

Protein kinase C- α downregulates estrogen receptor- α by suppressing c-Jun phosphorylation in estrogen receptor-positive breast cancer cells

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Abstract. Protein kinase C (PKC) activity is elevated in malignant compared with that in normal human breast tissue. In the present study, we investigated the regulatory mechanism and the co-relationship between PKC- α and estrogen receptor- α (ER- α) in ER- α -positive and tamoxifen-resistant (TAMR) breast cancer cells. Our results showed that the level of ER- α expression was significantly decreased in TAMR when compared with that in tamoxifen-sensitive (TAMS) breast cancer cells. However, PKC- α phosphorylation was increased in TAMR breast cancer cells when compared to that in TAMS breast cancer cells. Additionally, ER- α expression was significantly decreased due to the overexpression of constitutively active PKC- α (CA-PKC- α). Next, we investigated the effects of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a reversible activator of PKC, on ER- α expression in ER- α -positive breast cancer cells. TPA decreased the levels of ER- α expression in a time- and dose-dependent manner. In contrast, the TPA-induced downregulation of ER- α was prevented by Go6983, a specific PKC inhibitor. Notably, we found that CA-PKC- α suppressed c-JUN phosphorylation, which is a major activating protein-1 factor, and TPA-induced downregulation of ER- α was prevented by SR11302, a specific activator protein-1 inhibitor. Taken together, we demonstrated that PKC- α activity suppressed the level of ER- α expression by inhibiting c-JUN phosphorylation in ER- α -positive breast cancer cells. Therefore, we suggest that PKC- α may be a potential therapeutic target for treating ER-positive and TAMR breast cancer.

Introduction

The estrogen receptors (ERs) ER- α and ER- β are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors (1). The status of ER expression in human breast tumors is an extremely important prognostic marker for selecting the appropriate hormonal therapy (2,3). Approximately 75% of breast cancers express ER and/or the progesterone receptor (PR) and are treated with targeted anti-estrogen therapy such as tamoxifen (2,4).

Tamoxifen is widely used for treating hormone-dependent, ER/PR-positive breast cancer (5). It is effective for inducing the arrest of tumor progression in 50% of patients with breast cancer (6). However, although anti-estrogen therapies targeting ER- α prevent disease recurrence in patients with hormone-dependent breast cancer, *de novo* or acquired resistance remains a major problem (7,8). To date, many mechanisms have been suggested for the tamoxifen-resistant model, yet the mechanisms are not fully understood.

Protein kinase C (PKC) is a member of a family of serine/threonine protein kinases and is involved in a wide variety of fundamental physiological processes including cell proliferation and apoptosis (9,10). Estrogen-treated breast cancer cells such as MCF-7 and HCC38 show rapid increases in PKC activity (11). PKC activity is significantly elevated in malignant tumor tissues when compared with that in normal human breast tissues (12). We reported that PKC- α mediates cell invasion and migration by inducing matrix metalloproteinase (MMP)-1 and MMP-9 expression in breast cancer cells (13).

The aim of the present study was to investigate the effect of PKC- α on the level of ER- α expression and the regulatory mechanism of PKC- α -induced downregulation of ER- α in ER-positive breast cancer cells.

Materials and methods

Reagents. 4-Hydroxytamoxifen (4-OHT) was purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM) were purchased from Thermo Scientific (Hemel Hempstead,

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UK). Penicillin (100 U/ml) and 100 mg/ml streptomycin were purchased from Life Technologies (Rockville, MD, USA). Go6983 was purchased from Tocris (Ellisville, MO, USA). SR11304 and mouse monoclonal anti-ER- α and anti- β -actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was purchased from R&D Systems (Minneapolis, MN, USA). The ECLplus reagents were from Amersham (Buckinghamshire, UK).

