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Phytoestrogen-Induced Phosphorylation of MAP Kinase in Osteoblasts is Mediated by Membrane Estrogen Receptor

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We have previously demonstrated that phytoestrogens isolated from safflower seeds significantly attenuated bone loss in ovariectomized rats, and directly stimulated proliferation and differentiation of cultured osteoblastic cells. In an attempt to elucidate underlying cellular mechanisms, in the present study we investigated effects of 17 β-estradiol (E_2) and phytoestrogens such as matairesinol and acacetin, a type of lignan and flavonoid, respectively, on activation of mitogen activated protein (MAP) kinases, extracellular signal-regulated kinase 1 (ERK1) and ERK2, in cultured osteoblastic ROS 17/2.8 cells. Western blot analysis with anti-MAP kinase antibody showed that a wide range concentrations (10^{-14} to 10^{-6} M) of E_2 as well as both phytoestrogens induced rapid and transient activation of ERK1/2 through phosphorylation within minutes. Maximum activation of MAP kinases by E_2 and phytoestrogens were observed at 10 and 15 min, respectively. E_2-induced phosphorylation of ERK1/2 returned to the control level at 30 min, whereas phytoestrogen-induced phosphorylation was maintained at high level until 30 min. PD-98059, a highly selective inhibitor of MAP kinase, prevented phosphorylation of ERK1/2 in the cells treated either with E_2 or phytoestrogens. To examine a possible involvement of estrogen receptor in the activation process of MAP kinase, Western blot analysis was performed in the presence and absence of the estrogen receptor antagonists, ICI 182,780 and tamoxifen. These antagonists blocked MAP kinase phosphorylation induced not only by E_2, but also by the phytoestrogens. To the best our knowledge, this study is the first to demonstrate that phytoestrogens such as flavonoid and lignan extracted from safflower seeds produce a rapid activation of MAP kinase, at least partially via membrane estrogen receptor of the cultured osteoblastic cells.

Key Words: Lignan, Flavonoid, Estrogen, Osteoblast, ERK1/2

INTRODUCTION

The crucial role played by estrogen in bone remodeling is widely accepted. Postmenopausal osteoporosis is associated with estrogen deficiency (Ibrahim & Hortobagyi, 1999), and it is well documented that hormone replacement therapy (HRT) minimizes bone loss (Michaelsson et al, 1998). However, due to breast or endometrial cancer risks associated with long-term use of HRT, the use of estrogen has been restricted (Genant et al, 1989; Persson et al, 1999). To overcome such limitations, alternative natural substances minimizing bone loss have been extensively searched and beneficial health aspects of phytoestrogens have been suggested (Dwyer et al, 1994, Brezezinski & Debi 1999).

Phytoestrogens, found in plants and foods, are diphenolic compounds with structural similarities to natural and synthetic estrogens; they bind the estrogen receptors with much lower affinity than 17 β-estradiol (E_2) (Adams et al, 1989; Miksicek 1994; Brzezinski & Debi, 1999). There are evidences indicating that phytoestrogens, like certain selective estrogen receptor modulators, have estrogenic action on the bone and cardiovascular system, and have antiestrogenic action on the breast (Dwyer et al, 1994; Brezezinski & Debi 1999). Major classes of phytoestrogens are isoflavones, lignans and coumestans (Draper et al, 1997). Soybeans and soy foods are the most significant dietary sources of isoflavones, oilseeds such as flaxseed are of lignans, and sprouts of clover and alfalfa are of coumestants (Axelson et al, 1982; Setchell & Adlercreutz, 1988; Kurzer & Xu 1997).

Seeds of safflower (*Carthamus tinctorious* L.) have long been clinically used in Korea as a herbal medicine for promotion of bone formation (Dongeuibokam 1989). However, the effects have not been scientifically investigated. Six polyphenolic compounds were isolated from safflower seeds which were found to be a rich source of flavonoids and lignans (Kang et al, 1999). Recently, we demonstrated that feeding defatted safflower seeds attenuated ovariectomy-induced bone loss (Kim et al, 2002), and the polyphenolic compounds in safflower seeds enhanced proliferation and differentiation of osteoblastic cells (Kim, 2001).

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ABBREVIATIONS: MAP, mitogen activated protein; ERK, extracellular signal-regulated kinase; E_2, 17 β-estradiol; ER, estrogen receptor; HRT, hormone replacement therapy.

