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13. ABSTRACT (Maximum 200 Words)

17β-estradiol (E2) induces rapid, non-genomic effect in MCF-7 breast cancer cells, including rapid activation of MAPK, phosphorylation of adapter protein Shc, increase of the interaction between Shc and estrogen receptor (ERα). More strikingly E2 also induced a rapid membrane association of ERα (1). Further studying the structure and function of ERα, we demonstrated that only membrane-associated ERα, but not cytosol and nuclear ones, mediates E2 effect on MAPK activation (2). To study the mechanism of ERα membrane association, we further investigated the role of Shc in estrogen action. Here we demonstrated that in MCF-7 cells, Shc acts as a chaperon linking ERα to the cell membrane by binding to a transmembrane receptor IGF-1R. Further study is under the way to investigate the molecular mechanism among these protein complex formation and their biological effects in estrogen non-genomic action.

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Introduction

17β-estradiol (E2) induces rapid, non-genomic effect in MCF-7 breast cancer cells, including rapid activation of MAPK, phosphorylation of adapter protein Shc, increase of the interaction between Shc and estrogen receptor (ERα). More strikingly E2 also induced a rapid estrogen receptor membrane association (1). Further studying the structure and function of ERα, we demonstrated that only membrane-associated ERα, but not cytosol and nuclear ones, mediates E2 effect on MAPK activation (2). Since Shc physically interacts with many trans-membrane growth factor receptors, such as IGF-1 receptor (IGF-1R), EGF receptor (EGFR), PDGF receptor (PDGFR) and Insulin receptor (IR), it is conceivable that Shc might be a chaperon brings ERα to the membrane by binding to one of above receptors. So far only IGF-1R and EGFR are reported to be involved in rapid E2 action (3;4). Based on this, we reasoned that Shc might be a molecule serving two functions, one is to bring ERα to the membrane by interaction with either IGF-1R or EGFR, and the other is to activate IGF-1R-initiated MAPK signaling pathway.

Body

In Task 4 of my grant proposal, we proposed to investigate the role of adaptor protein Shc on its function to mediate ERα membrane association in MCF-7 breast cancer cells. Shc is a common substrate of many transmembrane growth factor receptors, such as IGF-1R, IR, EGFR and PDGFR, activation of these receptor will lead to Shc physically association with these membrane receptors. So far only IGF-1R and EGFR are reported to be involved in rapid E2 action (3;4). Accordingly, we tested several protein expression, such as IGF-1R, EGFR, ERα and Shc, in MCF-7, LTED and COS-1 cells. LTED cells were developed from MCF-7 cells by long-term estrogen-deprivation and characterized as supersensitive to E2 treatment than their parental cells due to elevated ERα levels (5;6). Interestingly, all cells express IGF-1R from medium to high levels and only COS-1 cells, but not MCF-7 and LTED cells, show the EGFR expression, indicating that IGF-1R, but not ERGR, might be a potential transmembrane receptor interacting with Shc (**Fig. 1**). To demonstrate that Shc plays an important role in mediating ERα membrane association, a gene silencing method was employed by using siRNA against Shc. We transfected a pool of 4 siRNA's, each which were designed to knock down Shc. We also used a

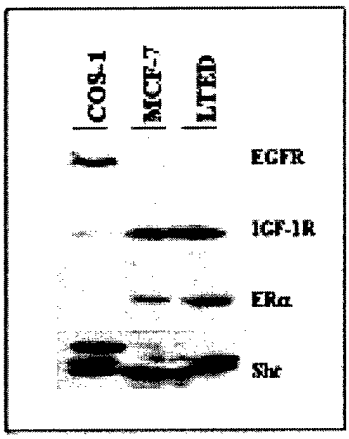

Fig. 1. Expression of proteins in MCF-7, LTED and COS-1 cells.

