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# Isoflavones suppress cyclic adenosine 3′,5′-monophosphate regulatory element-mediated transcription in osteoblastic cell line

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**Abstract**

Soy isoflavones have been implicated to exert benefit on bone loss in postmenopausal women. Isoflavones can induce estrogen response element-mediated transcription in osteoblastic cells. In the present study, we investigate whether isoflavones genistein and daidzein regulate target gene transcription through cAMP regulatory element (CRE) in osteoblastic cells. It was found that 17β-estradiol ($E_2$), genistein and daidzein suppressed the transcriptional activity of CRE-luciferase reporter gene in human osteoblastic cell line MG-63 cells. $E_2$ and genistein but not daidzein inhibited the cAMP analogue 8-Br cAMP-induced transcription of CRE reporter gene. Both genistein and $E_2$ inhibited basal and cAMP-induced mRNA levels of endogenous estrogen responsive genes containing CRE/CRE-like elements in their promoter regions, including interleukin (IL) 8 and serum- and glucocorticoid-inducible kinase 1 (SGK1). Daidzein inhibited basal and cAMP-induced IL-8, but not SGK1 mRNA expression. The inhibitory effects of $E_2$, genistein and daidzein on CRE-mediated transcription activity were enhanced by estrogen receptor (ER) α overexpression in MG-63 cells, which could be blocked by nonselective ER antagonists ICI182780, 4-OH tamoxifen and specific ERα antagonist MPP. Genistein and daidzein, but not $E_2$ treatment, caused a significant decrease in CRE-mediated transcription activity in ERβ-transfected MG-63 cells, which could be blocked by ICI182780, 4-OH tamoxifen and the selective ERβ antagonist (*R*,*R*)-5,11-diethyl-5.6,11,12-tetradro-2,8-chrysenediol. Our results indicate that isoflavones genistein and daidzein might modulate bone remodeling through ERs by regulating target gene expression through the CRE motifs.


*Keywords:* Isoflavone; Estrogen receptor; Cyclic adenosine 3′,5′-monophosphate regulatory element; Transcription

## 1. Introduction

Estrogen plays a key role in the maintenance of the adult skeleton [1,2]. Low circulating estrogen has been recognized as an important risk factor for peri- and postmenopausal osteoporosis [3,4]. Hormone replacement therapy is the most common therapy used for peri- and postmenopausal women to prevent bone loss [5]. However, accumulating evidence indicates that long-term use of estrogen has numerous side effects such as increased risks of breast cancer [6,7] and thromboembolic diseases [8]. Given the demonstrated risks of conventional estrogen therapy, a search for novel, cost-effective and safer alternatives for the prevention of bone loss in postmenopausal women is of importance [9,10].

Asiatic women with high intake of soy or soy products, which contain natural plant estrogens (phytoestrogens), have been shown to have decreased risks of osteoporosis, cardiovascular disease, breast and uterine cancer and climacteric symptoms [10-12]. Thus, isoflavones, the most abundant phytoestrogens in soy and soy products, have received considerable attention in the recent years. It has been shown that isoflavones stimulate osteoblastic differentiation and mineralization in vitro [13,14] and are endowed with anabolic activities on bone in vivo as estrogen does [15,16]. However, the molecular mechanisms whereby isoflavones exert their benefit on bone loss remain unknown.

Estrogen primarily acts by regulating gene transcription via ERα and ERβ. Classically, the receptor binds as homodimers or heterodimers to estrogen response elements (ERE) in the promoter of many, though not all, estrogen-response genes [17,18]. Increasing body of evidence suggests that estrogen receptors (ERs) can modulate gene expression through mechanisms that are independent of binding to classic ERE. ERs may also modulate transcription of genes that contain other response elements (e.g., AP-1, cAMP regulatory element, specific protein-1 and nuclear factor-κB) but not ERE [19,20]. ER-dependent activation or repression through a cAMP regulatory element (CRE) or CRE-like sequence motif has been demonstrated in the context of cyclin D1 [21], tumor necrosis factor α (TNFα) [22] and corticotropin-releasing hormone genes [23].

Isoflavones have a structure similar to that of estrogen and are capable of binding to both ERα and ERβ [24,25]. Isoflavones have been shown to induce transcriptional activity through ERE in many cell types including osteoblastic cell lines [26,27]. Isoflavones modulation

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of target gene transcription through CRE motif in osteoblastic cells remains unclear, although Liu et al. [28] have demonstrated that genistein stimulates CRE-mediated gene transcription in endothelial cells. Therefore, in the present study, we examined the effects of isoflavones on transcriptional activity of CRE-reporter gene in human osteoblastic cell line MG-63 cells, compared with the effects of 17β-estradiol ($E_2$) and clarified which subtype of ER was involved in the effects of isoflavones.

## 2. Materials and methods

### 2.1. Materials

17β-Estradiol and 4-hydroxytamoxifen were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). ICI 182780, 4-OH tamoxifen, dihydrochloride 1,3-bis(4-hydroxyphenyl)-4-methy-5-[4-(2-piperdinylethoxy)phenol]-1 h-pyrazole dihydrochloride (MPP) and (*R*,*R*)-5,11-diethyl-5.6,11,12-tetradro-2,8-chrysenediol (THC) was obtained from Tocris Bioscience (Bristol, UK). Daidzein and genistein were obtained from Tauto Biotech (Shanghai, China). cAMP response element-binding protein (CREB) and phospho-CREB antibodies were purchased from Cell Signal Technology (Beverly, MA, USA). Polyclonal antibodies against ERα (sc-543), ERβ (sc-53459) and secondary antibody for use with the chemiluminescent detection system were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### 2.2. Plasmids

The expression vector encoding human ERα (pCMV-ERα) and ERβ (pcDNA-ERβ) were generously provided by Dr. B.S. Katzenellenbogen (University of Illinois) and Dr. P. Fuller (Prince Henry's Institute of Medical Research, Melbourne, Australia), respectively. The CRE-reporter gene (*p2CRE-PGL3*) was created and provided by Dr. R.C. Nicholson (University of Newcastle, Australia).

### 2.3. Cell culture and transient transfections

Human osteoblastic cell line MG-63 were cultured in phenol red-free DMEM medium (Life Technologies, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% $CO_2$, 95% air.