The mechanisms by which E_2 and phytoestrogens affect the bone metabolism are not clear. The presence of functional estrogen receptors (ER) in osteoblasts (Komm et al, 1988) implies that E_2 acts directly on the function of bone cells. Besides the classical nuclear receptor-mediated E_2 action, increasing evidence for non-genomic effects and for specific binding sites for E_2 on various cells membranes has been accumulated (Lieberherr et al, 1993). E_2 in osteoblasts induces a rapid increase in intracellular Ca^{2+} (Lieberherr et al, 1993) and activates mitogen activated protein kinase (MAP kinase) (Endoh et al, 1997). MAP kinase p42 and p44, also described as extracellular signal-regulated kinases (ERK)1 and ERK2, are thought to play a pivotal role in integrating and transmitting transmembrane signals required for the growth and differentiation of osteoblasts (Cobb et al, 1991; Lai et al, 2001). Thus, the activation of this signaling pathway may represent a potential mechanism by which E_2 regulates functions of osteoblasts. In the present study, we investigated potential involvement of MAP kinase in the intracellular signaling induced in osteoblastic cells by phytoestrogens such as lignan and flavonoid extracted from safflower seeds. Possible involvement of the ER in the E_2- and phytoestrogen-induced MAP kinase activation was also examined.

METHODS

Cell culture

A rat osteoblast-like cell line, ROS17/2.8, was maintained in Dulbecco's Modified Eagles Medium (DMEM) (Gibco; Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (HyClone Lab Inc.) and 1% antibiotic-antimycotic (100 U/ml penicillin and 50 μg/ml streptomycin, Life Tech Inc.) at 37°C under a humidified atmosphere of 5% CO_2 in air

Western blot analysis of ERK expression

ROS 17/2.8 were plated on 60 mm^2-culture dish at a density of 4×10^5 cells and grown in phenol-red free DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic. The attached cells were starved to arrest cell growth with serum free DMEM media for 24 hours. The cells were treated with tamoxifen and ICI-182,780, estrogen receptor antagonists, and PD98059 (50 μM), a highly selective inhibitor of MAP kinase-extracellular signal regulated protein kinase ERK1 and ERK2, 30 min prior to stimuation with E_2 and E_2-BSA, and then were exposed to E_2, E_2-BSA and phytoestrogens (10^{-8} M). After the treatment, the cells were washed twice with PBS, and lysed with TPK lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 1 mM Na_3VO_4, 1 mM phenylmethyl-sulfonyl fluoride, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1% Nonidet P-40, pH 8.0). The cell lysates were centrifuged at 12,000 g for 20 min and the supernatant was then used. Protein concentration was quantified with Bradford assay (Bio-Rad Laboratory, CA).

For Western immunobloting, 30 μg of protein were denatured with SDS sample buffer, and subjected to electrophoresis on 10% SDS-polyacrylamide gel. Blots were transferred to nitrocellulose membrane (Bio-Rad Laboratory, CA). After blotting, the membrane was immediately placed into blocking buffer (5% nonfat milk in TBST solution (50 mM Tris, 10 mM NaCl pH 7.4 0.1% Tween 20)) and left for 1~2 hr at room temperature while shaking. The blocking buffer was discarded and replaced with new blocking buffer containing the primary antibody (1 : 1,000 dilution) (New England Biolab, USA). The membrane was incubated with the primary antibody solution on a shaker overnight. The membrane was then washed 3 times for 10 minutes with TBST solution on a shaker. The membrane was then placed in 5% nonfat milk in TBST solution containing secondary antibody anti-mouse IgG (1 : 2,000 dilution) conjugated to horseradish peroxidase (Amersham Pharmacia biotech, UK). The secondary antibody was incubated for 90 min at room temperature with agitation. After washing, each protein band was visualized on X-ray film using ECL system (Amersham Pharmacia biotech, UK) according to the manufacturer's instruction.

Primary antibodies used in the present studies, monoclonal anti-phospho-specific p44/p42 MAP kinase antibody, detect phosphorylated ERK1 and ERK2 (p44 MAP kinase and p42 MAP kinase), respectively, only when activated by phosphorylation at Thr202 and Tyr204 by the upstream kinase. However, this antibody does not cross-react with unphosphorylated MAP kinase.

