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Apigenin Inhibits Antiestrogen-resistant Breast Cancer Cell Growth through Estrogen Receptor-α-dependent and -independent Mechanisms

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Abstract

Breast cancer resistance to the antiestrogens tamoxifen and fulvestrant is accompanied by alterations in both estrogen-dependent and -independent signaling pathways. Consequently, effective inhibition of both pathways may be necessary to block proliferation of antiestrogen-resistant breast cancer cells. In this study, we examined the effects of apigenin, a dietary plant flavonoid with potential anticancer properties, on estrogen-responsive, antiestrogen-sensitive MCF7 breast cancer cells and two MCF7 sublines with acquired resistance to either tamoxifen or fulvestrant. We found that apigenin can function as both an estrogen and antiestrogen, in a dose-dependent manner. At low concentrations (1μM), apigenin stimulated MCF7 cell growth but had no effect on the antiestrogen-resistant MCF7 sublines. In contrast, at high concentrations (≥10μM), the drug inhibited growth of MCF7 cells and the antiestrogen-resistant sublines, and the combination of apigenin with either tamoxifen or fulvestrant demonstrated synergistic, growth-inhibitory effects on both antiestrogen-sensitive and -resistant breast cancer cells. To further elucidate the molecular mechanism of apigenin as either an estrogen or antiestrogen, effects of the drug on estrogen receptor-α (ERα) transactivation activity, mobility, stability, and ERα-coactivator interactions were investigated. Low-dose apigenin enhanced receptor transcriptional activity by promoting interaction between ERα and its co-activator AIB1 (amplified in breast cancer-1). However, higher doses (> 10μM) of apigenin inhibited ERα mobility (as determined by FRAP assays), downregulated ERα and AIB1 expression levels, and inhibited multiple protein kinases, including p38, PKA, MAPK and AKT. Collectively, these results show that apigenin can function as both an antiestrogen and protein kinase inhibitor with activity against breast cancer cells with acquired resistance to OHT or fulvestrant. We conclude that apigenin, through its ability to target both ERα-dependent and -independent pathways, holds promise as a new therapeutic agent against antiestrogen-resistant breast cancer.

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Keywords

apigenin; antiestrogen; fulvestrant; tamoxifen; breast cancer

Introduction

The majority of human breast tumors are estrogen receptor-alpha-positive (ERα+) and thus depend primarily on estrogens for growth (1). Currently, the first-line agent for treatment of ERα+ breast cancer in both pre- and post-menopausal women is the antiestrogen tamoxifen (OHT). The second-line drug given to post-menopausal women with receptor-positive, tamoxifen-resistant tumors is fulvestrant (Faslodex®; ICI 182, 780), a selective estrogen receptor downregulator (SERD) (2). These two antiestrogens are extremely important breast cancer therapeutics; however, not all ERα+ breast cancers respond to tamoxifen and fulvestrant, and for those women who do respond, initial positive responses can be of short duration (3), with most tumors eventually developing complete resistance to both of these agents (4). Clearly, more effective drugs are needed to enhance the efficacy of tamoxifen and fulvestrant in antiestrogen-insensitive and/or -resistant breast tumors.

The development of antiestrogen-resistant breast cancer is associated with a myriad of cellular, molecular and biochemical alterations (5). Multiple changes in ERα signaling and protein kinase pathways, such as mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase/AKT, and cyclic AMP (cAMP) protein kinase A (PKA) (6,7), have all been reported. The receptor co-activator AIB1(amplified in breast cancer-1), which is often overexpressed in breast cancer, functions as an oncogene by transmitting kinase-mediating growth factor signaling to the ERα (8). Previously, we established and characterized both tamoxifen-resistant (MCF7-T) and fulvestrant-resistant (MCF7-F) breast cancer cell lines (9). We further demonstrated significant changes in ERα, AIB1, protein kinases, and growth factor pathways in the antiestrogen-resistant sublines, as compared to the parental MCF7 cell line (9). As ERα and protein kinases play critical roles in breast cancer cell proliferation and antiestrogen resistance, targeting both pathways simultaneously could likely inhibit both genomic as well as non-genomic effects of estrogen, and also serve as a potential strategy for the treatment or prevention of antiestrogen-resistant breast cancer.

Apigenin (chemical name 5,7-dihydroxy-2- (4-hydroxyphenyl)-4H-1-benzopyran-4-one), a known phytoestrogenic compound (10-12), is a naturally occurring, nontoxic, nonmutagenic, plant flavonoid commonly present in various fruits and vegetables (13). Apigenin has demonstrated both anti-inflammatory and anticarcinogenic effects in various animal tumor model systems, including breast, colon, skin, thyroid, leukemia, and prostate (14-16). Epidemiological studies suggest that flavonoids play an important role in reducing the risk of breast cancer (17,18). Although the effects of apigenin and other flavonoids appear to be mediated through ERα binding-dependent and independent pathways (19), the precise mechanism of apigenin on breast cancer cell growth inhibition is not clear. Interestingly, apigenin displays dose-dependent alternate effects, enhancing E2-induced DNA synthesis at low concentrations, while inhibiting DNA synthesis at high concentrations (12). Furthermore, by competing with ATP, apigenin has been shown to be an inhibitor of protein kinases (20). Although the anti-proliferative activity of apigenin is widely accepted, it has also been shown to stimulate breast cancer cell growth (21), further demonstrating its complexity.

In the current study, we examined the effect of apigenin on antiestrogen-sensitive and -resistant breast cancer cells. We investigated whether apigenin can enhance the growth inhibitory efficiency of both tamoxifen and fulvestrant in hormone-sensitive and antiestrogen-resistant breast cancer cells. We demonstrated that low concentrations of apigenin stimulated

proliferation of hormone-sensitive MCF7 cells, that high doses of apigenin inhibited both hormone-sensitive and drug-resistant breast cancer cell growth, and synergistic action with OHT and fulvestrant in antiestrogen-resistant breast cancer cells. We further showed that high doses of apigenin blocked ERα mobility and transcriptional activity, induced degradation of ERα and its co-activator AIB1, and inhibited the activities of multiple protein kinases involved in antiestrogen resistance, including MAPK, PKA, P38 and AKT. This is the first study to show that apigenin can function as both an antiestrogen and protein kinase inhibitor and has activity against breast cancer cells with acquired resistance to tamoxifen and fulvestrant.

Materials and Methods

Materials

The following antibodies and reagents were used in this study: anti-PKAβ cat (C-20); anti-ERα (HC20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); monoclonal anti-human ERα (Chemicon International, Inc., Temecula, CA); mouse anti-glyceraldehyde phosphate dehydrogenase (GAPDH) (Chemicon International, Temecula, CA); monoclonal anti-AIB1 antibody (BD Biosciences); protease inhibitor mixture set III (Calbiochem-Novabiochem); Lipofectamine Plus reagent, cell culture medium (Invitrogen, Carlsbad, CA); FuGene (Roche Molecular Biochemicals, Indianapolis, IN); Apigenin, 17β-estradiol (E2), 4-hydroxytamoxifen (4-OHT), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and MG132 (Sigma, St. Louis, MO); ICI 182,780 (Tocris Cookson Ltd., Ellisville, MO); passive lysis buffer and Luciferase Assay System (Promega Corp., Madison, WI), Galaco-Star luminescent assay kit (PE Applied Biosystems, Foster City, CA); IRDye® 700DX Conjugated Affinity Purified Anti-MOUSE IgG (H&L), IRDye® 800 Conjugated Affinity Purified Anti-RABBIT IgG (H&L) (Rockland Immunochemicals, Inc., Gilbertsville, PA).

Plasmid Construction

pBD-Gal4-ERαAF2 was constructed by cloning ERαAF2 into pBD-Gal4 vector (Stratagene) (22). Creation of the ERE2pS2-Luc reporter plasmid was described previously (22). Wild-type ERα pSG5-ERα (HEGO) was kindly provided by Dr. Pierre Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France) and GFP-ERα by Dr. Michael Mancini (Baylor College of Medicine, Houston, TX) (23). CA-AKT was kindly provided by Dr. Harikrishna Nakshatri (Indiana University School of Medicine) (24). The plasmids pFA-CHOP, pFA2-CREB, pFA2-Elk1 and pFR-Luc for PathDetect® Trans-Reporting Systems were purchased from Stratagene (La Jolla, CA). pcDNA3-AIB1 was kindly provided by Dr. Myles Brown (Dana–Farber Cancer Institute, Harvard Medical School), The plasmid pAD-Gal4-rAIB1 was described previously by cloning rat coactivator AIB1 (rAIB1) into pAD-Gal4 vector (22).

Cell Culture and Proliferation Assays

The tamoxifen- and fulvestrant-resistant sublines MCF7-T, MCF7-F have been described previously (9). MCF7 human breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA). To assess the effects of apigenin, E2, ICI 182,780, or 4-OHT on cell proliferation, cells (2000/well) were plated in 96-well dishes in hormone-free medium for three days before drug exposure. Cell numbers were determined by MTT (25) assay at the indicated times after drug treatment. Following drug treatment, cells were exposed to MTT for 4 h, solubilized in DMSO, and the MTT metabolite formazan quantitated at 600 nm using a Bio-Tek (Winooski, VT) ELX-800 microplate absorbance reader.

