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Delayed activation of extracellular-signal-regulated kinase 1/2 is involved in genistein- and equol-induced cell proliferation and estrogen-receptor-α-mediated transcription in MCF-7 breast cancer cells

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Abstract

The aim of this study was to determine whether the extracellular-signal-regulated kinase 1/2 (ERK1/2) pathway is involved in genistein- and equol-induced cell proliferation and estrogen receptor (ER) α transactivation. For MCF-7 human breast cells, low concentrations of genistein and equol enhanced proliferation and induced MCF-7 cells to enter the S-phase. Genistein- and equol-induced cell proliferation and S-phase entry were blocked by the ERα antagonists 4-hydroxytamoxifen and ICI 182,780 and by the mitogen-activated protein kinase 1/2 inhibitor U0126. These data indicated that ERα and mitogen-activated protein extracellular kinase/ERK signaling were required for the effects of genistein/equol on cell growth and cell cycle progression. Genistein and equol induced delayed and prolonged activation of ERK1/2. Inhibition of ERK1/2 phosphorylation by U0126 led to complete suppression of genistein- and equol-induced estrogen response element reporter activity and to suppression of the estrogen-responsive gene *pS2*. The anti-estrogen ICI had no effect on genistein- and equol-induced ERK1/2 phosphorylation. These results suggest that activation of ERK1/2 lies upstream of ER-mediated transcription, and that ERK1/2 activation is necessary for the transactivation of ERα. In conclusion, genistein and equol elicit a delayed activation of ERK1/2, and this activation appears to be involved in the proliferation of breast cancer cells and estrogen-dependent transcriptional activation.

Keywords: Genistein; Equol; ERK1/2 signaling pathway; ER transactivation; MCF-7 breast cancer cells

1. Introduction

Soybeans provide a source of dietary isoflavones in the Asian diet [1]. The two major isoflavones in soybeans are genistein and daidzein, which have attracted attention because of their putative value as natural alternatives to hormone replacement therapy for relieving postmenopausal symptoms [2]. The major glycosides found in soybeans are daidzein, genistin and glycitein. These glucose-conjugated compounds are inactive estrogenically [3], but upon consumption are hydrolyzed by mammalian enzymes and the gut microflora to form the active aglycone isoflavone compounds daidzein, genistein and glycitein. Daidzein is further metabolized by the intestinal microflora to form the estrogenic compound equol. There is wide individual variation in the levels of excretion of isoflavone metabolites; only 33% of individuals convert daidzein into equol [4].

The estrogenic activity of genistein is the reason for its use as a dietary supplement in postmenopausal women. Genistein is estrogenic in estrogen-dependent human breast cancer (MCF-7) cells; at concentrations of 0.1–1.0 μM, it stimulates MCF-7 cell proliferation and induces an estrogen-responsive gene, *pS2* [5]. In vivo, genistein enhances uterine weight in rodents [6], stimulates estrogen-dependent human breast tumor growth [7] and negates the effectiveness of tamoxifen treatment [8]. Despite the limited estrogenicity of daidzein, its biologically active metabolite, equol, may have phytoestrogen effects [9]. Equol is one of the most biologically active metabolites of daidzein [10,11].

Estrogen signaling is involved in the proliferation and differentiation of estrogen-responsive cells [12]. The proliferative effect of estrogens on breast cancer cells is mediated mainly through estrogen receptors (ER), and the classical effects of estrogens and isoflavone phytoestrogens are mediated via transcriptional activation of estrogen-responsive genes [13]. The receptor–hormone complex binds to a specific estrogen response element (ERE) in the promoter region of target genes, leading to transcriptional activation [14]. However, activation of target genes by estrogens may also be mediated by other transcription factors [15] independent of the ERE. Recent evidence implicates cell surface receptors in rapid responses to estrogen and phytoestrogens [16]. Membrane-associated ERs interact with cell

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signaling pathways, such as the mitogen-activated protein extracellular kinase (MEK)/extracellular-signal-regulated kinase (ERK) and phosphatidylinositol kinase (PI3K) pathways, and thus initiate nongenomic effects [17].

Mitogen-activated protein kinase (MAPK) cascades transmit and amplify signals involved in cell proliferation and death. Among the three major MAP kinase pathways in human tissues, the one involving extracellular-signal-regulated kinase 1/2 (ERK1/2) is most relevant to breast cancer [18]. ERK1/2 is activated via a cascade involving ras/raf/mek proteins [19]. Signal transduction pathways may connect the nongenomic actions of estrogens to genomic responses. This type of nongenomic-to-genomic signaling is a distinct mechanism by which ERs regulate transcription at alternative response elements. At present, it is not clear how the genomic and nongenomic pathways of estrogen action interact to achieve full cellular response to the hormone or how these kinase cascades contribute to the activation of cell cycle genes in stimulated cells.

In this effort, ERα and the ERK signaling pathway were established as being involved in genistein- and equol-induced cell proliferation and cell cycle progression of breast cancer cells, and the mechanisms by which ERK signaling transactivates ERα were investigated.