RESULTS

Effects of E_2 and E_2-BSA

As shown in Fig. 1, Western blot analysis revealed rapid

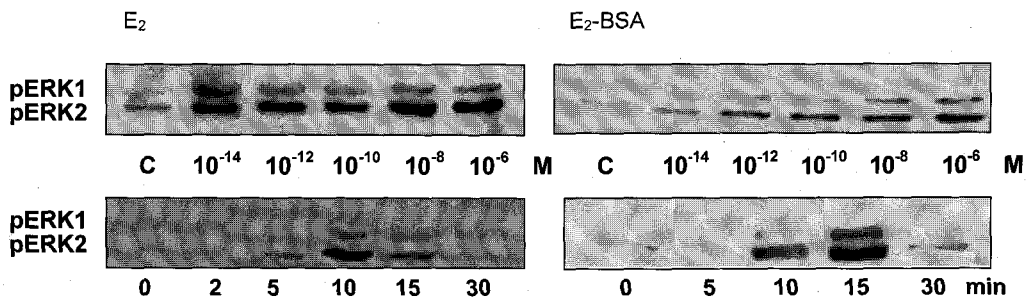

Fig. 1. Effect of 17 β-estradiol (E_2) and E_2-BSA on phosphorylation of ERK1/2 in ROS 17/2.8 cells. The cells were treated with E_2 or E_2-BSA for 10 or 15 min, respectively, at the concentrations indicated, and for the periods indicated at 10^{-8} M.

phosphorylation of MAP kinase in ROS17/2.8 cells by E_2, and the phosphorylation was dose-dependent of E_2 in the range between 10^{-14} and 10^{-6} M. Maximum phosphorylation of ERK1 and ERK2 by E_2 was achieved at 10 min and the phosphorylation returned to the control value within 30 min. The reference levels of phosphorylated ERK2 in the control untreated cultures were higher than phosphorylated ERK1 levels.

Possible involvement of putative membrane surface receptors was tested by using E_2-BSA. As shown in Fig. 1, E_2-BSA also induced rapid phosphorylation of MAP kinase in a dose-dependent manner, and the maximum response was shown at 15 min.

ER antagonists, tamoxifen and ICI-182,780, and MEK inhibitor, PD-98059, abolished the phosphorylation of ERK1 and ERK2 induced by E_2 and E_2-BSA (Fig. 2).

Effects of phytoestrogens: flavonoid and lignan

Both acacetin, a flavonoid, and matairesinol, a lignan, induced rapid phosphorylation of ERK1 and ERK2. In contrast to E_2, acacetin- and matairesinol-induced phosphorylation of ERK1 and ERK2 peaked at 5~10 min and the peaks were maintained up to 30 min (Fig. 3). When the effect of acacetin and matairesinol concentrations was examined at 10 min, a wide range of concentrations (10^{-14}~

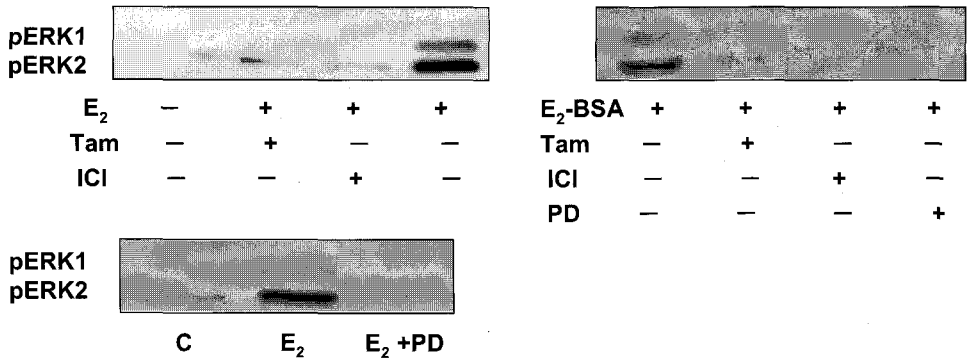

Fig. 2. Effect of estrogen receptor antagonists, tamoxifen and ICI-182780, and MAP kinase inhibitor, PD-98059, on phosphorylation of ERK1/2 induced by E_2 and E_2-BSA in ROS 17/2.8 cells. The cells were treated with 10^{-8} M E_2 and E_2-BSA for 10 min in the absence or presence of 10^{-8} M tamoxifen, ICI-182780 and PD-98059.

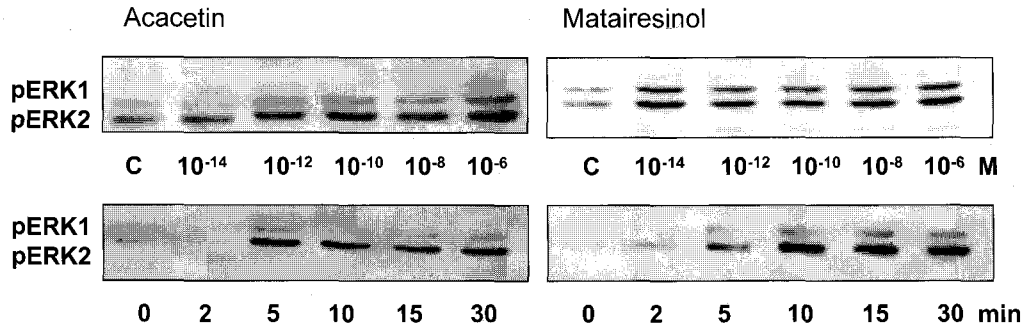

Fig. 3. Effect of acacetin and matairesinol on the phosphorylation of ERK1/2 in ROS 17/2.8 cells. The cells were treated with E_2 and E_2-BSA for 10 min at the concentrations indicated, and for the periods indicated at 10^{-8} M.