Cell culture and establishment of tamoxifen-resistant (TAMR) MCF-7 breast cancer cells. MCF-7 breast cancer cells were grown in a humidified atmosphere of 95% air and 5% CO₂ at 37°C in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin and 100 μ g/ml streptomycin. T47D and ZR75-1 breast cancer cells were cultured in RPMI-1640. The TAMR breast cancer cell line was kindly provided by Professor Keun Wook Kang (Seoul National University, Seoul, Korea). The TAMR cell line was established using a previously reported methodology (14). Briefly, MCF-7 cells were washed with PBS, and the culture medium was replaced with phenol red-free DMEM containing 10% charcoal-stripped steroid-depleted FBS (HyClone, Logan, UT, USA) and 0.1 mM 4-OHT. The cells were continuously exposed to this treatment regimen for 2 weeks, and the 4-OHT concentration was increased gradually up to 3 mM over a 9-month period. Initially, cell growth rates were depressed. However, after exposure to the medium for 9 months, the rate of cell growth increased gradually, indicating the establishment of tamoxifen-resistant cells.

Cell proliferation. Cell proliferation was measured using a Countess[®] automated cell counter (Invitrogen, Carlsbad, CA, USA). Cells were plated at 5x10⁴/well in 6-well plates. Tamoxifen-sensitive (TAMS) and TAMR cells were incubated in phenol red-free DMEM containing 10% charcoal-stripped steroid-depleted FBS with or without 3 mM 4-OHT for the indicated times.

Western blotting. The cell lysates were used in the immunoblot analysis for ER- α , PKC- α and β -actin. The proteins were boiled for 5 min in Laemmli sample buffer and then electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. The proteins were transferred to PVDF membranes, and the membranes were blocked with 10% skim milk in TBS with 0.01% Tween-20 for 15 min. The blots were incubated with anti-matrix metalloproteinase (MMP)-1, PKC- α and β -actin antibodies (1:1,000 dilution) in 1% TBS/T buffer (0.01% Tween-20 in TBS) at 4°C overnight. The blots were washed three times, in TBS with 0.01% Tween-20, and they were subsequently incubated with anti-rabbit peroxidase-conjugated antibody (1:2,000 dilution) in TBS/T buffer. After a 1-h incubation at room temperature, the blots were washed three times and enhanced chemiluminescence reagents (Amersham Bioscience) were used for development.

Real-time polymerase chain reaction (RT-PCR). Total RNA was extracted from the cells using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. Isolated RNA

samples were then used for RT-PCR. Samples (1 μ g total RNA) were reverse-transcribed into cDNA in 20- μ l reaction volumes using a First-Strand cDNA Synthesis kit for RT-PCR, according to the manufacturer's instructions (MBI Fermentas, Hanover, MD, USA).

Gene expression was quantified by real-time PCR using a SensiMix SYBR kit (Bioline Ltd., London, UK) and 100 ng of cDNA per reaction. The sequences of the primer sets used for this analysis were: human ER- α (forward, 5'-CGC TAC TGT GCA GTG TGC AAT-3' and reverse, 5'-CCT CAC AGG ACC AGA CTC CAT AA-3') and GAPDH as an internal control (forward, 5'-ATT GTT GCC ATC AAT GAC CC-3' and reverse, 5'-AGT AGA GGC AGG GAT GAT GT-3'). An annealing temperature of 60°C was used for all primers. PCRs were performed in a standard plate format with an ABI 7900HT real-time PCR detection system. The raw threshold cycle (C_T) value was first normalized to the housekeeping gene for each sample to obtain Δ C_T. The normalized Δ C_T was then calibrated to the control cell samples to obtain $\Delta\Delta$ C_T. All cDNA samples were analyzed in three independent experiments.

PKC- α siRNA and myr-PKC- α transfection. PKC- α siRNA was purchased from Bioneer Corp. (Daejeon, Korea). Myr-PKC- α FLAG was a gift from Dr R.R. Hodges (Addgene plasmid #10807) (15). We found that the optimal siRNA knockdown and overexpression conditions involved transfection of the MCF-7 breast cancer cells at 80% confluence, and the cells were maintained in DMEM with 10% FBS; Effectene[®] (Qiagen, Valencia, CA, USA) was used for the transfections with PKC- α siRNA (25 and 50 nM, or as noted) or Myr-PKC- α FLAG following the manufacturer's protocols. Fresh serum-free media with or without 20 nM TPA were added 24 h after the 48-h transfection. The level of PKC- α protein expression was analyzed by western blotting.

