Endocr (2008) 34:29–35
DOI 10.1007/s12020-008-9099-1

ORIGINAL PAPER

Phytoestrogens induce differential estrogen receptor β-mediated responses in transfected MG-63 cells

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Received: 21 May 2008 / Accepted: 24 August 2008 / Published online: 21 October 2008

Abstract Phytoestrogens may function as partial agonists or antagonists of estrogen in many tissues including bone. Five phytoestrogens, belonging to the isoflavones and the flavonoids groups, were assayed in the human MG-63 osteoblastic cell line for their ability to stimulate transcriptional activity of an estrogen-response element (ERE)-luciferase reporter gene via the estrogen receptor β (ERβ). Although MG-63 cells were shown to express endogenous estrogen receptors, estradiol (E_2) did not affect transcriptional activity of an ERE reporter in these cells. However, E_2 did activate the ERE-reporter significantly in MG-63 cells where ERβ was overexpressed. The isoflavones, genistein and daidzein, caused a dose-dependent increase in the ERE-reporter activity in MG-63 cells overexpressing ERβ. Among the flavonoids, kaempferol activated ERE-reporter activity, whereas puerarin inhibited ERE-reporter transcription in cells overexpressing ERβ. Quercetin had no effect on ERE-reporter activity over a concentration range of 10^{-10}–10^{-6} mol/l. The ERE-reporter activity induced by daidzein, genistein, and kaempferol was blocked by both ICI 182780 and 4-hydroxytamoxifen and partly blocked by puerarin. Our results demonstrated that different phytoestrogens exhibited differential transcription activity of an ERE-reporter via ERβ-mediated mechanisms in MG-63 cells.

Keywords Phytoestrogens · Estrogen receptor β · Transcription

Introduction

Estrogens are used in hormone replacement therapy (HRT) to prevent hot flashes, urogenital atrophy, and osteoporosis in postmenopausal women [1, 2]. HRT may also reduce the risk of heart disease [3], Alzheimer's disease [4], and colon cancer [5]. However, available evidence appears to suggest that long-term use of estrogens has numerous side effects including uterine bleeding and hyperplasia, and an increased risk of breast [6, 7] or endometrial cancer [8]. In this regard, there has been an intense pursuit for selective estrogen receptor modulators (SERMs) that have a safer profile, such as tamoxifen and raloxifene. Although treatment with these compounds may provide partial benefits with respect to the risk of breast cancer and bone loss, they may also be associated with an increase in endometrial cancer (tamoxifen) and hot flashes (raloxifene) [9, 10]. Thus, the search for superior SERMs for use in HRT continues to be of intense interest.

There is also an increasing interest in the use of phytoestrogens as substitutes for traditional estrogen replacement therapies in menopausal women [11]. Interest in phytoestrogens has been fueled by observational studies showing a lower incidence of menopausal symptoms, osteoporosis, cardiovascular disease, and breast and endometrial cancers in Asian women who have a diet rich in soy products [12–17]. Phytoestrogens are nonsteroidal, diphenolic structures found in many plants (e.g., fruits,

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vegetables, legumes, wholegrains, and especially soy food products) that have similar chemical and structural properties to that of estrogens. In vitro studies have demonstrated that a variety of phytoestrogens can bind to estrogen receptors and exhibit estrogenic activity [18, 19].

Two different estrogen receptor (ER) subtypes, different gene products, are known: ERα, the first ER discovered, and ERβ, which was discovered in 1996 [20, 21]. ERα and ERβ have similar modular domain structures and very high amino acid identity in their DNA-binding domains (97%), although they are more divergent in their N-terminal A/B domains (only 18% amino acid identity) and in their ligand-binding domains (59% amino acid identity) [22]. The tissue distribution patterns of ERα and ERβ are also quite different. It is notable that ERβ is more highly expressed in the prostate, ovary, colon, urinary tract, and some brain regions, but expressed at lower levels in certain reproductive organs [22, 23]. Hence, a selective ERβ agonist might maintain the beneficial effects of estrogen therapy in these ERβ-rich tissues without increasing the risk of cancer in other organs that are ERα rich, such as the breast and uterus. In addition, ERβ has been shown to be anti-proliferative when present along with ERα in breast cancer cells [24, 25]. Interestingly, the soy isoflavones, such as daidzein and genistein, were among the first compounds noted to be ligands with selective affinity for ERβ [26, 27]. The relatively selective binding of soy isoflavones to ERβ indicates that isoflavones may produce clinical effects distinct from estrogens by either selectively or differentially triggering ERβ-mediated transcriptional activation or repression pathways.

