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Molecules and Cells

Tectoridin, a Poor Ligand of Estrogen Receptor α, Exerts Its Estrogenic Effects via an ERK-Dependent Pathway

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Phytoestrogens are the natural compounds isolated from plants, which are structurally similar to animal estrogen, 17β-estradiol. Tectoridin, a major isoflavone isolated from the rhizome of *Belamcanda chinensis*. Tectoridin is known as a phytoestrogen, however, the molecular mechanisms underlying its estrogenic effect are remained unclear. In this study we investigated the estrogenic signaling triggered by tectoridin as compared to a famous phytoestrogen, genistein in MCF-7 human breast cancer cells. Tectoridin scarcely binds to ER α as compared to 17β-estradiol and genistein. Despite poor binding to ER α, tectoridin induced potent estrogenic effects, namely recovery of the population of cells in the S-phase after serum starvation, transactivation of the estrogen response element, and induction of MCF-7 cell proliferation. The tectoridin-induced estrogenic effect was severely abrogated by treatment with U0126, a specific MEK1/2 inhibitor. Tectoridin promoted phosphorylation of ERK1/2, but did not affect phosphorylation of ER α at Ser[118]. It also increased cellular accumulation of cAMP, a hallmark of GPR30-mediated estrogen signaling. These data imply that tectoridin exerts its estrogenic effect mainly via the GPR30 and ERK-mediated rapid nongenomic estrogen signaling pathway. This property of tectoridin sets it aside from genistein where it exerts the estrogenic effects via both an ER-dependent genomic pathway and a GPR30-dependent nongenomic pathway.

INTRODUCTION

Phytoestrogens are the natural phenolic compounds isolated from plants, and are composed of several chemical groups, such as isoflavonoids, flavonoids, lignans, coumestanes and stilbenes. They are structurally similar to animal estrogen 17β-estradiol (E2) and influence several biological events, including reproduction, development and metabolism. Phytoestrogens are often used for estrogen replacement therapy replacing E2. Estrogen replacement therapy has been shown to be effective at preventing osteoporosis, and protecting against cardiovascular disease and neurodegeneration in postmenopausal women (Manavathi and Kumar, 2006; Moutsatsou, 2007). However, recent reports suggest that estrogen replacement therapy not only increases the incidence of breast cancer, but also has other undesirable side effects (Grodstein et al., 2000).

A well-known example of a phytoestrogen is genistein, a soy isoflavone that is a strong ligand of the classical estrogen receptor (ER) (Moutsatsou, 2007). Tectoridin and its aglycon, tectorigenin, are isolated from the rhizome of *Belamcanda chinensis*, a flowering perennial growing on the hillsides of East Asia, including the Korean peninsula (Shin et al., 1999). Besides their phytoestrogenic effects, tectorigenin and tectoridin are known to have several pharmacological effects, including anti-cancer, anti-inflammatory, antioxidant, and hepatoprotective (Jung et al., 2003; Kang et al., 2005; Kim et al., 1999; Lee et al., 2005; Monthakantirat et al., 2005; Thelen et al., 2007). As determined by a competition assay evaluating the ER-binding affinity of tectoridin, tectorigenin and genistein, genistein showed the strongest binding affinity for ER α and ER β, with a binding affinity comparable to that of E2. In contrast, tectorigenin had a moderate binding affinity for ER α and ER β, and tectoridin scarcely bound to ER α and ER β (Morito et al., 2002). Nevertheless, despite having weak binding affinities for ER, both tectorigenin and tectoridin produced strong estrogenic effects (Monthakantirat et al., 2005; Seidlova-Wuttke et al., 2004).

Estrogen signaling is known to be exerted via two different receptors, namely the classical estrogen receptor and the transmembrane receptor. When estrogen binds with high affinity to cytoplasmic ER, the complex undergoes a conformational change and interacts with specific DNA sequences, known as the Estrogen Responsive Element (ERE), in the nucleus, resulting in diverse physiological effects. This is often referred to as the genomic estrogen signaling pathway (Manavathi and Kumar, 2006; Simpson et al., 2005). In contrast, in the rapid nongenomic pathway, estrogenic effects are exerted via a novel transmembrane G-protein-coupled receptor, named GPR30 (Prossnitz et al.,

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2008; Revankar et al., 2005). Estrogen binds to GPR30 and triggers rapid intracellular signaling pathways, including epidermal growth factor receptor (EGFR) dependent extracellular signal-regulated kinase (ERK) activation (Filardo et al., 2000). Other studies have shown that estrogen stimulates GPR30-dependent adenylyl cyclase activity and production of intracellular cAMP (Filardo et al., 2002; Nakhla et al., 1994). The genomic and nongenomic pathways mediated by estrogen via ER and GPR30 are connected to each other and regulate estrogen signaling in a coordinated fashion (Prossnitz et al., 2008).

