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Growth factor signaling enhances aromatase activity of breast cancer cells via post-transcriptional mechanisms

Bin Su[a,*], Cynthie Wong[b], Yanyan Hong[b], Shiuan Chen[b,**]

[a] Department of Chemistry, College of Sciences and Health Professions, Cleveland State University, 2121 Euclid Ave., Cleveland, OH 44115, USA
[b] Division of Tumor Cell Biology, Beckman Research Institute of the City of Hope, 1500 E Duarte Road, Duarte, CA 91010, USA

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ABSTRACT

It has been demonstrated that growth factors produced by breast cancer cells stimulate aromatase expression in both breast cancer and adjacent adipose fibroblasts and stromal cells. However, whether these growth factors affect aromatase activity by other mechanisms still remain unclear. In the current study, MCF-7aro and T47Daro aromatase transfected breast carcinoma cells were used to explore the mechanisms of post-transcriptional regulation of aromatase activity by growth factor pathways. Our study reveals that PI3K/Akt and MAPK inhibitors suppressed aromatase activity in MCF-7aro cells. However, PI3K/Akt pathway inhibitors stimulated aromatase activity in T47Daro cells. This is due to enhanced MAPK phosphorylation as compensation after the PI3K/Akt pathway has been blocked. IGF-1 treatment increased aromatase activity in both breast cancer cell lines. In addition, LTEDaro cells (long-term estrogen deprived MCF-7aro cells) which have enhanced MAPK activity, show higher aromatase activity compared to parental MCF-7aro cells, but the aromatase protein level remains the same. These results suggest that aromatase activity could be enhanced by growth factor signaling pathways via post-transcriptional mechanisms.

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1. Introduction

Estrogen plays a critical role in the growth of estrogen-dependent breast carcinoma. Approximately 60% of premenopausal and 75% of postmenopausal patients have estrogen-dependent breast cancer [1,2]. Clinical studies demonstrate that in postmenopausal breast cancer patients, concentration of estrogen in breast tumor tissues is several-fold higher than it in plasma [3]. These results support the hypothesis that estrogen is made and accumulates in the tumor. Aromatase is one of the key enzymes for the biosynthesis of estrogen. The enzyme expression is highly elevated in human breast cancer tissue than in normal breast tissue, as measured by enzyme activity assay and reverse transcription-polymerase chain reaction (RT-PCR) analysis [2,4–8]. High expression of aromatase in breast cancer cells and surrounding adipose stromal cells contributes significantly to breast tumor development and growth in the patients [2,6]. Logically, aromatase is a particularly attractive target in the treatment of estrogen receptor (ER) positive breast cancer [9].

Aromatase is encoded by the *CYP19* gene, and is expressed in a tissue-specific manner. The regulation of aromatase in various tissues is different. So far, several tissue-specific promoter regions have been identified, which include promoters PI.1, PI.3, PI.4, PI.6, PI.7, and PII [10]. PI.4 is the main promoter used in normal adipose tissue and is responsive to glucocorticoids and cytokines such as IL-1β, IL-6 and TNFα. Normal breast cell also utilizes this promoter for aromatase expression [11]. However, the increased expression of aromatase in breast cancer tissues is associated with a switch, from promoter I.4 to promoters I.3 and II, in the major promoter region utilized in *CYP19* gene expression. As a result of the switch, the regulation of estrogen biosynthesis changes from one controlled primarily by glucocorticoids and cytokines to a promoter regulated by cAMP (adenosine cyclic 3′,5′-monophosphate)-mediated pathways [7,8,11].