Transient transfections were performed using Sofast (Sunma Biotech, Xiamen, China) cationic polymer transfection reagent according to the manufacturer's protocols. Briefly, 1 day before transfection, $1\times10^5$ cells/well MG-63 cells were seeded in 48-well plates and cultured in phenol red-free DMEM containing 10% charcoal-stripped FBS. Each well was then transfected with 0.4 μg DNA and 10 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. $E_2$, genistein, daidzein, ICI 182780, 4-OH tamoxifen, MPP and THC were added 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

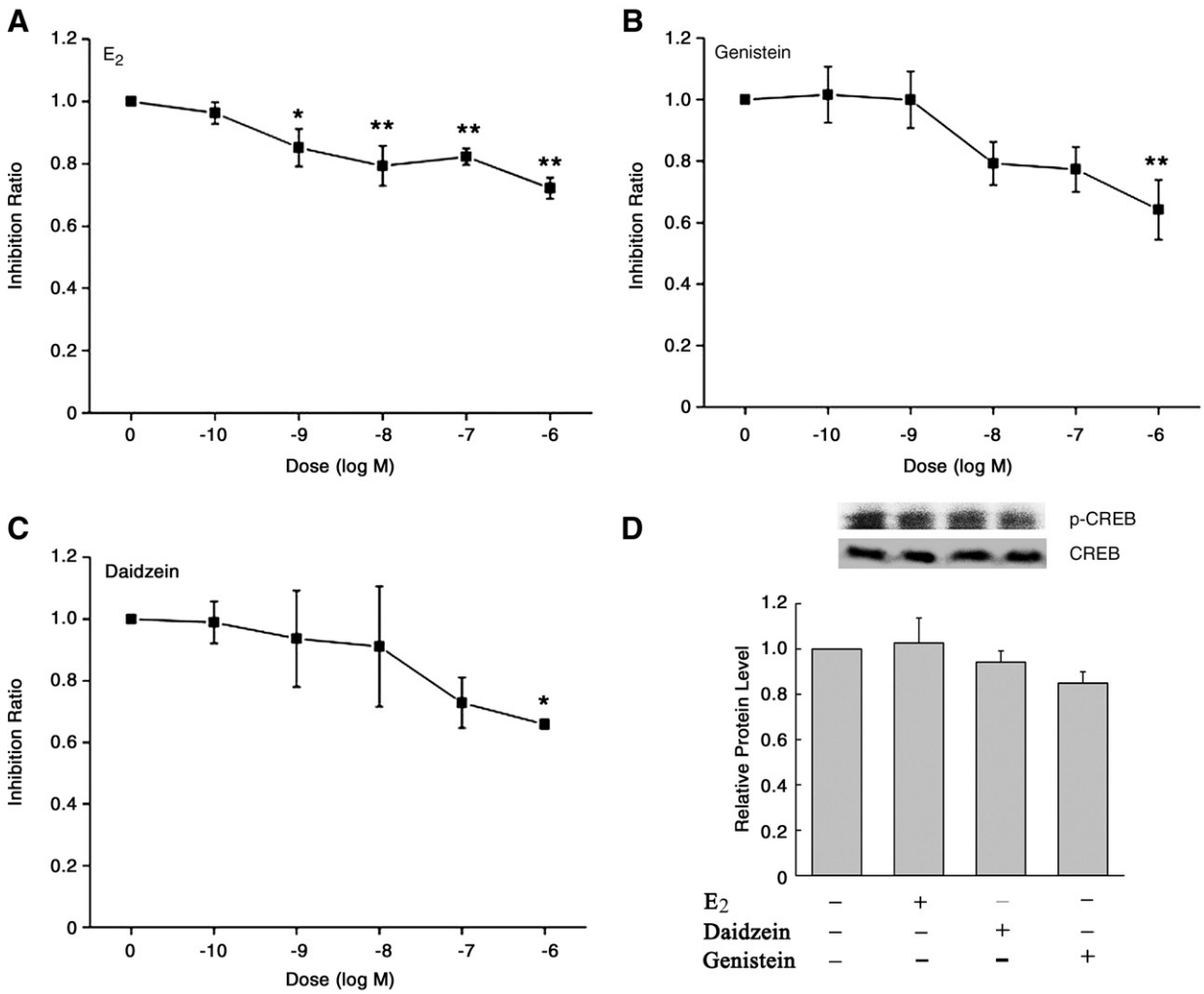


Fig. 1. Effects of $E_2$ and isoflavones on CRE-mediated transcription and CREB phosphorylation in MG-63 cells. A-C, MG-63cells were transfected with CRE reporter plasmid and then exposed to $E_2$, genistein and daidzein at the indicated concentrations for 24 h. Results are expressed as the inhibition ratio compared to the vehicle control. Each value represents the mean±S.E.M. from five independent experiments. D, MG63 cells were treated with $E_2$ ($10^{-7}$ mol/L), genistein ($10^{-6}$ mol/L) and daidzein ($10^{-6}$ mol/L) for 24 h, then phosphorylated CREB protein (p-CREB) level was determined using Western blot analysis. Results are expressed as the fold induction of control. Each value represents the mean±SEM from four independent experiments. **P*<.05; ***P*<.01 compared with vehicle control.

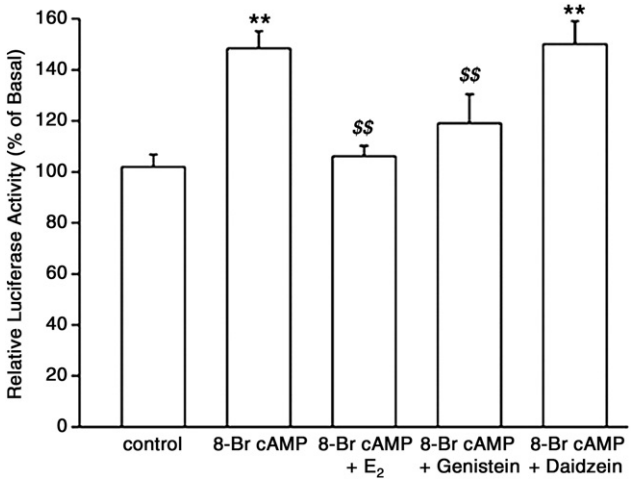


Fig. 2. Effects of $E_2$ and isoflavones on 8-Br cAMP-induced CRE promoter activity. MG-63 cells were transfected with CRE reporter plasmid and then treated with $E_2$ ($10^{-7}$ mol/L), genistein ($10^{-6}$ mol/L) and daidzein ($10^{-6}$ mol/L) in the presence or absence of 8-Br cAMP ($10^{-5}$ mol/L) for 24 h. Results are expressed as the percentage of control. Each value represents the mean±S.E.M. from five independent experiments. $^{**}P<.01$ compared with vehicle control; $\$\$P<.01$, compared with 8-Br cAMP treatment.

luciferase activity is presented as firefly luciferase values normalized to renilla luciferase activity.

