The estrogen 17ß-estradiol and phytoestrogen genistein mediate differential effects on osteoblastic NF-κB activity

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Abstract. Estrogen (17ß-estradiol) and genistein, a phytoestrogen, are both endowed with anabolic activities on bone *in vivo* and stimulate osteoblastic differentiation and mineralization *in vitro*. However, the mechanisms by which these agents promote osteoblastic differentiation and bone anabolic responses are multifactorial and only partly understood. Recently, the NF-κB signal transduction pathway was implicated as a negative regulator of osteoblastic differentiation and suppression of this pathway leads to osteoblastic differentiation and mineralization *in vitro*. To examine whether estrogen and/or genistein regulate osteoblast differentiation by modulating the NF-κB pathway, we examined the effect of 17ß-estradiol and genistein on basal and TNFα-stimulated NF-κB activity in the preosteoblastic cell line MC3T3. MC3T3 cells were transiently transfected with an NF-κB responsive luciferase reporter and cultured for 24 h with either vehicle, or physiological doses of 17ß-estradiol (10^{-9} to 10^{-7} M), or genistein (10^{-6} to 10^{-5} M). Our data reveal that while 17ß-estradiol had no effect on basal NF-κB activity in MC3T3 cells, it significantly antagonized NF-κB activity induced by TNFα (1 or 10 ng/ml). By contrast, genistein (10^{-6} or 10^{-5} M) significantly increased NF-κB activity, and showed no antagonistic effects on TNFα-induced NF-κB promoter activity. These studies suggest that the estrogenic compounds, 17ß-estradiol and genistein, mediate very different actions on osteoblastic cells. While 17ß-estradiol may stimulate bone anabolism, in part, by antagonizing TNFα-induced NF-κB activation, genistein not only fails to prevent cytokine-induced NF-κB activation, but directly promotes NF-κB activation in MC3T3 cells. These data suggest important mechanistic differences in the mechanisms by which 17ß-estradiol and genistein promote osteoblast differentiation.

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Introduction

Many edible plants contain small quantitates of diverse phytoestrogens that are believed to promote bone health. Phytoestrogen molecules can be divided into two major chemical classes: isoflavones and coumestans. The isoflavones are found predominantly in soybeans (*Glycine max*), whereas coumestans are produced primarily by clovers (*genus Trifolium*) and some legumes. These molecules function as antioxidants in plants, but in mammalian tissues act as agonists, or partial agonists, of estrogen receptors.

Soybean-derived isoflavones are under intense investigation for both enhancing human health and preventing several common diseases such as cardiovascular disease, cancers of reproductive tissues, and osteoporosis (1-5). However, their mechanisms of action are poorly characterized.

Isoflavones, including genistin and genistein, are present in soybeans at a comparatively high concentration. Genistin is hydrolyzed to genistein by ß-glucosidase in the gastrointestinal system. Genistein was demonstrated to have an anabolic effect on bone metabolism of rats (6-8), suggesting a potential benefit in the prevention of osteoporosis, which is widely recognized as a major public health problem, and leads to increased risk of fracture. Bone fractures can be debilitating and significantly reduce quality of life. Fractures of the proximal femur are particularly devastating and the incidence increases dramatically with age (9). Hip fractures almost always require major surgery, often lead to permanent disability, and have a high rate of mortality in the aged population (10).

Nutritional and pharmacological factors such as isoflavones may be an important tool for preventing bone loss with aging. The dietary intake of genistein was shown to prevent bone loss in ovariectomized rats, an animal model of postmenopausal osteoporosis (11). Furthermore, genistein stimulates osteoblastic bone formation and mineralization *in vitro* (6-8,12), and can stimulate protein synthesis in osteoblastic cells (13-15). Moreover, genistein was shown to inhibit osteoclastogenesis (16), in part through inhibition of protein kinase activation and suppression of protein tyrosine phosphatase activity (17), as well as by inducing apoptosis of mature osteoclasts through a Ca^{2+} signaling mechanism (18).