Statistical analysis. Statistical significance was determined using the Student's t-test. Results are presented as means \pm standard errors. All P-values are two-tailed, and differences were considered significant at P<0.05.

Results

Inverse co-relationship between PKC- α and ER- α in the TAMR cell line. We chose TAMS and TAMR MCF-7 breast cancer cells to verify the relationship between PKC- α and ER- α . We treated each cell type with 3 mM 4-OHT for the indicated times. As shown in Fig. 1A, the proliferation of TAMS cells in response to 3 mM 4-OHT was significantly suppressed. However, the proliferation of 3 mM 4-OHT-treated TAMR cells increased in a time-dependent manner, and the gap in proliferation was significantly different after 6 days (Fig. 1A). We also compared the expression level of PKC- α and ER- α in TAMS and TAMR cells. The levels of ER- α protein and mRNA expression were significantly decreased in the TAMR cells when compared with the levels in TAMS cells (Fig. 1B and C). In contrast, PKC- α phosphorylation was significantly increased in the TAMR cells (Fig. 1B). Therefore, an inverse correlation was noted between PKC- α and ER- α in the TAMR MCF-7 breast cancer cell line.

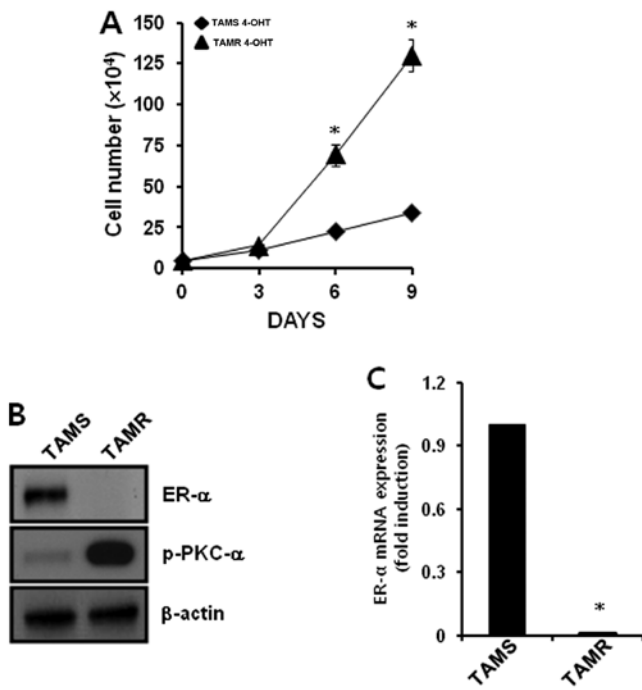


Figure 1. Inverse co-relationship between protein kinase (PKC)- α and estrogen receptor (ER)- α in tamoxifen-resistant MCF-7 breast cancer cells. (A) Established tamoxifen-sensitive (TAMS) and tamoxifen-resistant (TAMR) MCF-7 cells were seeded in a 6-well plate and treated with 3 mM 4-OHT for the indicated times after a 24-h incubation. Cell number was measured using the Countess automated cell counter. (B and C) Established TAMS and TAMR MCF-7 cells were seeded and harvested at 24 h. (B) The levels of ER- α , p-PKC- α and β -actin were analyzed by western blotting. (C) The level of ER- α mRNA was analyzed by real-time polymerase chain reaction analysis. Results are representative of three independent experiments. Values shown are means \pm standard errors. * P <0.01 vs. control.

