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- Note -

BCAR3 Activates the Estrogen Response Element through the PI3-kinase/Akt Pathway in Human Breast MCF-12A Cells

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Breast cancer anti-estrogen resistance 3 (BCAR3) has been identified as one of the genes that induces anti-estrogen resistance in breast cancer. We have previously reported that BCAR3 activates promoters of c-Jun, activator protein-1, and the serum response element. In this study, we investigated the functional role of BCAR3 in the activation of the estrogen response element (ERE) in normal human breast MCF-12A cells. Transient expression of BCAR3 induced ERE activation, which was further increased by 17β-estradiol treatment but was not blocked by the anti-estrogen tamoxifen. Next, we studied the signaling pathway of BCAR3 leading to ERE activation. BCAR3-mediated ERE activation was inhibited by LY294002 and AZD5363, inhibitors of the phosphatidylinositol (PI) 3-kinase pathway, but not by PD98059 and U0126, inhibitors of the mitogen-activated protein kinase pathway. ERE activation was induced by the catalytic subunit p110α of PI3-kinase or the active mutant of Akt, and this activation was not further increased by additional BCAR3 transfection. Based on these results, we propose that BCAR3 plays an important role in ERE activation through the PI3-kinase/Akt pathway in human breast MCF-12A cells.

Key words : Anti-estrogen, BCAR3, estrogen response element, signaling pathway, PI3-kinase

Introduction

Nearly 80% of breast cancer are estrogen receptor (ER)-positive and estrogen-dependent [7]. Anti-estrogen drugs such as tamoxifen has been used as an effective endocrine treatment in ER-positive breast cancer patients, but the patients up to 50% do not respond to endocrine therapies due to the acquired resistance to anti-estrogen [13]. Among several mechanisms underlying anti-estrogen resistance, it has been proposed that newly expressed genes including breast cancer anti-estrogen resistance (BCAR's) play an important role in the resistance [32]. Out of six BCAR genes identified, BCAR3 overexpression stimulates breast cancer cell growth and induces anti-estrogen resistance by avoiding estrogen-dependent cell proliferation [2, 10, 33]. We reported that expression of BCAR3 in normal breast MCF-12A cells induces cell cycle progression, c-Jun expression, and cell proliferation [23, 25].

BCAR3 is composed of an N-terminal SH2 domain, a proline/serine-rich central domain and a C-terminal guanine exchange factor (GEF) domain [17]. We have shown that the SH2 domain of BCAR3 directly interacts with auto-phosphorylated EGF receptor resulting in regulation of EGF-mediated cell cycle progression [23]. We and others reported that the C-terminal GEF domain of BCAR3 directly interacts with BCAR1, also known as p130Cas, and this interaction regulates breast cancer progression, cell adhesion, cytoskeleton reorganization and invasion [6, 18, 24, 25, 33]. These actions of BCAR3 are shown to be mediated through the signal transducing proteins including phosphatidylinositol (PI) 3-kinase/Akt, c-Src, PAK1, and small GTPase including Ras, Rac and Cdc42 [3, 10, 25, 29]. Although many studies have been conducted on the signaling pathway of BCAR3 leading to regulation of breast cancer cell growth, the functional role of BCAR3 on the activation of ER and the subsequent activation of estrogen response elements (ERE) is yet to study.

The physiological effects of estrogen are mediated through two intracellular ERs, ERα and ERβ. Estrogen-mediated signaling effects of ERs can be divided into genomic and

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non-genomic effect [27]. Genomic effects occur when the ER-estrogen complex moves to the nucleus and binds directly to the specific DNA sequence known as ERE. Alternatively, this ER-mediated ERE can be activated in an estrogen-independent manner by EGF and cAMP [9]. Non-genomic effects of ER indirectly regulate gene expression through various signaling cascades including the PLC/PKC pathway [20], the MAPK pathway [8], the PI3-kinase pathway [20], the PKA pathway [12].