The molecular mechanisms by which genistein mediates its anabolic effect on bone, however, remain to be elucidated. Recently, we (19) and others (20) reported that the NF-κB signal transduction pathway antagonizes osteoblastic differen-

tiation *in vitro*, through suppression of TGFß- and BMP-induced SMAD signal transduction, and that pharmacological suppression of NF-κB activation promotes osteoblastic differentiation and mineralization *in vitro* (19). Interestingly, genistein was reported to inhibit NF-κB activation in the macrophage cell line J774 (21) suggesting a potential mechanism for the capacity of genistein to promote osteoblastic differentiation. The 5 members of the mammalian NF-κB family RelA/p65, RelB, c-Rel, NF-κB1/p50, and NF-κB2/p52 are activated through one of two specific pathways, the canonical NF-κB pathway or an alternative pathway. In the canonical pathway, activation of the inhibitor of IκB kinase (IKK) complex leads to phosphorylation of the NF-κB-associated IκBα, catalyzing its ubiquitination and proteasomal degradation, and in the process releases active NF-κB dimers that translocate to the nucleus and enhance transcription of target genes. In the alternative NF-κB pathway, NF-κB-inducing kinase (NIK) and IKKα target p100 for proteolytic processing, thereby releasing active RelB-containing dimers (22).

In this study, we investigated whether 17ß-estradiol and/or genistein modulate NF-κB activation in MC3T3 preosteoblastic cells. Our data reveal that although both 17ß-estradiol and genistein augmented mineralization of MC3T3 cells, their actions on NF-κB activation under basal conditions and following TNFα-induction were completely different. While 17ß-estradiol antagonized TNFα-induced NF-κB activation, genistein not only failed to prevent cytokine-induced NF-κB activation, but directly stimulated NF-κB activation in MC3T3 cells.

Materials and methods

Chemicals. α-Minimal essential medium (α-MEM) and antibiotics (penicillin and streptomycin) were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). All other reagents were purchased from the Sigma Chemical Corporation (St. Louis, MO), unless otherwise indicated. All water used was glass distilled.

Cell culture. The preosteoblastic cell line MC3T3-E1, clone 14 (MC3T3), was purchased from the American Type Culture Collection (Manassas, VA) and was previously described in detail (23). Cells were cultured at 37˚C in a humidified 5% CO_2 incubator in plastic dishes containing α-MEM supplemented with 10% FBS, with 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged every 3 days using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS).

Osteoblast differentiation assays and Alizarin Red-S staining. MC3T3 cells ($1.0x10^5$ cells per well) were cultured for 72 h in α-MEM (1.0 ml/well) containing 10% FBS in 12-well dishes. Medium was aspirated and changed to mineralization medium (phenol Red-free α-MEM supplemented with 10% FBS, L-ascorbic acid (100 μg/ml) and 4 mM ß-glycerophosphate as previously described (13,19). Cells were cultured with fresh medium every 3 days. At 28 days, cells were rinsed with PBS and calcium deposition was visualized by fixing the cells in 75% ethanol for 30 min at 4˚C followed by staining with Alizarin Red-S (40 mM, pH 6.2) for 30 min at room temperature. Excess stain was removed by copious washing with distilled water.

NF-κB reporter constructs and luciferase assays. The NF-κB responsive reporter (pNF-κB-LUC) was purchased from BD Biosciences. Reporter plasmids (pNF-κB-LUC) or empty vector control (pGL3-Basic) were transfected into MC3T3 cells using Lipofectamine 2000 reagent (Invitrogen) as previously described (19). Briefly, MC3T3 cells ($1.0x10^5$ cells/well) were cultured for 24 h in α-MEM, and then changed to α-MEM without FBS and antibiotics and transfected with pNF-κB-LUC reporter plasmid or empty vector. Five hours later the medium was changed to α-MEM containing 10% FBS plus antibiotics and wells were treated with vehicle or 17ß-estradiol (10^{-9} or 10^{-8} M) or genistein (10^{-6} or 10^{-5} M), and/or TNFα (1 or 10 ng/ml), as indicated in the figures. The cells were further cultured for 24 h.

Luciferase activity was measured on a microplate luminometer (Turner Designs, Sunnyvale, CA, USA). Equal transfection efficiency was validated for all plasmids using Renila luciferase reporter plasmid pRL-SV40, using the dual-luciferase assay system (Promega, Madison, WI).

