Biochanin A Stimulates Osteoblastic Differentiation and Inhibits Hydrogen Peroxide-Induced Production of Inflammatory Mediators in MC3T3-E1 Cells

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Phytoestrogens are plant chemicals that are structurally analogous to estrogen and are known to affect estrogenic activity. Biochanin A, a naturally occurring isoflavone, has been identified and detected in various diets and plant species. We examined the effects of biochanin A on the differentiation of osteoblastic MC3T3-E1 cells and the production of local factors in osteoblasts. Biochanin A (1—50 μM) caused a significant elevation of cell growth, alkaline phosphatase (ALP) activity, collagen content, and osteocalcin secretion in osteoblastic MC3T3-E1 cells (p<0.05). The effect of biochanin A (10 μM) in increasing ALP activity and collagen content was completely prevented by the presence of 10⁻⁸ M cycloheximide and 10⁻⁸ M tamoxifen, suggesting that biochanin A's effect results from a newly synthesized protein component and might be partly involved in estrogen action. We then examined the effect of biochanin A on the H₂O₂-induced production of inflammatory mediators in osteoblasts. Biochanin A (1—10 μM) decreased the 0.2 mM H₂O₂-induced production of TNF-α, IL-6 and NO in osteoblasts. These results suggest that biochanin A may be useful as potential phytoestrogens, which play important physiological roles in the prevention of postmenopausal osteoporosis.

Key words biochanin A; osteoblastic MC3T3-E1 cell; differentiation

Osteoporosis associated with estrogen deficiency is the most common cause of age-related bone loss. Hormone replacement therapy (HRT) can resolve most postmenopausal problems. However, compliance with HRT is poor because of risks of breast and endometrial cancers associated with long-term use of HRT.¹ In an effort to search for an alternative treatment, potential health benefits of phytoestrogens have been suggested.² Phytoestrogens are naturally occurring plant-derived nonsteroidal estrogens which are present in the human diet. Their chemical structure is similar to that of estrogen, what enables them to bind the estrogen receptor thus acting as estrogen agonists or antagonists.³ Isoflavonoids are a group of natural plant substances that share structural similarity to natural animal estrogens, such as estradiol, and exhibit affinity for the estrogen receptor (ER).⁴ Dietary consumption of isoflavones has been linked to lower risks for breast cancer⁵ and prostate cancer,⁶ as well as protection from osteoporosis and post-menopausal “hot flashes”.⁷

Biochanin A, one of the major isoflavonoids in pasture legumes is a methoxylated isoflavone not present in soy foods, but rather is the major isoflavone constituent in red clover and in commercially available extracts of this plant.⁸ The presence of biochanin A in mammalian cell cultures reduced the metabolism of benzo[a]pyrene (B[a]P) to mutagenic intermediates by 54% and the binding of metabolites to DNA by 30% to 40%.⁹ In vivo carcinogenic studies in a mouse B[a]P-induced lung tumor model revealed that biochanin A significantly reduced the incidence of tumor as well as the mean number of tumors.¹⁰ Biochanin A showed transactivation potentials at 10⁻⁸ M concentrations and had efficiencies of 51% compared to 17β-estradiol (efficiency 100%) in yeast system containing human ER.¹¹ In the HepG2 cell based transactivation assay, biochanin A had efficiencies of 150% compared to 17β-estradiol (efficiency 100%). Also, biochanin A that activate the estrogen receptor in both yeast and mammalian cells bind efficiently to hER.¹²

Normal bone remodeling is achieved by a balance of bone formation and bone resorption. These processes are closely regulated and are under the control of both systemic hormones as well as locally derived cytokines.¹² The inflammatory cytokine tumor necrosis factor (TNF) have been shown to exert complex effects on bone remodeling.¹³ As the bone cell responsible for bone formation, the osteoblast appears to be the bone cell targeted by TNF-α, in mediating their inhibitory effect on bone formation. However, it is now clearly established that the process of osteoclastic bone resorption mediated by TNF-α is also dependent on the coexistence of osteoblasts, suggesting that TNF-α stimulate the release of soluble factors from osteoblasts, which, in turn, stimulate osteoclasts to resorb bone.¹⁴ MC3T3-E1 cells, an osteoblast-like cell line, have been reported to retain the capacity to differentiate into osteoblasts¹⁵ and to have both estrogen receptors (ER) α and β.¹⁶,¹⁷ Those cells may provide very useful information about the effects of phytoestrogens on the differentiation of osteoblasts. In the present study, the in vitro effect of biochanin A on the activity of osteoblasts and the production of bone-resorbing mediators in osteoblastic MC3T3-E1 cells was investigated in order to determine the possible bioactivities of biochanin A on bone metabolism.