The signaling pathways of ER and BCAR3 leading to regulation of breast cancer cell proliferation have been shown to share similarity, but the relationship between these two proteins is not elucidated. In particular, a functional role of BCAR3 in ERE activation has not been studied. In this study, we assessed effects of expressed BCAR3 on ERE activation in normal human breast epithelial MCF-12A cells. We found that BCAR3 induces activation of ERE in the absence of estrogen and estrogen addition further increases the BCAR3-mediated ERE activation. Furthermore, we found that BCAR3 activates ERE in a PI3-kinase/Akt-dependent manner

Materials and Methods

Materials

PD98059, U0126, LY294002, and AZD5363 were obtained from Selleck Chemicals (Houston, TX). Rabbit polyclonal anti-BCAR3 antibodies were produced by Eurogentec (Belgium). Monoclonal antibodies against focal adhesion kinase (FAK) and paxillin were purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies were obtained from Amersham (Piscataway, NJ). Goat anti-mouse antibody conjugated with tetramethyl rhodamine isothiocyanate (TRITC) was from Jackson Immunoresearch Laboratories Inc. (West Groove, PA). All other reagents were purchased from Sigma-Aldrich. (St Louis, MO).

Cells culture

Immortalized normal human breast MCF-12A cells were purchased from the ATCC (Manassas, VA) and grown as suggested by the ATCC. The cells were maintained as described previously [25].

Plasmid constructs, transfection

The full-length BCAR3 was constructed by inserting the PCR product of BCAR3 into EcoRI/XhoI cloning sites of pCMV-Myc vector or pEGFP vector (Clontech, Mountain View, CA) as previously described [25]. The plasmids containing catalytic active subunit p110a of PI3-kinase (PI3 KCA), the active mutant of Akt (Akt-EE), and inhibitory p85 subunit of PI3-kinase deleted N-terminal SH2 domain of p85 (p85DSH2) were kindly supplied from Prof. J.H. Kim (Dept. of Biology, Korea University, Seoul, Korea). MCF-12A cells were transfected with the indicated amount of myc-tagged plasmid or GFP-tagged plasmid using DMRIE-C reagent (Invitrogen, Waltham, MA) and further incubated for 20 hr as described previously [25].

Luciferase assays

MCF-12A cells were grown on a 12-well plate and transfected with the luciferase reporter containing 100 ng of ERE (3×ERE-TATA-Luc) or c-jun promoter construct using DMRIE-C reagent as described previously [25]. The transfection mixture also included 50 μg of BCAR3, PI3KCA, Akt-EE, p85DSH2, or pCMV-myc vector. After 4 hr of transfection, the cells were treated with or without 10 nM of 17β-estradiol (E2) in the presence or absence of 1 mM of 4-hydroxytamoxifen (OHT) for 20 hr. The treated cells were then lysed with reporter lysis buffer, and luciferase activities were determined using Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions.

Immunostaining and confocal microscopy

MCF-12A cells were grown on acid-washed coverslips for 24 hr and transfected with pEGFP-BCAR3 (50 mg/ml). The cells were then fixed, permeabilized, and incubated with either anti-FAK antibody or anti-Paxillin for 1 hr at 37°C, followed by incubation with TRITC-conjugated anti-mouse IgG antibody for 30 min. For a live cell analysis, the transfected MCF-12A cells were transferred in a confocal microscope equipped with CO2 supply and further stabilized for 4 hr. The live cells' fluorescence and phase contrast images were captured at 3 min intervals.

Statistical analysis

Results are obtained from three independent experiments. Data are expressed as means ± standard error of the mean (SEM). The results were statistically analyzed by the one-way ANOVA method. The results were analyzed by one-way ANOVA, and the significance was examined with Fischer's Protected LSD post-hoc test. A p value < 0.05 was considered statistically significant.

Results

Transfection of BCAR3 promotes ERE activation

To understand the functional roles of BCAR3, we first examined whether BCAR3 can activate ERE in non-tumorigenic human mammary epithelial MCF-12A cells. Increasing amount of BCAR3 was cotransfected with the luciferase ERE plasmid in MCF-12A cells. As shown in Fig. 1A, BCAR3 increased ERE activation in a dose-dependent manner about 6-fold. Next, we treated BCAR3-transfected cells with 17β-estradiol (E2). E2 treatment further increased ERE activation approximately 70% more. As control, we examined the activation of *c-jun* promoter, and found that BCAR3 similarly activated *c-jun* promoter (Fig. 1B). Unlike results of ERE promoter, E2 treatment did not induce activation of *c-jun* promoter and did not affect BCAR3-induced activation of *c-jun* promoter. Fig. 1C shows dose-dependent expression of BCAR3 protein. These results suggest that BCAR3 can activate ERE with additive effect of additional E2.