Prostaglandin E2 (PGE2), the product of cyclooxygenase-2 (COX-2) in the breast cancer cells, binds to EP1 (PGE2 receptor 1) and EP2 receptors to increase the cAMP level and stimulates aromatase gene expression in breast cells [12,13]. In addition, growth factors secreted by breast cancer cells could also stimulate aromatase expression in both breast cancer and adjacent adipose fibrob-

Abbreviations: PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; MAPK, mitogen activated protein kinase; COX2, cyclooxygenase 2; PGE2, prostaglandin E2; EP, PGE2 receptor; ER, estrogen receptor; EGF, epidermal growth factor; TGFα, transforming growth factor α; IGF-1, insulin-like growth factor-1; TNFα, tumor necrosis factor α; FGF, fibroblast growth factor; IL-1β, interleukin-1β; IL-6, interleukin-6; cAMP, adenosine cyclic 3′,5′-monophosphate; Her2, human epidermal growth factor receptor; mTOR, mammalian target of rapamycin; LTED, long-term estrogen deprivation; STIs, signal transduction inhibitors.

* Corresponding author. Tel.: +1 216 687 9219; fax: +1 216 687 9298.
** Corresponding author. Tel.: +1 626 256 4673x63454; fax: +1 626 301 8972.
E-mail addresses: B.su@csuohio.edu (B. Su), Schen@coh.org (S. Chen).

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lasts and stromal cells [11,14]. It has been demonstrated that both insulin and insulin-like growth factor-1 are able to potentiate dexamethasone stimulated aromatase activity in human skin fibroblasts. However, they do not directly stimulate aromatase activity in these cells [15]. Epidermal growth factor (EGF) has been found to increase aromatase activity and expression in the human adrenocortical carcinoma cell line NCI-H295R [16]. TGFα is able to enhance both basal and FSH-stimulated aromatase activity after long time treatment in human granulosa cells [17]. In addition, TGFα, EGF and FGF also stimulate aromatase activity in MCF-7 and T47D breast cancer cells [18]. Through the use of a transgenic mouse model, one group found that Her2 status is a determinant of mammary aromatase activity [19]. MCF-7 cell conditioned medium can stimulate aromatase activity in breast adipose fibroblasts at the transcriptional level [20]. All the studies suggest a correlation between growth factor pathways activation and increased aromatase activity. However, it is difficult to distinguish the transcriptional or post-transcriptional regulation of aromatase by growth factors in most of the studies.

Although estrogen concentration is higher in the breast cancer tissue of ER+ breast cancer patients, aromatase protein level does not always proved to be higher than it in the normal breast tissue [5]. One of the reasons is the low efficiency of the current aromatase antibody used to check the aromatase protein level in the studies. Secondly, it is also possible that aromatase activity has been significantly improved by growth factors secreted by the cancer tissue, but aromatase protein level does not increase dramatically. In another word, growth factors might enhance aromatase activity via a post-transcriptional mechanism. If this is the case, it is normal that no high aromatase protein is detected with these ER+ breast cancer samples, but the aromatase activity and estrogen concentration both have been elevated. A systematic study is needed to elucidate the possible aromatase post-transcriptional regulation by growth factors.

In the current study, we used MCF-7aro and T47Daro aromatase transfected breast carcinoma cells to explore the mechanisms of posttranscriptional regulation of aromatase activity. The basal aromatase activity in the original cells is almost undetectable, and the high aromatase activity in the stably transfected cells is artificially controlled and the expression is less likely to be regulated by the cellular signaling pathways [21]. These cells are good model to study the post-transcriptional regulation of aromatase. EGFR and IGFR family are the major growth factor receptors expressed in the breast cancer cell surface. We will mainly focus the study on how these pathways regulate aromatase activity in breast cancer cells. Specific signal transduction inhibitors and growth factors such as IGF were used in the study to investigate how aromatase has been affected. Aromatase protein level was also checked by western blotting to exclude the possibility that aromatase protein degradation could be affected by the growth factor pathways. The results suggest that aromatase activity can be enhanced by growth factor signaling pathways via post-transcriptional mechanisms.