The phytoestrogenic activities of tectorigenin and tectoridin have already been reported (Monthakantirat et al., 2005), however, the molecular mechanisms underlying their estrogenic effect remain unclear. In this study, we hypothesized that tectoridin could trigger estrogen signaling via the nongenomic pathway, which could be related to GPR30. Our data provide new insight into the mechanisms of nongenomic estrogen signaling triggered by a poor ligand of ER, tectoridin.

MATERIALS AND METHODS

Materials

Tectorigenin and tectoridin were isolated from the rhizome of *B. chinensis*, as previously described (Jung et al., 2003). Genistein, dimethyl sulfoxide, propidium iodide and U0126 were purchased from Sigma-Aldrich (USA). The antibodies against ER α, phosphorylated ER α (Ser^{118}), ERK1/2 and phosphorylated ERK1/2 (Thr202/Tyr204) were purchased from Cell Signaling Technology (USA).

Cell culture

Human breast adenocarcinoma MCF-7 cells were obtained from the American Type Culture Collection (USA). MCF-7 cells were maintained in maintenance medium (Minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 100 units/ml penicillin and 100 μg/ml streptomycin). For phytoestrogen stimulation, MCF-7 cells were cultured in experimental medium (phenol red free-MEM supplemented with 10% charcoal dextran treated-FBS (Biotechnics Research, USA), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin) during the experimental period. The cells were cultured in a humidified atmosphere (95% air, 5% CO_2) at 37°C.

ER α binding assay

Binding affinity of phytoestrogens to ER α was measured using the EnBio Estrogen Receptor/Coactivator Ligand Assay System according to the manufacturer's protocol (EnBioTec Laboratories, Japan). Briefly, we added biotinylated coactivator peptide to the avidin-coated plate and incubated the plate for 1 h at room temperature. We mixed estrogenic compounds (E2, genistein, tectorigenin and tectoridin) with recombinant human ER α. Then, the sample-ER α mixture was added to the biotinylated coactivator-coated plate and the plate was incubated for 1 h at room temperature. After washing the plate three times with wash buffer provided by the manufacturer, anti-ER α/HRP antibody was applied to the wells. After 30 min of incubation at room temperature, the plate was washed three times and 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate solution was added. The absorbance at 450 nm was measured using the Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, USA).

Flow cytometric cell cycle analysis

MCF-7 cells (5×10^5) were seeded on 60 mm dishes with hormone-free experimental medium and incubated for 48 h at 37°C in order to synchronize the cells in the G0/G1 phase. The cells were then treated with experimental medium supplemented with various concentrations (0-10 μM) of genistein, tectorigenin and tectoridin for 96 h. The cell cycle was analyzed as previously described (Kang et al., 2007).

Luciferase reporter gene assay

MCF-7 cells (1×10^5 cells per well) were plated into a 24-well plate with maintenance medium and incubated for 24 h. Then, the cells were co-transfected with 0.2 μg of pERE-Luc (Maeng et al., 2005) and 5 ng of pRL-CMV (control vector for transfection efficiency, Promega, USA) using the Fugene 6 Reagent (Roche Diagnostics, Germany). Twenty-four hours after transfection, the cells were cultured in experimental medium supplemented with various concentrations (0-10 μM) of genistein, tectorigenin and tectoridin for 72 h. To investigate ERK1/2 dependency of tectoridin-induced estrogen signaling, the cells were pre-treated with U0126 (20 μM) or PD98059 (50 μM) for 1 h before tectoridin was added. Luciferase activity was then measured using the Dual Luciferase Kit (Promega, USA) and Synergy HT Multi-Mode Microplate Reader.