Western Blot and Quantitation

Whole cell lysates were prepared in 1X SDS sample buffer by sonication, and the supernatant protein concentration then determined using a Bio-Rad protein assay kit. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were then incubated with primary antibody followed by incubation with infrared dye IR700-labeled goat anti-mouse IgG or IR800-labeled goat anti-rabbit IgG (LI-COR) secondary antibodies and quantified with LI-COR imaging system and Odyssey software. All Western blots were done at least two times.

Co-immunoprecipitation

Lysates from MCF7 cells were prepared in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% Triton X-100, 1 mM Na_3VO_4, protease inhibitor) and incubated with protein G-agarose for 30 min at 4 °C. After centrifugation at 12,000 × *g* for 15 s, the precleared supernatants were incubated with 5 μl of anti-ERα antibody or IgG at 4°C for 3 h followed by incubation with 30 μl of protein G-agarose beads for 30 min. The beads were then pelleted by brief centrifugation, washed three times with Tris-buffered saline (TBS), and finally, resuspended in 30 μl of SDS-PAGE loading buffer for SDS-PAGE and Western blotting.

Transient Transfection Assay and Reporter Enzyme Assays

MDA-MB-231 or HeLa cells were cultured in hormone-free medium for three days and then transfected with equal amounts of total plasmid DNA (adjusted by the corresponding empty vectors) using Lipofectamine Plus reagent or FuGene according to the manufacturer's guidelines. Unless stated otherwise, 24 h after transfection, cells were treated with the specified drug. At the end of the experiment, cell lysates were prepared for reporter enzyme assays using passive lysis buffer, according to the manufacturer's instructions. Luciferase and β-galactosidase activities were determined using the Luciferase Assay System (Promega) and Galaco-Star assay kit (Applied Biosystems), respectively. Luciferase activity was corrected for transfection efficiency by expression as ratio of luciferase to β-galactosidase activities.

Quantitative Real-Time PCR

Cells were cultured in basal medium for 3 days and treated with apigenin (10 μM). Total RNA was prepared by a RNAeasy Mini Kit (QIAGEN, Valencia, CA), according to the manufacturer's protocol. RNA (2 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), according to the instructions of the manufacturer. The resulting cDNA (equivalent to 40 ng total RNA) was used in quantitative real-time PCR using FastStart DNA MasterPLUS SYBR Green I (Roche Applied Science, Indianapolis, IN) and LightCycler according to the instructions of the manufacturer.

Yeast Two-Hybrid Reporter Assays

The yeast two-hybrid reporter assay was performed as described previously (26). Briefly, yeast cells (strain PJ69-4A, a gift from Dr. Philip James, University of Wisconsin, Madison) were transformed with pAD-Gal4-rAIB1 and pBD-Gal4-ERαAF2. To measure the strength of the interaction of AIB1 with ERα in the presence of apigenin, β-gal expression levels in liquid yeast cultures from three independent transformants, were determined using Galaco-Star luminescent assay kit (Applied Biosystems).

Yeast Estrogen Reporter Assay

The yeast estrogen-responsive reporter assay was performed as described previously (22). Briefly, yeast cells (RS188N) were transformed with vectors for ERα, ERE-β-gal reporter gene

and either empty pAD-Gal4 vector or pAD-Gal4-rAIB1. Cells were grown overnight in selection medium (lacking uracil, tryptophan, and/or leucine) and then divided into treatment groups. Test compounds were dissolved in DMSO. The β-gal activity in yeast was assayed 18 h later using a Galacto-Star luminescence assay kit (Applied Biosystems).

Live Cell Microscopy and FRAP (Fluorescence Recovery After Photobleaching)

Live cell fluorescence microscopy and FRAP were performed on MCF7 cells transfected with GFP-ERα. Cells were grown on 12-mm cover slips in 6-well plates and transfected with GFP-ERα plasmid using FuGene (27), and subsequently maintained in minimum Eagle's medium with 5% dextran-coated charcoal-stripped fetal bovine serum at 37 °C. Transfected cells were then treated for 1 h with apigenin (10 μM), E2 (10 nM), 4-OHT (100 nM), or ICI (100 nM), and FRAP analysis was carried out on a Spinning disk confocal microscope (Yokogawa CSU10/Nikon TE 2000) using Metamorph7.1 software (Molecular Devices, Sunnyvale, CA) and the MOSAIC® Digital Diaphragm System (Photonic Instruments, St. Charles, IL). A single z-section was imaged before and at various time intervals after the 2-s bleach. Images were taken every 5 s for a 5 min period.

Statistical Analyses

P-values were determined by ANOVA statistical test and Student's t-test.

Results

Dose-dependent effects of apigenin on growth of breast cancer MCF7 cells

The common flavonoid apigenin has been well established to have antiproliferative activity against numerous cancer types, including breast cancer (28,29), and several mechanisms have been proposed for its antineoplastic effects (15,30). However, apigenin was also paradoxically shown to stimulate proliferation of breast cancer cells (21), and consequently, we sought to further investigate the divergent effects of this drug. To examine the effects of apigenin on the growth of breast cancer cells, ERα+ MCF7 breast cancer cells were treated with various concentrations of apigenin. At low (0.1 and 1 μM) concentrations, apigenin stimulated MCF7 cell growth (Fig. 1A); in contrast, apigenin inhibited cell growth at a higher (10 μM) concentration (Fig. 1A), revealing a dose-dependent activity of apigenin on cell growth. To test the effect of apigenin on E2-induced proliferation, MCF7 cells were treated with a combination of E2 and various doses of apigenin. Treatment with 10 μM apigenin blocked E2-induced cell proliferation, while 1 μM apigenin had no effect (Fig. 1B). To examine whether the growth stimulatory effect of apigenin was mediated by ERα, MCF7 cells were treated with 1 μM apigenin in the presence of increasing doses of ICI (fulvestrant) or OHT. Apigenin-induced cell growth was inhibited by both ICI and OHT (Fig. 1C & D), demonstrating that the growth stimulatory effect of apigenin was mediated by ERα. It was also notable that only 1-10 nM of antiestrogen was required to inhibit 1 μM apigenin, suggesting that ERα has a weak binding affinity for this flavonoid.

Growth inhibition of antiestrogen-resistant breast cancer cells by apigenin

We next investigated whether apigenin can inhibit proliferation of antiestrogen-resistant breast cancer cells. MCF7 and its tamoxifen- and fulvestrant-resistant derivatives (MCF7-T and MCF7-F, respectively) were grown in hormone-free medium for three days, and then treated with various doses of apigenin. Low concentrations of apigenin again stimulated MCF7 cell growth but had no effect on growth of either MCF7-T or MCF7-F (Fig. 1E). High doses of apigenin inhibited proliferation of all three cell lines, but to a differing extent. Apigenin at 20 μM inhibited the growth of MCF7 cells and MCF7-T cells by 80%, but only by 40% for MCF7-F cells (Fig. 1E). Because the level of ERα in MCF7-F is very low compared to MCF7 and

MCF7-T (Fig. 1F), this observation suggests that ERα levels may determine cell sensitivity to apigenin.

Previously, it was suggested that the antiestrogenic activity of flavonoid phytochemicals may be mediated by ERα-independent pathways (19). The above results, including the ability of apigenin to inhibit the growth of MCF7-F cells at high doses, suggests that apigenin may act by both ERα-dependent and -independent mechanisms. Our previous results (9), including gene expression analysis demonstrating overexpression of protein kinase A, MAPK kinase, PI3 kinase and co-activator AIB1 in MCF7-T and MCF7-F compared to MCF7 (Supplemental Table 1), suggested that these antiestrogen-resistant sublines were not completely dependent on ERα for growth. To validate those microarray results at the protein level, we performed Western blot analysis for AIB1 and the PKAβ catalytic subunit. We observed overexpression of AIB1 in MCF7-F cells, and both MCF7-F and MCF7-T cells displayed upregulation of the PKAβ catalytic subunit (Fig. 1F), consistent with our previous observation (9).

Effects of apigenin combined with OHT or ICI on breast cancer cell growth

We next investigated whether apigenin can enhance the inhibition of breast cancer cell growth by the antiestrogens OHT or ICI in a synergistic manner. We first evaluated the effects of various combinations of apigenin (1.0, 2.5, 5, 10, and 20 μM) with OHT or ICI on the growth of MCF7 cells. Again, apigenin treatment alone showed dose-dependent effects on MCF7 cell growth (Fig. 2A, B). Treatment of MCF7 cells with apigenin (2.5 – 10 μM) and OHT (100 nM) resulted in greater ($P<0.01$) growth inhibition than apigenin or OHT alone (Fig. 2A). The combination of apigenin (10 μM) plus ICI (100 nM) also showed synergistic growth inhibition of MCF7 cells ($P<0.01$) (Fig. 2B).

We next examined the effects of combinations of apigenin with OHT or ICI on the growth of MCF7-F cells. We had previously reported that this subline was refractory to the inhibitory effects of both fulvestrant and tamoxifen (9). As shown in Figure 2C, MCF7-F cell growth was inhibited by 20 μM apigenin, and treatment of MCF7-F cells with both apigenin and OHT showed greater ($P<0.05$) growth inhibition than either apigenin or OHT alone (Fig. 2C). However, treatment with ICI showed no effect on apigenin-mediated growth inhibition (Fig. 2D).