2. Materials and methods

2.1. Chemicals and reagents

Genistein, (±)-equol, 17β-estradiol (E2), 4-hydroxytamoxifen (OHT) and ICI 182,780 (ICI) were purchased from Sigma (St. Louis, MO, USA). U0126 and PD98059 were purchased from Calbiochem (San Diego, CA, USA). Fetal bovine serum (FBS) was obtained from PAA Laboratories (Pasching, Austria). Charcoal-stripped FBS (CS-FBS) was obtained from Biological Industries (Kibbutz Beit Haemek, Israel). The rabbit polyclonal antibody against ERα was obtained from NeoMarkers (Fremont, CA, USA), and the antibody against β-actin was obtained from Boster Corporation (Wuhan, China). ERK1/2 and phospho-ERK1/2 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). The rabbit polyclonal antibody and horseradish peroxidase conjugate of anti-rabbit immunoglobulin G were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Enhanced chemiluminescence detection reagents were obtained from Amersham Biosciences (Piscataway, NJ, USA).

2.2. Plasmids

The luciferase reporter plasmid pERE-TATA-Luc$^+$ and the rat ERα expression vector rERa/pCI were provided by Dr. M. Takeyoshi (Chemicals Assessment Center, Chemicals Evaluation and Research Institute, Oita, Japan). The plasmids were constructed as previously described [20]. The plasmid phRL-tk (used as internal control for transfection efficiency and cytotoxicity of test chemicals) containing the Renilla luciferase gene was purchased from Promega (Madison, WI, USA).

2.3. Cell culture

MCF-7 (an ER$^+$ human breast cancer cell line) and MDA-MB-231 cells were obtained from ATCC (Rockville, MD, USA). MCF-7 cells routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 nM E2, 2 mg/ml insulin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 4 mM glutamine and 10% FBS, and MDA-MB-231 cells were cultured in DMEM with 4 mM glutamine and 10% FBS. Exponentially growing cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. For cell proliferation assays, Western blot analysis, real-time polymerase chain reaction (PCR), cell cycle and report gene analysis, cells were switched to phenol-red-free MEM (without E2) supplemented with 5% CS-FBS at 5 days before exposure to equol and genistein.

2.4. Cell growth assay

Experiments were accomplished in 96-well plates containing phenol-red-free MEM supplemented with 5% CS-FBS. MCF-7 cells were seeded at a plating density of 2×10^3 cells/well in 200 μl of medium. Two days later, the medium was replaced, and the cells were exposed to genistein, equol or its vehicle (ethanol; 0.1%) for 3 days. At appropriate times, MTT stock solution (20 μl, 5 mg/ml; Sigma) was added to each well, and the plates were further incubated for 4 h at 37°C. The supernatant was removed, and DMSO (200 μl) was added to each well to solubilize formazan crystals. The absorbency at 490 nm was measured with a Multiscan MCC 340 microplate reader (Titertek, Huntsville, AL, USA). All measurements were performed in triplicate. Data points represented the means of the values for the four wells. Cellular proliferation was expressed as the mean numbers of cells ±S.E.M.

2.5. Cell cycle analysis

Cells (5×10^5) were incubated at 37°C overnight in triplicate 6-cm plastic dishes in phenol-red-free MEM supplemented with 5% CS-FBS and then for 2 days with genistein or equol. Cells were trypsinized, washed in cold phosphate-buffered saline (PBS; pH 7.4), fixed in 70% ethanol/30% PBS and stored at 4°C until processing. A portion (1 ml) of the fixed cell suspension containing 1×10^6 cells was washed twice in cold PBS. The fixed cells were treated for 30 min at 4°C in the dark with fluorochrome DNA staining solution (1 ml) containing 40 μg of propidium iodide and 0.1 mg of RNase A. The stained cells were analyzed by flow cytometry.

2.6. Western blot analysis

The cells were washed twice with ice-cold PBS and then scraped off in 0.2 ml of buffer [20 mM HEPES (pH 6.8), 5 mM EDTA, 10 mM EGTA, 5 mM NaF, 0.1 μg/ml okadaic acid, 1 mM dithiothreitol, 0.4 M KCl, 0.4% Triton X-100, 10% glycerol, 5 μg/ml leupeptin, 50 μg/ml phenylmethanesulphonylfluoride, 1 mM benzamidine, 5 mg/ml aprotinin and 1 mM Na orthovanadate] and incubated on ice for 30 min, followed by centrifugation at 12,000 rpm for 15 min. The supernatant was stored at −70°C. Protein concentrations were measured with the BCA Protein Assay (Pierce, Rockford, IL, USA). Afterwards, proteins were diluted to equal concentrations, boiled for 5 min and separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes, which were probed with ERα primary antibodies (NeoMarkers) or phospho-ERK1/2 (Cell Signaling Technology) overnight at 4°C. Membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies for 1 h at room temperature to enhance chemiluminescence (Amersham Biosciences) before exposure to film. β-Actin was used to normalize for protein loading. All experiments were performed at least twice with similar results.

2.7. Real-time PCR

Cells were grown in 6-cm plastic dishes (1×10^6 cells/dish in 5 ml of estrogen-free medium). At 24 h after plating, test compounds (dissolved in ethanol) were added. The medium was changed every 24 h after plating; various test compounds were added fresh with each change. After 48 h, total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA, USA), and 5 μg of each sample was reverse-transcribed using the M-MLV first-stand synthesis system (Promega). cDNAs were analyzed in triplicate with the MJ Real-Time PCR System (Bio-Rad). For *pS2* and the internal control gene *GAPDH*, the primers were 5′-TTCTATCCTAATACCATCGACG-3′ (pS2 forward) and 5′-TTTGAGTAGT-CAAAGTCAGAGC-3′ (pS2 reverse), and 5′-GAAGGTGAAGGTCGGAGTC-3′ (GAPDH forward) and 5′-GAAGATGGTGATGGGATTTC-3′ (GAPDH reverse), respectively. Normalization was achieved by dividing the expression level of mRNA by its respective *GAPDH* expression level. The results were expressed as fold inductions.