Clonogenic assay

MCF-7 cells (1000 cells per well) were seeded into a 24-well plate with maintenance medium and incubated for 24 h. For estrogen starvation, each well was washed with experimental medium three times and the cells were further incubated in experimental medium for 48 h. Then, the cells were treated with new experimental medium supplemented with estrogenic compounds (1 nM E2, 10 μM genistein, 10 μM tectoridin) and U0126 (0-20 μM). The cells were re-treated with the same medium every 3-4 days. Twelve days after phytoestrogen stimulation, the cell colonies were stained with Coomassie Brilliant Blue R-250. Spot density was measured using i-MAX™ Gel Image Analysis System (Core Bio System, Korea) and Alpha View software (Alpha Innotech, USA).

Intracellular cAMP accumulation

MCF-7 cells (5×10^5 cells per well) were seeded into a 6-well plate with maintenance medium and incubated for 24 h. For estrogen starvation, each well was washed with the experimental medium three times and the cells were incubated in experimental medium for 24 h. Then, the cells were stimulated with various concentrations (0-10 μM) of genistein and tectoridin for 10 min. After phytoestrogen stimulation, the cells were lysed with 0.1 M HCl. The cell lysate (5 μl) was directly used for cAMP measurements using the Cyclic AMP EIA Kit (Cayman, USA), according to manufacturer's instructions. Protein concentration was determined using the Bio-Rad Protein Assay Kit (USA) and was used to normalize the cAMP levels.

Western blot analysis

MCF-7 (1×10^6) cells were plated into 60 mm dishes with maintenance medium and incubated for 24 h. For estrogen starvation, each well was washed with the experimental medium three times and the cells were incubated in experimental medium for 24 h. Then, the cells were stimulated with various concentrations (0-10 μM) of phytoestrogen and U0126 (20 μM) for various amounts of time (0-60 min). After treatment, the cells were washed with ice-cold PBS and cell pellets were lysed for 10 min in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF), followed by brief sonication. Protein samples (15 μg) were subjected to electrophoresis on NuPAGE 10% Bis-Tris gels

(Invitrogen, USA), and transferred to a polyvinylidene fluoride membrane (GE Healthcare, UK). The membrane was probed with primary antibodies overnight at 4°C, and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Chemiluminsecent signals were developed with the ECL Plus Western Blotting Detection System (GE Healthcare, UK) and detected with LAS-4000 Luminescent Image Analyzer and Multi Gauge version 3.1 software (Fujifilm, Japan).

Statistical analysis

Data were expressed as means ± SD. The statistical significance of the differences was determined by Student's *t*-test. *P* values less than 0.05 were considered as statistically significant.

RESULTS

Tectoridin bound poorly to estrogen receptor α and yet exerted a strong estrogenic effect

Figure 1A shows the chemical structures of genistein, tectorigenin and tectoridin used in this study. Binding affinities of these phytoestrogens to ER α have been reported previously by performing a competitive ER α binding assay (Morito et al., 2002). Since tectorigenin and tectoridin showed potent estrogenic effects via ERE activation among isoflavones isolated from *B. chinensis* (data not shown), we further confirmed the binding affinities of phytoestrogens to the ER α via a different method, receptor/coactivator ligand ELISA assay, and found that tectoridin is indeed a poor ligand. While the binding affinity of genistein was comparable to that of E2, tectorigenin had a lower binding affinity for ER α and tectoridin had almost no binding affinity for ER α (Fig. 1B).

Surprisingly, in spite of poor binding to ER α, both tectoridin and tectorigenin exerted strong estrogenic effects. When MCF-7 cells are cultured in hormone-free medium, their growth is arrested at the G0/G1 phase and the cell population in the S-phase almost disappears. Exposure of such cells to estrogen causes cell cycle progression to recommence and increases the cell population in the S-phase. Therefore, flow cytometric cell cycle analysis of G0/G1 synchronized MCF-7 cells is a good method to verify estrogenicity of xenoestrogens and phytoestrogens (Vanparys et al., 2006). Genistein induced a dose-dependent increase of cells in the S-phase (21.3% of the cells were in the S-phase when treated with 10 μM genistein), as expected. Tectoridin and tectorigenin treatment (10 μM) increased the percentage of cells in the S-phase to 19.6% and 21.3%, respectively, while only 4.4 % of cells in the vehicle control were in the S-phase (Fig. 1C). Genistein, tectorigenin and tectoridin also induced strong transcriptional activation of ERE in a dose-dependent manner, as measured by a transient transfection experiment using the pERE-Luc construct. At higher concentrations (5, 10 μM), tectorigenin and tectoridin had similar potencies of luciferase induction to those of genistein (Fig. 2A). Furthermore, using a clonogenic assay, we confirmed that tectoridin strongly stimulated proliferation of MCF-7 cells (Fig. 3). From these data, we hypothesized that the estrogenic effects of tectoridin could be resulted from other mechanisms without a strong ligand binding to ER α.