There is growing evidence to indicate that SERMs provide tissue-selective effects even while interacting with the same subtype of estrogen receptor. For instance, raloxifene exhibits agonist activity in some tissues, such as the bone [28, 29], but acts as an antagonist in other tissues, including the breast [30]. A number of investigations using distinct in vitro and in vivo models have demonstrated that some phytoestrogens modulate estrogen-sensitive molecular parameters in a SERM-like manner [31–34]. Nevertheless, the tissue-selective effects of phytoestrogens are not yet well documented. Therefore, we chose a number of phytoestrogens belonging to the isoflavones and flavonoid groups, and tested their estrogenic activity through ERβ in MG-63 cells using reporter-gene assays.

Results

Expression of estrogen receptors in MG-63 cells

As shown in Fig. 1a, MG-63 cells expressed both ERα and ERβ using Western blot analysis. When these cells were transfected with a simple ERE-containing promoter (p2ERE-PGL3) driving luciferase reporter expression, estradiol (E_2) treatment did not significantly affect transcriptional activity of the ERE reporter over the concentration range of 10^{-10}–10^{-6} mol/l, compared with vehicle in eight individual experiments (data not shown). Similarly, treatment of these cells with genistein, daidzein, kaempferol, puerarin, or quercetin did not influence ERE-reporter transcription over the concentration range 10^{-10}–10^{-6} M ($n = 8$, data not shown).

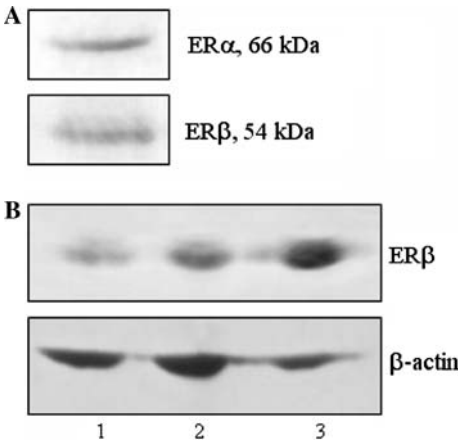

Fig. 1 Identification of ER proteins in MG-63 cells by Western blot analysis. **a** The expression of ERα and ERβ proteins were identified in MG-63 cells. **b** Overexpression of ERβ protein in MG-63 cells. 1, Cells without transfection; 2, MG-63 cells transfected with pcDNA plasmid; and 3, Cells transfected with pcDNA-ERβ plasmid

Effects of E_2 and phytoestrogens on transcriptional activity of estrogen-response reporter in MG cells overexpressing ERβ

To further elucidate the ERβ-mediated transcriptional activities, cotransfection of the ERE-reporter plasmid along with an ERβ expression plasmid was performed. Western blot analysis showed that the expression level of ERβ in cells transfected with the ERβ expression plasmid was greatly increased compared with control cells (Fig. 1b).

Treatment of these cells with increasing concentrations of E_2 resulted in activation of ERE-reporter transcription by 3.6-fold and 6.4-fold at 10^{-9} and 10^{-7}, respectively (Fig. 2a). Daidzein, at 10^{-7} and 10^{-6} M, induced 3-fold and 5-fold increase in luciferase transcription, respectively (Fig. 2b). Genistein (10^{-8}–10^{-6} M)showed a dose-dependent stimulation of transcriptional activity of the ERE reporter (Fig. 2c). Genistein, at 10^{-8} M, significantly induced ERE promoter activity 4.5-fold whereas daidzein did not induce transcriptional activity at a dose of 10^{-8} M (Fig. 2b, c). Among the flavonoids, puerarin had a non-dose-dependent inhibitory effect on ERE-reporter activity