2.8. Transfection and reporter gene assays

MDA-MB-231 cells were placed in 24-well microplates (Corning, Acton, MA, USA) at a density of 1.0×10^5 cells/well in the phenol-red-free MEM containing 5% CS-FBS. Following 12 h of incubation, the cells were transfected with 0.5 μg of pERE-TATA-Luc$^+$, 0.2 μg of rERa/pCI and 0.1 μg of phRL-tk, with 5 μg of Sofast™ (Sunma Co., Xiamen, China) transfection reagent per well. After an incubation period of 12 h, the transfection medium was replaced. The cells were harvested after being exposed to the test chemicals for 24 h. After three rinses with PBS (pH 7.4), the cells were lysed with 1× passive lysis buffer (Promega). The cell lysates were analyzed immediately with a 96-well plate luminometer (Berthold Detection System, Pforzheim, Germany). The amounts of luciferase and Renilla luciferase were measured with the Dual-Luciferase Reporter Assay System Kit (Promega) following the manufacturer's instructions. The value of luciferase activity for each lysate was normalized to the Renilla luciferase activity. The relative transcriptional activity was converted into fold induction above the vehicle control value (*n*-fold).

2.9. Statistical analyses

Data were expressed as mean±SD. Statistical differences were analyzed by one-way analysis of variance. $P<.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. ERα and the MEK/ERK pathway are involved in genistein- and equol-induced cell proliferation of MCF-7 cells

The effects of genistein and equol on cell proliferation were examined first. In these experiments, MCF-7 cells were exposed for 72 h to different concentrations of genistein and equol ranging from 0.01 to 50 μM. As shown in Fig. 1A, genistein and equol stimulated cell

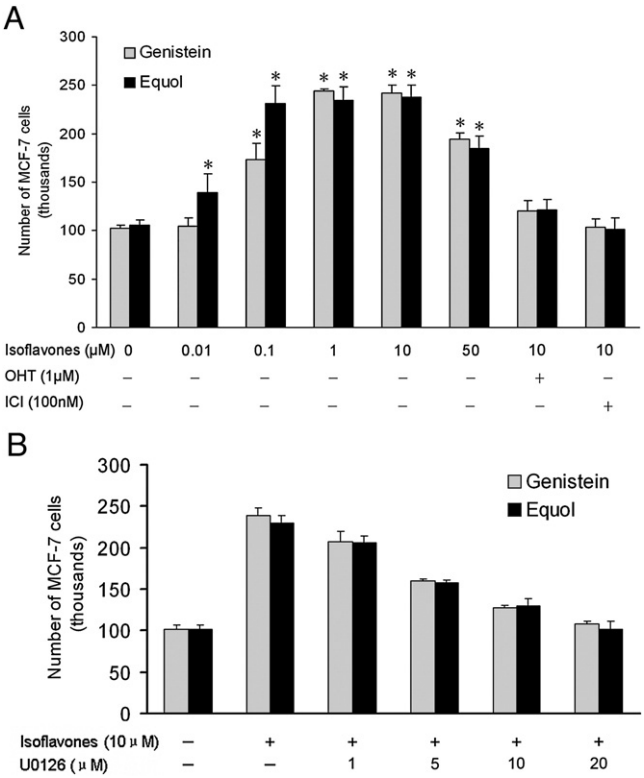

Fig. 1. (A) Dose responses of genistein and equol to cell growth. MCF-7 cells were plated in phenol-red-free MEM plus 5% CS-FBS in the absence or in the presence of genistein and equol at concentrations ranging from 0.01 to 50 μM, or in combination with the anti-estrogen ICI (100 nM) or OHT (1 μM) for 72 h. Colorimetric MTT assays were then performed. (B) Inhibition of genistein- and equol-induced cell growth by the MEK1/2 inhibitor U0126. MCF-7 cells were plated in phenol-red-free MEM plus 5% CS-FBS in the absence or in the presence of 10 μM genistein and equol, or in combination with the MEK1/2 inhibitor U0126 at concentrations ranging from 1 to 20 μM for 72 h. Colorimetric MTT assays were then performed. Data points represented the means of the values for the four wells. Cellular proliferation was expressed as the mean number of cells ±S.E.M. (n=4). *Significantly different from control, P<.05.

proliferation in a dose-dependent manner at relatively low and physiologically relevant concentrations (<10 μM). A stimulatory effect was apparent even upon exposure to 0.01 μM equol or 0.1 μM genistein. Equol exerted a growth-stimulatory effect earlier than and more substantial than that of genistein. At a high concentration (50 μM), these stimulations were less, but cell proliferation was still greater than that for the control. The maximum stimulatory growth of 10 μM genistein and equol was inhibited by the addition of an ERα antagonist (ICI or OHT).

Since MEK/ERK signaling is associated with cell proliferation and survival, the effects of a specific MEK1/2 inhibitor on genistein- and equol-mediated cell proliferation were examined. Addition of U0126 eliminated genistein- and equol-mediated cell proliferation in a concentration-dependent manner (Fig. 1B). Thus, these data showed that an ERα-mediated mechanism and the MEK/ERK pathway were involved in genistein- and equol-induced cell proliferation.

