

Original Article

Suppression of NFκB related signal transduction pathway by daidzein in MC3T3-E1 cells

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Abstract: *Objective:* Postmenopausal osteoporosis (PMOP) is among one of the common metabolic bone diseases within primary osteoporosis. In recent years, isoflavone phytoestrogens have been widely adopted to treat PMOP due to their bone protective effect similar to estrogen and their having little side effect. Previous studies have shown that isoflavones might play a role in bone protection through the estrogen receptor (ER) in osteoblast cells. However, the downstream molecular mechanism of isoflavones action remains unclear. *Methods:* We investigated daidzein (DAI)'s influence on mouse osteoblast MC3T3-E1 cells. Our results demonstrate that both DAI and 17-β estradiol (E2) could promote proliferation of osteoblast cells. The expression level of the differentiation marker alkaline phosphatase (ALP) was also investigated, and our results further support that appropriate concentration of E2 could promote differentiation of MC3T3-E1 cells. In order to verify that isoflavones is capable of promoting bone formation through ERs and downstream inhibition of the NFκB signaling pathway, we activated the NFκB pathway with tumor necrosis factor (TNF-α) and then determined the expression level of related signaling factors. *Results:* Results from Western blot demonstrate that upon stimulation with TNF-α, the expression level of ERβ was significantly up-regulated, while the expression levels of NFκB pathway core proteins, IKKβ and RelA (p65), were down-regulated. *Conclusion:* Taken together, this study suggests that DAI might promote bone formation of osteoblast cells through binding to and triggering the overexpression of ERs, and ultimately, inhibiting the downstream NFκB pathway.

Keywords: Postmenopausal osteoporosis (PMOP), 17-β estradiol (E2), estrogen receptor, isoflavones, daidzein (DAI), NFκB pathway

Introduction

Postmenopausal osteoporosis (PMOP) is one of the common metabolic bone diseases within primary osteoporosis [1, 2]. As the lifespan of the global population becomes longer, more women are affected by PMOP as they age. Although estrogen replacement therapy (ERT) could effectively control PMOP, long-term use of estrogen has been shown to increase the risk of other diseases including breast cancer [3]. In recent years, researchers have started to focus on isoflavone phytoestrogens, due to their bone protective effect similar to estrogen and their having little side effect [4-6]. Studies have shown that isoflavones might also play a role in bone protection through the estrogen receptor (ERs) [7-11], however, the downstream molecular mechanism is still unclear. Elucidating the molecular effect of isoflavones on

bone protection would provide further evidence for the effectiveness of plant-based estrogen for the prevention and treatment of PMOP, in addition to providing new targets in new drug research and development.

Daidzein (DAI) is a typical isoflavone that potentially exerts its bone protective effect by stimulating osteoblast cells and inhibiting osteoclast cells via the estrogen receptor related pathway [12-16]. By combining our results with those from previous studies, we wish to examine whether daidzein is capable of promoting bone formation through ERs while also inhibits downstream NFκB signaling pathway activity like 17-β estradiol (E2).

We first investigated the effect of daidzein on the proliferation of mouse osteoblastic cell line MC3T3-E1 using 17β-estradiol (E2) as control.

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The proliferation of MC3T3-E1 cells, and the expression level of alkaline phosphatase (ALP), an osteoblast cell differentiation marker, at different drug concentrations were tested. Our results showed that both DAI and E2 could promote proliferation of osteoblast and increase ALP level. We then tested the expression levels of the signaling molecules of the intracellular ERs and NFκB signal factors after activating the NFκB pathway with tumor necrosis factor (TNF-α). Our result showed that the expression levels of ERβ and IκB were significantly up-regulated, while the expression levels of IKKβ and RelA (p65) were down-regulated.

Our results demonstrate that daidzein, similar to E2, might promote bone formation by suppressing NFκB related signal transduction pathway in MC3T3-E1 cells. However, future research is needed to elucidate the detailed molecular mechanism of how daidzein affect various components of the whole signaling pathway.