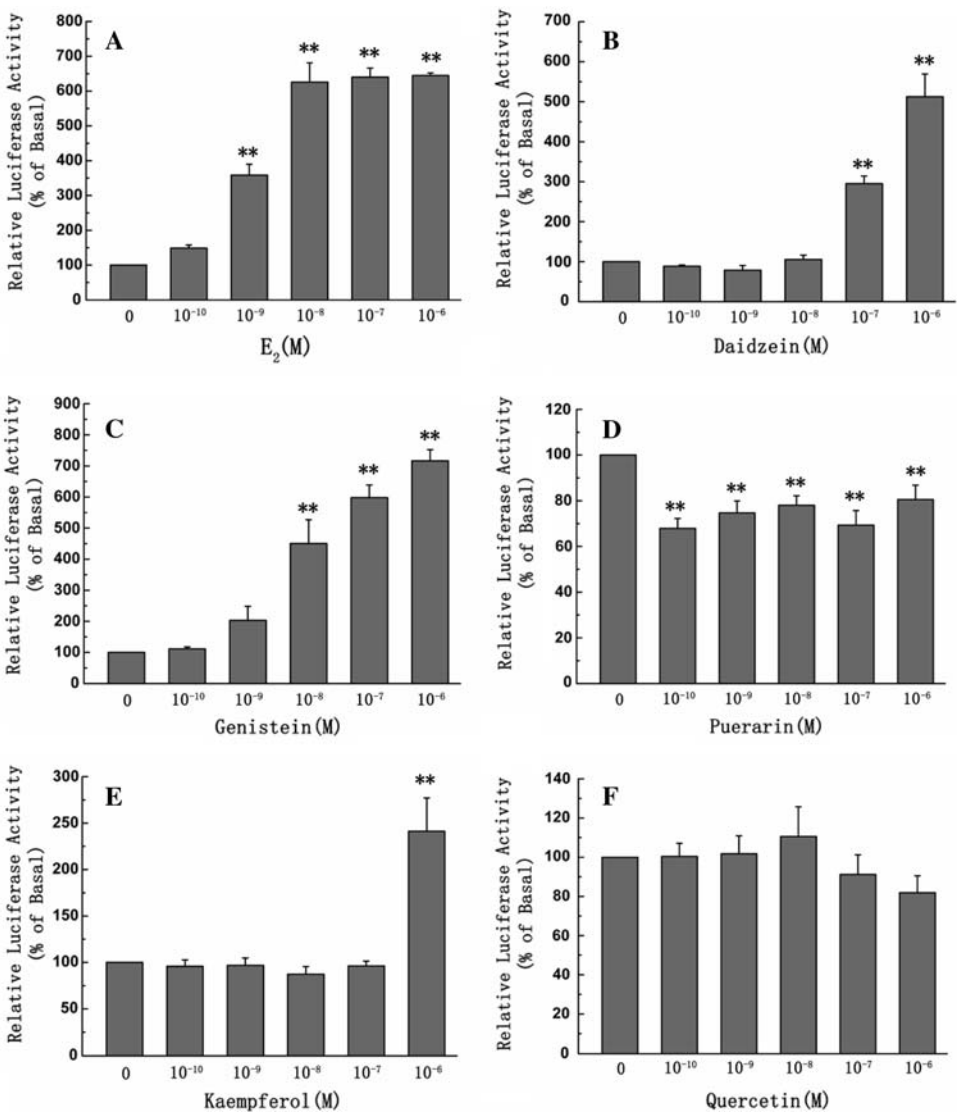

Fig. 2 The effects of phytoestrogens on ERE-reporter gene activity. MG-63 cells were co-transfected with ERE-PGL3 reporter plasmid and pcDNA-ERβ (ERβ), and then exposed to E_2 and phytoestrogens at the indicated concentration. Relative luciferase activities were shown as percentage of vehicle treated cells. Values represent the mean ± SEM from five independent experiments. *$P < 0.05$; **$P < 0.01$, compared with vehicle

(Fig. 2d). Kaempferol exhibited a weak estrogenic activity as indicated by the 2.5-fold increase in transcriptional activity of ERE reporter at a high concentration (10^{-6} M) (Fig. 2e). In contrast, quercetin showed no significant effects on ERE-reporter activity through the range of 10^{-10}–10^{-6} M (Fig. 2f).

Cotransfection of ERE-reporter plasmid along with pcDNA empty vector into MG-63 cells caused no significant changes in responsiveness to E_2 or phytoestrogens compared with nontransfected cells (data not shown).

The effect of ER antagonists on phytoestrogen-induced transcriptional activity of an ERE-reporter

In MG-63 cells overexpressing ERβ, the ER antagonist ICI 182780 dose dependently reduced ERE-reporter activity (Fig. 3a). 4-Hydroxytamoxifen (4-OH-Tam) exhibited an inhibitory effect on promoter activity of ERE-reporter in a non-dose-dependent manner (Fig. 3b). The ERE-reporter activity induced by E_2, daidzein, genistein, and kaempferol was blocked by both ICI 182780 and 4-OH-Tam (Fig. 4a, b).