3.2. Genistein and equol stimulate the cell cycle progression of MCF-7 cells in an ERα- and MEK/ERK-dependent manner

It is generally assumed that the growth-stimulatory properties of phytoestrogens are mediated by their binding to ERs. Similar to the action of estrogens, such binding may stimulate events in the G1-to-S-phase entry in the cell cycle. Thus, the effects of genistein and equol on the cell cycle were determined by flow cytometry (Fig. 2). Addition of 10 μM genistein or equol promoted cell cycle progression, with 40% or 44% of the cell population accumulating in S-phase, respectively, significantly different from the control value of 16% (P<.05).

To assess the contribution of ERα and the ERK pathway to genistein- and equol-stimulated cell cycle entry, the response of MCF-7 cells to genistein or equol in the presence of an ER antagonist (ICI or OHT) or the MEK1/2 inhibitor U0126 was investigated. The genistein- and equol-induced S-phase cycle entry was completely blocked by addition of OHT. Furthermore, U0126 and ICI not only blocked the genistein- and equol-induced increase in cells accumulating in S-phase but also reduced the basal level of cells in the S-phase by about 60%.

3.3. MEK/ERK signaling is involved in genistein- and equol-induced expression of pS2

ERα is a ligand-dependent transcription factor that regulates gene expression through interaction with DNA sequence EREs located within the regulatory regions of target genes. The human *pS2* gene was initially characterized as a gene whose expression is specifically controlled by estrogen in the breast cancer cell line MCF-7. Accordingly, the effects of genistein and equol exposure on the expression of *pS2* in MCF-7 cells were investigated. Genistein and equol (10 μM) induced the expression of pS2 mRNA in a time-dependent manner (Fig. 3A). Exposure of cells for 48 h to 10 μM genistein and equol increased the expression of pS2 mRNA by approximately14-fold (significantly different from control, P<.05). The increase was inhibited by addition of the ER antagonist ICI (Fig. 3B). However, OHT, another ERα antagonist, reduced the stimulatory effect of genistein on *pS2* expression from 14-fold to 5-fold of the control (significantly different from control, P<.05) and completely inhibited the effect of equol.

To determine whether the ERK1/2 pathway was linked to genistein- and equol-mediated expression of the ERα target gene *pS2*, estrogen-dependent MCF-7 cells were exposed to genistein or equol in the presence of MEK1/2 inhibitors. PD98059 reduced genistein- and equol-induced up-regulation of pS2 mRNA expression from 14-fold or 16-fold to 8-fold of the control (significantly different from control, P<.05), but U0126 inhibited the up-regulation (Fig. 3B), suggesting that the MEK/ERK pathway up-regulates ERα transcription activity.

3.4. MEK/ERK signaling is involved in genistein- and equol-induced ERE reporter activity in MDA-MB-231 cells

To evaluate the specificity of genistein and equol in transactivating the ERα, the plasmid pERE-TATA-Luc$^+$ and the rat ERα expression vector rERa/pCI were transiently cotransfected into ERα$^-$ MDA-MB-231 cells. The plasmid phRL-tk was used as internal control for transfection efficiency and cytotoxicity. Enhancement of pERE-TATA-Luc$^+$ expression was observed in the transfected cells exposed to 10 μM genistein or equol for 24 h (Fig. 4). Genistein and equol caused inductions of 2.5-fold and 2-fold (significantly different from control, P<.05), respectively, and exerted weak estrogenic effects relative to E2, which produced a 3-fold induction. The ER antagonist ICI partially reduced genistein-induced luciferase expression and completely eliminated equol-induced expression of luciferase. In addition, the MEK1/2 inhibitor U0126 reduced the stimulatory effect of genistein and equol on ERα-regulated transactivation, but the induction remained greater than the value for the control.

3.5. U0126, but not ICI, blocks genistein- and equol-induced delayed activation of ERK1/2

Genistein and equol stimulate proliferation and exert their estrogenic activities through ERK1/2 signaling and ER signaling. To determine their specific association, the activity of ERK was assayed at