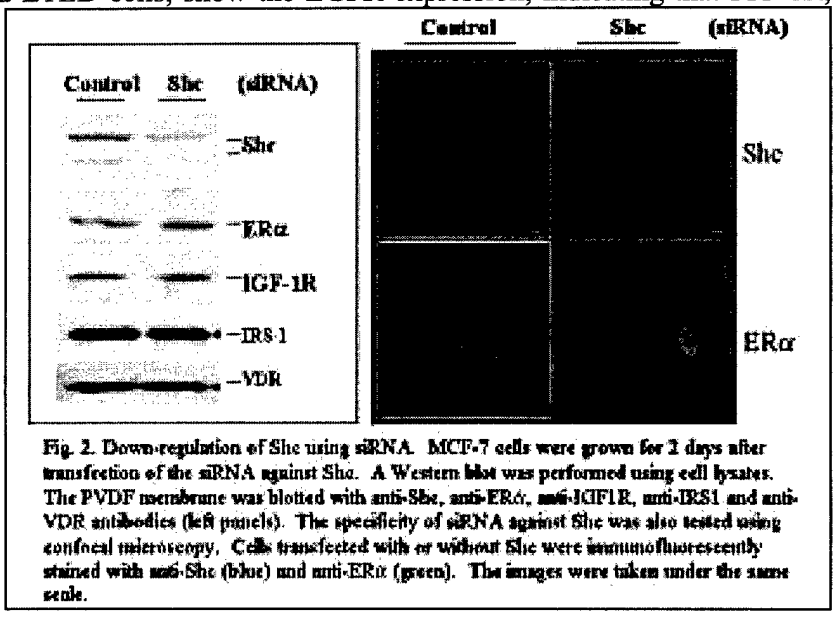

Fig. 2. Down-regulation of Shc using siRNA. MCF-7 cells were grown for 2 days after transfection of the siRNA against Shc. A Western blot was performed using cell lysates. The PVDF membrane was blotted with anti-Shc, anti-ERα, anti-IGF1R, anti-IRS1 and anti-VDR antibodies (left panels). The specificity of siRNA against Shc was also tested using confocal microscopy. Cells transfected with or without Shc were immunofluorescently stained with anti-Shc (blue) and anti-ERα (green). The images were taken under the same scale.

control pool of irrelevant siRNA molecules as negative control. Both siRNA were purchased from Dharmacon Inc. As shown in **Fig. 2** (left panels), expression of siRNA against Shc exclusively knocked down the Shc protein without altering the levels of any other proteins (ERα, IGF-1R, IRS-1 and VDR) tested in this experiment. The specificity of Shc siRNA was also further confirmed by confocal microscopy method, showing that down-regulation of Shc did not change ERα expression in the cells (right panels in Fig. 2). The interaction between ERα and IGF-1R has been reported in ERα-transfected COS-1 cells (3). Since we demonstrated that ERα physically interacted with Shc, we were wondering if both ERα and IGF-1R interaction is Shc-dependent. To do so, one of our strategies is to knock down Shc in MCF-7 cells and test if this can alter the IGF-1R and ERα association. **Fig. 3.** shows that siRNA against Shc decreased Shc protein expression in MCF-7 cells (lower panel). Estrogen treatment increased the ERα and IGF-1R interaction in control siRNA expressed cells. Down-regulation of Shc blocked ERα interaction with IGF-1R, indicating that Shc is an intermediate protein linking both ERα and IGF-1R association. Further study on the functions of this triple protein formation is currently under active investigation.

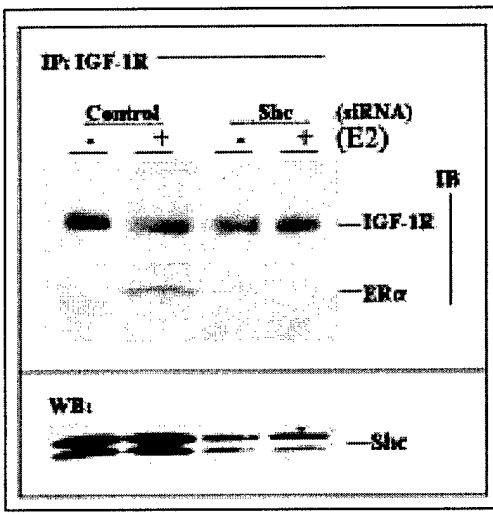

Fig. 3. Knocking down Shc blocked the interaction between ERα and IGF-1R. Cells were transfected with or without siRNA against Shc. The protein levels of Shc was shown in Western blot (lower panel). The ERα and IGF-1R interaction was measured by immunoprecipitation of IGF-1R and detection of ERα on Western blot (upper panel).