As shown above, puerarin exhibited inhibitory effects on ERE-reporter activity similar to those of 4-OH-Tam. Therefore, we tested the effect of puerarin on E_2- and phytoestrogen-induced ERE-reporter activity in MG-63 cells overexpressing ERβ. It was shown that puerarin partly blocked E_2-, daidzein-, genistein-, and kaempferol-induced ERE-reporter activity in cells overexpressing ERβ (Fig. 4c).

The effect of E_2, ER antagonists, and phytoestrogens on cell viability

To determine whether concentrations of phytoestrogens that affected ERβ-dependent transcription were detrimental

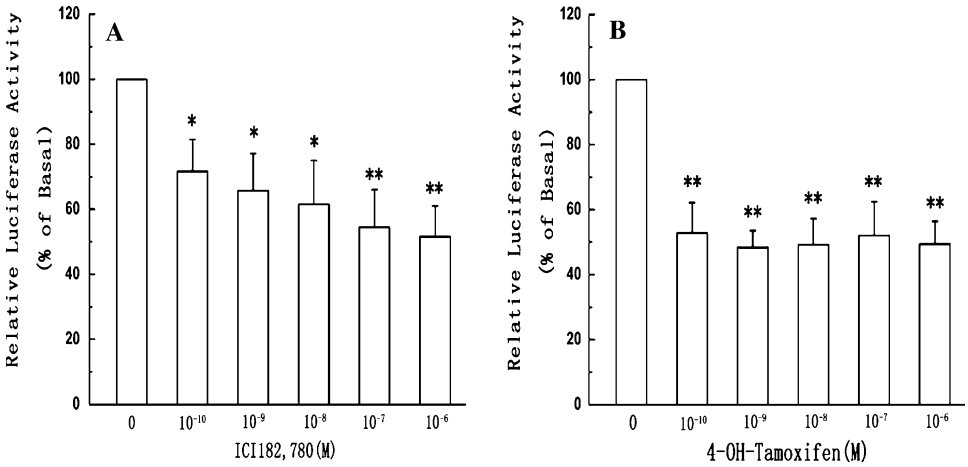

Fig. 3 The effects of ER antagonists on ERE-reporter transcription. MG-63 cells were co-transfected with ERE-PGL3 reporter plasmid and pcDNA-ERβ (ERβ), and then exposed to ICI 182780 (**a**) or 4-OH-Tam (**b**) at the indicated concentration. Relative luciferase activities were shown as percentage of vehicle treated cells. Values represent the mean ± SEM from four independent experiments. $^{**}P < 0.01$ compared with vehicle

to cells, cell growth, and survival was examined in cells which were co-transfected with pERE-PGL3 and ERβ plasmids. Treatment of these cells for up to 24 h with E_2, ER antagonists, or phytoestrogens had no significant effect on cell growth and viability at a dose of 10^{-6} M compared with vehicle control (Fig. 5).

Discussion

In this study, we have shown that E_2, daidzein, genistein, and kaempferol stimulated ERE-reporter activity, whereas puerarin, ICI 182780, and 4-OH-Tam repressed ERE-dependent transcription in MG-63 cells overexpressing ERβ. Moreover, these phytoestrogens, E_2 and SERMs, ICI182780, and 4-OH-Tam, did not affect the viability and growth of transfected MG-63 cells. These data suggested that daidzein, genistein, kaempferol, and puerarin act through ERβ to modulate ERE-mediated transcription in MG-63 cells.