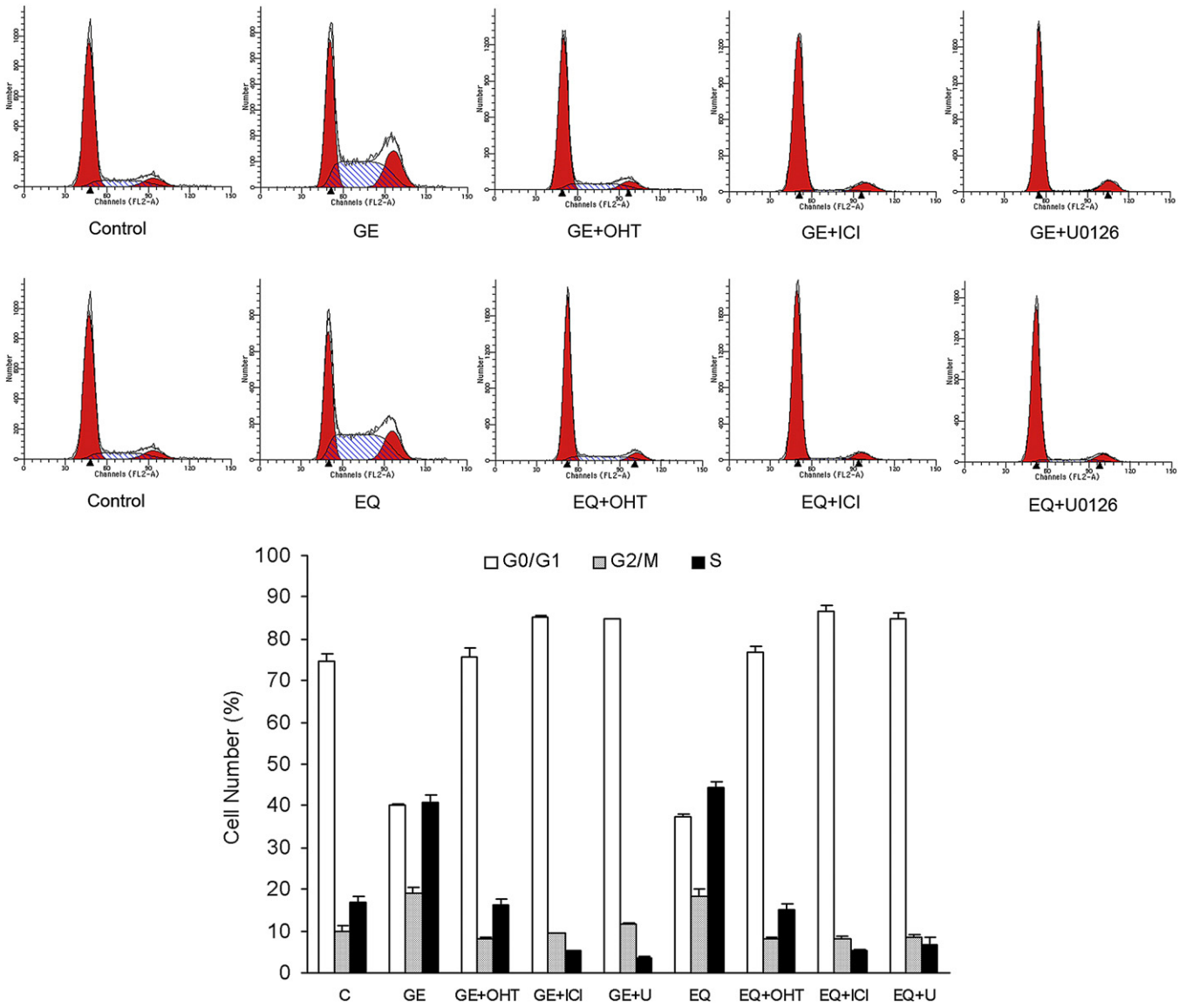

Fig. 2. Effects of genistein and equol on cell cycle distribution. MCF-7 cells were incubated with 10 μM genistein or equol, or in combination with the anti-estrogen ICI (100 nM) or OHT (1 μM) or with the MEK1/2 inhibitor U0126 (10 μM) for 48 h, and the percentage of cells in each cell cycle phase (G1, S and G2M) was determined by flow cytometry (mean±SD; $n=3$).

3, 6, 12 and 24 h after stimulation (Fig. 5A). Genistein induced delayed and persistent activation of phosphorylated ERK1/2 in a time-dependent fashion, with ERK activity peaking at 24 h. In contrast, equol stimulated sustained activation of ERK, which peaked at 12 h and regressed at 24 h. The MEK1/2 inhibitor U0126 (Fig. 5B), but not the ER antagonist ICI (Fig. 5C), blocked genistein- and equol-induced phosphorylation of ERK1/2. These data indicated that ERK/12 signaling was upstream and could regulate ERα signaling.

3.6. Genistein and equol down-regulate ERα protein in MCF-7 cells

E2 down-regulates the levels of ERα in breast cancer cells through an increased turnover of the E2-activated ERα protein and a reduced transcription rate of its own gene [21]. This down-regulation represents an additional characteristic of ERα activation by an agonist. Genistein and equol down-regulated the ERα protein in MCF-7 cells in a time-dependent manner (Fig. 6A). Furthermore, the MEK1/2 inhibitor U0126 further reduced genistein- and equol-mediated down-regulation of the ERα protein (Fig. 6B).

4. Discussion

The growth-promoting effects of genistein and other isoflavones in soy products (such as daidzein and biochanin A) and a metabolite of daidzein, equol, have been widely investigated. In the present study, genistein and equol promoted the growth of ER-dependent MCF-7 breast cancer cells; this stimulation was blocked by the anti-estrogens OHT and ICI. Equol induced cell growth at a concentration lower than that of genistein, indicating that equol has higher proliferation-inducing activity. Nevertheless, the proliferative pattern of cells exposed to genistein and equol was biphasic. Genistein and equol at low concentrations stimulated the proliferation of MCF7 breast cancer cells, whereas they were cytotoxic at high levels. Our data support the hypothesis that the actions of phytoestrogens not only are mediated via the ER as estrogen agonists but also interact with other ER-independent cellular mechanisms at higher concentrations to inhibit cell proliferation [22].