MATERIALS AND METHODS

Reagents Biochanin A was purchased from Wako Pure Chemicals, Industries, Ltd. (Japan). Biochanin A was dissolved in dimethylsulfoxide (DMSO) and then diluted with the medium (final DMSO concentration=0.05% (v/v)). All other reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise stated.

Cell Culture MC3T3-E1 cells (RCB1126, an osteoblast-
like cell line from C57BL/6 mouse calvaria) was obtained from the RIKEN Cell Bank (Tsukuba, Japan). MC3T3-E1 cells were cultured at 37°C in 5% CO₂ atmosphere in α-modified minimal essential medium (α-MEM; GIBCO). Unless otherwise specified, the medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin.

**Cell Viability: MTT Assay** The cells were suspended in medium and plated at a density of 7.0 × 10⁶ cells/well into a 96-well culture dish (Costar, Cambridge, MA, U.S.A.). After 24 h, the medium was replaced with phenol red-free media containing 5% charcoal-dextran-treated FBS (CD-FBS) supplemented with sample. After 2 d of culture, cell proliferation was measured by MTT assay. This assay is based on the ability of viable cells to convert soluble 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble dark blue formazan reaction product. In the bulk cell photometric MTT assay, the bulk conversion of MTT in the well plate was measured photometrically. MTT was dissolved in DPBS at a concentration of 5 g/l and sterilized by autoclaving. The stock solution was added (one part to 10 parts medium) to each well of culture plate, and the plate was incubated at 37°C for 2 h. DMSO was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature, to ensure that all the crystals were dissolved, the plates were read on a microplate reader at a wavelength of 570 nm.

**Alkaline Phosphatase (ALP) Activity** After the cells were cultured at a density of 10⁶ cells into culture dish for 7 d, the medium was replaced with phenol red-free α-MEM containing 5% CD-FBS. After the cells rinsed with PBS, the medium was exchanged for medium containing 10 mM β-glycerophosphate plus various concentrations of biochanin A, and in combination with biochanin A and 10⁻⁶ M tamoxifen, and the cells were cultured further 3 d; β-glycerophosphate was added to initiate in vitro mineralization. The medium was removed and the cell monolayer was gently washed twice with PBS. The cells were lysed with 0.2% Triton X-100 and the lysate was centrifuged at 14000 × g for 5 min. The clear supernatant, optical density at 540 nm was measured by using a microplate reader. Nitrite concentrations were calculated from the standard curve of sodium nitrite prepared in culture medium.

**Statistics** The results are expressed as mean ± S.E.M. (n=6). Statistical analysis was performed using one-way ANOVA (p<0.05). The analysis was performed using SAS statistical software.

RESULTS

**Effect of Biochanin A on the Growth of MC3T3-E1 Cells** MC3T3-E1 cell growth was promoted by stimulation with biochanin A at 1—50 μM up to approximately 2.3-fold (Fig. 1). Based on this preliminary observation, we evaluated the differentiation-inducing activities of biochanin A on MC3T3-E1 cells by assessing for intracellular ALP activity, collagen synthesis, and osteocalcin secretion.

**Effect of Biochanin A on ALP Activity in MC3T3-E1 Cells** ALP activity was measured to study the effect of biochanin A on the osteoblastic differentiation in MC3T3-E1 cells (Fig. 2). Culture in the presence of biochanin A (1—50 μM) caused a significant increase in the ALP activity of osteoblastic cells (Fig. 2A). The effect of biochanin A (10 μM) in increasing ALP activity was eliminated in the presence of cycloheximide (10⁻⁶ M) or tamoxifen (10⁻⁶ M) (Fig. 2B).
Effect of Biochanin A on Collagen Synthesis in MC3T3-E1 Cells

The effect of biochanin A on collagen content in osteoblastic MC3T3-E1 cells is shown in Fig. 3. The presence of 1—50 μM biochanin A caused a significant increase in collagen content (Fig. 3A). The biochanin A (10 μM)-induced increase in collagen synthesis was completely eliminated by the presence of cycloheximide (10−6 M) or tamoxifen (10−6 M) (Fig. 3B). The data are expressed as a percentage of control and the control value was 2.83 ± 0.43 μg per 10^7 cells. *p<0.05 vs. control.