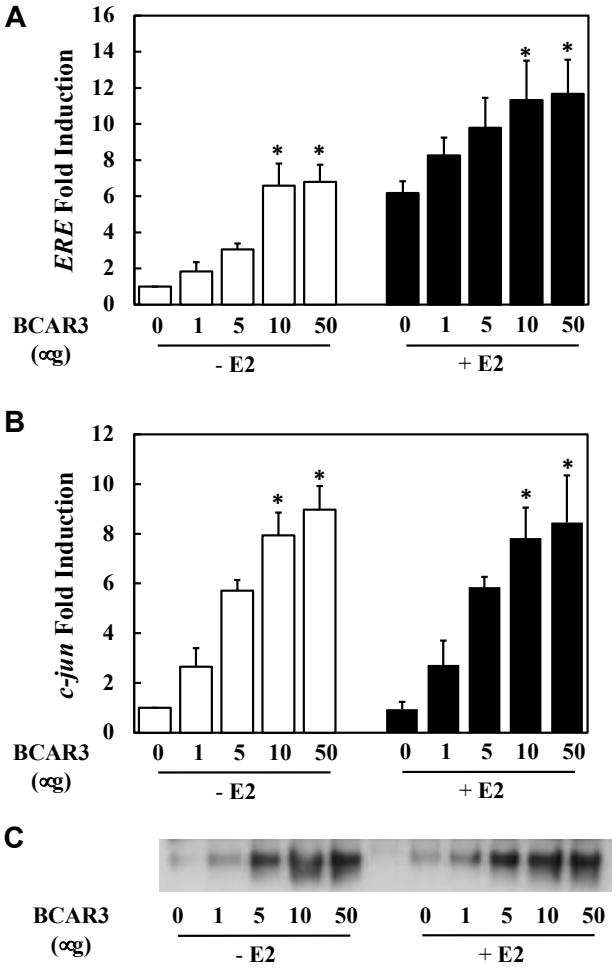

Fig. 1. Activation of ERE and *c-jun* promoter by BCAR3 in MCF-12A cells. MCF-12A cells were transfected with increasing amount of BCAR3 plasmid; 0 μg, 1 μg, 5 μg, and 10 μg. All transfections included 100 ng of ERE promoter (A) or *c-jun* promoter (B). 4 hr after transfection, the cells were further incubated with or without 10 nM of E2 for 20 hr. Luciferase activity was measured and normalized to a myc plasmid-transfected or vehicle-treated control set to a value of 1. (C) The cell lysates were immunoblotted with antibodies against BCAR3. Each data point is the average ±SEM of triplicate determinations. * $p<0.05$ relative to control plasmid transfection and vehicle treatment of cells.

Effect of tamoxifen on BCAR3-induced ERE activation

Initially, BCAR3 was identified as a gene to induce anti-estrogen resistance including tamoxifen in breast cancer. Therefore, we next examined the effect of tamoxifen on the BCAR3-mediated ERE activation (Fig. 2). BCAR3-transfected MCF-12A cells were treated with the 4-hydroxytamoxifen (OHT), active metabolic tamoxifen, in the presence or absence of E2. Tamoxifen treatment did not inhibit BCAR3-mediated ERE activation in the absence of E2 but blocked only additive ERE activation by E2. These results suggest no inhibitory effect of tamoxifen on BCAR3-mediated ERE activation. As a control, we examined the effect of OHT on the activation of the *c-jun* promoter induced by BCAR3. We found no inhibitory effect of OHT on BCAR3 activation of the *c-jun* promoter. Taken together, these results imply that BCAR3 activates the ERE promoter through the different mechanisms of E2 activation of ERE.