Key research accomplishments

1. We successfully constructed an ERα-expression vector by ligation of DNA sequence coding for a membrane-signaling sequence of GAP-43 protein on the C-terminal of ERα insert (Task 1) (2).
2. Functional testing the expressed membrane-targeted ERα has been done using confocal immunofluorescence microscopy. Comparing with wild type ERα that show 99% expressed in the nucleus, our data show that expression this vector in COS-1 cells lead to 45% of ERα expressed on the cell membrane and rest of it in the cytosol (Task 2) (2).
3. We then tested the ERα-mediated MAPK activation in COS-1 cells. Compared with the wild type one (99% in nucleus), Cytosol (99% in cytosol), the membrane expressed ERα is the only form that mediated estrogen effect on MAPK phosphorylation (Task 3) (2).
4. We are currently working on the mechanism how adapter protein Shc mediates ERα on its membrane association and MAPK activation.

Reportable outcomes:

1) We have one paper published under this grant supporting mechanism. Title: Membrane association of estrogen receptor α mediates estrogen effect on MAPK activation. BBRC, 294: 926-933, 2002 (2).

2) A manuscript is under writing, entitled "Adapter protein Shc as a chaperon mediates estrogen receptor membrane association in breast cancer cells".
3) Three abstracts were submitted to Endo2003 in Philadelphia, PA

1, IGF-1 receptor activation and its physical interactions with adapter protein Shc and ER alpha mediate the non-genomic action of estradiol in breast cancer cells. Song, RX, Zhang, Z, Boa, Y, Black, MJ, and Santen, RJ. Department of internal medicine, UVA, Charlottesville, VA 22903

2. Antiestrogen and estrogens regulate cell proliferation and apoptosis differently in long-term estrogen-deprived and wild type MCF-7 cells. Zhang, Z, Boa, Y, Black, MJ, Santen, RJ and Song, RX, Department of Internal Medicine, UVA, Charlottesville, VA 22903

3. MAP kinase negatively regulates estrogen receptor-mediated transcriptional activity in human breast cancer cells. Zhang, Z, Boa, Y, Black, MJ, Santen, RJ and Song, RX, Department of Internal Medicine, UVA, Charlottesville, VA 22903

4) The results from this grant was presented in the following meetings:
   1. Invited speaker in one of symposiums "Recognition of estrogen receptor in estrogen non-genomic action". Title: Involvement of Shc in estrogen receptor membrane association in breast cancer cells. FASEB – Cell Biology, San Diego, 4-14-2003.
   2. Invited speaker. Title: Upstream signaling pathways in estrogen action on MAPK activation in breast cancer cells. Department of Biochemistry, University of California – Riverside, 4-16-2003.
   3. Invited speaker. Title: Upstream signaling pathways in estrogen action on MAPK activation in breast cancer cells. Department of Microbiology of UVA, Charlottesville, VA, 4-8-2003.
   4. Presenting in Division of Endocrinology, Department of internal medicine, UVA, Charlottesville, 6-3-2003. Title: Non-genomic signaling pathway of estrogen and breast cancer.

Conclusions

Adapter protein Shc acts as a chaperon and it involves in rapid estrogen action linking ERα to the cell membrane by binding on IGF-1 receptor.

Reference List

1. Song RX, McPherson RA, Adam L, Bao Y, Shupnik M, Kumar R, Santen RJ 2002 Linkage of Rapid Estrogen Action to MAPK Activation by ERalpha-Shc Association and Shc Pathway Activation. Mol Endocrinol 16:116-127

2. Zhang Z, Maier B, Santen RJ, Song R.X 2002 Membrane association of estrogen receptor alpha mediates estrogen effect on MAPK activation. Biochem Biophys Res Commun 294:000

3. Kahlert S, Nuedling S, van Eickels M, Vetter H, Meyer R, Grohe C 2000 Estrogen receptor alpha rapidly activates the IGF-1 receptor pathway. J Biol Chem 275:18447-18453

4. Richards RG, DiAugustine RP, Petrusz P, Clark GC, Sebastian J 1996 Estradiol stimulates tyrosine phosphorylation of the insulin-like growth factor-1 receptor and insulin receptor substrate-1 in the uterus. Proc Natl Acad Sci U S A 93:12002-12007

5. Martin LA, Farmer I, Johnston SR, Ali S, Marshall CJ, Dowsett M 2003 Enhanced ERalpha ERBB2 and MAPK signal transduction pathways operate during the adaptation of MCF-7 cells to long term oestrogen deprivation. J Biol Chem

6. Santen R, Jeng MH, Wang JP, Song R, Masamura S, McPherson R, Santner S, Yue W, Shim WS 2001 Adaptive hypersensitivity to estradiol: potential mechanism for secondary hormonal responses in breast cancer patients. The Journal of Steroid Biochemistry and Molecular Biology 79:115-125