Materials and methods

Reagents

Daidzein, 17β-estradiol, Tumor Necrosis Factor-α, MTT, pNPP, DMSO and all other related reagents were purchased from Sigma-Aldrich; BCA protein assay reagent kit was purchased from Pierce; ECL Western blotting detection reagents were from GE healthcare; IκB α polyclonal antibody, NFκB1 (p50) polyclonal antibody, IKKβ (IKKβ) polyclonal antibody, RelA (p65) polyclonal antibody, ERβ polyclonal antibody, β-actin monoclonal antibody and GAPDH monoclonal antibody were obtained from Protein Tech; Polyclonal goat anti-rabbit/goat-anti mouse immunoglobulins/HRP were acquired from Invitrogen.

Cell culture

MC3T3-E1 cells were obtained from ATCC. MC3T3-E1 cells were cultured at 37°C and 5% CO₂ in α-MEM medium, supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM glutamine, 100 U/ml penicillin-streptomycin, non-essential amino acid solution and sodium pyruvate.

Cell proliferation assessment by MTT assay

MC3T3-E1 cell proliferation upon treatment with E2 and DAI were analyzed by the

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MC3T3-E1 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and cultured for 24 hours. Then, various concentrations of E2 and DAI were added and cells were cultured for 44 hours. In addition, 10 μl MTT (5 mg/ml) was added to the cell culture. Four hours later, the culture medium was removed and 150 μl DMSO was added to each well to dissolve the resulting formazan crystals. Absorbance was measured at 490 nm using an enzyme-linked immunosorbent assay reader (Thermo MULTISKAN MK3). Data were collected from three separate experiments. DMSO-treated cells were used as negative control.

Alkaline phosphatase activity assay

MC3T3-E1 cells at density of 1×10^4 per well were seeded in 96-well plate containing media with α-MEM and 10% FBS with or without E2 and DAI (Concentration ranging from 10^{-6} M to 10^{-12} M) and cultured for 48 hrs. Then, the culture medium was removed, and total ALP activity was measured using PNPP as substrate. After addition of 150 μl reaction mixture containing 25 mM diethanol amine, 1 mM MgCl₂, 6.7 mM PNPP and 0.2% Triton X-100, cells were cultured at 37°C for 30 min in a dark room. Next, 100 μl stop buffer containing 0.1 M NaOH was added, and ALP activity was measured colorimetrically at 405 nm.

Western blot assay

Cells were washed, collected and homogenized in lysis buffer (10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 2 mM dithiothreitol, 1% Triton X-100 and protease inhibitor cocktail), then centrifuged (16,000 g, 10 min, 4°C). The protein-containing supernatant was used for Western blot. To ensure that an equal amount of proteins was loaded into each case, total protein was quantified by BCA protein assay. Equal amounts of proteins (50 μg) were subjected to SDS-PAGE (10-12% gel). The gel-separated proteins were transferred to nitrocellulose membranes (GE Healthcare). Membranes were blocked with 10% bovine serum albumin in TBST [10 mM Tris-HCl (pH 8.0), 137 mM NaCl, and 0.05% Tween-20 by vol] overnight at 4°C and then probed with primary antibodies at room temperature (25°C) for 2 h. Each of the targeted proteins was subjected to immunostaining with specific antibodies. The membranes were

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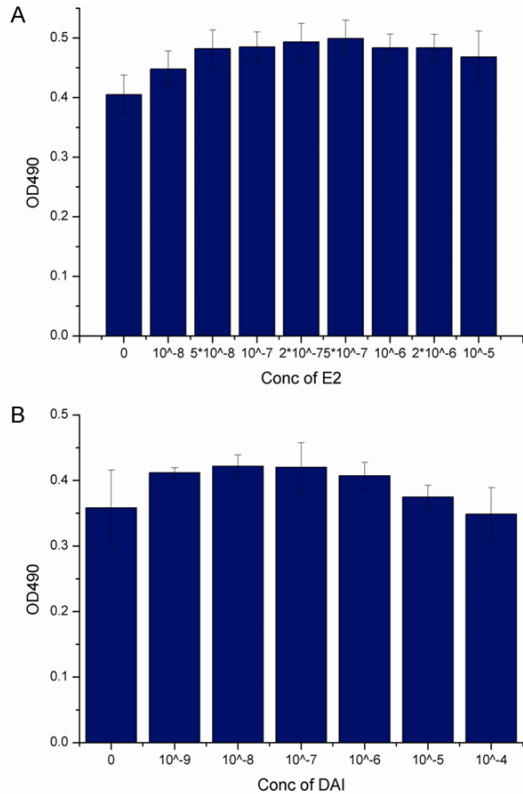