Activation of MAP protein kinase is an essential signal transduction event for E2-mediated cell proliferation. Three major MAP kinase

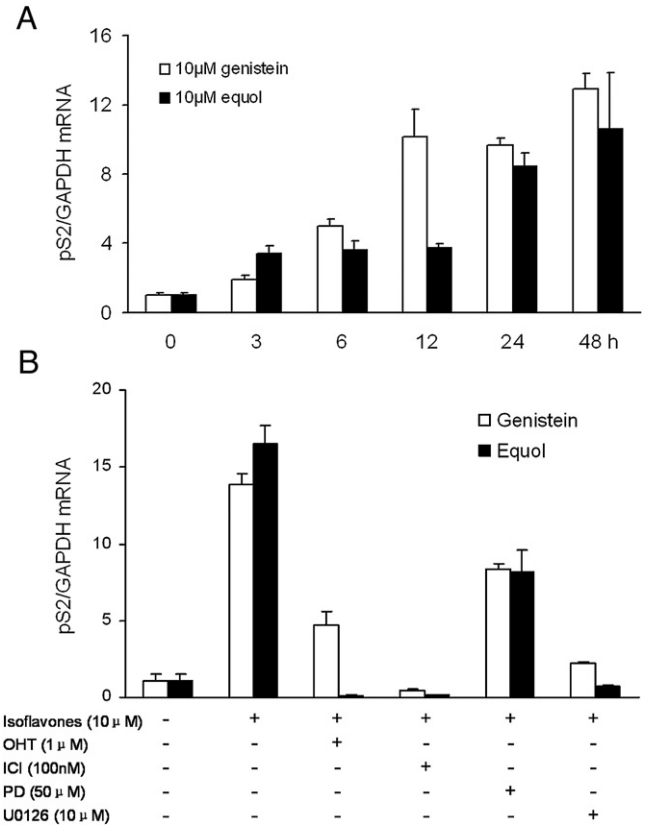

Fig. 3. (A) Effect of genistein and equol on the expression of pS2 mRNA in MCF-7 cells. MCF-7 cells were incubated with 10 μM genistein or equol for 0, 3, 6, 12, 24 or 48 h. Total RNAs were isolated and examined by real-time PCR for the expression of pS2 mRNA. (B) Effects of anti-estrogens or MEK1/2 inhibitors on genistein- and equol-induced expression of pS2 mRNA in MCF-7 cells. Cells were plated in phenol-red-free MEM plus 5% CS-FBS in the absence or in the presence of indicated concentrations of genistein or equol alone, or in combination with the anti-estrogen ICI (100 nM) or OHT (1 μM) or with the MEK1/2 inhibitor PD98059 (50 μM) or U0126 (10 μM). Total RNAs were isolated at 48 h and examined by real-time PCR for the expression of pS2 mRNA. The signals were normalized to a *GAPDH* internal control, and the results were expressed as fold induction in comparison to controls (mean±SD; n=3).

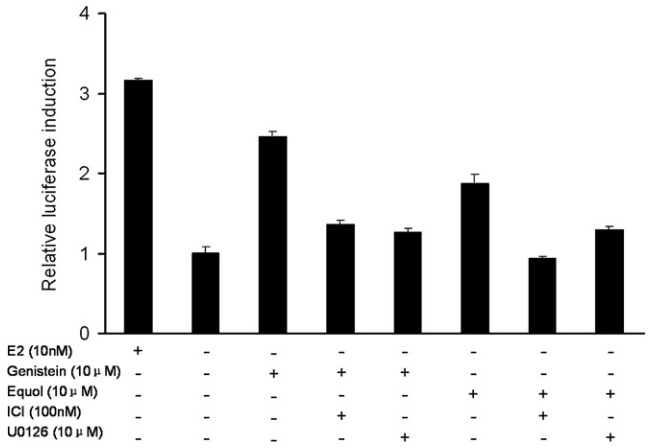

Fig. 4. The estrogenic activities of genistein and equol examined by reporter gene assay in MDA-MB-231 cells. Cells were transiently cotransfected with 0.5 μg of pERE-TATA-Luc$^+$, 0.2 μg of rERa/pCI and 0.1 μg of phRL-tk, and then exposed to 10 μM genistein and equol alone, or in combination with the pure anti-estrogen ICI (100 nM) or the MEK1/2 inhibitor U0126 (10 μM) for 24 h. The cell lysates were analyzed by the Dual-Luciferase Reporter Assay System Kit (Promega). Ethanol solvent was used as control, and estrogenic activity was presented as fold of control. Values are presented as the mean±SD of three independent experiments.

pathways exist in human tissues, but the one involving ERK1/2 is most relevant to breast cancer [18]. Our results show that the MEK1/2 inhibitor U0126 inhibits genistein- and equol-induced cell proliferation in a dose-dependent manner; this suggests that MEK/ERK signaling is involved in cell stimulation by genistein and equol.

Estrogens regulate various events leading cells to the transition from G1 to S [23,24]. In the present study, genistein and equol promoted S-phase entry and G2/M arrest in ER$^+$ MCF-7 cells, and this promotion was blocked by the anti-estrogens OHT and ICI. In ER$^-$ MDA-MB-231 breast cancer cells, however, genistein induced only G2/M cell cycle arrest and had no effect on S-phase entry [25,26]. Our study demonstrated that the molecular mechanisms whereby genistein and equol promote S-phase entry are similar to those of E2, and that these actions are exerted through the ER. To verify the role of MEK/ERK signaling in the genistein- and equol-induced cell cycle progression in MCF-7 cells, the inhibitor U0126 was used. Genistein- and equol-induced S-phase entry was completely blocked by U0126. These observations indicate that, in MCF-7 cells, the ERα is needed for S-phase entry and DNA synthesis through MEK/ERK pathway activation.