### 2.4. Western blot analysis

Western blot analysis was carried out as described previously [26]. Briefly, cells were scraped off the plate in the presence of lysis buffer. The cell lysates were quickly sonicated and centrifuged, and the supernatants were then collected. The samples were separated on an SDS-8% polyacrylamide gel, and the proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was then blocked in 5% skim milk powder in 0.1% Tris-buffered saline/Tween 20 (TBST) for 2 h at room temperature. After three washes with TBST, the nitrocellulose filters were incubated with primary antibody for ERα, ERβ (1:500), CREB, phospho-CREB and β-actin (1:1000) at 4°C overnight, then the filters were incubated with a secondary antibody, horseradish peroxidase-conjugated IgG, (1:1000). 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.

### 2.5. Total RNA extraction and quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA of cells was isolated by using TRIzol reagent according to the manufacturer's instructions. Two micrograms of RNA was reverse-transcribed using superscript reverse transcriptase and stored at −20°C. The primers for real-time RT-PCR

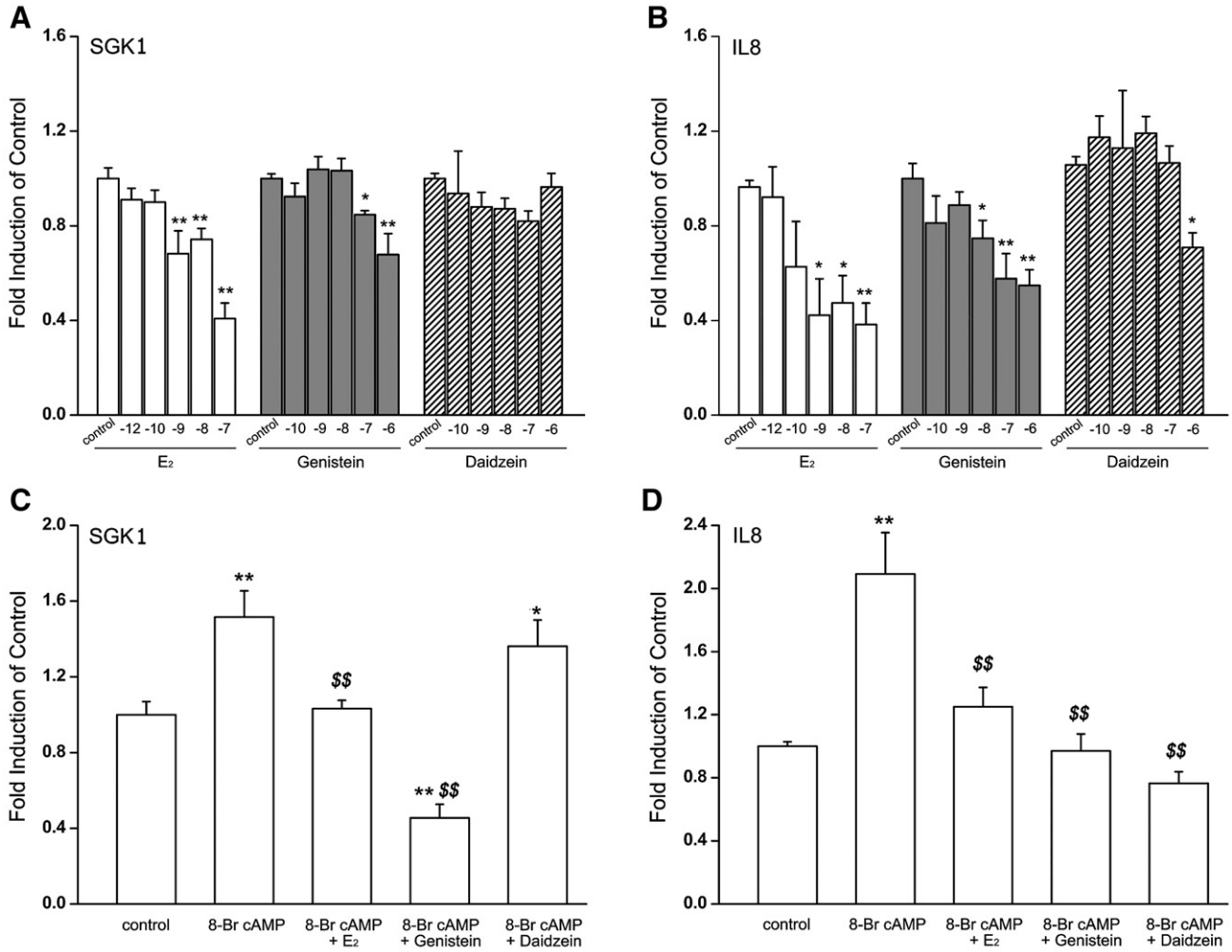


Fig. 3. Effects of $E_2$ and isoflavones on mRNA levels of SGK1 and IL-8 in MG-63 cells. A and B, Cells were exposed to $E_2$, genistein and daidzein at the indicated concentrations for 24 h. C and D, cells were exposed to $E_2$ ($10^{-7}$ mol/L), genistein ($10^{-6}$ mol/L) and daidzein ($10^{-6}$ mol/L) in the presence or absence of 8-Br cAMP ($10^{-5}$ mol/L) for 24 h. Quantitative real-time RT-PCR was used to determine SGK1 (A, C) and IL-8 (B, D) mRNA expression. Data were expressed as fold induction of control±S.E.M. ($n=4$). $^{*}P<.05$; $^{**}P<.01$ compared with vehicle control; $\$\$P<.01$ compared with 8-Br cAMP treatment.

were serum- and glucocorticoid-inducible kinase 1 (SGK1, accession number NM_005627): sense 5′-AAGCAGAGGAGGATGGGT-3′ and antisense 5′-GAACATTCCGCTCCGACA-3′; interleukin (IL) 8 (accession number NM_000584): sense 5′-TGGCAGCCTTCCTGATTT-3′ and antisense 5′-AACTTCTCCACAACCCTC-3′. Quantitative real-time PCR analysis was carried out in duplicates using Rotor Gene 3000 (Corbett Research, Sydney, Australia). Real-time PCR solution consisted of 40 ng diluted cDNA product, 0.1 μM of each paired primer, 1.5–2.5 mM $Mg^{2+}$, 100 μM deoxynucleotide triphosphates, 2 U Taq DNA polymerase and 1×PCR buffer. SYBR green (BMA, Rockland, ME, USA) was used as detection dye. Quantitative real-time PCR conditions were optimized according to preliminary experiments to achieve linear relationships between initial RNA concentration and PCR product. The amplification cycles were set at 40 cycles. The temperature range to detect the melting temperature of the PCR product was set from 60–95°C. Amplification of the housekeeping genes β-actin and GAPDH were measured for each sample as an internal control for sample loading and normalization. The specificity of the primers was verified by examining the melting curve as well as subsequent sequencing of the real-time RT-PCR products. To determine the relative quantitation of gene expression for both target and housekeeping genes, the comparative Ct (threshold cycle) method with arithmetic formulae ($2^{-\Delta\Delta Ct}$) was used [29]. Because very similar data were obtained by using either β-actin or GAPDH as an internal control, GAPDH was used for calculation of ΔCt in presentation of results.