2. Materials and methods

2.1. Reagents

Radiolabeled [1β-^{3}H]-androst-4-ene-3,17-dione was obtained from NEN Life Science Products (Boston, MA). PI3K inhibitor LY294002, Akt inhibitor Triciribine and MAPK inhibitor U0126 were purchased from Cayman Chemical (Ann Arbor, MI). mTOR inhibitor RAD001 was from Novartis (Basel, Switzerland). IGF-1, trypsin and all enzymes were obtained from Invitrogen (Carlsbad, CA). Testosterone and 17β-estradiol were from Sigma Chemical (St. Louis, MO). For *in vitro* experiments, these agents at various concentrations were dissolved in DMSO. Mouse anti-aromatase was from Serotec (Raleigh, NC). All other antibodies were from Cell Signaling (Danvers, MA) or Santa Cruz Biotechnology (Santa Cruz, CA). Radioactive samples were counted on a LS6500 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Scintillation solution ScientiSafe 30% was obtained from Fisher Scientific (Pittsburgh, PA).

2.2. Cell culture

The ER-positive aromatase-overexpressing MCF-7 and T47D cell lines, MCF-7aro and T47Daro, were prepared by stable transfection with the human placental aromatase gene and neomycin selection, as described previously [21]. Her-2-over-expressing MCF-7 cells were kindly provided by Dr. Dihua Yu (The University of Texas M.D. Anderson Cancer Center, Houston, TX). All three breast cancer cell lines were cultured in MEM, supplemented with 10% fetal bovine serum (FBS), 100 mg/L sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin-streptomycin, and 200 mg/L G418 for MCF-7aro, MCF-7/Her2 and T47Daro cells (Invitrogen, Carlsbad, CA). LTEDaro cells (long-term estrogen deprivation MCF-7aro cells) developed in our laboratory were cultured in the same media, but charcoal stripped fetal bovine serum (CSFBS) was used. Cell cultures were grown at 37 °C, in a humidified atmosphere of 5% CO_2 in a Hereaus CO_2 incubator.

2.3. Tritiated water-release assay in cells

Measurement of aromatase enzyme activity in cells was based on the tritium water release assay [22,23]. For all the experiments, the regular cell culture media with 10%FBS was used. For the IGF-1 treatment, cells were starved of FBS for 24 h before the treatment. Cells in 12-well plates were treated with DMSO (control), various signal transduction inhibitors at different concentrations for 24 h. Then, the cells were incubated for 1 h (24 h incubation for MCF-7 and MCF-7/Her2 cells, and 6-well plates were used) with fresh media containing the inhibitors and 2 μCi [1β-^{3}H]-androst-4-ene-3,17-dione (100 nM). Subsequently, the reaction mixture was removed, and cellular proteins were precipitated using 10% trichloroacetic acid at 42 °C for 20 min. After a brief centrifugation, the media was extracted three times with an equal amount of chloroform to extract unused substrate, and the aqueous layer subsequently treated with 1% dextran-treated charcoal. After centrifugation, a 300-μL aliquot containing the product was counted in 3 mL of liquid scintillation mixture. Each sample was prepared in triplicate and results were corrected for blanks and for the cell contents of culture flasks. One milliliter of 0.5N NaOH was added to each well and the plates were shaken overnight at room temperature to solubilize cell proteins. Protein concentrations were determined by using the Bradford assay method to normalize measured radioactivity.

2.4. Expression and Purification of NmChAro

The design of recombinant human aromatase NmChAro was described by our previous study [24]. The *Escherichia coli* BL21 (DE3) strain was used for the expression of NmChAro. Bacteria was harvested, incubated on ice for 30 min with 0.5 mg/mL lysozyme in buffer A [100 mM potassium-phosphate buffer (pH 7.4), 20% glycerol, 1 mM DTT, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μM androstenedione], and disrupted by sonication on ice (Branson Sonifier 450, 70% full power, 3× 1 min). NmChAro was isolated from the pelleted membranes with buffer B (buffer A containing 0.1% Tween 20 and 0.5 M NaCl), and purified by metal-ion affinity chromatography (Ni Sepharose 6 Fast Flow; Amersham). After elution of NmChAro with a linear imidozole gradient from 50 mM to 300 mM in buffer B, the red colored fractions were pooled,