Effect of Biochanin A on Osteocalcin Secretion in MC3T3-E1 Cells

The MC3T3-E1 cells were cultured with biochanin A, and the content of osteocalcin in medium were measured (Fig. 4). The increase in the osteocalcin content was significant at a biochanin A concentration of 1—50 μM in MC3T3-E1 cell culture. The data are expressed as a percentage of control and the control value was 0.11 ± 0.002 ng per 10^6 cells. *p<0.05 vs. control.

Effect of Biochanin A on TNF-α, IL-6, and NO Production

We investigated whether biochanin A modulates osteoblast production of TNF-α, IL-6, and NO (Fig. 5). When 0.2 mM H2O2 was added to cells, production of TNF-
α, IL-6, and NO increased significantly. However, H$_2$O$_2$-induced TNF-α, IL-6, and NO productions were inhibited by treatment of biochanin A (1, 10 µM).

DISCUSSION

The increase in bone mass can be achieved by increasing the osteoblastic bone formation, decreasing osteoclastic bone resorption, or both. Therefore, in this study, the effects of biochanin A on osteoblast activity and secretion of bone-resorbing agents in osteoblasts were investigated. Osteoblast activity was investigated by the following measurements: cell growth, ALP activity, collagen synthesis, and osteocalcin contents. The generation of bone-resorbing agents in osteoblasts was studied by measuring TNF-α, IL-6, and NO after culture of osteoblasts with biochanin A.

The enhancement in new bone formation is obtained by the increase in the number of osteoblasts and/or the degree of osteoblast differentiation. In our study, there was an increase in the growth rate of osteoblastic cells after the biochanin A treatment. The cells treated with biochanin A showed a maximal increase of 2.3-fold in their growth rate compared to the control cells. MC3T3-E1 cells treated with biochanin A showed a statistically significant increase in ALP activity, collagen synthesis, and osteocalcin content, which are widely accepted phenotype marker of differentiated osteoblastic cells.20) The effects of biochanin A in increasing ALP activity and collagen synthesis in osteoblastic MC3T3-E1 cells were blocked completely by the presence of cycloheximide, an inhibitor of protein synthesis. The anabolic effect of biochanin A may be based partly on a newly synthesized protein component. Moreover, the effects of biochanin A in elevating cell survival, ALP activity and collagen synthesis in osteoblastic cells were blocked completely by the anti-estrogen tamoxifen. This suggests that the differentiation-promoting effect of biochanin A, like that of other phytoestrogens, is estrogen receptor mediated.21) These results not only confirm that biochanin A acts through the estrogen receptor but also suggest that it has the potential to mimic the beneficial activities of estrogen in bone.

Isoflavones are compounds in plant foods, that are structurally similar to the mammalian estrogens22) and that have received considerable attention for their potential bone-sparing properties. Loss of lumbar spine bone mineral content and bone mineral density was significantly lower in the women taking the isoflavone supplement than in those taking the placebo.23) Biochanin A, a naturally occurring isoflavone, has been identified and detected in various diets and plant species.24) Biochanin A is a genistein precursor, which is not present in soy foods. Structurally, it differs from genistein only in a single methoxy group at the 4' position, but it inhibits tyrosine-specific protein kinase activity less effectively than genistein.25) Plant isoflavones are typically detected in food products as substituted glucose conjugates. The bioavailability and biological activity of isoflavones found in foods typically require hydrolysis of glycoside bonds to liberate the free aglycons. Additional metabolism of biochanin A usually involves O-methylation,26) which leads to an increase in the extent of binding to estrogen receptor molecules, presumably through the 4'-hydroxyl group.27) However, because the efficiency of anaerobic demethylation by acetogenic bacteria is relatively low, the possibility that human hepatic enzymes may be capable of demethylating to isoflavone biochanin A to yield the more estrogenic derivative genistein should be considered.

The mechanism of action of isoflavones in the prevention of bone loss appears to differ from that of estrogen. Whereas estrogen prevent bone loss by reducing bone resorption,28,29) the isoflavones genistein and biochanin A are antiresorptive only at chronic high doses and therefore have been reported to either inhibit or not affect the bone turnover rate.30,31) Interestingly, neither of these isoflavones blocks the elevated bone resorption rates induced by ovariectomy or menopause.32,33) Thus while isoflavone treatment may prevent bone loss, in part, because the enhanced bone formation exceeds resorption, the mechanism underlying the enhanced bone formation by isoflavone treatment in vivo is largely unknown. In immature and mature osteoblast cell cultures certain isoflavones have been shown to modestly stimulate differentiated osteoblast characteristics, such as the synthesis of total proteins,34) type I collagen, alkaline phosphatase activity,35) and mineralized matrix deposition.36) Although the affinity of genistein for ER$\alpha$ and ER$\beta$ is lower than that of 17β-estradiol, the relative binding affinity of genistein for ER$\beta$ is greater than for ER$\alpha$.37) The two ER isoforms exhibit differential ligand-dependent regulation not only of transcription from synthetic inducible promoter elements38) but also of endogenous genes and cellular functions in osteoblasts in vitro.39) Thus, the repertoire of target genes affected, as well as the response of any specific gene, will be dependent on the particular ligand bound to the receptor isoforms and the ratio of ER$\alpha$ and ER$\beta$. Thus, the divergent effects of isoflavones on bone metabolism in vivo, including their unique regulation of osteoblast and osteoclast functions, could be caused by the relative concentrations of the ER$\alpha$ and ER$\beta$ isoforms in the osteoblasts and other skeletal cells.39)