### 2.6. Cell viability assay

After cells were transfected with CRE-reporter gene, cells were treated with $E_2$, genistein, daidzein, ICI 182780, 4-OH-Tam, MPP and THC 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 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) by functional mitochondria to formazan [30]. After a 2 h incubation of the cells with MTT at 37°C, the cells were lysed with dimethyl sulphoxide in Sorensen's glycine buffer and the formazan crystals solubilized. Absorbance was read at 550 nm using a spectrophotometric microplate reader (Bio-Rad).

### 2.7. Statistical analysis

For illustrative purposes, the results are presented as the mean percentage of control±S.E.M. Statistical analyses were carried out using one-way analysis of variance followed by the LSD-*t* test. Significance was set at $P<.05$.

## 3. Results

### 3.1. $E_2$ and isoflavones repress CRE-mediated transcription in MG-63 cells

MG-63 cells were transient transfected with a simple CRE-containing promoter (p2CRE-GL3)-driven luciferase reporter expression and then treated with increasing concentrations of $E_2$, genistein and daidzein for 24 h, respectively. As shown in Fig. 1A, $E_2$ treatment produced significant decreases in promoter activity of CRE-reporter gene over the concentration range of $10^{-9}$ to $10^{-6}$ mol/L. Maximal inhibition was obtained at $10^{-6}$ mol/L, with approximately 30%

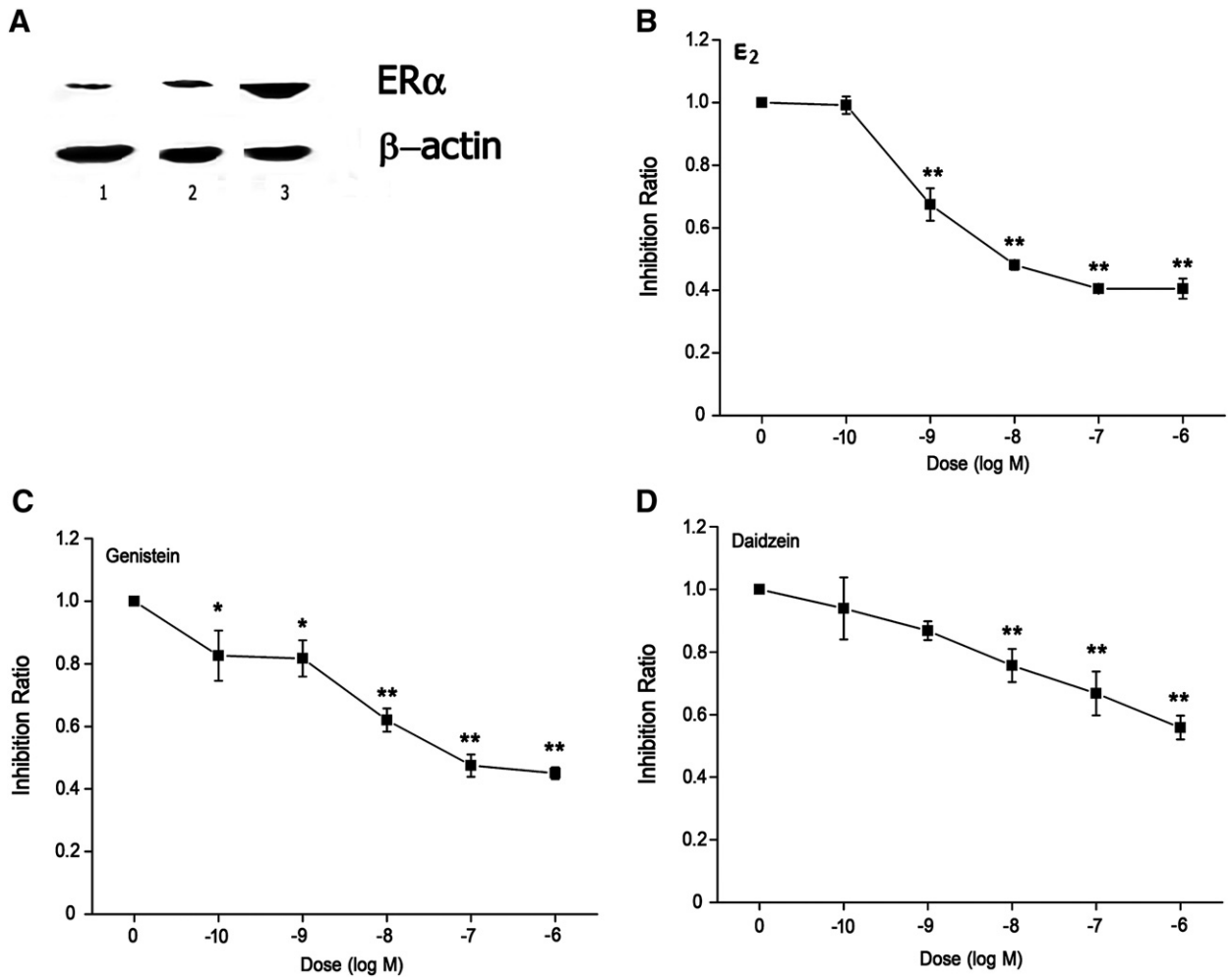


Fig. 4. $E_2$ and isoflavones repress CRE-mediated transcription in MG-63 cells transfected with ERα. MG-63 cells were cotransfected with CRE reporter plasmid and pCMV-ERα. (A) ERα protein expression was determined using Western blot analysis: 1, Cells without transfection; 2, Cells transfected with pCMV plasmid; and 3, Cells transfected with pCMV-ERα plasmid. (B–D) transfected MG-63 cells were exposed to $E_2$ (B), genistein (C) and daidzein (D) at the indicated concentrations for 24 h. Results are expressed as the inhibition ratio compared to the vehicle control. Each value represents the mean±S.E.M. from five independent experiments. *$P<.05$; **$P<.01$ compared with vehicle control.

inhibition. Treatment of cells with either genistein or daidzein also caused a reduction of CRE-promoter activity. The maximal decrease was seen at $10^{-6}$ mol/L of genistein and daidzein, with about 36% and 34% inhibition, respectively (Fig. 1B and C).