Overexpression of constitutively active PKC- α (CA-PKC- α) decreases the level of ER- α expression in ER- α -positive breast cancer cells. Based on Fig. 1, we examined the direct relationship between PKC- α and ER- α in ER-positive breast cancer cells. The cells were transfected with CA-PKC- α for 48 h, and the cell lysates were harvested to detect PKC- α and ER- α expression. The results showed that the level of ER- α expression decreased due to CA-PKC- α overexpression in MCF-7 and ZR75-1 breast cancer cells (Fig. 2A and B). Therefore, we demonstrated that the level of ER- α expression was regulated through a PKC- α -dependent mechanism in ER-positive breast cancer cells.

Expression of ER- α was decreased in ER- α -positive breast cancer cells by TPA in a time- and dose-dependent manner. Next, we investigated the effect of TPA on ER- α expression. TPA is a natural molecule that is a well-known tumor promoter and reversible activator of PKC (16). We treated MCF-7 cells with 10 nM TPA for the indicated times. As shown in Fig. 3A, the level of ER- α protein expression was decreased in a time-dependent manner following TPA treatment. Furthermore, ER- α protein expression in response to TPA was significantly decreased in T47D and ZR75-1 breast cancer cells (Fig. 3B). In addition, we confirmed the level of ER- α mRNA expression following TPA treatment. The level of ER- α mRNA expression was also significantly decreased to 0.2 ± 0.06 -fold (in

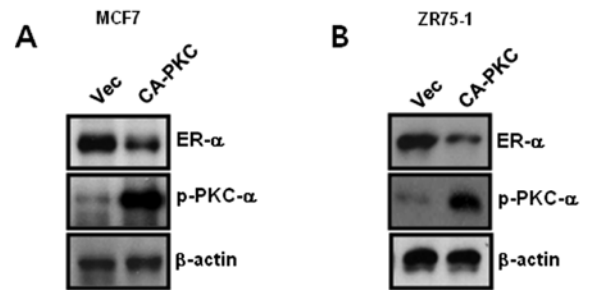


Figure 2. Overexpression of constitutively active protein kinase C (CA-PKC)- α decreases estrogen receptor (ER)- α expression in ER- α -positive breast cancer cells. After (A) MCF-7 and (B) ZR75-1 breast cancer cells were transfected with pCMV6 (Vec) or CA-PKC- α (0.4 μ g) for 48 h, respectively, they were further incubated for 24 h in serum-containing media. ER- α , PKC- α and β -actin expression was analyzed by western blotting. Results are representative of three independent experiments.