ERE activation by BCAR3 is mediated by the PI3-kinase/Akt pathway but not by the MAPK pathway

To understand the signaling pathway of BCAR3-mediation ERE regulation, we examined the involvement of the signaling cascades of PI3-kinase and MAPK in the activation. To do this, we utilized various inhibitors and activators of the PI3-kinase pathway and the MAPK pathway. As shown in Fig. 2A, BCAR3-induced ERE activation in the absence of E2 was reduced by inhibitors of the PI3-kinase pathway including LY294003 (PI3-kinase inhibitor) and AZD5363 (Akt inhibitor), but not by those of the MAPK pathway including PD98095 and U0216 (MEK1/2 inhibitor). In the E2-stimulated cells, ERE activation was not affected by these inhibitors, but ERE activation stimulated by E2 treatment with BCAR3 was partially inhibited by LY294003 and AZD5363 but not by PD98095 and U0216. These results implicate that the sig-

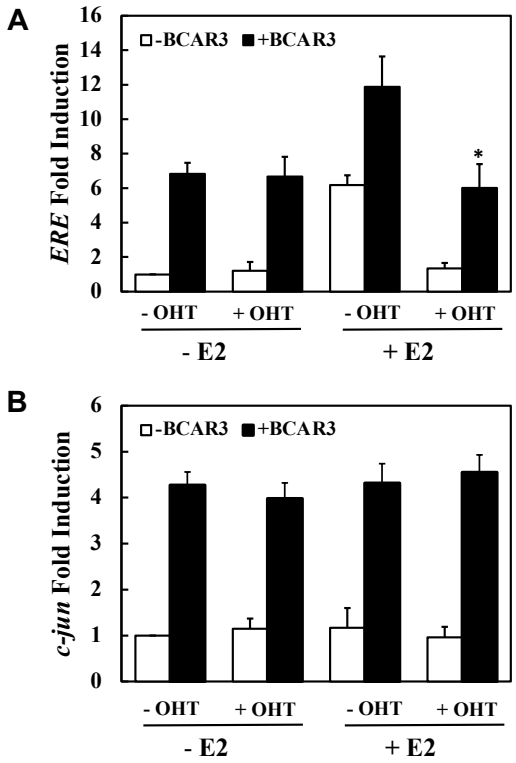

Fig. 2. Effect of tamoxifen on activation of ERE promoter or *c-jun* promoter BCAR3. MCF-12A cells were transfected with 50 μg of BCAR3 or control plasmid with 100 ng of reporter plasmid of ERE (A) or *c-jun* (B). 4 hr after transfection, the cells were further incubated with or without 1 μM of 4-hydroxytamoxifen (OHT) in the absence or presence of 10 nM of E2 for 20 hr. Luciferase activity was measured and normalized to a myc plasmid-transfected or vehicle-treated control set to a value of 1. Each data point is the average ±SEM of triplicate determinations. * $p<0.05$ relative to BCAR3 plasmid transfection with E2 treatment.