Generally, phosphorylated CREB is the active form of CREB and can bind to CRE motif, then induce the gene transcription. To investigate whether $E_2$ and isoflavones inhibited CRE-promoter activity through modulating CREB phosphorylation, MG63 cells were treated with $E_2$

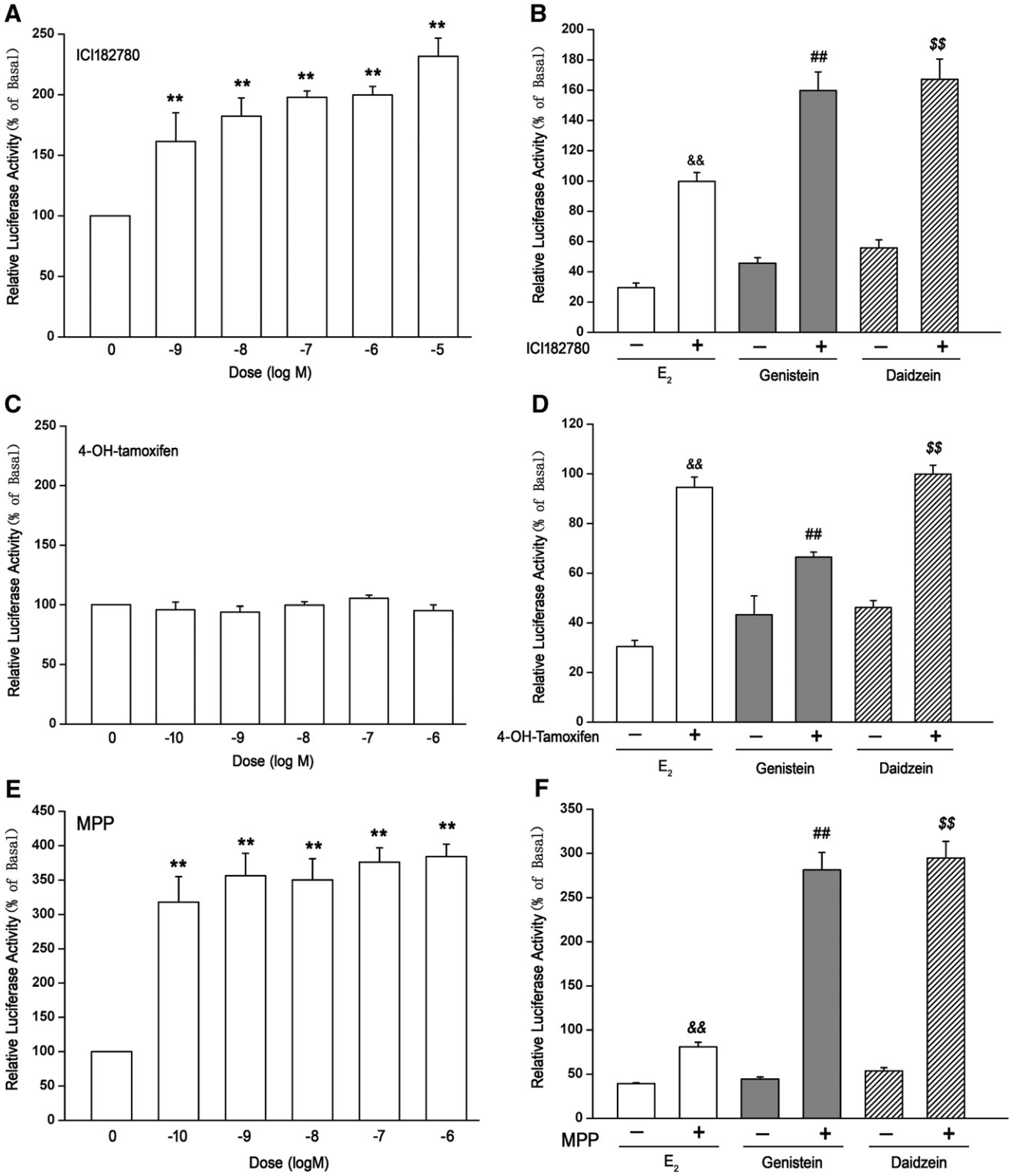


Fig. 5. Effects of ICI 182780, 4-OH tamoxifen and MPP on CRE-mediated transcription in MG-63 cells transfected with ERα. MG-63 cells were cotransfected with CRE reporter plasmid and pCMV-ERα. (A, C and E) Cells were exposed to ICI 182780 (A), 4-OH tamoxifen (C) and MPP (E) at the indicated concentrations for 24 h. (B, D and F) Cells were exposed to $E_2$ ($10^{-7}$ mol/L), genistein ($10^{-7}$ mol/L) and daidzein ($10^{-7}$ mol/L) with or without $10^{-6}$ mol/L ICI 182780 (B), $10^{-6}$ mol/L 4-OH-tamoxifen (D) or $10^{-6}$ mol/L MPP (F) for 24 h. Results are expressed as the percentage of vehicle control. Each value represents the mean±S.E.M. from four independent experiments. $^{**}P<.01$, compared with vehicle control. &&,##,$$$P<.01$, compared with $E_2$, genistein or daidzein treatment, respectively.

($10^{-7}$ mol/L), genistein ($10^{-6}$ mol/L) and daidzein ($10^{-6}$ mol/L) for 24 h. As shown in Fig. 1D, neither $E_2$ nor two isoflavones affected CREB phosphorylation in MG63 cells.

Activation of cAMP-dependent protein kinase (PKA) signaling pathways can turn on targeted genes by trans-activation through a consensus CRE motif. Thus, we observed the effects of $E_2$ and isoflavones on 8-Br cAMP-induced transcriptional activity of CRE reporter. As shown in Fig. 2, $E_2$ ($10^{-7}$ mol/L) significantly inhibited 8-Br cAMP-induced transcriptional activity of CRE reporter gene. Genistein ($10^{-6}$ mol/L) also produced an inhibition on cAMP-induced transcription whereas daidzein ($10^{-6}$ mol/L) had no effect on cAMP-induced transcription.

To determine whether concentrations of $E_2$ and isoflavones that affected CRE-dependent transcription were detrimental to cells, cell growth and survival were examined. Treatment of these cells for up to 24 h with $E_2$ and isoflavones had no significant effect on cell growth and viability at a dose of $10^{-6}$ mol/L compared with vehicle control (data not shown).