Finally, we tested the effects of combinations of apigenin with OHT or ICI on the growth of OHT-resistant MCF7-T cells, which express high levels of ERα. At doses of 10-20 μM, apigenin alone inhibited growth of MCF7-T cells (Fig. 2E), and the presence of OHT showed no effect on apigenin-mediated growth inhibition of this subline (Fig. 2E). The combination of apigenin (5–10 μM) and ICI (100 nM) resulted in greater ($P <0.05$) growth inhibition of MCF7-T, as compared to apigenin or ICI alone (Fig. 2F). These results demonstrate that apigenin alone can inhibit cell proliferation of MCF7-F and MCF7-T cells and is also capable of acting synergistically with both OHT and ICI in these drug-resistant cells.

Dose-dependent effect of apigenin on ERα transcription activity

Based on the observation that a low concentration of apigenin stimulated proliferation of hormone-sensitive MCF7 cells, we hypothesized that apigenin may activate E2-responsive genes. To test this hypothesis, we investigated the effect of apigenin on ERα-mediated gene transcription using estrogen-responsive reporter assays. For these experiments, we utilized MDA-MB-231 cells transfected with wild type ERα (pSG5-ERα) and estrogen-responsive reporter gene (ERE2pS2-Luc) and then treated with various concentrations of either E2 or apigenin. As expected, a dose-dependent increase in reporter gene activity was observed after E2 treatment (Fig. 3A). Consistent with the dose-dependent effect of apigenin on the growth of MCF7 cells (Fig. 1A, B), a biphasic effect of apigenin on ERα activity was observed. Low

concentrations (0.1-1 μM) of apigenin increased ERE2pS2-Luc expression (Fig. 3B), while high concentrations (10-20 μM) decreased expression.

To further investigate whether apigenin-induced ERE2pS2-Luc is ERα-dependent, MDA-MB-231 cells were transfected with or without receptor and then treated with apigenin. Apigenin did not induce luciferase activity in the absence of ERα (Fig. 3C, lane 2), while apigenin-induced luciferase activity in the presence of ERα was inhibited by ICI (Fig. 3C), demonstrating that low-dose apigenin activity is ERα-dependent. To examine whether apigenin can eliminate E2-induced ERα activity, MDA-MB-231 cells transfected with pSG5-ERα and ERE2pS2-Luc were treated with E2, either alone or with various doses of apigenin. As shown in Figure 3D, apigenin also inhibited E2-induced luciferase activity in a dose-dependent manner.

To understand whether the antagonistic activity of apigenin is dependent upon binding to ERα, MDA-MB-231 cells transfected with pSG5- ERα and ERE2pS2-Luc were treated with various concentrations of E2, alone or in combination with apigenin or OHT. While the antagonistic effect of OHT was reduced by increasing E2 concentration, the antagonistic activity of apigenin was independent of E2 concentration (Fig. 3E). Therefore, unlike OHT, whose antagonistic activity is dependent on competing with estrogen for ERα binding, the antiestrogenic effect of apigenin does not appear to be due to competition with estrogen for ERα binding.

Apigenin reduces steady state protein levels of ERα and AIB1

We and others have previously shown that agonist/antagonist binding can lead to changes in ERα stability (27,31). To examine whether apigenin can affect ERα degradation, we utilized three cell lines with different levels of receptor: MCF7-T cells, having a higher level of ERα than MCF7 cells, and MCF7-F cells, having very low ERα levels (Fig. 1F). To test receptor stability in the presence or absence of apigenin, MCF7, MCF7-T, and MCF7-F were treated with 10 μM apigenin, 10 nM E2, 100 nM ICI, or 100 nM OHT for 24 h, and Western blot analysis for ERα was then performed. Steady-state levels of ERα were decreased in all three cell lines after apigenin treatment (Fig. 4A); E2 and ICI also induced ERα degradation, while OHT stabilized ERα levels (Fig. 4A). A dose-response study of MCF7 cells revealed that apigenin markedly decreased steady-state ERα levels at 10-20 μM (Fig. 4B), doses that also inhibit both ERα transcriptional activity and cell growth (Figs. 1, 3), further supporting our observations on the dose-dependent effects of apigenin on MCF7 cell growth and estrogen-responsive gene expression. Unlike E2 and the antiestrogens, apigenin decreased the level of AIB1 in MCF7 cells (Fig. 4C), suggesting that apigenin can block estrogen signaling by downregulating both ERα and AIB1.

To further examine the effect of apigenin on AIB1 and ERα protein degradation, MCF-7 cells were treated with the proteasome inhibitor MG132, prior to apigenin treatment. As shown in Fig 4D, treatment with MG132 blocked downregulation of ERα and AIB1 by apigenin. Treatment with the protein synthesis inhibitor CHX, however, had no effect on apigenin-induced downregulation of AIB1 and ERα (Fig 4E), suggesting that *de novo* protein synthesis is not required for apigenin-induced protein degradation. We then examined the effects of apigenin on ERα and AIB1 mRNA levels by real-time RT-PCR. Apigenin decreased ($P<0.01$) the level of AIB1 mRNA, but had no effect on ERα mRNA level (Fig. 4F). Taken together, these results demonstrated that apigenin induces proteasomal degradation of both ERα and AIB1, as well as inhibits *AIB1* transcriptional activity.

Apigenin induces ERα–co-activator binding and AIB1 enhances estrogen reporter gene response to apigenin

It is now well known that estrogen promotes physical interactions between ERα and its coactivators, while antiestrogens inhibit such interactions. As AIB1 is the dominant ERα coactivator in ERα(+) breast cancer (32), we examined the effect of apigenin on the interaction between AIB1 and ERα using a yeast two-hybrid assay, to further elucidate the estrogenic and antiestrogenic characteristics of apigenin. To quantify the interaction of AIB1 and ERα, we measured β-galactosidase activity in liquid yeast culture. Yeast transformed with pBD-Gal4-ERαAF2 and pAD-Gal4-AIB1 displayed increased activity in the presence of apigenin (Fig. 5A), suggesting that apigenin can promote an ERα-AIB1 interaction. This interaction was further examined by co-immunoprecipitation, where even low doses of apigenin (1 μM) were sufficient to induce the receptor-coactivator interaction in MCF7 cells (Fig. 5B).

As AIB1 is overexpressed in breast cancer cells, and ERα enhances gene expression by forming a complex with the transcriptional machinery through a bridge formed by coactivator proteins (32), we examined whether AIB1 can enhance apigenin-induced ERα transcriptional activity. For this purpose, we used yeast cells transformed with ERα and ERE-β-Gal, HeLa cells transfected with ERα and ERE2pS2-Luc and also tested the effect of apigenin on ERα transcriptional activity in the presence or absence of AIB1. In both systems, the response of the estrogen reporter gene to apigenin was greatly enhanced by the addition of co-activator AIB1 (Fig. 5C, D), suggesting that apigenin, at lower concentrations, may enhance ERα transcriptional activity and stimulate breast cancer cell growth by recruiting AIB1 to ERα. High concentration (10 μM) of apigenin also induced AIB1 interaction with ERα in yeast cells transformed with ERα and ERE-β-Gal (Supplementary Fig. 1); however, at this concentration, apigenin inhibited ERα transcription activity (Fig. 3), perhaps by downregulating both ERα and AIB1 (Figure 4) and inhibiting protein kinases activity (Figure 6).

Effects of apigenin on ERα mobility

Depending on the nature of the ligand, ERα mobility and nuclear localization can be significantly altered (23), and we thus examined the effect of apigenin on ERα mobility. To characterize ERα mobility in the presence of apigenin and to compare the differences in ERα mobility induced by apigenin, E2, tamoxifen and ICI, we treated GFP-ERα tranfected MCF7 cells with those agents, followed by FRAP analysis. In cells treated with apigenin, E2, or OHT, a distinct dark zone was detected immediately after 2s bleaching, while fluorescence was fully recovered by 30s after bleaching (Fig. 6A). In contrast, cells treated with ICI showed very little recovery in a 5-min time frame (Fig. 6A, bottom panel). As it has been reported that ERα is extremely mobile in absence of ligand (23), and FRAP analysis of DMSO-treated control cells resulted in immediate recovery of the bleach zone (data not shown), our results demonstrate that the mobility of ERα is similar in the presence of apigenin, E2 and OHT. Although apigenin and ICI can both downregulate ERα protein levels, they have different effects on ERα mobility, suggesting that these compounds may reduce ERα protein levels by different mechanisms (27).

Effects of apigenin on kinase activity

The antiproliferative effects of apigenin in prostate cancer appear to be mediated in part by inhibition of MAPK and PI3K-AKT (33), and genistein, an isoflavone, has been shown to inhibit breast cancer growth by inhibiting of p38, MAPK, and AKT kinase activity (34). As these kinases have been shown to become dramatically altered as breast cancers develop drug resistance (6,9,35), we next examined whether apigenin can inhibit the activity of p38, PKA, MAPK and AKT. Here, PathDetect Trans-Reporting Systems and reporter genes for CHOP, CREB, and Elk1 were used to monitor the activity of p38, PKA and MAPK, respectively. Each of the ELK-1, CREB and CHOP plasmids were cotransfected into MDA-MB-231 cells with

a pFR-Luc reporter gene. As shown in Fig. 6B, apigenin inhibited CHOP (p38 kinase pathway), CREB (PKA pathway), and Elk1 (MAPK pathway) activities in a dose-dependent manner; these doses were consistent with a recent report showing kinase inhibition by apigenin in cancer cells (36). It has been previously reported that AKT protects breast cancer cells from antiestrogen-induced apoptosis (24), and increased AKT kinase activity was also reported in both tamoxifen and ICI-resistant cell lines (7). To test the effect of apigenin on AKT activity, a construct expressing constitutively active AKT (CA-AKT) was co-transfected with ERE2pS2-Luc and ERα into MDA-MB-231 cells. As shown in Fig. 6C, AKT enhanced E2-induced ERE-Luc activity, and this enhancement was inhibited by apigenin. Thus, by inhibiting the activity of p38, PKA, MAPK and AKT, all of which have been associated with breast cancer drug resistance (24,37), apigenin is capable of both blocking the growth of drug-resistant breast cancer and resensitization to OHT and ICI.