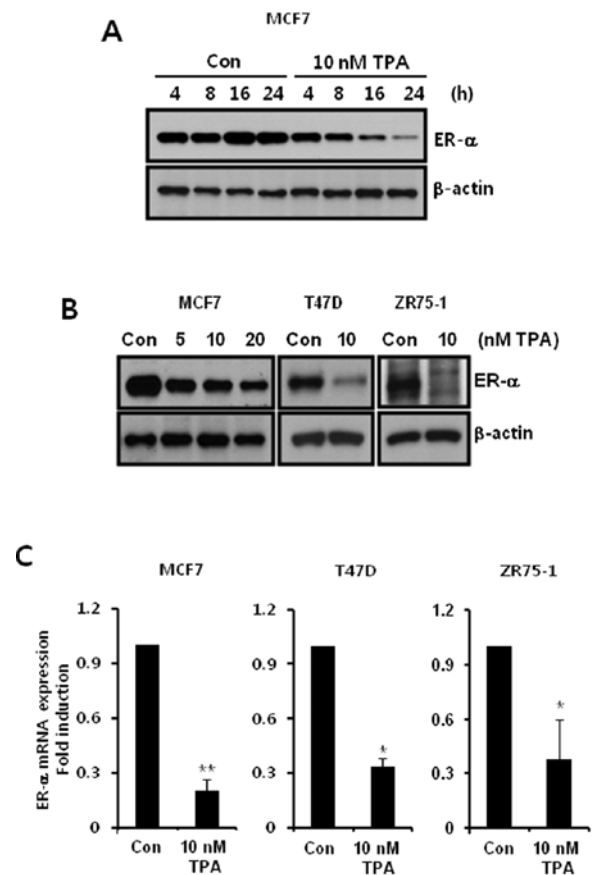


Figure 3. Estrogen receptor (ER)- α protein and mRNA expression was decreased following 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment in a time- and dose-dependent manner. (A) MCF-7 cells were treated with 10 nM TPA for the indicated times after serum-starvation for 24 h. ER- α protein was analyzed by western blotting. (B and C) The cells were treated with the indicated concentrations of TPA. ER- α protein and mRNA expression was analyzed by (B) western blotting and (C) real-time polymerase chain reaction analysis, respectively. Results are representative of three independent experiments. Values shown are means \pm standard errors. * P <0.05, ** P <0.01 vs. control. Con, control.

MCF-7 cells), 0.34 ± 0.04 -fold (in T47D cells) and 0.39 ± 0.23 -fold (in ZR75-1 cells) of the control level following 10 nM TPA treatment, respectively (Fig. 3C). These results demonstrated

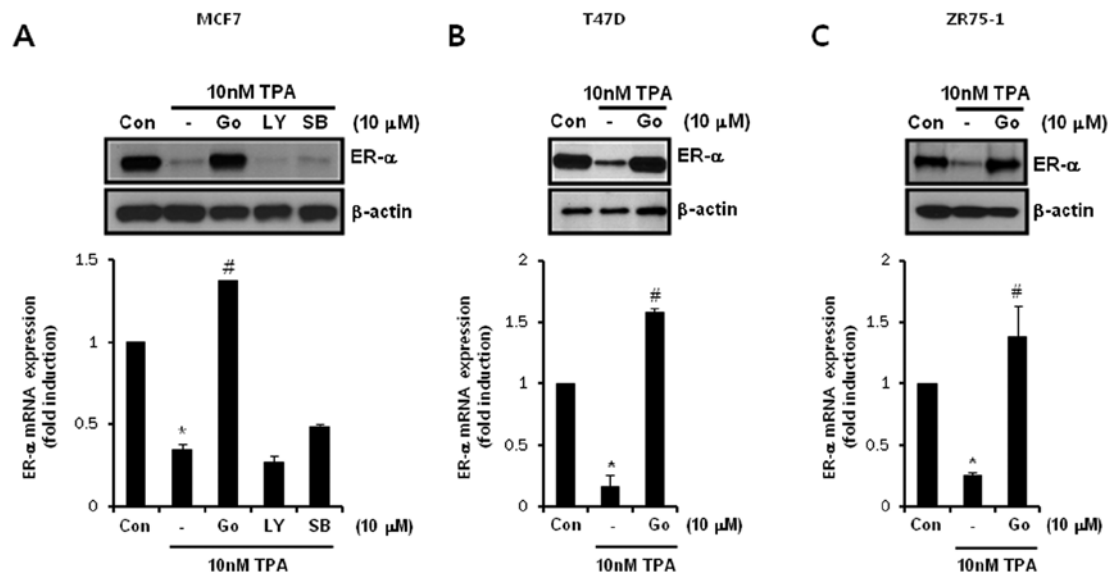


Figure 4. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA)-induced downregulation of estrogen receptor (ER)- α is prevented by Go6983 but not by LY294002 or SB203580. (A) After serum-starvation for 24 h, MCF-7 cells were pretreated with 10 μ M Go6983 (Go), 10 μ M LY294002 (LY), and 10 μ M SB203580 (SB), respectively, for 30 min and were then treated with 10 nM TPA for 24 h. ER- α protein and mRNA expression was analyzed by western blotting and real-time polymerase chain reaction analysis, respectively. (B and C) After serum-starvation for 24 h, (B) T47D and (C) ZR75-1 cells were pretreated with 10 μ M (Go), respectively, for 30 min and then treated with 10 nM TPA for 24 h. ER- α protein and mRNA expression was analyzed by western blotting and real-time polymerase chain reaction analysis, respectively. Results are representative of three independent experiments. Values shown are means \pm standard errors. * P <0.05 vs. control, # P <0.05 vs. TPA-treated cells. Con, control.