### 3.2. $E_2$ and isoflavones modulate mRNA expression of SGK1 and IL-8

To determine whether the effects of isoflavones on model promoters were similar to their effects on endogenous estrogen responsive genes in MG-63 cells, we examined steady state mRNA levels of two genes SGK1 and IL-8, both of which contain CRE-like elements in their promoter region [31, 32]. As shown in Fig. 3 A&B, $E_2$ at $10^{-9}$–$10^{-7}$ mol/L significantly inhibited mRNA levels of SGK1 and IL-8. At $10^{-7}$ mol/L, $E_2$ resulted in a 59% decrease in SGK1 and a 62% decrease in IL-8. Geinstein treatment caused a decrease in SGK1 and IL-8 mRNA level. At $10^{-6}$ mol/L, genistein inhibited SGK-1 mRNA expression by about 32% and IL-8 mRNA expression by about 45%. Daidzein did not affect SGK1 mRNA expression, whereas it at a $10^{-6}$ mol/L inhibited IL-8 mRNA expression by 29%. 8-Br cAMP ($10^{-5}$ mol/L) significantly simulated SGK1 and IL-8 mRNA expression. The effects of 8-Br cAMP on SGK1 and IL-8 mRNA expression were inhibited by $E_2$ ($10^{-7}$ mol/L) and genistein ($10^{-6}$ mol/L). Daidzein ($10^{-6}$ mol/L) inhibited 8-Br cAMP-induced IL-8 mRNA expression but did not affect 8-Br cAMP-induced SGK1 mRNA expression (Fig. 3C and D).

### 3.3. $E_2$ and isoflavones repression of CRE-mediated transcription with ERα

In order to elucidate if ERα mediated the effects of $E_2$ and isoflavones on CRE-mediated transcription, cells were cotransfected with CRE-reporter plasmid and ERα expression plasmid. Western blot analysis showed that the expression level of ERα in cells transfected with the ERα expression plasmid was greatly increased compared with control cells (Fig. 4A).

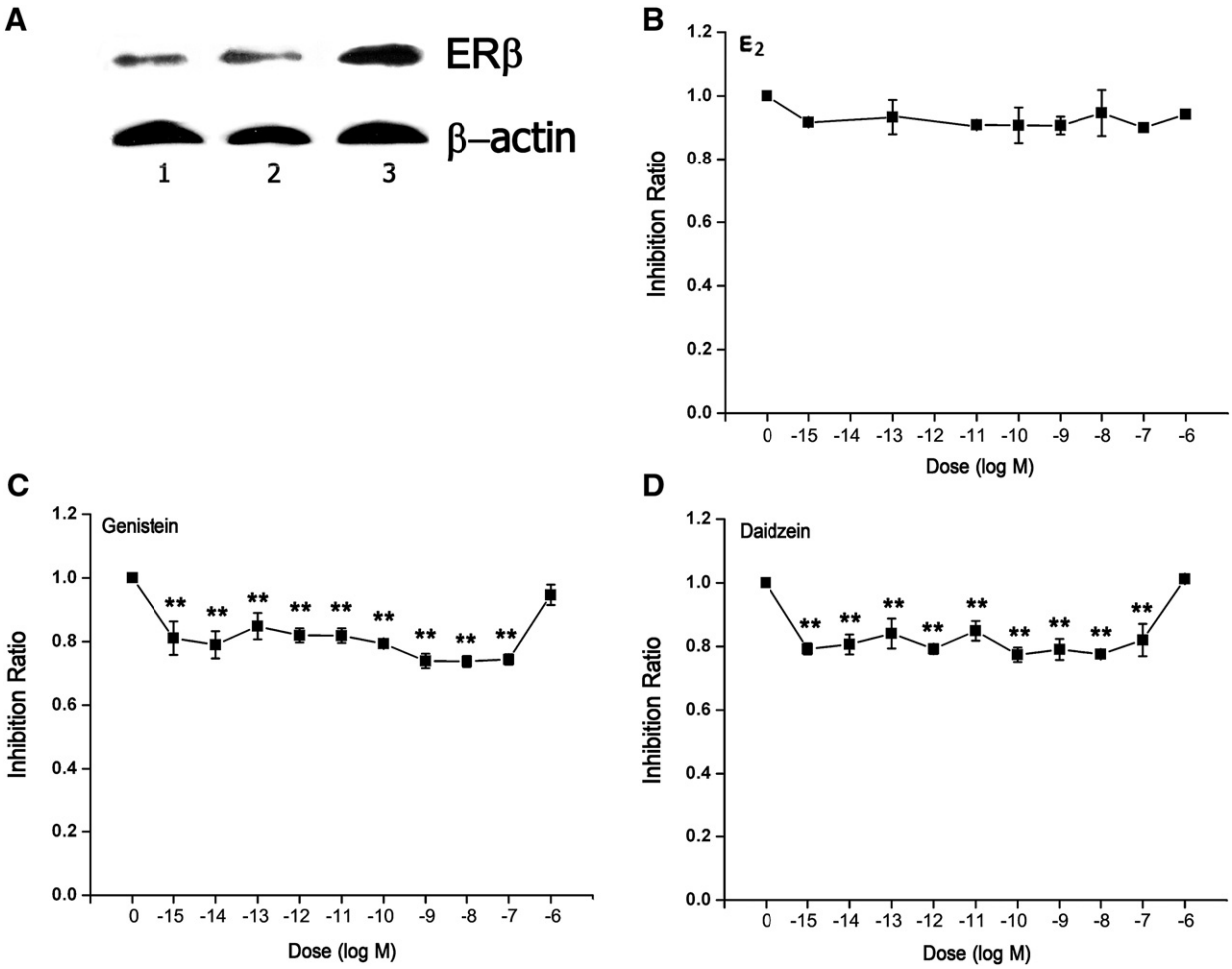


Fig. 6. Effects of $E_2$ and isoflavones on CRE-mediated transcription in MG-63 cells transfected with ERβ. MG-63 cells were cotransfected with CRE reporter plasmid and pcDNA-ERβ plasmid. (A) ERβ protein expression was determined using Western blot analysis: 1, cells without transfection; 2, cells transfected with pcDNA plasmid; 3, cells transfected with pcDNA-ERβ plasmid. (B–D) transfected MG-63 cells were exposed to $E_2$ (B), genistein (C) and daidzein (D) at the indicated concentrations for 24 h. Results are expressed as the inhibition ratio compared to the vehicle control. Each value represents the mean±S.E.M. from four independent experiments. $^{**}P<.01$, compared with vehicle control.

Treatment of these cells with increasing concentrations of $E_2$ resulted in greater inhibition of CRE-promoter activity compared to the cells without ERα overexpression. In ERα-transfected MG-63 cells, $E_2$ at $10^{-7}$ mol/L elicited maximal inhibition of CRE-promoter activity, up to about 60% inhibition (Fig. 4B); Genistein ($10^{-10}$–$10^{-6}$ mol/L) produced significant repression of CRE-promoter activity. At $10^{-6}$ mol/L, genistein reached the maximal inhibition by about 55% (Fig. 4C). Daidzein ($10^{-8}$-$10^{-6}$ mol/L) also showed a dose-dependent inhibition of CRE promoter activity, and the maximal inhibition occurred at $10^{-6}$ mol/L, with approximately 45% inhibition (Fig. 4D).