The tectoridin-induced estrogenic effect was abrogated by treatment with U0126

Since we confirmed that tectoridin is a poor ligand of ER α, we next examined whether tectoridin induces estrogenic effects via a nongenomic ERK-dependent estrogen signaling pathway. We measured estrogenic activity in the presence of U0126 (20 μM) or PD98059 (50 μM), specific MEK1/2 inhibitors. Induction of ERE activation by treatment with genistein and tectoridin decreased in the presence of U0126 or PD98059 (Fig. 2B and Supplemental Fig. 1). In contrast, the ERE activation induced by genistein and tectoridin was not changed in the presence of chemical inhibitors of p38 mitogen-activated protein kinase (p38), c-Jun N-terminal kinase (JNK), and protein kinase C (PKC) (data not shown). These data suggest that tectoridin-induced estrogen signaling operates via the ERK-dependent pathway. In addition, cell proliferation activities of tectoridin, genistein and E2 almost disappeared in the presence of U0126 (20 μM) (Figs. 3A and 3B). Inhibitory activity against estrogenic compound-induced cell proliferation was less in the presence of

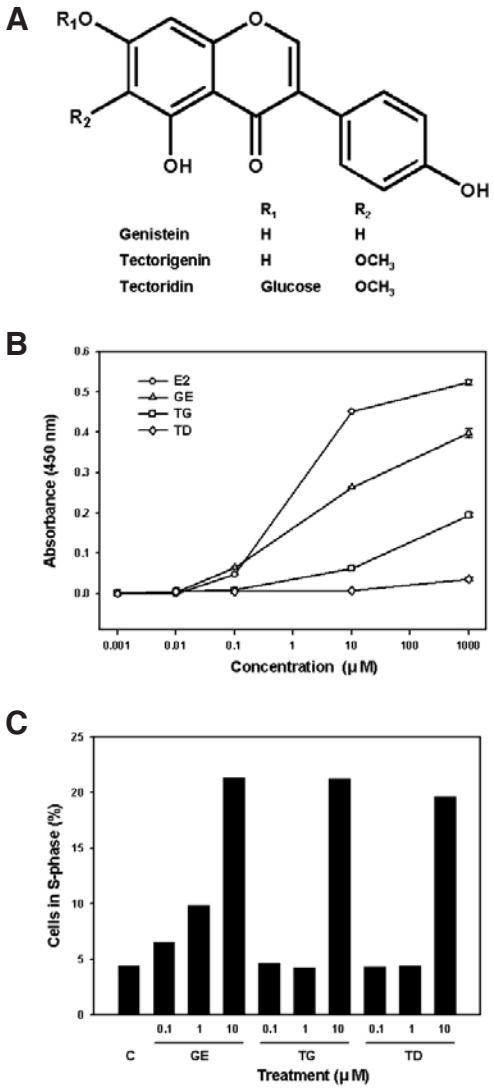

Fig. 1. (A) Chemical structures of genistein, tectorigenin and tectoridin used in this study. (B) Binding affinities of 17β-estradiol (E2), genistein (GE), tectorigenin (TG) and tectoridin (TD) to ER α. The values are means ± SD from triplicate experiments. (C) Effect of GE, TG and TD on cell cycle distribution. The G0/G1 synchronized MCF-7 cells were treated with various concentrations (0-10 μM) of GE, TG or TD for 96 h. The cells were stained with propidium iodide and analyzed by using flow cytometry. Each bar represents cells in the S-phase (%). C, the vehicle control.