A number of studies have showed that phytoestrogens such as genistein, daidzein, quercetin, and kaempferol influence ER-mediated transcriptional activity of ERE-reporter systems. Mueller et al. [19] found that genistein displayed a preference for transactivation of ERβ-ERE responses compared to ERα-ERE response in the endometrial Ishikawa cell line. Harris et al. [34], in MCF-7 cells transfected with ERα or ERβ, evaluated the dose-dependent responses of various phytoestrogens and revealed that genistein and daidzein had a differential and robust transactivation of ERα- and ERβ-induced transcription, with up to 100-fold stronger activation of ERβ. Kostelac and coworkers demonstrated that the isoflavones genistein and daidzein were able to modulate the binding of both ERα and ERβ to estrogen-response elements (EREs), but preferentially activated the binding of ERβ to ERE [35]. There are conflicting results in the literature regarding the effect of kaempferol and quercetin on ERE-reporter activity. Van der Woude et al. [36] showed that quercetin induced ERE-dependent transactivation in MCF-7 cells through both ERα and ERβ but with a higher capacity, like genistein, to stimulate ERβ responses as compared to the ERα stimulation responses. In contrast, Harris et al. [34] reported that, in MCF-7 cells, kaempferol and quercetin has minimal agonist activity, and act as antagonists through both ERα and ERβ at the highest dose (10 μM) in the presence of E_2 (0.5 nM). In the present study, we found that kaempferol had weak activity for transactivating through ERβ, while quercetin did not affect transcriptional activity measurably in MG-63 cells that were overexpressing ERβ. Taken together these results lead to the suggestion that the estrogenic activity of phytoestrogens might be dependent on cell context and/or the concentration of other ER ligands.

We have also demonstrated that puerarin has an antagonistic activity similar to 4-OH-Tam, in cells overexpressing ERβ, suggesting that puerarin can act as an antagonist of ERβ in MG-63 cells. Of note, 4-OH-Tam totally blocked E_2-, and other phytoestrogen, mediated transcriptional activity of the ERE-reporter, whereas puerarin only partly blocked the effects of E_2, genistein, and daidzein. Such data suggests that 4-OH-Tam and puerarin differ in the mechanisms that underlie their antagonistic activity.

Bone is an active organ, which undergoes continuous remodeling cycles of resorption and formation processes during its entire life [37]. Estrogen has been implicated as being especially responsible for maintaining bone metabolism in women [38, 39]. Although the mechanisms that underlie estrogen-mediated modulation of bone metabolism remain unclear, it has been found that a reduction in the circulating level of estrogen results in bone loss after the onset of menopause in women [39–41]. In recent years, increasing evidence has indicated that phytoestrogens