Figure 1. Effects of E2 and DAI treatment on the proliferation of MC3T3-E1 cells as assessed by MTT assay. A. Treatment with E2 at concentrations ranging from 10⁻⁸ M to 10⁻⁵ M; B. Treatment with DAI at concentrations ranging from 10⁻⁹ M to 10⁻⁴ M. OD490 was monitored for MTT assay. Data are shown as mean ± SD.

washed three times with TBST and then incubated at room temperature with HRP-conjugated secondary antibodies for 1 hour before being visualized with an ECL chemiluminescence detection kit.

Statistical analysis

All experiments were carried out in triplicates. Statistical significance was assessed with one-way ANOVA using the SPSS software system. Data were presented as mean values ± standard deviation. *P* values of <0.05 were considered as statistically significant.

Results

Appropriate concentration of DAI and E2 promotes MC3T3-E1 cell proliferation

In order to verify that DAI and E2 affect the proliferation rate of osteoblast cells, we chose

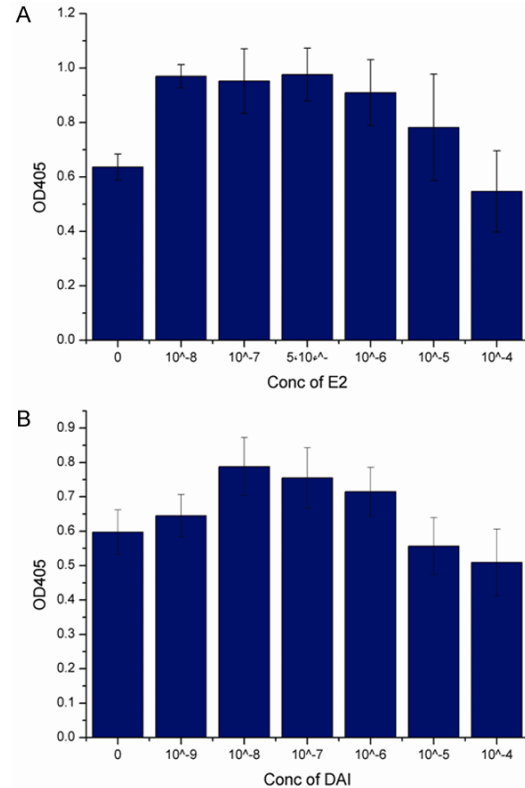


Figure 2. Effects of E2 and DAI on osteoblast differentiation MC3T3-E1 were seeded in 96-well plate and exposed to various concentrations of E2 and DAI for 48 hours. ALP activity was determined spectrophotometrically at 405 nm. Data are shown as mean ± SD.

mouse MC3T3-E1 cells as our model, and assessed their proliferation upon treatment with different concentrations of DAI. We also tested the proliferation of MC3T3-E1 upon E2 treatment as a control. Ten thousand cells per well were cultured for 24 hours after passage, then different concentrations of DAI and E2 ranging from 10⁻⁸ M to 10⁻³ M were added and the cells were cultured for another 48 hours. Cell proliferation was assessed by MTT assay. Results from the MTT assay were summarized in **Figure 1**. Both E2 and DAI affected the proliferation of MC3T3-E1 cells, with maximal effects observed at around 10⁻⁸-10⁻⁷ M. Further experiments showed DAI had the highest stimulation effect at around 5 × 10⁻⁷ M. On the other hand, higher concentrations of E2 and DAI inhibited proliferation of MC3T3-E1 cells. Statistical analysis demonstrated that treatment with E2 and DAI at 10⁻⁸-10⁻⁷ M led to significant improvement in proliferation rate.