Inhibition of the MEK/ERK pathway prevents growth of hormone-mediated breast cancer cells [27]. Jones and Kazlauskas [28] have demonstrated that only growth factors capable of inducing prolonged ERK1/2 activation (>90 min) promote S-phase entry in target cells, whereas those that induce short-lasting responses by these enzymes (<30 min) fail to act as full mitogens, suggesting that ERK or another signaling enzyme activation is dissociated from the promotion of G1-phase completion. This is explained by the fact that only their strong and prolonged activation allows translocation of ERKs and other MAP kinases to the cell nucleus, where they control gene transcription by phosphorylating and thereby activating DNA-bound transcription factors [29]. In MCF-7 cells, genistein and equol induced prolonged ERK1/2 activation, an essential step in cell proliferation and cell cycle progression.

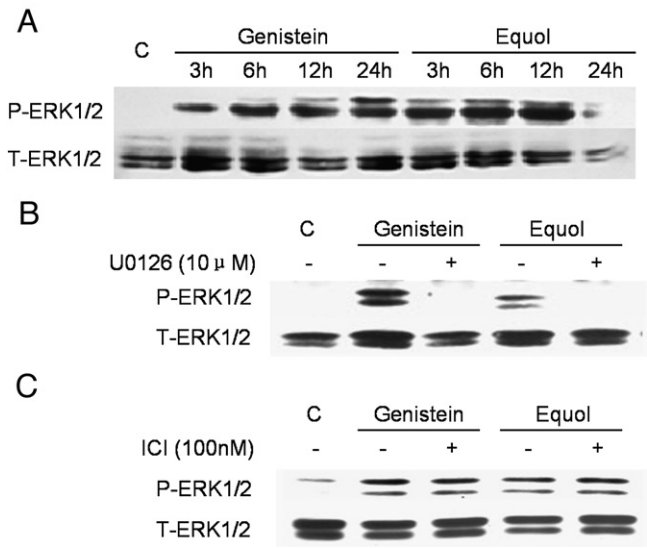

Fig. 5. (A) Effects of genistein and equol on ERK1/2 phosphorylation in MCF-7 cells. After 48 h of serum starvation, the cells were exposed to 10 μM genistein or equol for 0, 3, 6, 12 or 24 h. (B) Effects of the MEK1/2 inhibitor U0126 on the expression of the ERα protein. After 48 h of serum starvation, the cells were exposed to 10 μM genistein or equol, or in combination with the MEK1/2 inhibitor U0126 (10 μM) for 24 h. (C) Effects of the ER antagonist ICI on the expression of the ERα protein. After 48 h of serum starvation, the cells were exposed to 10 μM genistein or equol, or in combination with the ER antagonist ICI (100 nM) for 24 h. Lysates (50 μg of protein) were immunoblotted with specific antibodies against the total or phosphorylated form of ERK1/2. This experiment was repeated twice with similar results.

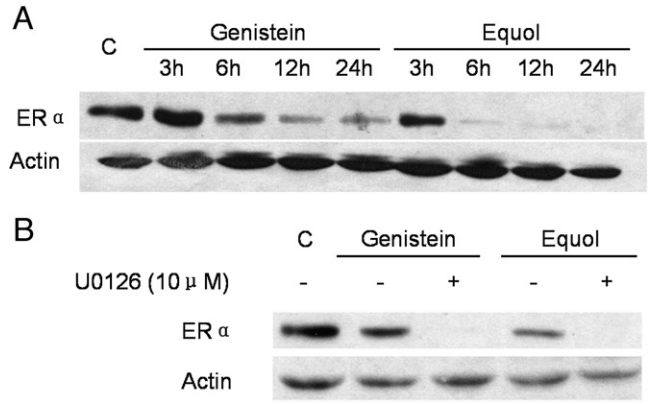

Fig. 6. (A) Effects of genistein and equol on the expression of the ERα protein. Cells were incubated with 10 μM genistein or equol for 0, 3, 6, 12 or 24 h. Expression of the ERα protein was analyzed by Western blot analysis with β-actin as control. This experiment was repeated twice with similar results. (B) Effects of an MEK1/2 inhibitor on the expression of the ERα protein. MCF-7 cells were incubated with 10 μM genistein or equol, or in combination with the MEK1/2 inhibitor U0126 (10 μM) for 24 h. Expression of the ERα protein was analyzed by Western blot analysis with β-actin as control. This experiment was repeated twice with similar results.

Furthermore, the genistein- and equol-induced ERE-directed transcription was abolished after coincubation of MCF-7 cells with an inhibitor of ERK1/2 activation, indicating that the ERK signaling pathway is involved in ER-dependent transcription. This action also was confirmed by effects on mRNA expression of the estrogen-responsive gene *pS2*. pS2 is a 6.7-kDa protein secreted by MCF-7 cells in response to estrogens [30] and serves as a marker for estrogen-like activity. Genistein (1–50 μM) increases pS2 levels in the growth medium of MCF-7 cells [31], as does daidzein and equol (1 μM) [32]. The anti-estrogen tamoxifen inhibits *pS2* expression stimulated by genistein, daidzein and equol [5], suggesting that these compounds stimulate *pS2* expression via an ER-mediated mechanism. In the present study, genistein and equol induced *pS2* mRNA expression in a time-dependent manner, and this induction was blocked by the anti-estrogen ICI and the MEK inhibitor U0126.