Discussion

Acquired resistance of breast cancer cells to the antiestrogens tamoxifen and fulvestrant is accompanied by the dysregulation of ERα-dependent signaling molecules, such as co-activators, as well as altered receptor-independent growth pathways, such as protein kinases (6,9). To effectively block proliferation of antiestrogen-resistant breast cancer cells, it may be necessary to identify a drug(s) that can target both of these important pathways. Apigenin, the most common flavonoid present in fruits and vegetables, has been shown to inhibit the growth of human tumor cell lines (16), including breast cancer cells (15,18,28,38). However, the molecular mechanisms underlying that antiproliferative effect are not well understood. Recent evidence suggests that some plant flavones and isoflavones that prevent cancer can enhance the efficacy of cancer therapeutics by modifying the activity of cell survival pathways. The antiproliferative effects of apigenin in prostate cancer have been proposed to be mediated in part by inhibition of MAPK and PI3/AKT (33,38,39). In this study, we demonstrated a biphasic effect of apigenin on MCF7 breast cancer cell growth. At lower concentrations, apigenin stimulated cell growth by activating ERα-mediated gene expression. However, at high concentrations, apigenin inhibited cell growth by reducing ERα and AIB1 protein levels and inhibiting multiple kinases. These observations are consistent with a recent report that antiestrogenic effects of flavonoids are mediated by both ERα -dependent and -independent pathways (19). While a recent study reported that ERβ activation by apigenin suppresses prostate and breast cancer growth (40), whether the apigenin-elicited effects on ERβ is dose-dependent remains to be determined.

Since more than 60% of human breast tumors are ERα(+) and dependent on estrogens for growth (17), the first-line drug for these tumors is the selective estrogen receptor modulator (SERM) OHT, while fulvestrant is the second-line drug for ERα(+) tumors that are resistant to tamoxifen. However, drug-sensitive breast cancer can eventually acquire resistance to both drugs, presenting a major challenge in disease management. Therefore, therapeutic agents that can be used to treat antiestrogen-resistant breast cancer and/or enhance sensitivity to tamoxifen or fulvestrant would be of great value. Toward this objective, we recently generated breast cancer cell lines that recapitulate acquired resistance to tamoxifen and fulvestrant, and we also reported dramatic changes in ERα- and growth factor-signaling pathways in those cell lines (9). The current study demonstrates various differential effects of apigenin on the growth of MCF7, tamoxifen-resistant MCF7-T, and fulvestrant-resistant MCF7-F cells. At lower concentrations, apigenin stimulated MCF7 cell growth but had no growth stimulatory effects on drug-resistant MCF7-F and MCF7-T (Fig. 1E). At higher concentrations, apigenin inhibited the growth of all three breast cancer cell lines, with ERα(+) MCF7 and MCF7-T showing a greater response to high dose apigenin than ERα(−) MCF7-F (Fig. 1E). In hormone-sensitive MCF7 cells, the combination of apigenin with antiestrogens resulted in greater growth inhibition than either drug alone. In addition, apigenin showed synergistic activity with

antiestrogens in drug-resistant breast cancer cells (Fig. 2), indicating that apigenin has potential as a new therapeutic agent for antiestrogen-resistant breast cancer, and further suggesting that apigenin can target both ERα-dependent and -independent pathways. These hypotheses are further supported by a recent report demonstrating that the isoflavone genistein can augment the inhibitory effect of tamoxifen on various breast cancer cells (17).

We also observed that ERα mobility was similar in the presence of apigenin, E2 and OHT, but markedly different from receptor mobility in the presence of ICI (Fig. 6A). This observation suggests that the mechanism of receptor downregulation induced by apigenin is different from ICI, which induces ERα degradation by immobilizing the receptor to the nuclear matrix (27). Currently, the mechanism of ERα downregulation by apigenin is unknown. However, a recent study reported that apigenin induces hypoxia-inducible factor 1, alpha subunit (HIF-1A) degradation (41) by disrupting HIF-1A association with chaperone complexes through competition with ATP (41). Furthermore, as heat shock protein 90-binding agents (geldanamycin and its derivative 17AAG) can inhibit breast cancer cell growth by ERα destabilization (42,43), it is possible that apigenin induces ERα degradation by targeting heat shock and/or other chaperone proteins.

AIB1, a major ERα co-activator and oncogene in breast cancer (32,44) that supports estrogen-independent growth in antiestrogen-resistant disease (45), is overexpressed in fulvestrant-resistant MCF7-F cells (see Fig. 1F and ref. (9)). Apigenin, at low concentrations, promoted the interaction of ERα with AIB1 and also enhanced ERα transcriptional activity, resulting in overall growth stimulation. However, at high concentrations, apigenin downregulated the level of AIB1 protein (Fig. 4C), suggesting that AIB1 is a potential target of apigenin. Degradation of AIB1 by RAD001, a derivative of the mTOR inhibitor rapamycin, has been reported to play a key role in breast tumor growth inhibition, and the combination of RAD001 with tamoxifen was more effective than tamoxifen alone (46), suggesting that degradation of AIB1 can contribute to inhibition of breast cancer cell proliferation. Although the mechanism of AIB1 downregulation by apigenin is not known, the effect of the drug on heat shock protein and phosphorylation pathways (41,47) may contribute to degradation of this coactivator.

In prostate cancer, apigenin was reported to induce cell cycle arrest by inhibiting both MAPK and PI3K/AKT (33), and the constitutive activation and increased activities of protein kinases p38, PKA, MAPK and PI3K/AKT have also been associated with drug resistance in breast cancer (1,6,7,24,35,48-50). Increased protein kinase activity not only enhances growth factor-dependent breast cancer survival but can also increase estrogen-stimulated breast cancer growth by modulating ERα activity (24). Thus, by inhibiting these protein kinases, apigenin has the potential to block both estrogen-dependent and –independent signaling pathways. The mechanism of protein kinase inhibition by apigenin is not clear, although some studies suggest that by competing with ATP, apigenin may inhibit phosphorylation of kinases (51). This possibility is further supported by the structure of apigenin being similar to the drug PD98059, an inhibitor of MAPK phosphorylation (52). Additional studies suggest that apigenin can induce degradation of protein kinases by targeting heat shock proteins (18,36). Apigenin has been reported to inhibit breast cancer cell growth by blocking proteasome activity to (53). However, much higher concentrations of apigenin (50-100 μM) are required to inhibit proteasome activity compared to those sufficient to induce degradation of ERα and AIB1 (10-20 μM apigenin; Fig 4).

In summary, this is the first study to demonstrate a biphasic effect of apigenin in hormone-sensitive breast cancer cells. At lower concentrations, apigenin activates ERα-mediated gene transcription by promoting AIB1 binding to ERα, resulting in growth stimulation of ERα(+) breast cancer cells. At higher concentrations, apigenin downregulates protein levels of ERα and AIB1 and inhibits protein kinases p38, MAPK, PKA and AKT/PI3K, leading to growth

inhibition. An exciting aspect of this study is that apigenin has the potential to inhibit both ERα-dependent pathway and protein kinase-mediated growth factor signaling pathways. As both pathways are commonly altered in antiestrogen-resistant breast cancer, these broad effects of apigenin may be synergistic in combination with antiestrogens in growth inhibition of antiestrogen-resistant breast cancer cells. Apigenin thus fits into a recently proposed novel paradigm for the treatment of drug-resistant breast cancer by signal transduction inhibitors in combination with antiestrogen therapy (7,46). Based on its ability to target both ERα-dependent and -independent pathways, apigenin warrants further investigation as a therapeutic agent for both antiestrogen-sensitive and -resistant breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

