 containing 5% normal FBS for 48 h and then in phenol red-free medium supplemented with 5% charcoal-stripped FBS for an additional 24 h before estrogen stimulation. Different concentrations of estrogen were used in the absence or presence of other inhibitors to treat cells for 30 min or for the times indicated. For treatment with inhibitors, cells were pretreated with the respective inhibitors for 30 min and cotreated with estrogen for an additional 30 min. In the experiments, 10 nM wortmannin, 1 μM LY 294002, 40 μM emodin, or 20 μg/ml genistein was used. All of the experiments described in this report have been repeated two to three times, and the representative results are shown.

Immunoblot Analysis. Cells were harvested and lysed in ice-cold NETN buffer [150 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8.0), and 0.5% NP40]. Cell lysates with equal amounts of protein were loaded in SDS-polyacrylamide gels and separated with electrophoresis. The separated proteins were transferred to polyvinylidene difluoride membranes and probed with antibodies.

Results

The potential ER-independent function of estrogen was tested in the breast cancer cell line MDA-MB-435, in which ER-α and ER-β are undetectable (8, 9). Treatment with estrogen resulted in activation of Akt as indicated by the levels of phosphorylated Akt protein recognized by an antibody raised against Ser473 phosphorylation of Akt. Activation of Akt by estrogen is a relatively fast response, and the peak level of phospho-Akt can be observed within 30 min of incubation with estrogen (Fig. 1A) in a dose-dependent manner (Fig. 2). The rapid kinetics of Akt activation by estrogen suggest that the nuclear event of estrogen-ER signaling was not involved. Under the experimental conditions, estrogen also transiently
activated Akt phosphorylation in MCF-7 cells, which express functional ER (10), within 15 min (Fig. 1B). The expression of ER-α in the cell lines used in this study, MDA-MB-435, MDA-MB-231, and MCF-7, was confirmed by Western blotting analysis (Fig. 1C). ER-α expression can be readily detected in MCF-7 cells, whereas no ER-α expression can be detected in MDA-MB-435 and MDA-MB-231 cells even after a longer exposure, consistent with previous reports that MDA-MB-435 and MDA-MB-231 are breast cancer cell lines lacking expression of endogenous ER-α (8, 9). The antibody detected a significant level of ER-α in a stable transfectant of ER-α derived from 293 cells, whereas no ER-α was detected in the parental cells, confirming the specificity of the antibody used in this analysis.

Akt is a downstream factor activated by PI3K. We further explored the possible involvement of the PI3K pathway in ER-independent induction of Akt by cotreatment with PI3K inhibitors wortmannin and LY 294002. In both cases, the induction of Akt by estrogen was blocked by the PI3K inhibitors, indicating that estrogen regulates Akt activity through the PI3K pathway (Fig. 3A). On the other hand, activation of Akt by estrogen was not inhibited by the ER-α antagonist ICI 182,780, consistent with our observation that estrogen induced Akt phosphorylation in an ER-independent manner (Fig. 3B). The same dose of ICI 182,780 actively inhibited Akt activation in the ER-α-positive MCF-7 cells, confirming the activity of the estrogen antagonist used in this study (Fig. 3C). Similarly, the other estrogen antagonist, tamoxifen, also failed to inhibit estrogen-mediated Akt activation (data not shown).
The tyrosine kinase Src has been shown to activate PI3K (11). We therefore tested whether estrogen-mediated PI3K activation requires Src activity by cotreatment of estrogen with the Src kinase inhibitor PP2 or its inactive analogue PP3 (12, 13). Treatment of PP2 significantly inhibited estrogen-mediated Akt activation, whereas the control compound PP3 did not have any effect (Fig. 4A). The observation of Src-dependent, estrogen-mediated Akt activation can be extended to other breast cancer cell lines such as MDA-MB-231, which also lacks a detectable level of ER-α (Fig. 4B).

Discussion

In the traditional model, estrogen modulates the expression of downstream genes by binding to the ER and inducing subsequent nuclear translocation of the receptor dimers. It has been commonly believed that estrogen affects cell signaling mainly through nuclear events. Recently, evidence of the cytoplasmic function of estrogen has started to emerge. The most striking example is the direct association between the ER and PI3K in cells stimulated by estrogen (4). Activation of PI3K by this mechanism resulted in activation of Akt and downstream antiapoptotic signaling. Consistent with this previous report, we also observed estrogen-mediated Akt activation in ER-α-positive MCF-7 cells (Fig. 3C). Furthermore, this activation can be blocked by the estrogen antagonist ICI 182,780, which further supports the involvement of ER in the signaling pathway. On the other hand, exposure to estrogen activates the PI3K pathway and attenuates glutamate-induced toxicity in neuronal cells (5). Although the precise mechanism for estrogen-mediated protection in neuronal cells is not clear, the activation of PI3K signaling by estrogen appears to be ER-independent because estrogen antagonist ICI 182,780 failed to block the protective effect of estrogen. Given the pleiotropic nature of the estradiol hormone, it is possible that estrogen may convey its multiple functions through both ER-dependent and ER-independent pathways. Recently, the ER-α-independent function of estrogen in neuronal cells was reported (7); in this previous study, estrogen induced the activation of mitogen-activated protein kinase signaling in the cerebral cortex of ER-α knockout mice. Other evidence supporting the ER-independent mechanism of estrogen came from the growth-inhibitory activity of the estrogen antagonist tamoxifen and its active metabolite, 4-hydroxytamoxifen, in MDA-MB-435 cells (8). In addition, it was recently reported that tamoxifen induces p21WAF1 gene transcription through the Sp1 binding site of the promoter in ER-negative lung cancer cells (14). In this study, we show for the first time that estrogen activated the PI3K-Akt pathway in breast cancer cells lacking expression of the known ERs.

The actual mechanism triggering the ER-α- and ER-β-independent estrogen activity remains to be determined. Our results suggest that the activation appeared to be mediated through the Src-PI3K signaling cascade. When the specific Src kinase inhibitor PP1 was used, the phosphorylation of Akt was down-regulated, whereas the inactive compound, PP2, did not have any effect on Akt phosphorylation. Therefore, our study demonstrates that estrogen can activate the PI3K-Akt pathway through an ER-independent mechanism. Further study is required to understand the downstream signaling consequences of the estrogen-activated Akt pathway.

References

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