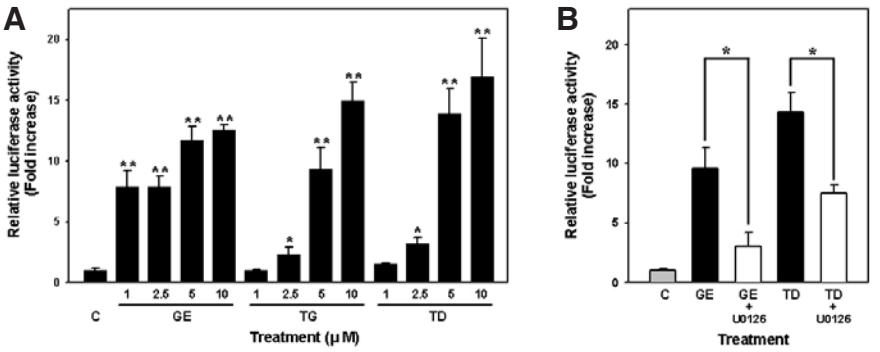

Fig. 2. Effects of phytoestogens on transactivation of the estrogen responsive element (ERE). (A) Luciferase activities of MCF-7 cells transfected with an ERE-containing construct were measured after treatment with various concentrations of genistein (GE), tectorigenin (TG) or tectoridin (TD) for 72 h. Relative luciferase activity was calculated as a fold-increase over the vehicle control, C. Each bar represents the mean ± SD from triplicate experiments. **P < 0.01 and *P < 0.05, for significant differences from the vehicle control. (B) Luciferase activities of MCF-7 cells transfected with an ERE-containing construct were measured after treatment with GE (10 μM) or TD (10 μM) alone or together with U0126 (20 μM) for 24 h. Relative luciferase activity was calculated as a fold-increase over the vehicle control, C. Each bar represents the mean ± SD from triplicate experiments. *P < 0.05, for significant differences between GE or TD single treatments without U0126 and respective co-treatments with U0126.

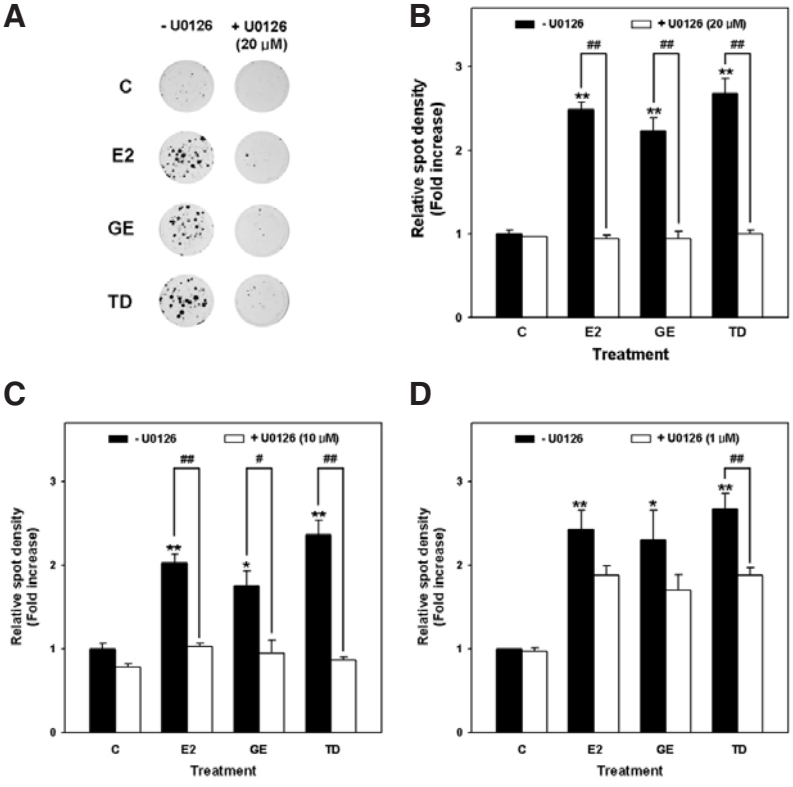

Fig. 3. Effects of 17β-estradiol (E2), genistein (GE) and tectoridin (TD) on proliferation of MCF-7 cells. MCF-7 cells were treated with E2 (1 nM), GE (10 μM) or TD (10 μM) alone or together with three different concentrations (A, B, 20 μM; C, 10 μM; D, 1 μM) of U0126 for 12 d. The cell colonies were stained with Coomassie Brilliant Blue R-250 (A). Spot densities of the stained cell colonies were measured using an image analysis system (B-D). Relative spot density was calculated as a fold-increase over no estrogen stimulation, C, in the absence of U0126. Each bar represents the mean ± SD from triplicate experiments. **P < 0.01 and *P < 0.05, for significant differences from no estrogen stimulation in the absence of U0126. $^{\#\#}$P < 0.01 and $^{\#}$P < 0.05, for significant differences between E2, GE or TD single treatments without U0126 and respective co-treatments with U0126.

lower concentrations (1, 10 μM) of U0126 (Figs. 3C and 3D).