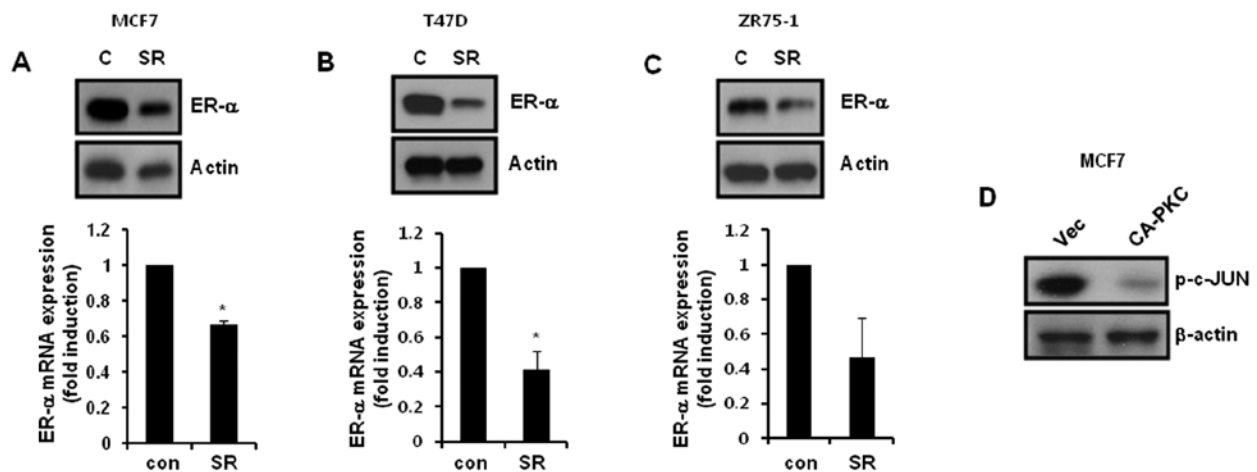


Figure 5. Estrogen receptor (ER)- α expression is suppressed in breast cancer cells by an inhibitor of activating protein-1 (AP-1). After serum-starvation for 24 h, (A) MCF-7, (B) T47D and (C) ZR75-1 breast cancer cells were treated with 3 μ M SR11302 (SR) for 24 h. ER- α protein and mRNA expression was analyzed by western blotting and real-time polymerase chain reaction analysis, respectively. (D) We confirmed the level of c-Jun phosphorylation in constitutively active-protein kinase C (CA-PKC)- α -overexpressing MCF-7 cells. c-JUN phosphorylation levels were analyzed by western blotting. Values shown are means \pm standard errors. * P <0.05 vs. control. C, control.

that TPA downregulates the expression of ER- α by activating PKC- α in ER-positive breast cancer cells.

TPA-induced downregulation of ER- α is mediated by a PKC- α -dependent pathway but not by a PI-3K/Akt-dependent pathway or p38-dependent pathway. To investigate the regulatory mechanisms of TPA-induced downregulation of ER- α , we treated ER-positive breast cancer cells with specific inhibitors such as the PKC inhibitor Go6983, the PI-3K inhibitor LY294002, and the p38 inhibitor SB203580. Our results showed that TPA-induced downregulation of ER- α protein and mRNA expression was prevented by Go6983 in MCF-7

cells but not by LY294002 or SB203580 (Fig. 4A). The level of ER- α mRNA expression was significantly decreased to 0.35 ± 0.03 -fold of the control level following TPA treatment (Fig. 4A). In contrast, TPA-induced downregulation of ER- α mRNA expression was suppressed to 1.37 ± 0.01 -fold of the control level by 10 μ M Go6983 treatment (Fig. 4A). Under the same conditions, our results also showed that TPA-induced downregulation of ER- α protein and mRNA expression was prevented by Go6983 in T47D (Fig. 4B) and ZR75-1 (Fig. 4C) breast cancer cells. Therefore, we demonstrated that TPA also regulates ER- α expression through a PKC-dependent pathway in ER-positive breast cancer cells.