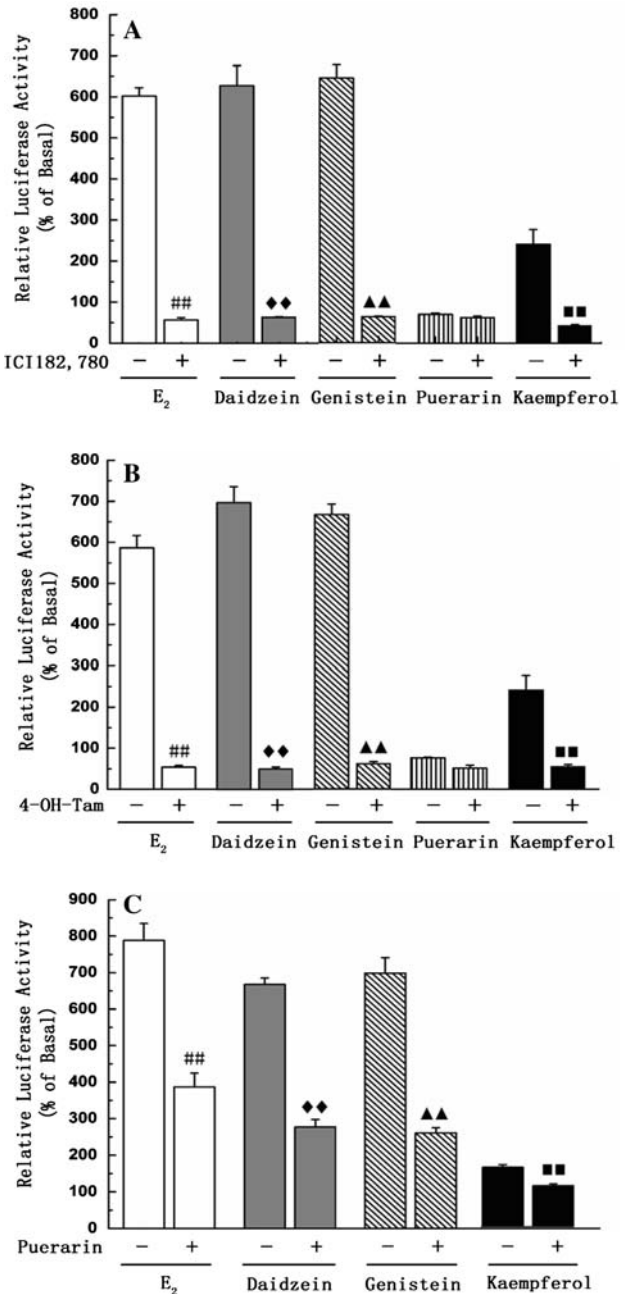

Fig. 4 The effect of ICI 182780, 4-OH-Tam, and puerarin on ERβ-mediated transcriptional activity induced by phytoestrogens. MG-63 cells were co-transfected with ERE-PGL3 reporter plasmid and pcDNA-ERβ (ERβ). Cells were then exposed to E_2 (10^{-7} M), daidzein (10^{-6} M), genistein (10^{-6} M), kaempferol (10^{-6} M) with or without the indicated antagonists (10^{-6} M), or puerarin (10^{-6} M). Relative luciferase activities were shown as percentage of vehicle. Values represent the mean ± SEM from four independent experiments. ##, ◆◆, ▲▲, ■■ $P < 0.01$, compared with E_2, daidzein, genistein, or kaempferol treatment, respectively

might prevent bone loss in postmenopausal women. A number of observational epidemiological studies showed the existence of associations between isoflavone intake and bone density [42–45]. A number of animal studies also

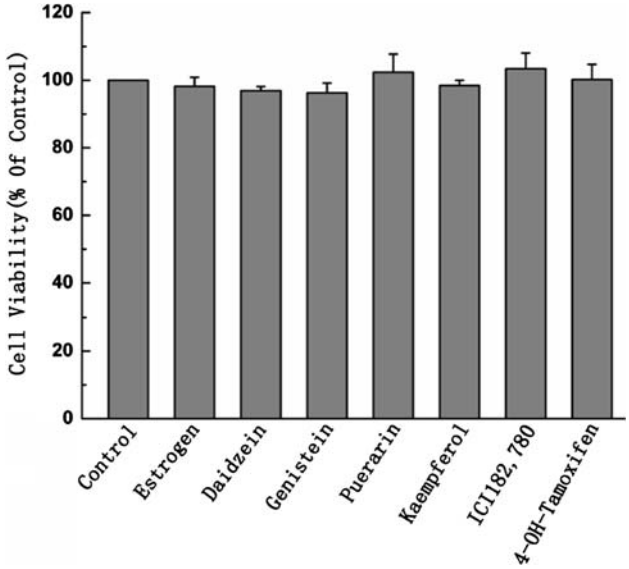

Fig. 5 The effect of E_2, ER antagonists, and phytoestrogens on cell viability. MG-63 cells were co-transfected with ERE-PGL3 reporter plasmid and pcDNA-ERβ (ERβ). Cells were then exposed to various reagents (10^{-6} M) as indicated for 24 h. Values represent the mean ± SEM from four independent experiments

support this hypothesis, showing that isoflavones can help to preserve bone mass [46–48]. Our findings that isoflavones genistein and daidzein could induce ERβ-mediated transcriptional activity in the human osteoblastic cell line, MG-63, suggest that isoflavones may exert effects on bone metabolism. Although other recent studies have demonstrated that puerarin enhances bone formation [49, 50], as does estrogen, our present study showed that puerarin exhibited an antagonistic effect on transcriptional activity of ERE reporter via ERβ.

Our present study has shown in MG-63 cells that the isoflavones genistein and daidzein induce ERE-reporter activity via ERβ. Among the flavonoids, kaempferol is shown to be a weak agonist, whereas puerarin acts as an antagonist of ERβ, which suggests that different phytoestrogens can induce differential transcriptional activity through ERE via ERβ.

Materials and methods

Materials

17β-Estradiol and 4-hydroxytamoxifen were purchased from Sigma–Aldrich Chemicals (St. Louis, MO). ICI 182780 was from Tocris Cookson (Bristol, UK). Daidzein, genistein, puerarin, kaempferol, and quercetin were obtained from Tauto Biotech (Shanghai, China). Polyclonal antibody against ERβ and secondary antibody for

use with the chemiluminescent detection system were supplied by Santa Cruz Biotechnology (sc-53459, Santa Cruz, CA).

Cell culture and transient transfections

Human osteoblastic cell line MG-63 was originally obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in phenol red-free DMEM medium (Sigma–Aldrich) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2 and 95% air.