In order to confirm the role of ERα on the repression of CRE-promoter activity induced by $E_2$ and isoflavones, nonselective ER antagonists and specific ERα antagonist were used. ICI 182780 at $10^{-6}$

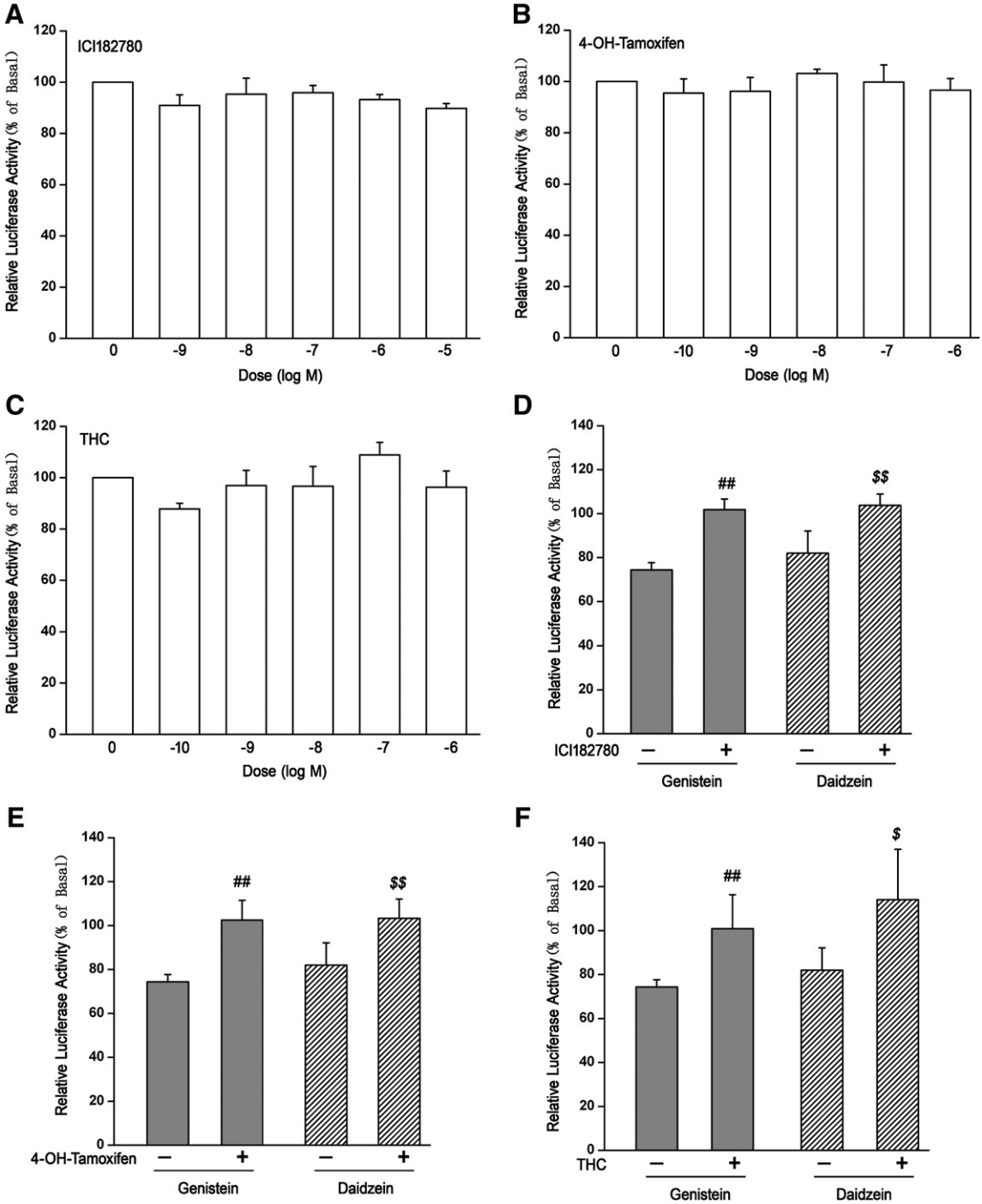


Fig. 7. Effects of ICI 182780, 4-OH tamoxifen and THC on CRE-mediated transcription in MG-63 cells transfected with ERβ. MG-63 cells were cotransfected with CRE reporter plasmid and pcDNA-ERβ plasmid. (A–C) Cells were exposed to ICI 182780 (A), 4-OH tamoxifen (B) and THC (C) at the indicated concentrations for 24 h. (D) Cells were exposed to genistein ($10^{-7}$ mol/L) and daidzein ($10^{-7}$ mol/L) with or without ($10^{-6}$ mol/L) ICI 182780 for 24 h. (E) Cells were treated with genistein ($10^{-7}$ mol/L) and daidzein ($10^{-7}$ mol/L) in the presence or absence of ($10^{-6}$ mol/L) 4-OH-tamoxifen for 24 h. (F) Cells were treated with genistein ($10^{-7}$ mol/L) and daidzein ($10^{-7}$ mol/L) in the presence or absence of ($10^{-6}$ mol/L) THC for 24 h. Results are expressed as the percentage of vehicle control. Each value represents the mean±S.E.M. from four independent experiments. ##$P$<.01 compared with genistein treatment; \$$P$<.05; \$\$$P$<.01; compared with daidzein treatment.

mol/L blocked the actions of $E_2$ ($10^{-7}$ mol/L), genistein ($10^{-6}$ mol/L) and daidzein ($10^{-6}$ mol/L) in ERα transfected cells (Fig. 5B). Treatment of cells with ICI 182780 alone caused stimulatory effect on CRE-mediated transcription with ERα (Fig. 5A). 4-OH tamoxifen at $10^{-6}$ mol/L blocked $E_2$ ($10^{-7}$ mol/L)- and daidazien ($10^{-6}$ mol/L)-induced inhibition of CRE-promoter activity and partly blocked genistein ($10^{-6}$ mol/L)-induced inhibition (Fig. 5D). 4-OH tamoxifen alone had no effect on CRE promoter activity (Fig. 5C). MPP, the selective ERα antagonist, at $10^{-6}$ mol/L blocked $E_2$ ($10^{-7}$ mol/L)-, genistein ($10^{-6}$ mol/L)- and daidzein ($10^{-6}$ mol/L)-induced inhibition of CRE-promoter activity in ERα-transfected cells (Fig. 5F). Treatment of cells with MPP alone resulted in an increase in CRE-mediated transcription (Fig. 5E).