The osteoblast is the main bone cell type that is primarily responsible for bone formation. However, this cell also possesses receptors for calcitropic hormones with bone-resorbing activity, and it is therefore assumed that the osteoblast also serves as a mediator in the process of bone resorption, which is ultimately carried out by the osteoclast.40) Several lines of evidence have found a tight association between oxidative stress and pathogenesis of osteoporosis. Marked decrease in plasma antioxidants was found in aged osteoporotic women41); there is also a biochemical link between increased oxidative stress and reduced bone mineral density (BMD) in aged men and women42); dietary antioxidant vitamin intake has a beneficial effect on BMD in postmenopausal women43); oxidative stress increases differentiation and function of osteoclasts.40) In respiratory cells, a small amount of the consumed oxygen is reduced in a specific way, yielding a variety of highly reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anion.45) ROS are capable of causing oxidative damage to biomacromolecules, leading to lipid peroxidation, the oxidation of amino acid residues, the formation of protein–protein cross-link and DNA oxidative damage. Under certain pathological conditions, the dynamic balance between the generation and elimination of ROS may be broken and thus the cellular ROS levels increase significantly. High levels of ROS may cause the oxidative damage of various cellular components, and finally result in cell
ROS enhance osteoclastic activity, but inhibit the differentiation of osteoblasts. ROS might also indirectly stimulate osteoclasts by augmenting expression of resorptive cytokines such as TNF-α, IL-1, and IL-6 that have been strongly implicated in estrogen-deficiency bone loss. ROS are potent inducers of these cytokines in many cells through activation of NF-kB. Therefore, ROS might induce bone hyper-resorption in estrogen deficiency. In our study, we examined the production of TNF-α, IL-6, and nitric oxide (NO) in osteoblastic MC3T3-E1 cells and found that H2O2 upregulated the production of these local factors in osteoblasts. Furthermore, biochanin A (1 and 10 µg/ml) inhibited the H2O2-induced production of TNF-α, IL-6, and NO. These results indicate that biochanin A might be useful for diseases associated with the excessive production of ROS, and skeletal tissues may benefit from the consumption of biochanin A-containing foods.

Nitrict oxide (NO) can be generated endogenously in several types of cells and plays diverse biological roles. On the other hand, when produced in large excess or produced with ROS concurrently, NO also displays cellular toxicity and can induce apoptotic cell death in different types of cells. NO has been shown to modulate osteoclast recruitment and activity. In vitro studies suggest that high levels of NO suppress osteoblast proliferation and differentiation.

Cytokines such as IL-1 and TNF-α induce NO production in osteoblasts. NO produced in bone exerts an inhibitory effect on bone resorption mediated by osteoclasts. TNF-α and IL-6, which are produced by inflammatory cells in the proximity of osteoblasts, have adverse effects on bone formation and stimulate osteoclastic bone resorption. This may be relevant to states characterized by an excess of local production of cytokines in bone, such as may be seen during inflammation, e.g., rheumatoid arthritis or metastatic bone diseases. TNF-α and IL-6 are also produced by osteoblasts and therefore may exert an autocrine/paracrine effect on bone remodeling. Since enhanced IL-6 production by osteoblast lineage cells has been strongly implicated in causing osteoclast differentiation and thus increased bone resorption and rate of bone loss in estrogen deficiency, the biochanin A-mediated suppression of TNF-α and IL-6 may be an important additional mechanism explaining the anti-resorptive effects of isoflavones.

In summary, the data described in this report suggest that biochanin A exerts beneficial effects on osteoblast-like cells. In addition, inhibition of H2O2-induced production of TNF-α, IL-6, and NO by biochanin A may be partly related to inhibition of osteoclastogenesis.

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REFERENCES