ERα
: estrogen receptor-α

E2
: 17β-estradiol

GFP
: green fluorescent protein

ICI
: ICI 182,780

4-OHT
: 4-hydroxytamoxifen

SERD
: selective estrogen receptor down-regulator

SERM
: selective estrogen receptor modulator

GAPDH
: glyceraldehyde phosphate dehydrogenase

AF2
: activating function-2

wt
: wild type

ERE
: estrogen response element

MEM
: minimal essential medium

DMEM
: Dulbecco's modified Eagle's medium

DMSO
dimethyl sulfoxide

CHX
cycloheximide

FCS
fetal calf serum

MTT
5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

MAPK
mitogen-activated protein kinase

PKA
protein kinase A

FRAP
fluorescence recovery after photobleaching

LUC
luciferase

AIB1
amplified in breast cancer-1

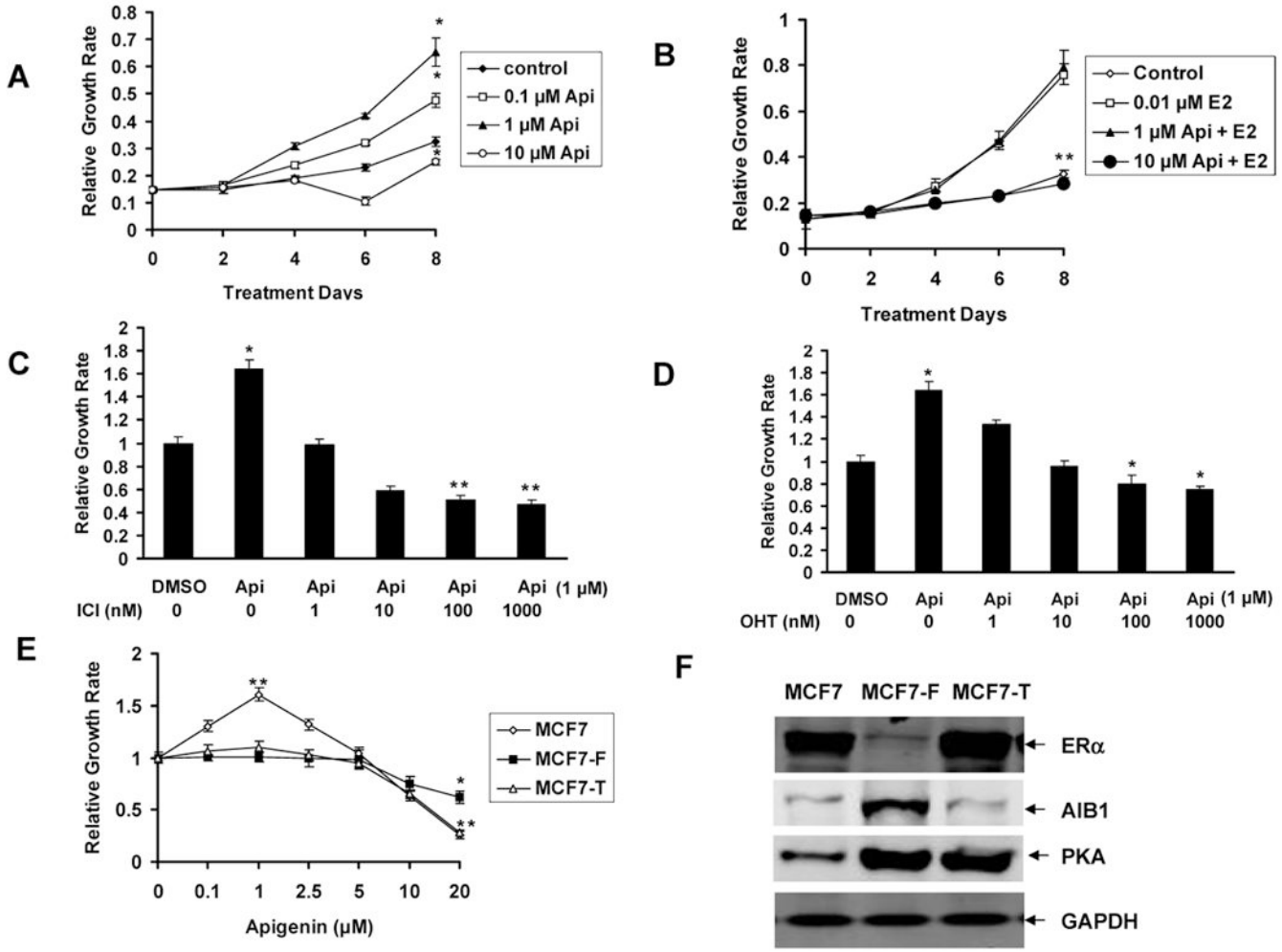


Figure 1.

A, Dose-dependent effects of apigenin on breast cancer MCF7 cell growth. To determine growth rates in the presence of apigenin (Api), cells were plated in 96-well dishes (2,000 per well) in basal medium for the indicated times and cell numbers were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-, diphenyltetrazolium bromide (MTT) assay. Relative cell growth rates (drug vs. vehicle) are shown in the presence of the indicated doses of apigenin, (*, P<0.05), as compared with DMSO-treated controls, Student's *t* test. **B, Dose-dependent effects of apigenin on E2-induced breast cancer cell growth.** MCF7 cells were treated with E2 or combinations of E2 with the indicated concentrations of apigenin and cell numbers determined by MTT assay after treatment. *Points*, mean (n = 6); *bars*, SE. **, P<0.01, 10 μM Api+E2 compared with 10 nM E2 (control), Student's *t* test. **C, D, Fulvestrant and OHT inhibit apigenin-induced breast cancer cell growth.** MCF7 cells were treated with combinations of apigenin and various concentrations of fulvestrant (ICI) or OHT. Cell numbers were determined by MTT assay after treatment. *Points*, mean (n = 6); *bars*, SE. *, P<0.05, **P<0.01, compared with DMSO treated controls, Student's *t* test**. E, Dose-dependent effects of apigenin on MCF7, MCF7-F and MCF7-T cells and differential inhibitory effects of apigenin on growth of drug-sensitive and -resistant breast cancer cells.** To determine growth rates in the presence of apigenin, MCF7, MCF7-T, and MCF7-F cells were plated in 96-well dishes (2,000 per well) in basal medium for the indicated times and cells treated with various doses of apigenin for 7 days. Cell numbers were then determined by MTT assay and relative cell

growth rates (drug *vs.* vehicle) then determined. *Points*, mean (n = 6); *bars*, SE. *, P<0.05, **P<0.01, compared with DMSO treated controls, Student's *t* test**. F, Differential expression of ERα, AIB1, PKA in drug-resistant breast cancer cell lines.** ERα, AIB1 and PKA protein levels in MCF7, MCF7-F and MCF-T cells were determined by immunoblotting using specific antibodies. GAPDH was used as a loading control. Representative results of two independent experiments, each performed in duplicate, are shown.

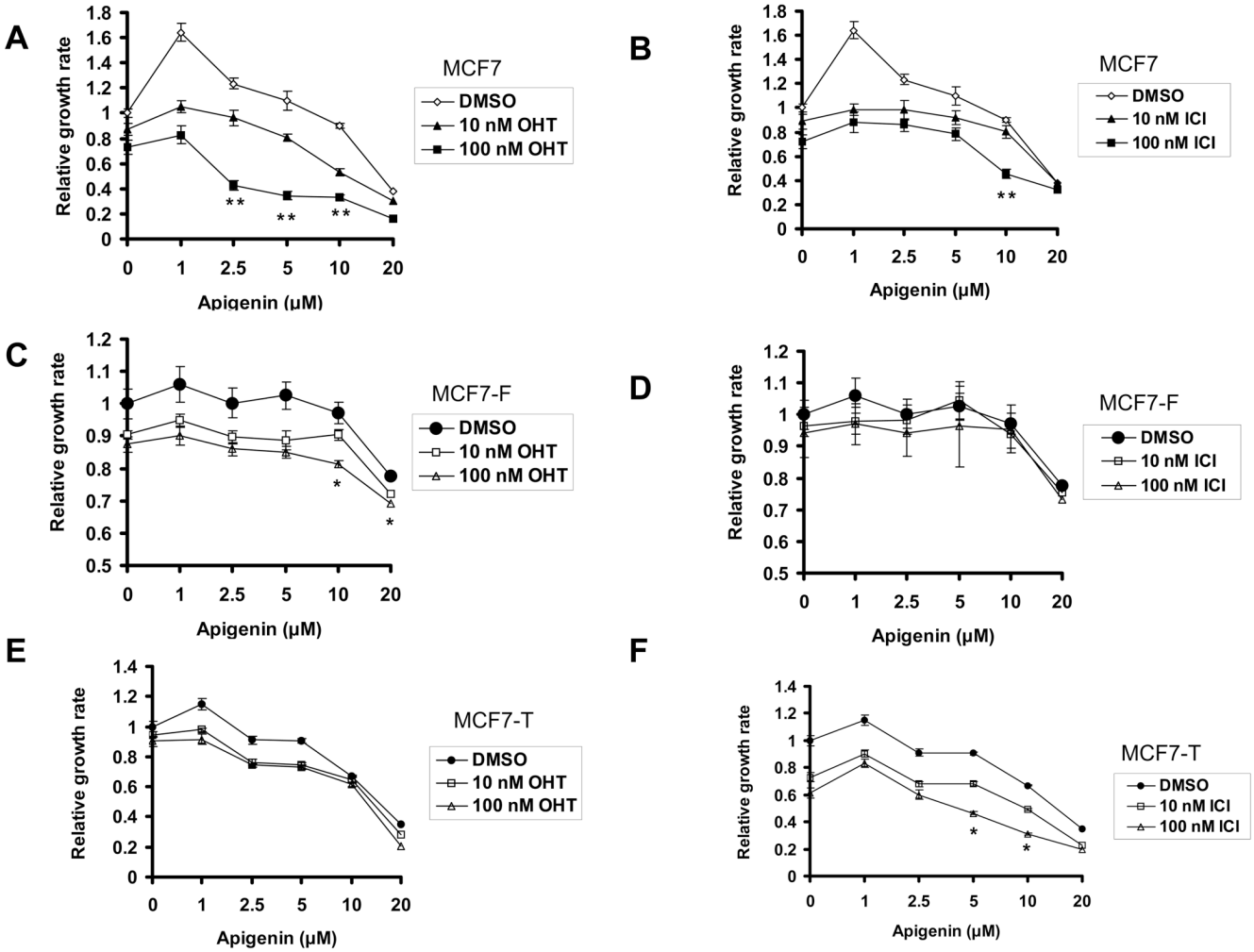


Figure 2. Synergistic effects of apigenin and OHT or fulvestrant (ICI) on MCF7 (A, B), MCF7-F (C, D), and MCF7-T (E, F) cells

Growth curves for 0, 1, 2.5, 5, 10, 20 μM apigenin in combination with varying dosages of OHT (A, D, E) and ICI (B, D, F). Growth was determined by MTT assay. *Points*, mean of at least six replicates; *bars*, SE. *, P<0.05, **P<0.01, compared with DMSO-treated controls by ANOVA.