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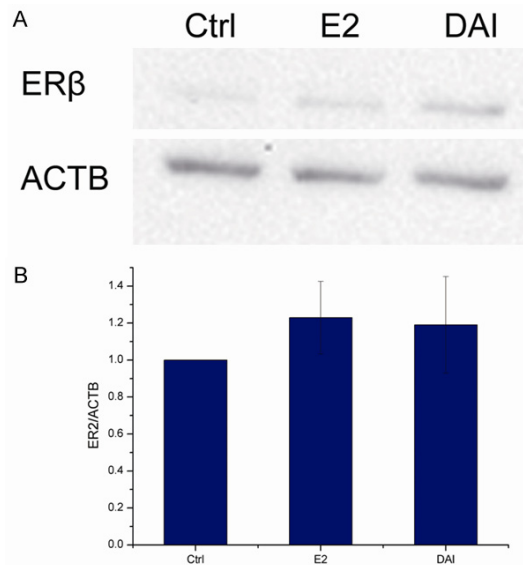


Figure 3. Influence of E2 and DAI treatment on the expression level of ERβ as demonstrated by Western blot. A. Western blot results showed that E2 and DAI treatments affected the expression of ERβ. The Ctrl, E2 and DAI sets are the negative control (0.1% DMSO), E2 (5×10^{-7} M) treatment and DAI (5×10^{-7} M) treatment group. Cells were cultured for 24 hours after passage, and then treated with the respective drugs. Cells were collected and analyzed by Western blot 48 hours later. Beta-actin antibody was used as an internal control; B. Quantification of Western blot results (n=8), data are shown as mean \pm SD.

E2 and DAI treatments increase ALP activity in MC3T3-E1 cells

Since proliferating osteoblasts exhibit ALP activity that is greatly enhanced during in vitro bone formation. ALP activity is therefore, a feasible marker for investigating the effect of E2 and DAI treatment on MC3T3-E1 cells. ALP catalyzes the hydrolysis of p-nitrophenyl phosphate (pNPP) to p-nitrophenol. Since pNPP is colorless while p-nitrophenol demonstrates strong absorbance at 405 nm, ALP activity can be easily detected as the increase in rate of absorbance at 405 nm, which is proportional to ALP enzyme activity.

As shown in **Figure 2**, both E2 and DAI significantly increased ALP activity in MC3T3-E1 cells. Our result showed that daidzein stimulates ALP activity with the highest efficiency observed at around 10^{-8} M.

Similar to the trends demonstrated by the cell proliferation assay, effect of E2 and DAI on ALP activity was also dose-dependent, with ALP

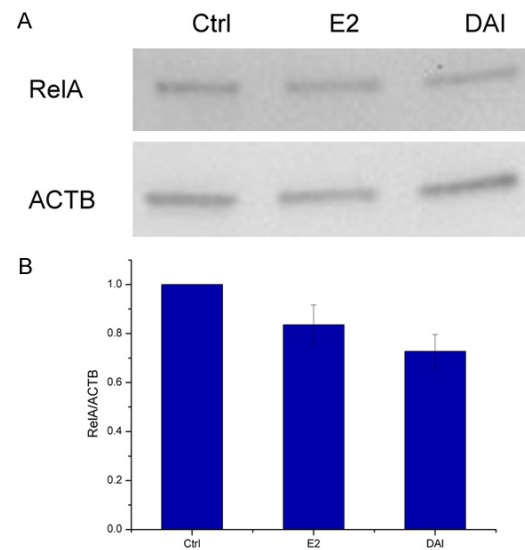


Figure 4. Effect of E2 and DAI treatment on the expression level of RelA (p65) as measured by Western blot. A. Western blot results showed that E2 and DAI treatments affected the expression of RelA (p65). The Ctrl, E2 and DAI sets are the negative control (0.1% DMSO), E2 (5×10^{-7} M) treatment and DAI (5×10^{-7} M) treatment group. Cells were cultured for 24 hours after passage and then treated with the respective drugs. Cells were collected and analyzed by Western blot 48 hours later. Beta-actin antibody was used as an internal control; B. Quantification of Western blot results (n=15), data are shown as mean \pm SD.

activity continued to increase until treatment at around 10^{-8} M, followed by a gradual decline upon treatment at above that concentration. These results showed E2 and DAI significantly contribute to osteoblast ALP activity.

Influence of E2 and DAI on the estrogen receptors (ERs) and NFκB related signaling pathway

Previous study has shown that E2 can promote bone formation through ERs. In order to verify that isoflavones can also promote bone formation through ERs, we measured the expression level of ERβ upon addition of DAI. As shown in **Figure 3**, Western blot results demonstrated that both E2 and DAI treatment significantly improved the expression level of ERβ.