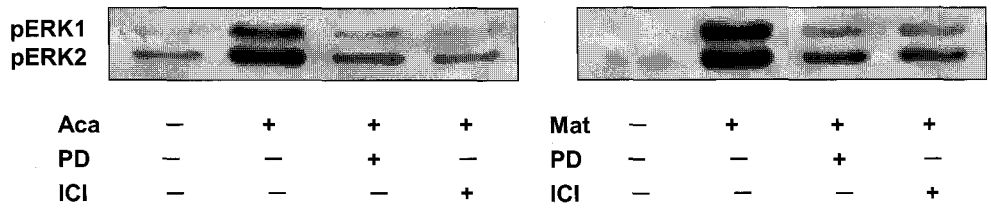

Fig. 4. Effect of ICI-182780, an estrogen receptor antagonist, and PD-98059, a MAP kinase inhibitor, on phosphorylation of ERK1/2 induced by acacetin and matairesinol in ROS 17/2.8 cells. The cells were treated with 10^{-8} M acacetin and matairesinol for 10 min in the absence or presence of 10^{-8} M ICI-182780 and PD-98059.

10^{-6} M) were active in inducing phosphorylation of ERK1 and ERK2, which was more pronounced for ERK2 than for ERK1.

Both acacetin- and matairesinol-induced MAP kinase phosphorylation was abolished not only by MEK inhibitor, PD-98059, but also by an estrogen receptor antagonist, ICI-182,780 (Fig. 4).

DISCUSSION

We previously demonstrated that similar to estrogen, naturally occurring phytoestrogens such as flavonoids and lignans extracted from safflower seeds stimulated proliferation and differentiation of osteoblastic cells (Kim, 2001). To the best of our knowledge, the study described herein is the first to demonstrate that phytoestrogens induced rapid phosphorylation of MAP kinases via estrogen receptor in osteoblastic ROS 17/2.8 cells. Phosphorylation of the MAP kinases, ERK1 and ERK2, could be induced even with very low concentration of 10^{-14} M phytoestrogens.

Estrogen induced MAP kinase phosphorylation in osteoblasts within 5 min. This action was most likely mediated through a plasma membrance receptor, which was again confirmed in the present study by using membrane impermeable BSA bound to estrogen. Other previous studies also showed a link between the surface estrogen receptor and the MAP kinase signaling cascade in various cell types such as osteoblast cells (Endoh et al, 1997), endothelial cells, neurons and breast cancer cells (Migliaccio & Di, 1996; Collins & Webb 1999; Goetz 1999). In osteoblasts, estrogen rapidly (5~120 sec) increases intracellular Ca^{2+} concentration via Ca^{2+} influx from extracellular fluid and via Ca^{2+} mobilization from the endoplasmic reticulum (Lieberherr et al. 1993). Estrogen also rapidly increases inositol 1,4,5-triphosphate and diacylglycerol production by activating phospholipase C in the membrane of osteoblasts. Taken together, it is clear that the estrogen receptor is not only a classical ligand-induced transcriptional enhancer, but also a mediator of common intracellular signaling pathways in multiple cell types.

Although the present study demonstrated that phytoestrogens like estrogen induced phosphorylation of the MAP kinase in the osteoblastic cells, the underlying mechanisms remain unclear. One possibility may be related to the agonistic effect of phytoestrogens on the estrogen receptor (Adams et al, 1989; Miksicek 1994; Brzezinski & Debi, 1999), since estrogen receptor antagonists, tamoxifen and ICI-182,780, clearly inhibited by phytoestrogens-induced MAP kinase phosphorylation. Furthermore, both flavones and lignans, like estrogen, enhanced gene expression of estrogen receptor subtypes of α and β (Kim, 2001). These results suggest that phytoestrogens regulate functions of osteoblasts via estrogen receptors, at least in part. However, possibilities of activating intracellular signaling pathways through other mechanisms should also be elucidated.

Physiological relevance of the membrane-associated phenomena in osteoblasts is not obvious. Upon binding to estrogen, both the membrane estrogen receptors α and β activate ERK-related kinases, leading to cell proliferation in multiple cell types (Razandi et al, 1999). However, further studies are required to investigate any link between phytoestrogen-initiated MAP kinase activation and phytoestrogen-mediated proliferation and differentiation of osteoblasts.

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