Tectoridin increased intracelluar cAMP accumulation

Since the estrogen-induced activation of ERK occurs via GPR30, we examined cAMP accumulation in phytoestrogen-treated cells. Cyclic AMP is known to play a critical role in GPR30-mediated estrogen signaling (Filardo et al., 2002; Nakhla et al., 1994). Both genistein and tectoridin (1-10 μM) significantly increased the intracellular cAMP content 10 min after treatment (Fig. 4).

Tectoridin rapidly increased the phosphorylation of ERK1/2, but did not induce phosphorylation of ER α at Ser^{118}

Both genistein and tectoridin rapidly increased expression of phosphorylated ERK1/2, while the expression level of total ERK did not changed. The ratio of phosphorylated ERK1/2 to total ERK1/2 peaked 5 min after the start of treatment, and was sustained to 30 min (Fig. 5). In addition, phosphorylation of ERK1/2 almost vanished when the cells were treated with U0126 (20 μM) (Fig. 6). While genistein strongly induced phosphorylation of ER α at Ser^{118}, tectoridin could not induce the rapid phosphorylation of ER α. Specifically, treatment with genistein (10 μM) strongly increased the level of phosphorylated-ER α at Ser^{118} in a time-dependent manner (Fig. 5A). Treatment with various concentrations (1-10 μM) of genistein for 10 min also strongly induced phosphorylation of ER α at Ser^{118}. In contrast, treatment with tectoridin for 10 min did not trigger the phosphorylation of ER α at Ser^{118} at any of the concentrations tested (Fig. 6). This result may be related to the binding affinity

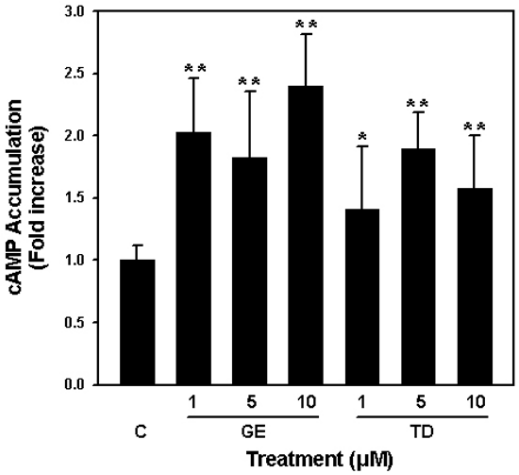

Fig. 4. Effect of genistein (GE) and tectoridin (TD) on intracellular cAMP accumulation. MCF-7 cells were stimulated with various concentrations (1-10 μM) of GE or TD for 10 min. Relative cAMP content was calculated as a fold-increase over the vehicle control, C. Each bar represents the mean ± SD (n = 9). **P < 0.01 and *P < 0.05, for significant differences from the vehicle control.