We also determined the expression levels of other important components in the NFκB pathway. As shown in **Figures 4** and **5**, both E2 and DAI treatments significantly decreased the expression level of RelA (p65) and IKKβ. It is possible that treatment with E2 and DAI suppressed the expression of IKKβ, which then

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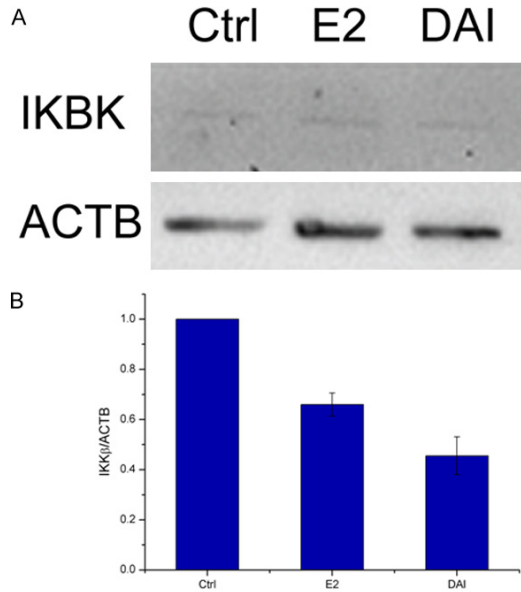


Figure 5. Effect of E2 and DAI treatment on the expression level of IKKβ as assessed by Western blot. A. Western blot results showed that E2 and DAI treatments affected the expression of IKKβ. The Ctrl, E2 and DAI sets are the negative control (0.1% DMSO), E2 (5×10^{-7} M) treatment and DAI (5×10^{-7} M) treatment group. Cells were cultured for 24 hours after passage and then treated with the respective drugs. Cells were collected and analyzed by Western blot 48 hours later. Beta-actin antibody was used as an internal control; B. Quantification of the Western blot results (n=4), data are shown as mean \pm SD.

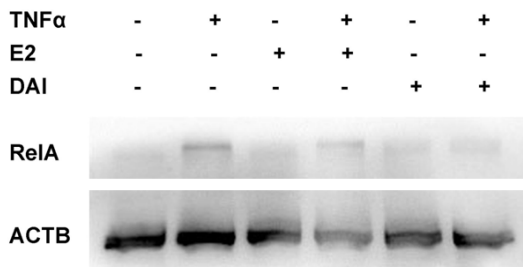


Figure 6. Effect of E2 and DAI treatment on the expression level of RelA (p65) after TNF-α (20 ng/ml) activation as measured by Western blot. Lane 1, 3, 5, contained control without TNF-α treatment, lane 2, 4, 6, were samples treated with 20 ng/ml TNF-α. Lane 1 and 2 were not subjected to drug treatment; lane 3 and 4 were treated with E2 (5×10^{-7} M); lane 5 and 6 were treated with DAI (5×10^{-7} M). Cells were cultured for 24 hours after passage, then treated with E2, DAI or control DMSO. After 47 hours, TNF-α or control DMSO were added. An hour later, the cells were collected and analyzed by Western blot. Beta-actin antibody was used as an internal control.

resulted in the decrease in RelA (p65) expression and ultimately inhibited the NFκB pathway in osteoblast cells.

We employed the NFκB signaling pathway inducer, TNF-α, to verify the effect of DAI treatment. As shown in **Figure 6**, after 1 hour of TNF-α treatment, RelA (p65) expression increased significantly. However, when cells were treated with E2 or DAI, the expression level of RelA (p65) decreased significantly compared to the control group, indicating that DAI is capable of inhibiting the NFκB signaling pathway even when the pathway is activated.

Discussion

Our results showed that daidzein could potentially promote bone formation by suppressing the expression of NFκB related proteins in MC3T3-E1 cells. However, the detailed mechanism of such effect, especially the post-translational modification such as the phosphorylation, of related proteins, still requires further investigation. We plan to follow up our findings using Western blot and other related techniques.

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Disclosure of conflict of interest

None.

Abbreviations

PMOP, postmenopausal osteoporosis; E2, 17-β estradiol; DAI, daidzein; EQ, equol; ERs, estrogen receptors; IκB, inhibitors of nuclear factor-kappa B; IKK, kappa B inhibitors kinase; ALP, alkaline phosphatase; TNF-α, tumor necrosis factor-α.

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