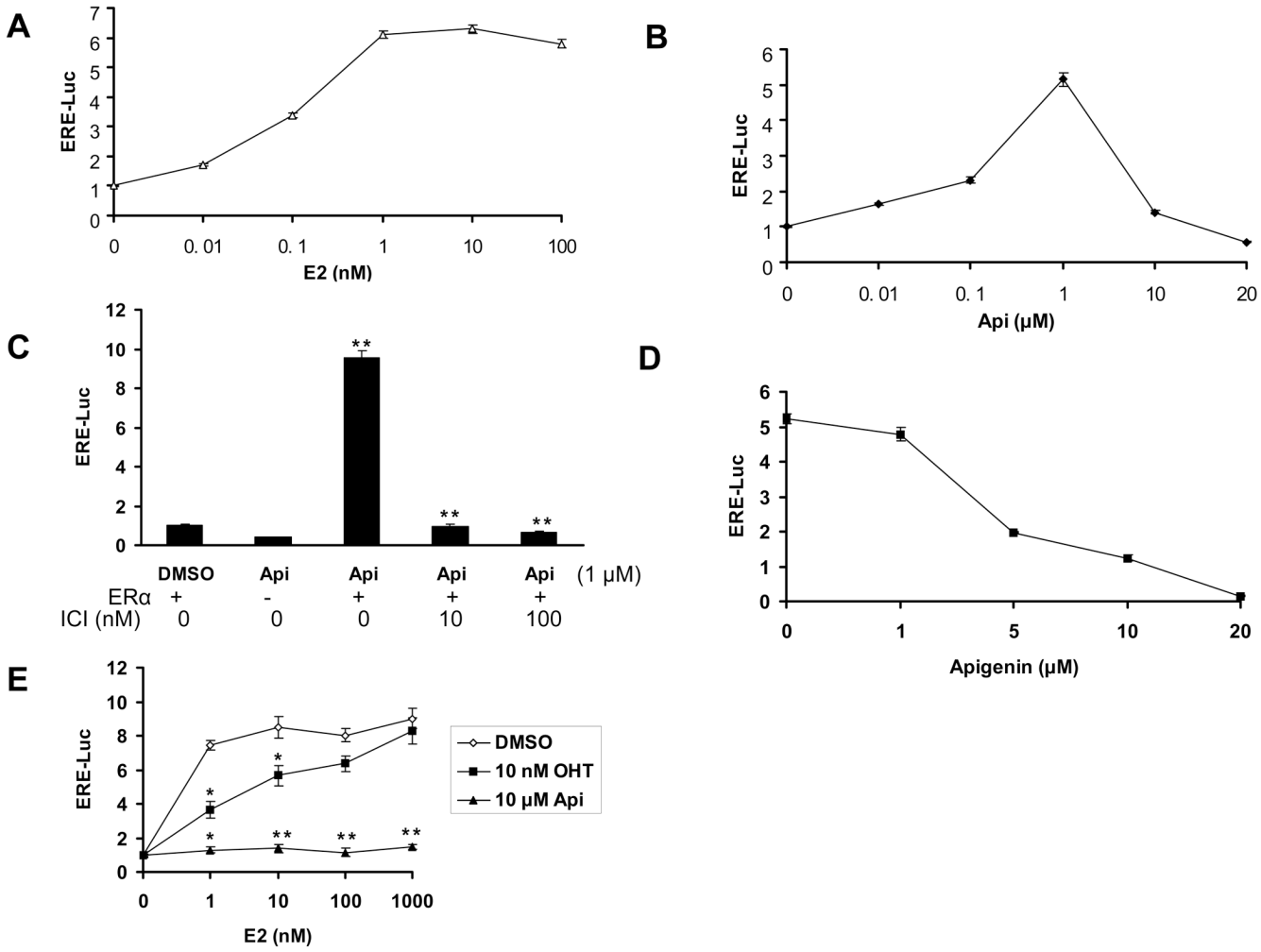
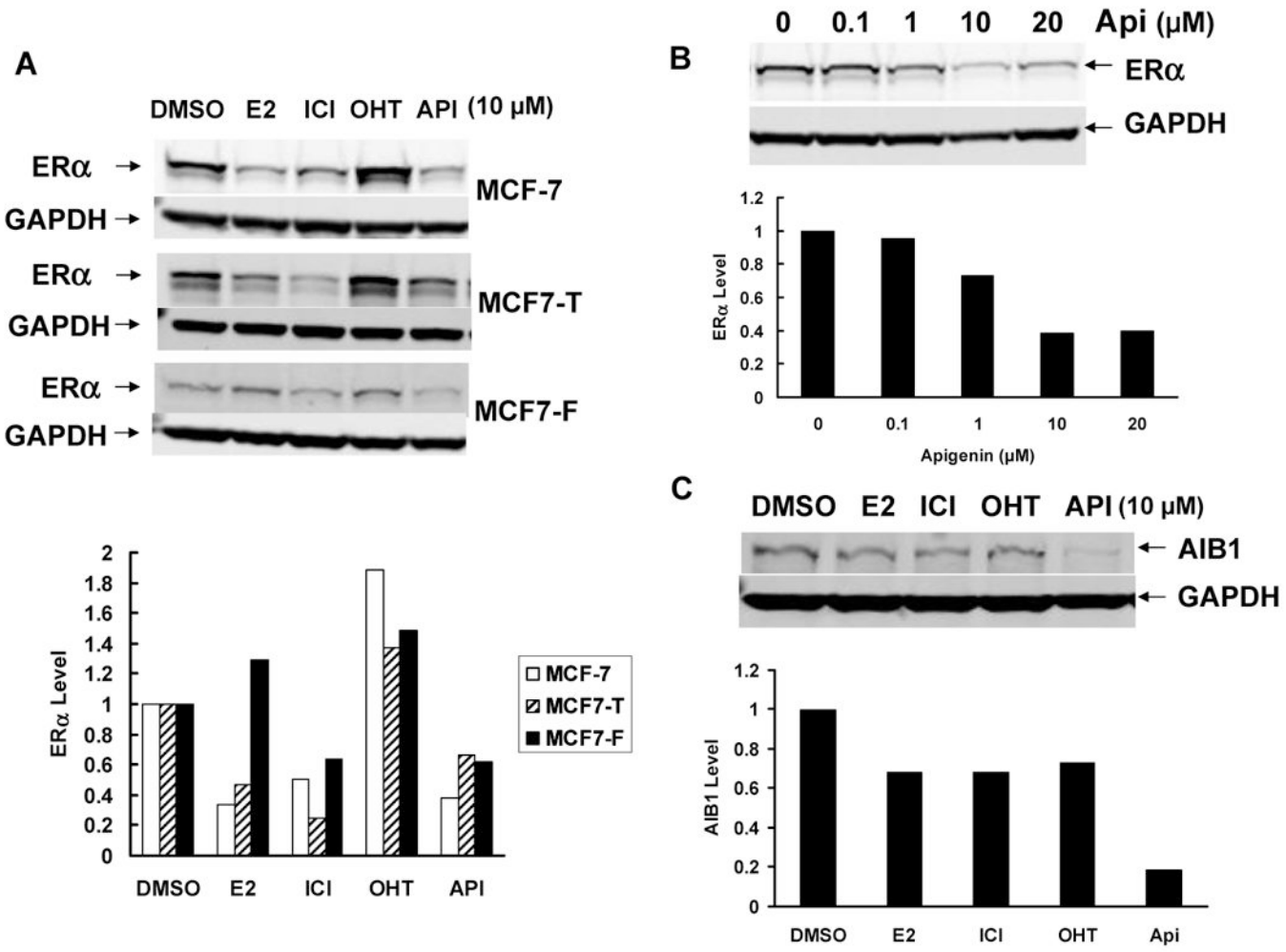


Figure 3.

A, B, Effects of apigenin on ERα transcriptional activity is dose-dependent. ERα transcriptional activity was determined by measuring luciferase activity in MDA-MB-231 cells transfected with ERE2pS2-Luc, pSG5-ERα and pCMV-β-Gal. Cells were treated with the indicated doses of E2 alone (A) or the indicated doses of apigenin alone (B) for 24 h. To correct for transfection efficiency, luciferase and β-galactosidase activities were expressed as a ratio. Values shown are means ± SD, n=4. **C, Apigenin-induced ERE2pS2-Luc expression is ERα-dependent and is blocked by the pure antiestrogen fulvestrant.** MDA-MB-231 cells were transfected with or without ERα and then treated with 1 μM apigenin alone or with a combination of apigenin and ICI (fulvestrant) for 24 h. To correct for transfection efficiency, luciferase and β-galactosidase activities were expressed as a ratio. Values are means ± SD, n=4. **D, Antiestrogenic effect of apigenin is dose-dependent.** MDA-MB-231 cells transfected with ERα were treated with 0, 1, 5, 10, 20 μM apigenin in the presence of 10 nM E2. To correct for transfection efficiency, luciferase and β-galactosidase activities were expressed as a ratio. Values are means ± SD, n=4. **E, Antiestrogenic activity of apigenin is not attributed to competition with estrogen for ERα binding.** Transfected MDA-MB-231 cells were treated with the indicated doses of E2 alone, 10^{-8} mol/L OHT in combination with indicated doses of E2, or 10^{-5} mol/L apigenin in combination with the indicated doses of E2. To correct for transfection efficiency, luciferase and β-galactosidase activities were expressed as a ratio.