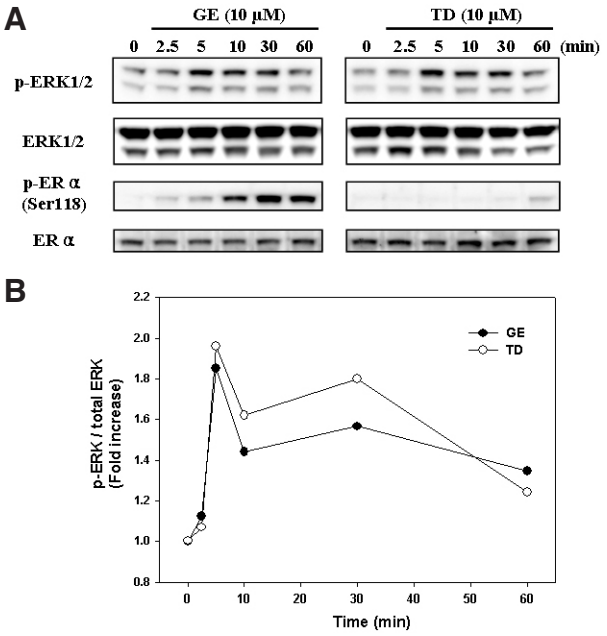

Fig. 5. The rapid phosphorylation of ERK1/2 and ER α at Ser[118] following treatment with genistein (GE) or tectoridin (TD). MCF-7 cells were treated with GE (10 μM) or TD (10 μM) for 0-60 min. (A) Cellular levels of phosphorylated-ERK1/2 (p-ERK1/2), ERK1/2, phosphorylated-ER α (at Ser118; p-ER α), ER α were measured by means of western blotting analysis. Representative immunoblots are shown from three independent experiments. (B) The band density of phosphorylated-ERK1/2 in each lane shown in A was measured and normalized to that of total ERK1/2. Each value is expressed as a fold increase compared to the value at 0 min.

of genistein and tectoridin for ER α. Interestingly, genistein-induced phosphorylation of ER α at Ser[118] was not diminished by the addition of U0126 (20 μM) (Fig. 6).

DISCUSSION

Post-translational modifications of proteins are known to affect protein activity, and ER α is known to be phosphorylated at multiple sites within the protein (Lannigan, 2003). Both ligand-dependent and -independent regulation of ER α has been reported to play a critical role in ER α activitiy. Phosphorylation of ER α at Ser[118] is an indicator, both *in vitro* and *in vivo*, of ligand-dependent ER α in breast tumors and can be used to predict responsiveness to hormone replacement therapy (Weitsman et al., 2006).

Genistein, a strong ligand of ER α, induced phosphorylation of ER α at Ser[118] in a time-dependent manner in this study. We further demonstrated that genistein-induced phosphorylation of ER α at Ser118 was not diminished by treatment with U0126 (Fig. 6). This result implies that genistein-dependent phosphorylation of ER α at Ser[118] is caused by kinases other than ERK1/2. Similar results were reported that showed that E2-induced phosphorylation of ER α at Ser[118] is independent of ERK1/2 (Joel et al., 1998; Weitsman et al., 2006).

As shown in Fig. 1A, genistein, tectorigenin and tectoridin have the same isoflavone scaffold. Tectorigenin and tectoridin are bulkier than genistein due to additional 6-methoxy and 7-β-D-glucosyl functional groups. Tectorigenin exhibited a lower binding affinity for ER α than genistein did, and tectoridin scarcely bound to ER α. This is probably due to the bulkier structures of tectorigenin and tectoridin. In spite of the poor binding affinity for ER α, tectoridin triggered potent estrogenic effects, including the recovery of the population of cells in the S-phase in G0/G1 synchronized MCF-7 cells, ERE-transactivation and the induction of MCF-7 cell proliferation. Tectoridin-induced estrogenic effects were severely abrogated by treatment with U0126. Tectoridin also rapidly induced phosphorylation of ERK1/2 within 5 min of application, while it did not induce phosphorylation of ER α at Ser[118] (Figs. 5 and 6). We suspect that tectoridin may phosphorylate other sites of ER α, such as Ser[104] and Ser[106]. A recent study reported that phosphorylation at Ser[104] and Ser[106] by ERK1/2 is important for ER α activity (Thomas et al., 2008). Another possible explanation is that tectoridin induces estrogenic effects solely via an ER α-independent mechanism, such as GPR30-mediated estrogen signaling. In our study, tectoridin increased intracellular cAMP accumulation, which is an important second messenger in GPR30-dependent estrogen signaling. This result suggests that tectoridin induced estrogenic effects mainly via GPR30-mediated rapid nongenomic signaling. This property of tectoridin sets it aside from genistein. Genistein also induced rapid phosphorylation of ERK1/2 and increase of intracellular cAMP content concurrently with an increase in phosphorylation of ER α at Ser[118]. These results support the hypothesis that genistein exerts its estrogenic effects via both an ER-dependent genomic pathway and a GPR30-dependent nongenomic pathway (Maggiolini et al., 2004; Moutsatsou, 2007).