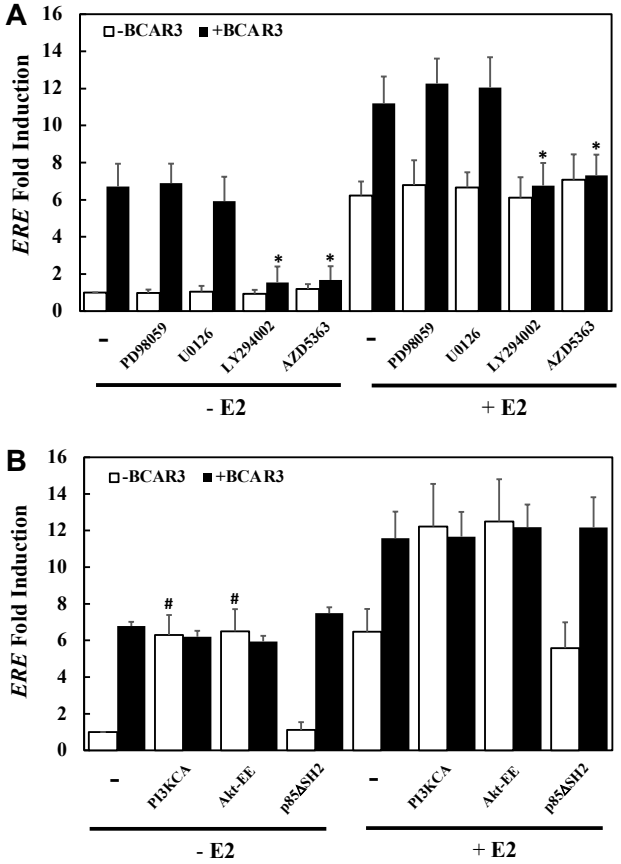

Fig. 3. Effects of inhibitors and activators of the MAPK pathway and the PI3-kinase pathway on BCAR3-induced ERE activation. (A) MCF-12A cells were transfected with 50 μg of the control myc plasmid or BCAR3. All transfections included 100 ng of ERE reporter plasmid. 4 hr after transfection, the cells were further incubated with inhibitors of PD98059 (50 mM), U0126 (15 mM), LY294002 (10 mM), or AZD5363 (3 mM) in the absence or presence of 10 nM of E2 for 20 hr. Luciferase activity was measured and normalized to a myc plasmid-transfected or vehicle-treated control set to a value of 1. (B) MCF cells were transfected with 50 μg of PI3KCA, Akt-EE, or p85ΔSH2 along with 50 μg of the control myc plasmid or BCAR3. All transfections included 100 ng of ERE reporter plasmid. 4 hr after transfection, the cells were further incubated with or without 10 nM of E2 for 20 hr. Luciferase activity was measured and normalized to a myc plasmid-transfected or vehicle-treated control set to a value of 1. Each data point is the average ±SEM of triplicate determinations. *$p<0.05$ relative to BCAR3 plasmid transfection and vehicle treatment, $^{\#}$ $p<0.05$ relative to control plasmid transfection and vehicle treatment.

naling cascade of PI3-kinase plays an important role in BCAR3 signaling leading to ERE activation rather than that of MAPK.

To confirm the functional role of the PI3-kinase pathway in BCAR3-mediated ERE activation, we utilized active mutants of the catalytic subunit p110a of PI3-kinase (PI3KCA) and Akt (Akt-EE) (Fig. 2B). Transfection of PI3KCA and Akt-EE stimulated ERE activation in the absence of BCAR3. There was no further ERE activation with the addition of BCAR3. Interestingly, BCAR3-induced ERE activation was not inhibited by the dominant-negative mutant of the p85 subunit of PI3-kinase (p85ΔSH2), suggesting that catalytic subunit p110a of PI3-kinase, not p85 subunit, is involved in the BCAR3-mediated ERE activation. Additional E2 treatment in transfection of PI3KCA or Akt-EE further induced ERE activation. However, this additive ERE activation by E2 treatment with active PI3KCA or Akt-EE was not further stimulated with the extra BCAR3 transfection. These results in-

dicate that ERE induction by BCAR3 in MCF-12A cells is mediated by the PI3-kinase/Akt pathway but not the MAPK pathway and that stimulatory action of E2 on ERE activation is independent of signaling of BCAR3 and the PI-3 kinase pathway.

BCAR3 co-localizes with FAK and paxillin at the focal adhesion and the leading edge

Having shown that BCAR3 activates nuclear-localized ERE, we wondered whether BCAR3 localizes at the nucleus and regulates ERE activation. Therefore, we examined the subcellular location of BCAR3 in MCF-12A cells. As shown in Fig. 4A, the transfected GFP-tagged BCAR3 was localized at the focal point and the leading edge in MCF-12A cells. When the cells were immunostained with antibodies against FAK and paxillin, co-localization of BCAR3 with FAK and paxillin was observed. Although these proteins were localized at the same location, a direct association between BCAR3 and FAK/paxillin was not observed (data not shown). Next, we examined the cellular movement of GFP-tagged BCAR3 using live cell image analysis in MCF-12A cells. As shown in Fig. 3B, BCAR3 continuously rearranged its cytoplasmic location and dynamically moved from cytoplasmic clusters into the protruding area of the cell surface. We previously reported that BCAR3 regulates insulin-mediated membrane ruffling, a characteristic feature of actively migrating cells [23]. Combined with the present results, we suggest that BCAR3 is present in cytosol rather than in the nucleus and induces ERE activation.