### *3.4. Isoflavones but not $E_2$ regulation of CRE-mediated transcription with ERβ*

MG-63 cells were cotransfected with CRE-reporter plasmid and ERβ expression plasmid. As shown in Fig. 6A, the expression level of ERβ in cells transfected with the ERβ expression plasmid was greatly increased.

$E_2$ treatment had no effect on CRE promoter activity in ERβ transfected cells (Fig. 6B). Both genistein and daidzein treatment resulted in a decrease in CRE-promoter activity with ERβ. The statistical effects were achieved at the concentration range of $10^{-15}$–$10^{-7}$ mol/L (Fig. 6C—D). At $10^{-6}$ mol/L, genistein and daidzein did not affect the transcriptional activity of CRE reporter gene. The effects of either genistein or daidzein could be blocked by ICI 182780, 4-OH tamoxifen and the selective ERβ antagonist THC (Fig. 7D—F). Treatment of cells with ICI 182780, 4-OH tamoxifen or THC alone did not affect CRE-promoter activity in ERβ transfected cells (Fig. 7A–C).

## 4. Discussion

It has been shown that genistein and daidzein activate gene transcription via classical estrogen response elements (EREs) in various cells including osteoblastic cell lines [26,27]. However, no published information is available on isoflavaones modulation of gene transcription from alternative response element in osteoblastic cells. In the present study, we found that isoflavones, genistein and daidzein suppressed CRE-mediated transcriptional activity. In addition, we also demonstrated that the effects of genistein and daidzein on model promoter were similar to their effects on endogenous estrogen responsive gene in MG-63 cells.

Isoflavones, genistein and daidzein, are shown to be capable of binding to two known ERs, ERα and ERβ [24, 25]. ERα- or ERβ-dependent transactivation of EREs signaling pathway by genistein and daidzein has been found in many cell types [26,33,34]. Recently, Liu et al. [28] have shown that genistein enhances CRE-mediated gene transcription in endothelial cells, and these effects are independent on estrogen receptors. We showed that, in the present study, greater inhibition of CRE-mediated transcription induced by genistein or daidzein were obtained in either ERα- or ERβ-transfected cells. Moreover, these effects were blocked by global antiestrogen as well as the selective ERα and ERβ antagonists. These results suggest that the suppressive effects of isoflavones on CRE promoter activity are specific to ER activation.

We also showed that estrogen and isoflavones elicited distinct transcriptional actions. $E_2$ effectively triggered ERα-mediated transcriptional repression of CRE signaling pathways but did not affect ERβ-mediated transcription of CRE reporter. In contrast, isoflavones not only triggered transcriptional repression of CRE-mediated transcription with ERα but also inhibited CRE-promoter activity through ERβ. Previous studies have demonstrated that isoflavones bind to ERβ more effectively than to ERα, whereas $E_2$ binds to ERα and ERβ with equal affinity [35,36], which might partly explain the above results. Moreover, previous studies also suggest that divergent transcriptional actions of estrogen and isoflavones probably result from the differences in their ability to recruit coregulators and trigger transcriptional functions of ERs [37,38]. In addition, we also found that isoflavones repression of CRE-mediated transcription with ERβ was not in a dose-dependent manner. At a very low concentration (e.g., $10^{-15}$ mol/L), either genistein or daidzein could induce modest inhibition of CRE-promoter activity, whereas at the higher concentration $10^{-6}$ mol/L, they did not have inhibitory effect. It is hard to understand such effects of isoflavones on CRE promoter activity with ERβ. The study by Mueller et al. had also shown no dose-dependent induction of ERE-mediated transcription with ERβ by some phytoestrogens and their metabolites, such as zearalenone, in endometrium cells [39]. Nevertheless, more works should be done to explore the mechanisms by which isoflavones repress CRE promoter activity with ERβ.

Isoflavones have been proposed as natural selective estrogen receptor modulators (SERMs) due to their binding to ERs [40]. SERMs can act like estrogen in some tissues but block the action of estrogen in others [41]. The results of the present study indicate that isoflavones geinstein and daidzein exhibit similar effects as $E_2$, repressing CRE-mediated transcription in MG-63 cells through ERα. In addition, our study also demonstrated that SERMs ICI 182780 and MPP modulate CRE-mediate transcription with ERα in an opposite fashion to $E_2$, stimulating CRE promoter activity. These results are also consistent with our previous and An et al's studies where it was shown that $E_2$ and ICI 182780 exhibit opposite effects on gene transcription through CRE or CRE like motif with ERα [22,23].

A variety of endogenous factors, e.g., parathyroid hormone, modulate bone turn over through cAMP-dependent PKA signaling pathways and turn on targeted genes by transactivation through CRE motifs in the promoter region [42]. CREB is a member of the bZIP or leucine zipper family of transcription factors, is phosphorylated by several protein kinases and modulates gene transcription in response to stimulation of the cAMP pathways [43]. Estrogen was reported to modulate bone remodeling through cAMP-PKA signaling pathway [44]. One of critical roles that estrogen influences bone resorption is down-regulating the production of bone resorbing cytokines such as TNFα, IL-1, IL-6 and IL-8 in osteoblasts [45]. It has been demonstrated that estrogen suppresses TNFα gene expression through AP1/CRE motif in its promoter region [22]. The present study demonstrated that, in osteoblastic cells, estrogen repressed not only CRE-mediated transcription but also basal and cAMP-induced mRNA expression of endogenous genes that contained CRE and CRE-like motifs in their promoters. These results support the idea that estrogen modulates bone remodeling by modulating CRE-mediated transcription of target genes in osteoblastic cells. In addition, we also found that genistein exhibited similar effects as estrogen, inhibiting CRE-mediated transcription and the mRNA expression of endogenous genes containing CRE or CRE-like motif. It suggests that genistein might exert similar effects as estrogen in osteoblastic cells.

We also found that daidzein did not exhibit fully consistent effects on transcription of CRE-reporter gene and mRNA expression of endogenous genes as $E_2$ and genistein did. For example, daidzein did not affect cAMP-induced transcription of CRE-reporter gene, whereas $E_2$ and genistein inhibited cAMP-induced transcription of reporter gene. This suggests that the effects of daidzein on bone might be different from estrogen and genistein. The chemical structure of daidzein differs from genistein, which might account for the different actions of daidzein and genistein. The molecular mechanisms by which daidzein and genistein exert differential effects on cAMP-induced transcription of CRE-reporter gene need to be further explored.

In conclusion, isoflavones genistein and daidzein suppress CRE-mediated transcriptional activity through ERs and the mRNA

expression of endogenous genes that contain CRE/CRE-like elements in their promoter in MG-63 cells. Our results suggest that isoflavones might modulate bone turnover through ERs by regulating target gene expression via the CRE motifs in their promoter.

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