GPR30 is involved not only in normal estrogen-dependent physiological responses but also in tumor progression, especially in ER-positive breast carcinomas, which do not respond to tamoxifen (an ER α antagonist) therapy. GPR30 is often overexpressed in high-risk breast and endometrial carcinomas (Prossnitz et al., 2008). In this study, we demonstrate that the estrogenic effects of tectoridin may be exerted mainly via GPR30. Therefore, tectorigenin and tectoridin as well as the total extract from the rhizome of *B. chinensis* have to be carefully considered for use as a selective estrogen receptor modulator (SERM) (Seidlova-Wuttke et al., 2004). Estrogenic com-

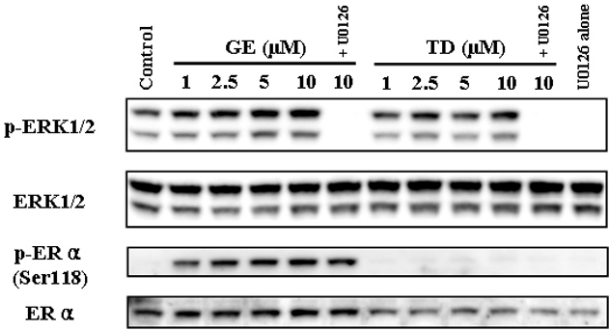

Fig. 6. The phosphorylation of ERK1/2 and ER α at Ser^{118} following treatment with various concentrations (0-10 μM) of genistein (GE) or tectoridin (TD). The MCF-7 cells were treated with GE or TD alone or together with U0126 (20 μM) for 10 min. Representative immunoblots are shown from three independent experiments.

pounds often possess biphasic properties. E2 induces apoptosis in hormone-dependent human breast cancer cells at a concentration much higher than normal physiological concentrations (20 μM as opposed to concentrations in the nanomolar range) (Altiok et al., 2007). Similarly, genistein shows a biphasic effect with regard to ER-positive breast cancer. It stimulates cell proliferation at physiologically relevant low concentrations while it inhibits cell proliferation at concentrations above 50 μM (Chen et al., 2003). Moreover, absorption and bioavailabilty studies reported that plasma concentrations of phytoestrogens in human range less than 10 μM (Moutsatsou, 2007; Setchell and Cassidy, 1999). At these concentrations, based on our study, not only genistein but also tectoridin can stimulate the estrogen-dependent MCF-7 cell proliferation. Therefore, consumption of phytoestrogens may be detrimental to people who have a predisposition for estrogen-dependent cancer and consumes phytoestrogens for estrogen replacement therapy. Development of genistein, tectorigenin and tectoridin as SERMs and the treatment/prevention of breast and endometrial cancer should be urged to take precautions. These concerns also lead us to emphasize a need for elucidating the molecular mechanisms underlying phytoestrogen-induced estrogen signaling.

We propose that tectoridin should rather be used as an improved option to treat diseases that are selective for GPR30, because tectoridin seems to exert its estrogenic effect mainly via the nongenomic pathway. A recent study reported that the estrogenic effect via GPR30, but not via ER α, attenuated hepatic liver injury after trauma-induced hemorrhage by inhibiting apoptosis (Hsieh et al., 2007; Prossnitz et al., 2008). In agreement with this, tectoridin and tectorigenin showed hepatoprotective effects against tetrachloride- and *tert*-butyl hyperoxide-induced liver injury (Lee et al., 2003; 2005).

In this study, we investigated the molecular mechanism of the estrogenic effects by tectoridin in MCF-7 human breast cancer cells. The isoflavone tectoridin significantly induced estrogenic activities despite its poor binding affinity for ER α. The tectoridin-induced estrogenic effect was severely reduced by treatment with U0126. Tectoridin induced rapid phosphorylation of ERK1/2 while, in contrast to genistein, it did not induce phosphorylation of ER α at Ser^{118}. In addition, tectoridin induced intracellular cAMP accumulation which is involved in GPR30-mediated estrogen signaling pathway. Therefore, we conclude that the estrogenic effect of tectoridin is exerted via GPR30-mediated nongenomic estrogen signaling involving ERK1/2 activation rather than via the ER α-dependent genomic pathway. These finding will provide a clue of nongenomic estrogen signaling triggered by phytoestrogens having poor binding affinities for ER, such as tectoridin. Furthermore, tectoridin might be used as a natural drug that is selective for the GPR30-mediated nongenomic estrogen signaling pathway for future clinic application.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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