Estrogens exert their effects through the action of ERs on gene expression. In addition to this "genomic" pathway of estrogen action, these hormones apparently act via additional signaling pathways involving plasma membrane ERs [33] because some effects of estrogens are so rapid that they cannot depend on the activation of RNA and protein synthesis. This hypothesis is supported by the recent demonstration of membrane-located ER in MCF-7 and other cell types [34]. These "nongenomic" actions of estrogen include immediate and transient activation of ERK1/2 MAP kinases.

Rapid E2-induced signaling varies according to cell type and may vary during ontogeny in the same cell. Among the several signaling pathways activated by E2, the ERK/MAPK pathway is commonly responsive to estrogens. This is basically in parallel with the results of recent studies reporting that E2 rapidly and transiently activates ERK1/2 in MCF-7 cells [35–37]. In these studies, the peak of ERK activation is observed between 1 and 15 min. The capacity of membrane-impermeable E2 to activate ERK signaling rapidly in these cell types indicates that such actions are initiated at the plasma membrane. Moreover, other researchers found that a delayed — rather than a transient — ERK1/2 activation was observed in MCF-7 cells after treatment with E2 [27,38]. This delayed ERK1/2 activation could be involved in estrogen-mediated transactivation of ERα.

Consistent with the classical concepts of steroid hormone action, the present results show that when MCF-7 cells were exposed to genistein and equol, they led to a slow but sustained activation of ERK1/2 up to 24 h. The induction observed at long time intervals (hours of treatment) is in accordance with a possible indirect mechanism. Cell modeling studies have shown that MKP activity can modulate the steady-state levels of phosphorylated ERK1/2 [39], such that when MKP activity is low, MAPK dephosphorylation is impaired, resulting in high ERK1/2 signaling activity. These findings are supported by recent studies showing that sustained ERK1/2 activation is the result of MKP1 degradation through the ubiquitin–proteasome pathway, and that overexpression of MKP1 directly decreases ERK1/2 phosphorylation [40]. Whether genistein- and equol-induced ERK1/2 activation is due to down-regulation of MKP1 expression in MCF-7 cells remains to be elucidated.

In our study, we also tested earlier time points ranging from 5 min to 1 h and did not observe rapid activation of ERK1/2 after treatment with genistein and equol in MCF-7 cells (data not shown). A nongenomic estrogenic effect was discounted because of the delayed, rather than rapid, ERK1/2 activation in response to genistein and equol stimulation of MCF-7 cells. Since membrane-associated ERs induce rapid and transient activation of ERK [16], our results suggest that the delayed ERK1/2 activation is related to nuclear ER, not to membrane ER. Some studies suggest that ERK signaling pathway is activated upon E2 binding to ERs [41,42]. However, our data show that the anti-estrogen ICI has no effect on genistein- and equol-induced ERK1/2 phosphorylation, suggesting that activation of ERK1/2 lies upstream of ER-mediated signaling.

Results from our study show that exposure of MCF-7 cells to genistein or equol results in down-regulation of the ER protein. Control of ER protein levels has been studied for various cell types [21,43], and it is now generally accepted that this protein is targeted for rapid degradation via the ubiquitin–proteasome pathway in response to E2 in breast cancer cells [44]. The activity of the proteasome pathway in controlling ER degradation in MCF-7 cells is directly linked to activation of transcription through the ERE. Degradation of E2-liganded ER requires transcriptional activity [45]. Thus, genistein- and equol-mediated loss of ER is dependent on proteasome activity and is necessary for ER-mediated transcription. The present results show, however, that U0126 degrades ER and blocks ER-mediated transcription. Loss of ER occurs when ER-mediated transcription is eliminated by the anti-estrogen ICI, and ICI-induced degradation of ER does not depend on transcription [45].

In this study, physiological concentrations of genistein and equol stimulate cell growth and S-phase entry in MCF-7 breast cancer cells. These actions are blocked by the ERα antagonists OHT and ICI and by the MEK1/2 inhibitor U0126, suggesting that an ER-dependent mechanism and the MEK/ERK signaling pathway are involved in these stimulations. ERα transactivation and expression of mRNA for the estrogen-responsive gene *pS2* are also induced by genistein and equol; these responses are completely blocked by U0126, indicating that the MEK/ERK signaling pathway is necessary for ER-mediated transcription. Genistein and equol induce delayed and prolonged phosphorylation of ERK1/2, showing that nuclear ER, not membrane ER, is related to this delayed phosphorylation of ERK1/2. Moreover, the anti-estrogen ICI has no effect on the genistein- and equol-induced delayed phosphorylation of ERK1/2; this suggests that activation of ERK1/2 lies upstream of ER-mediated signaling.

Taken together, our results suggest that delayed activation of ERK signaling is necessary for transactivation of ERα and support the hypothesis that low concentrations of genistein and equol (<10 μM) stimulate cell growth and cell cycle progression via ER-mediated transcription and activation of ERK1/2. ERα is a major growth regulator for many breast cancers and provides an exploitable target for therapy. A greater understanding of ER-mediated events, as well as the interactions of ER with ERK1/2 signaling pathways, should lead to more targeted strategies for the treatment of hormone-dependent breast cancer.

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