Values are means ± SD, n=4. *, P<0.05, **P<0.01, as compared with DMSO-treated controls using the Student's *t* test.



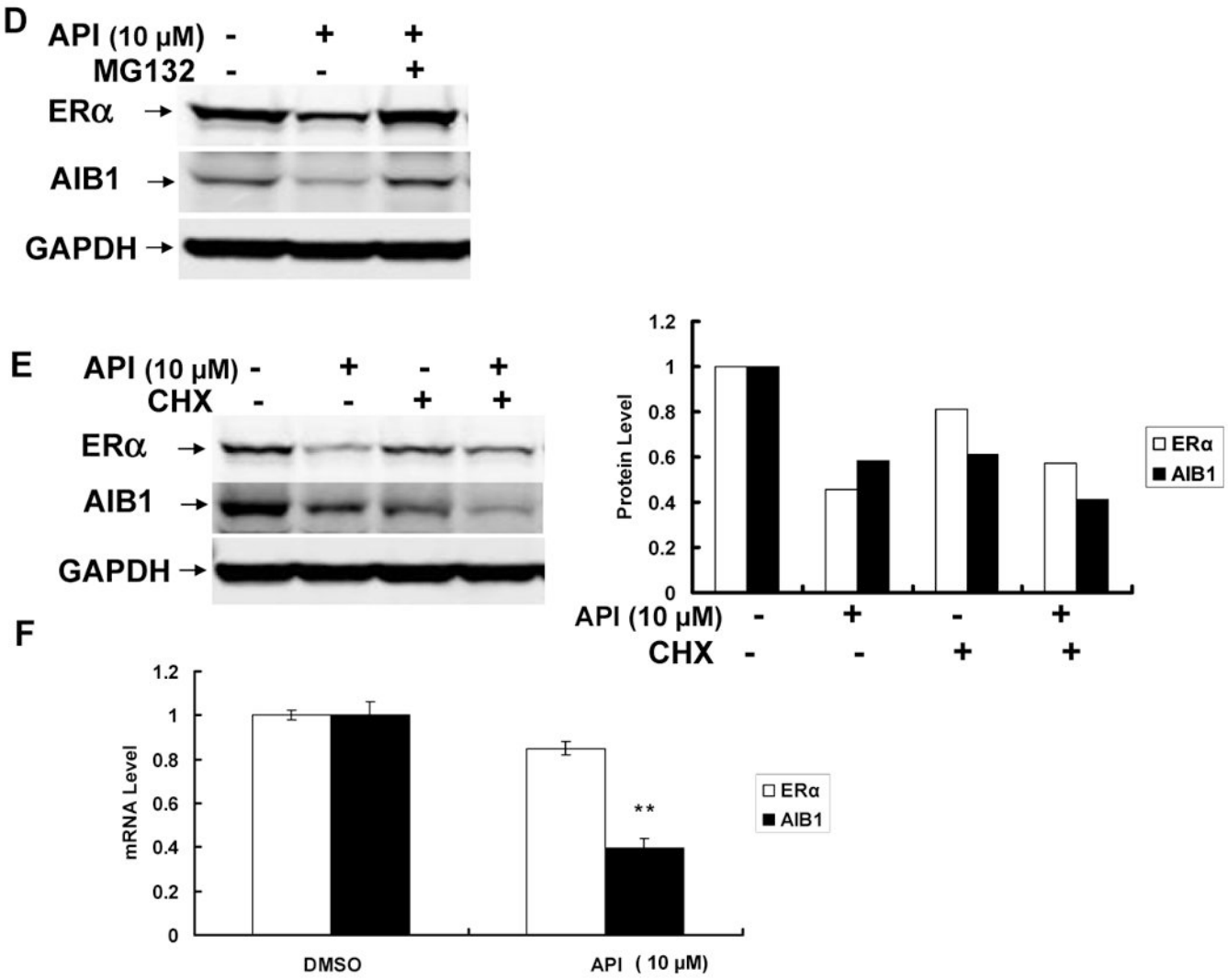


Figure 4.

A, Apigenin can induce ERα degradation in breast cancer cell lines. Breast cancer MCF7, MCF7-T, MCF7-F cells were treated with 10 nM E2, 100 nM ICI (fulvestrant), 100 nM OHT, or 10 μM apigenin for 24 h. Whole cell lysates were then prepared, subjected to SDS-PAGE, and blotted. ERα levels were measured and analyzed using Western blotting and a LICOR imaging system (described under "Materials and Methods"). Upper panel, Western blot image. Lower panel, quantitative analysis of Western blot of ERα protein from *upper panel*, normalized to GAPDH using LICOR Odyssey software. **B, Apigenin induced ERα degradation is dose-dependent.** MCF7 cells were treated with the indicated doses of apigenin for 24 h and ERα levels analyzed using Western blotting and LICOR imaging system (upper panel, Western blot image; lower panel, quantitative analysis of Western blot normalized to GAPDH). **C, Apigenin can induce co-activator AIB1 degradation.** Breast cancer MCF7 cells were treated with 10 nM E2, 100 nM ICI, 100 nM OHT, or 10 μM apigenin for 24 h. Whole cell lysates were prepared, subjected to SDS-PAGE, and blotted. AIB1 levels were measured and analyzed using Western blotting and LICOR imaging (upper panel, Western blot image; lower panel, quantitative analysis of Western blot of AIB1 protein from *upper panel*, normalized to GAPDH). For all experiments, representative results of two independent experiments, with each performed in duplicate, are shown. **D, Apigenin induces ERα and**

AIB1 degradation in breast cancer cell lines through the proteasome. Breast cancer MCF7 cells were treated with 10 μM apigenin and MG132 for 8 h as indicated. ERα levels were analyzed by Western blotting and a LICOR imaging system. **E, *De novo* protein synthesis is not required for apigenin-induced ERα and AIB1 degradation.** MCF7 cells were treated with apigenin and CHX for 8 h, as indicated. ERα and AIB1 levels were analyzed using Western blotting and LICOR imaging system. **F, AIB1 mRNA is down-regulated by apigenin.** MCF7 cells were treated by 10 μM apigenin for 8 h. ERα mRNA and AIB1 mRNA were quantified by RT-qPCR. **P<0.01, compared with DMSO-treated controls, Student's *t* test.