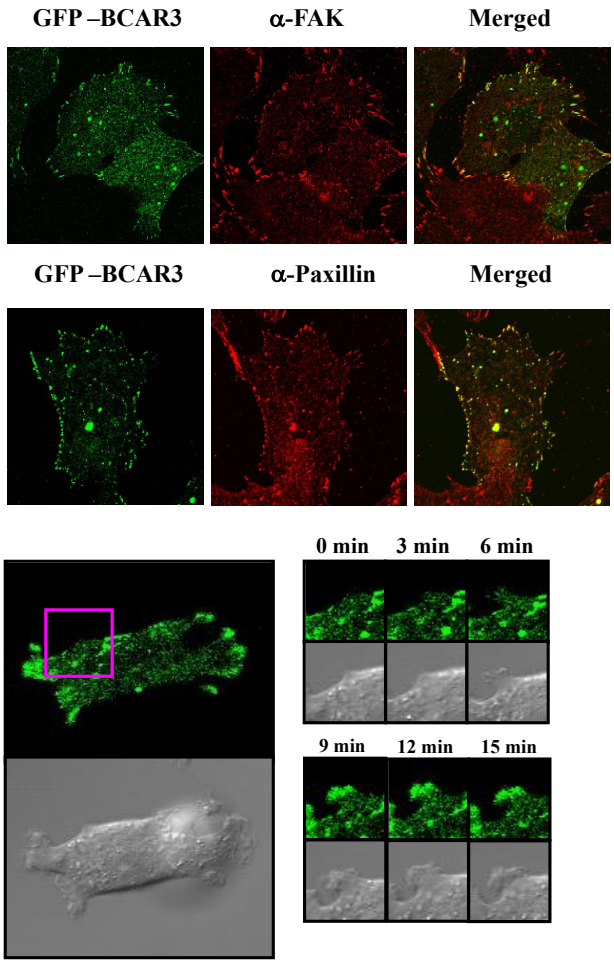

Fig. 4. Co-localization of BCAR3 with FAK or paxillin in MCF-12A cells. MCF-12A cells were transfected with 50 μg of pEGFP-BCAR3 for 24 hr. (A) The transfected cells were fixed and immunostained with antibodies against FAK and paxillin. (B) The transfected cells were further stabilized for 4 hr in a confocal microscope equipped with CO_2 supply, and live cell images were visualized at 0, 3, 6, 9, 12 and 15 min. Movement of the GFP-tagged BCAR3 was visualized, and the specific area of the cells in the box was enlarged.

Discussion

BCAR3 has been known to increase cell proliferation of normal and tumorigenic breast cells [10, 25]. However, an estrogenic effect of BCAR3 on ERE activation has not been elucidated. In the present study, we report that BCAR3 itself induces ERE activation in non-tumorigenic human breast MCF-12A cells and that E2 treatment further increases BCAR3-induced ERE activation. The estrogenic effect of BCAR3, which induces such ERE activation, was still maintained in the presence of anti-estrogen such as tamoxifen. Therefore, we conclude that BCAR3 plays an important functional role in ERE activation in breast cells.

ERE is activated through MAPK and PI3-kinase stimulated by growth factors such as EGF [8, 20]. Here, we report that the BCAR3-mediated ERE activation is blocked by inhibitors of the PI3-kinase pathway but not by those of the MAPK pathway. The involvement of the PI3-kinase pathway leading to BCAR3-mediated ERE activation was confirmed by the transfection of active mutants of PI3-kinase and Akt. The murine homolog of BCAR3 called AND-34 has been shown to activate PI3-kinase resulting in the induction of anti-estrogen resistance, where the p130Cas-BCAR3 complex plays an important role [10]. However, no studies have reported whether BCAR3 activates MAPK. Interestingly, prolonged activation of MAPK signaling shows the growth inhibitory effect on breast cancer cells. However, parallel activation of the PI3-kinase pathway is sufficient to overcome such an in-

hibitory effect of MAPK [34]. Taken together, we propose that the BCAR3-stimulated ERE activation is mediated through the PI3-kinase pathway, not the MAPK pathway and that this PI3-kinase pathway leading to ERE activation lies downstream of BCAR3 signaling.