PKC- α regulates ER- α expression by suppressing c-Jun activity in ER- α -positive breast cancer cells. Finally, we investigated whether the activator protein-1 (AP-1) transcriptional factor affects the level of ER- α expression in ER-positive breast cancer cells. After treatment with 3 μ M SR11302 for 24 h, the cells were harvested for detection of ER- α mRNA and protein expression. Our results showed that the levels of ER- α protein and mRNA expression was significantly decreased following SR11302 treatment of ER- α -positive breast cancer cells (Fig. 5A-C). In addition, we examined the level of c-JUN phosphorylation in CA-PKC- α -overexpressing MCF-7 cells. As shown in Fig. 5D, the level of c-JUN phosphorylation was decreased due to CA-PKC- α overexpression. Therefore, we demonstrated that PKC- α downregulated ER- α expression by suppressing AP-1 activity in ER- α -positive breast cancer cells.

Discussion

Although tamoxifen is widely used to treat all stages of breast cancer, almost 50% of patients with breast cancer fail to respond to tamoxifen and eventually acquire tamoxifen resistance, leading to tumor progression and death (8,17). The exact regulatory mechanism of tamoxifen resistance is not fully understood. In the present study, we investigated whether the level of ER- α expression is regulated by a PKC- α -dependent pathway in ER- α -positive breast cancer cells.

PKC, a serine/threonine kinase, regulates proliferation, differentiation and apoptosis in a variety of cells including breast and ovarian cancer cells (18,19). We previously reported that TPA-induced MMP-1 and MMP-9 expression is mediated through a PKC- α -dependent pathway in breast cancer cells (13). Upregulation of PKC- α and PKC- ϵ is correlated with stimulation of human endometrial cancer growth by tamoxifen (20). Consistent with this report, the PKC- α phosphorylation level was significantly augmented in the TAMR cell line compared with that in the TAMS cell line. Overexpression of CA-PKC- α significantly decreased the level of ER- α mRNA and protein expression in breast cancer cells. Thus, we suggest that PKC- α expression or activity may predict tamoxifen treatment failure.

Furthermore, the tumor promoter TPA is a natural molecule and reversible activator of PKC (16). TPA suppressed ER expression in MCF-7 cells, similar to estrogen treatment and increased ER phosphorylation (21). Elevated PKC activity suppressed ER expression in breast cancer (22,23). In addition, PKC- α levels may increase in patients with breast cancer resulting in low or negative ER levels compared to those in ER-positive patients (10,22,23). PKC- α overexpression is associated with a more aggressive neoplastic phenotype in MCF-7 breast cancer cells (24). Although we used treatment with TPA to activate PKC in breast cancer cells, our results showed that ER- α mRNA and protein expression decreased. Therefore, we suggest that activation of PKC- α may trigger tamoxifen resistance by downregulating ER- α in breast cancer cells.

AP-1 is a member of the Jun and/or Fos family. The AP-1 complex regulates transcriptional activity of a variety of genes including ER- α (25). ER- α efficiently binds to c-Jun and JunB but does not directly bind to any Fos family members (26). The induction of AP-1 transcriptional activity requires TPA-induced tumor promotion (27). Importantly, our results showed that CA-PKC- α overexpression significantly decreased

c-Jun phosphorylation. In addition, the ER- α expression level was suppressed by SR11302, an inhibitor of AP-1. Therefore, we demonstrated that activation of PKC- α suppressed the level of ER- α expression by inhibiting AP-1 activity in TAMR and ER-positive breast cancer cells.

In conclusion, we demonstrated an inverse relationship between PKC- α activity and the level of ER- α expression in TAMR and ER-positive breast cancer cells. PKC- α suppressed the level of ER- α expression by inhibiting c-Jun phosphorylation in ER-positive breast cancer cells. Therefore, we suggest that PKC- α may be a potential therapeutic target in TAMR and ER-positive breast cancer.

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