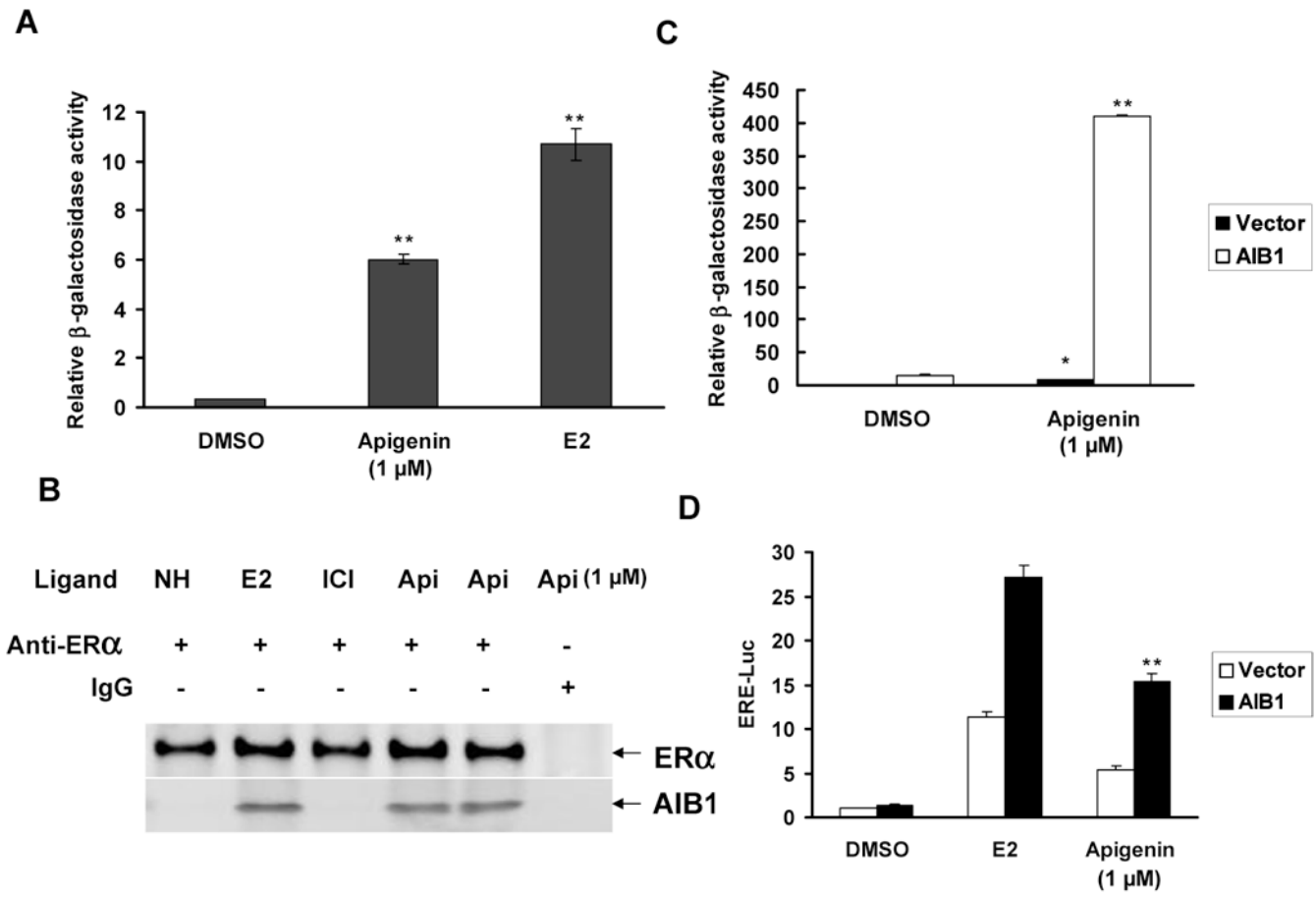


Figure 5.

A, Apigenin can induce an ERα-AIB1 interaction. Protein interactions were examined by yeast 2-hybrid system. The yeast strain PJ69–4A was transformed with pAD-GAL4-rAIB1 and pBD-GAL4-ERαAF2 and grown in liquid yeast culture with the appropriate ligand. The strength of the interaction of AIB1 with the receptor was determined by measuring β-gal activity from three independent transformants. β- galactosidase activity represents the level of interaction between AIB1 and ERα in the absence or presence or of Apigenin (10^{-6} M) or E2 (10^{-9} M). **P<0.01, compared with DMSO-treated controls, Student's *t* test. **B, Apigenin-induced ERα interaction with AIB1 in MCF7 cells.** Co-immunoprecipitation was used to examine the ERα-AIB1 interaction in MCF7 cells. ERα was precipitated from cell lysates using an anti-ERα antibody in the presence or absence of ligand (1 μM E2, ICI and apigenin; *NH*, no hormone). The presence of AIB1 in the pull-down complex was examined by immunoblotting using an AIB1-specific antibody. To assess the amount of precipitated ERα in the complex, the same membrane was then re-probed with an ERα antibody. Normal rabbit IgG was used as a negative control. Representative results of two independent experiments, each performed in duplicate, are shown. **C, AIB1 can enhance apigenin-induced ERα transcription activity in the yeast system.** ERα transcription activity was examined in a yeast estrogen reporter assay. Yeast cells (RS188N) were transformed with expression vectors for ERα and an estrogen-responsive reporter construct (ERE-β-Gal), in the absence or presence of the expression vector for AIB1. Cells were treated with or without apigenin. Values are means ± SD, n=4. *, P<0.05, **P<0.01, compared with DMSO-treated controls by Student's *t* test. **D, AIB1 can enhance apigenin-induced ERα transcription activity in HeLa cells.** ERα transcriptional activity was determined by measuring luciferase activity in HeLa cells

transfected with/without pcDNA3-AIB1, ERE2pS2-Luc, pSG5-ERα and pCMV-β-Gal. Cells were treated with 10 nM E2 or 1 μM apigenin for 24 h. Luciferase activity was corrected for transfection efficiency by expressing it as ratio of luciferase to β-galactosidase activities. Values are means ± SD, n=4. **P<0.01, with AIB1 compared with the vector control by Student's *t* test.

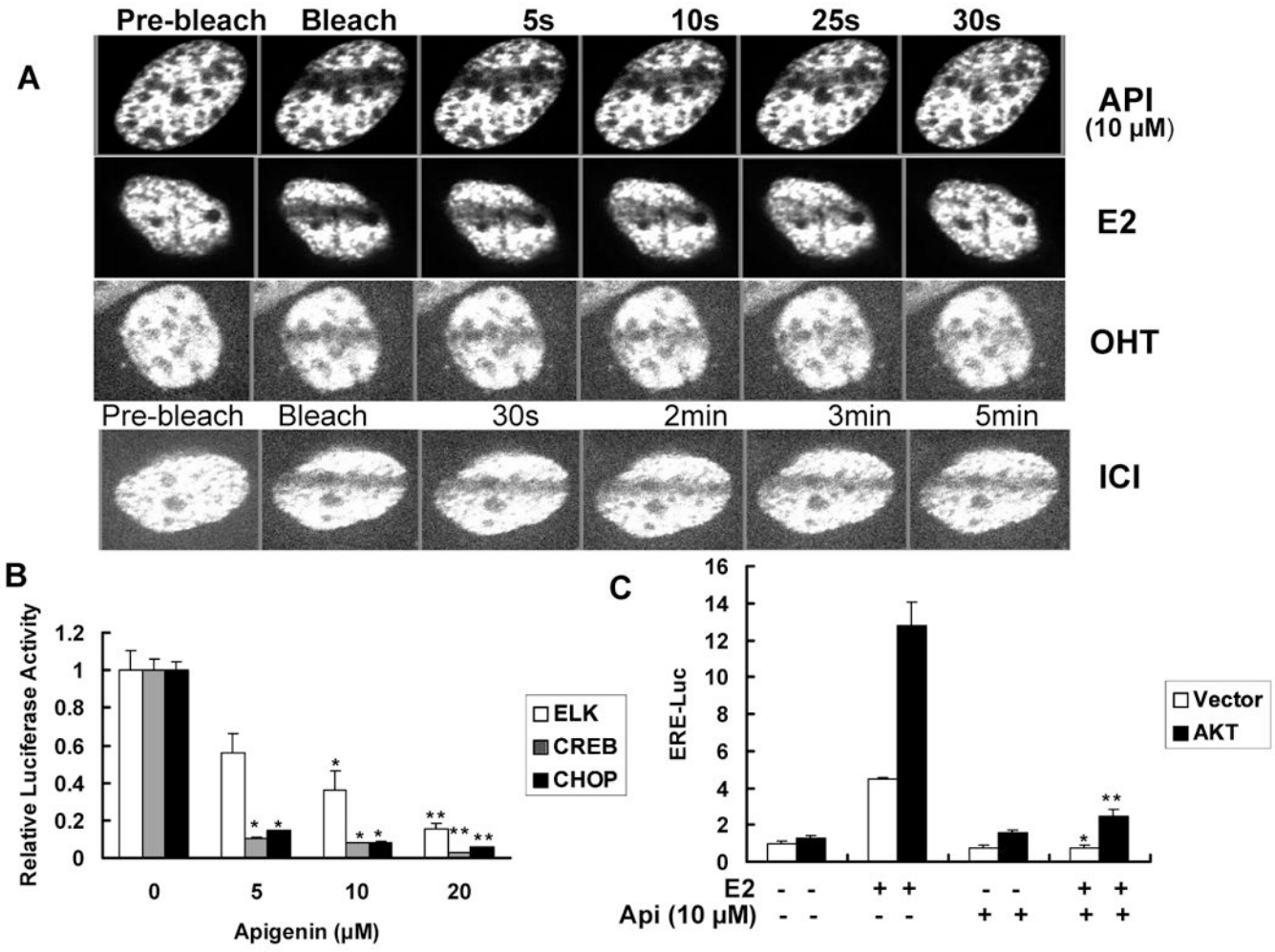


Figure 6.

A, FRAP analysis of ERα mobility in the presence of apigenin. MCF7 cells transfected with GFP-ERα were subjected to FRAP analysis. Images show a single z-section and were obtained before bleaching at the indicated time points. After treatment with 10 μM apigenin (Api) for 1 h, a clear bleach zone was detected. Recovery began within 5 s and full recovery was seen within 30 s. In cells treated with E2 and OHT (100 nM for 1 h), GFP-ERα shows similar dynamics to those observed in apigenin-treated cells. After addition of the pure antagonist, ICI 182,780 (ICI; 100 nM for 30 min), no fluorescence recovery was observed, even after 5 min, indicating that ERα was immobilized. **B, Dose-dependent inhibition of p38, MAPK and PKA pathways by apigenin.** Effects of apigenin on transcription factors CHOP (p38 pathway), CREB (PKA pathway) and ELK1 (MAPK pathway) was monitored using a PathDetect Trans-Reporter system. MDA-MB-231 cells were co-transfected with pFA2-CHOP, pFA2-CREB, or pFA2-ELK1, with pFR-Luc and pCMV-β-gal. After transfection, cells were treated with the indicated doses of apigenin for 20 h. To correct for transfection efficiency, luciferase and β-galactosidase activities were expressed as a ratio. **C, Inhibition of AKT activity by apigenin.** AKT enhances estrogen-dependent ERα transcriptional activity, and apigenin (10 μM) can inhibit this effect. MDA-MB-231 cells were transfected with ERE2pS2-Luc and pcDNA3 vector or CA-AKT. Values are means ± SD, n=4. *, P<0.05, **P<0.01, compared to E2 (10 nM) with no apigenin controls, Student's *t* test.