The ERE-binding activity of ERα is regulated by an estrogen-independent activation domain AF-1 and an estrogen-dependent AF-2 located within the ligand-binding and DNA-binding domains of ERα, respectively [16]. AF-1 domain suppresses the AF-2 domain and regulates ERα activity in the absence of a ligand [22]. AF-1 domain is phosphorylated by the PI3-kinase pathway stimulated by growth factors including EGF and IGF-1, resulting in the induction of estrogen-independent activation of ERα and subsequent ERE [1, 21, 31]. Therefore, it is of interest to elucidate whether the BCAR3-stimulated PI3-kinase pathway induces the phosphorylation of the AF-1 domain of ERα, and this phosphorylation plays an important role in BCAR3-mediated ERE activation.

Besides canonical nuclear ERα and ERβ, there is membrane-localized ER, named as a G-protein coupled estrogen receptor 1 (GPER1), which is structurally distinct from ERα and ERβ [14]. The GPER1 is expressed in 50% to 60% of breast cancer, induces ERE activation, and is associated with the development of tamoxifen resistance [15, 30]. The activation of GPER1 triggers the signaling cascades including MAPK, PI3-kinase, intracellular Ca mobilization, and cyclic AMP production [14]. This signaling pathway of E2-activated GPER1 occurs via the Gβγ-dependent, FAK-associated integrin αvβ1 pathway, resulting in the activation of gene expression [26]. In the present study, we observed that BCAR3 and FAK co-localizes at the focal adhesion and the leading edge of the membrane but not at the nucleus. We and others have reported that BCAR3 directly interacts with p130Cas [18, 23]. BCAR3-p130Cas complex activates PI3-kinase [10]. p130Cas and PI3-kinase have been shown to associate with FAK within focal adhesion resulting in the proliferation of breast cancer [4, 28]. Therefore, GPER1 and BCAR3 share the signaling transducers such as PI3-kinase and FAK, but the correlation between GPER1 and BCAR3 is unknown. further studies are required to establish which ER subtype, ERα or GPER1, is involved in the BCAR3-mediated ERE activation.

In summary, we demonstrate that BCAR3 plays an important role in ERE activation in a PI3-kinase/Akt-dependent manner in normal human breast MCF-12A cells. In addition, the anti-estrogen tamoxifen has no inhibitory effect on BCAR3-mediated ERE activation. Lastly, BCAR3 co-localizes with FAK and paxillin at the focal adhesion and the leading edge of cell movement.

Acknowledgment

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 인간 유방 MCF-12A 세포에서 PI3-kinase 경로를 통한 BCAR3의 estrogen response element 활성화

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Breast cancer anti-estrogen resistance 3 (BCAR3)는 유방암에서 항에스트로겐 내성을 유도하는 유전자들 중의 하나로 발견되었다. 우리는 이미 BCAR3가 *c-jun*, activator protein-1, serum response element의 promoter 등을 활성화하는 것을 보고하였다. 본 연구에서 우리는 정상 유방세포인 MCF-12A에서 estrogen response element (ERE) 활성에서의 BCAR3의 기능을 조사하였다. BCAR3의 발현이 ERE를 활성화하는 것을 발견하였다. 이 ERE 활성화는 17β-estradiol에 의해 더욱 증가하였고, 이는 항에스트론겐인 tamoxifen에 의해 억제되지 않았다. 다음으로 우리는 ERE 활성화를 이끄는 BCAR3의 신호전달 경로를 연구하였다. BCAR3에 의한 ERE 활성화는 phosphatidylinositol (PI) 3-kinase 경로 억제제인 LY294002와 AZD5363에 의해서는 억제되었으나, Mitogen-activated protein kinase 경로 억제제인 PD98059와 U0126에 의해서는 억제되지 않았다. ERE 활성화는 PI3-kinase의 catalytic subunit p110α와 Akt의 active mutant에 의해서는 유도되었고, 이 활성화는 추가적인 BCAR3에 의해서는 더욱 증가하지 않았다. 이러한 결과로부터 우리는 BCAR3가 PI3-kinase/Akt 신호전달경로를 통하여 ERE 활성화에 중요한 역할을 하는 것을 제시한다.