The expression vectors for ERβ (pcDNA-ERβ) and p2ERE-PGL3 were generously provided by Dr. P. Fuller (Prince Henry's Institute of Medical Research, Melbourne, Australia) and Dr. Richard N Day (University of Virginia, Charlottesville, VA), respectively. Transient transfections were performed using Sofast^{TM} (Sunma Biotech, Xiamen, China) cationic polymer transfection reagent according to the manufacturer's manual. Briefly, one day before transfection, 1×10^5 cells/well MG-63 cells were seeded in 48-well plates (Costar, Cambridge, MA) and cultured in phenol red-free DMEM containing 10% charcoal-stripped FBS. Each well was then transfected with 0.404 μg DNA containing 0.32 μg p2ERE-PGL3 and 0.08 μg pcDNA-ERβ as well as 4 ng of control DNA (pRL-TK-Renilla-luciferase vector, Promega) using 1.2 μl Sofast reagent. Eight hours later, culture media were changed to fresh media and cells were treated with various agents as indicated. E_2, ICI 182780, genistein, daidzein, puerarin, kaempferol, quercetin, and 4-OH-Tam were added to the treatment media as stock solutions in absolute ethanol or dimethylsulfoxide. Control media contained the same final solvent concentrations (0.01%). Each treatment was performed in triplicate for each preparation of cells. Luciferase assays were carried out 24 h later using the dual luciferase assay kit (Promega). Relative luciferase activity is presented as firefly luciferase values normalized to renilla luciferase activity.

Western blot analysis

Cells were scraped off the plate in the presence of lysis buffer consisting of 50 mM Tris–HCl, 150 mM NaCl,0.1% sodium dodecyl sulfate (SDS), 1% Triton-X 100, 1% sodium orthovanadate, 100 mM dithiothreitol, 0.6 mM phenylmethylsulfonyl fluoride (Sigma), 1.5 μg/ml aprotinin, and 50 mM leupeptin (Watson, Shanghai, China). The cell lysates were quickly sonicated and centrifuged at $12{,}000 \times g$ for 5 min at 4°C. The supernatant was collected and protein concentration was assayed using a modified Bradford assay. The samples were diluted in sample buffer (containing 250 mM Tris–HCl, 2% SDS, 10% glycerol, and 0.002% bromophenol blue) and boiled for 5 min. Samples were separated on an SDS-8% polyacrylamide gel, and the proteins were electrophoretically transferred to a nitrocellulose filter at 300 mA for 1.5 h in a transfer buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol. The filter was then blocked in TBS containing 0.1% Tween-20 (TBST) and 5% dried milk powder (w/v) for 2 h at room temperature. After three washes with TBST, the nitrocellulose filters were incubated with primary antibody for ERβ (1:500) and β-actin (1:1,000) at 4°C overnight. After another three washes with TBST, the filters were incubated with a secondary antibody, horseradish peroxidase-conjugated IgG, (1:1,000) for 1 h at room temperature and further washed for 30 min with TBST. Immunoreactive proteins were visualized using the enhanced chemiluminescence Western Blotting detection system (Santa Cruz). The light-emitting bands were detected with X-ray film. The resulting band intensities were quantitated using an image scanning densitometer (Furi Technology, Shanghai, China). To control sampling errors, the ratio of band intensities to β-actin was obtained to quantify the relative protein expression level.

Cell viability assay

After cells were transfected with ERβ (pcDNA-ERβ) and p2ERE-PGL3, cells were treated with E_2, ICI 182780, 4-OH-Tam genistein, daidzein, puerarin, and kaempferol at a dose of 10^{-6} M. Control media contained the same final solvent concentrations (0.01%). Each treatment was performed in triplicate for each preparation of cells for 24 h. The assay depends on the reduction of the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma) by functional mitochondria to formazan [46]. After a 2 h incubation of the cells with MTT at 37 °C, the cells were lysed with dimethyl sulfoxide in Sorensen's glycine buffer and the formazan crystals solubilized. Absorbance was read at 550 nm using a spectrophotometric microplate reader (Bio-Rad).

Statistical analysis

For illustrative purposes, the results are presented as the mean percentage of control ± SEM. Statistical analyses were carried out using one-way ANOVA followed by the LSD-t test. Significance was set at $P < 0.05$.

Acknowledgments The authors wish to thank Dr. P. Fuller, Prince Henry's Institute of Medical Research, Melbourne, Australia, and Dr. Richard N Day, University of Virginia, Charlottesville, VA, for generously providing pcDNA-ERβ and p2ERE-PGL3. This work is supported by the Science and Technology Committee of Shanghai (06jc140-83) and the National Science Foundation of China (No. 30800908).

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