Statistical analysis. Statistical significance was determined using GraphPad InStat version 3 for Windows XP (GraphPad Software). Multiple comparisons were performed by One-way ANOVA with Tukey-Kramer post test. $P<0.05$ was considered statistically significant. Data are presented as mean ± SD of 5 replicate wells and are representative of two or more independent experiments.

Results

Effect of 17β-estradiol and genistein on osteoblastic mineralization. MC3T3 cells were cultured with physiological concentrations of 17ß-estradiol (10^{-9} or 10^{-8} M) or genistein (10^{-6} or 10^{-5} M) for 28 days and stained for calcium deposition with Alizarin Red-S. The data show (Fig. 1) that both 17ß-estradiol and genistein had a stimulatory effect on MC3T3 mineralization *in vitro*. The magnitude of the increase in mineralization between genistein and 17ß-estradiol is comparable.

17β-estradiol and genistein mediate differential effects on basal NF-κB activity in MC3T3 cells. As suppression of NF-κB is now established to promote MC3T3 mineralization (19), we speculated that one mechanism by which 17ß-estradiol or genistein could stimulate osteoblastic mineralization is by downregulating NF-κB signal transduction. To investigate the action of 17ß-estradiol and genistein on NF-κB activity, we transiently transfected MC3T3 cells with an NF-κB luciferase reporter driven by 6 tandem copies of the NF-κB consensus sequence, thus providing a direct measurement of activation for this pathway.

Our data reveal that while 17ß-estradiol (10^{-9} to 10^{-7} M), had no effect on basal NF-κB activity in MC3T3 cells (Fig. 2A), genistein (10^{-6} or 10^{-5} M) significantly and dose-dependently stimulated NF-κB activity (Fig. 2B).

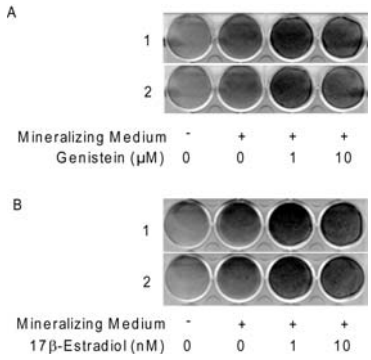

Figure 1. Genistein and 17ß-estradiol promote osteoblastic differentiation and mineralization in preosteoblastic MC3T3 cells. MC3T3 cells were differentiated into mineralizing osteoblasts by culture in α-MEM supplemented with 10% FBS, ascorbic acid (100 ng/ml) and 4 mM ß-glycerophosphate (mineralizing medium) in the presence or absence of either: (A) genistein (10^{-6} or 10^{-5} M); or (B) 17ß-estradiol (10^{-9} or 10^{-8} M). After 28 days, cells were washed with PBS and stained with Alizarin Red S to visualize calcium deposition. The figure shows the outcome of 2 independent experiments.

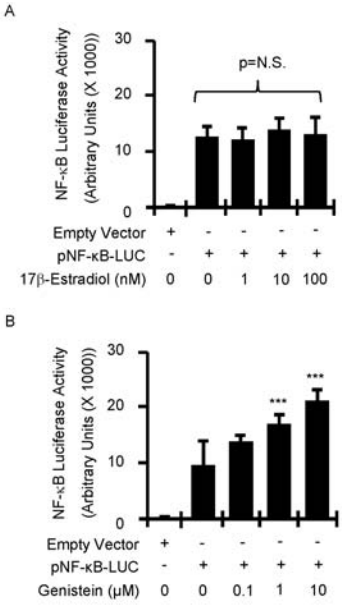

Figure 2. 17ß-estradiol and genistein mediate differential effects on NF-κB activation in preosteoblastic MC3T3 cells. MC3T3 cells were transfected with pNF-κB-LUC, an NF-κB activity reporter plasmid or empty vector and cells cultured in the presence or absence of either: (A) 17ß-estradiol (10^{-9} to 10^{-7} M), or (B) genistein (10^{-7} to 10^{-5} M). Luciferase activity was determined after 24 h. Data represent mean ± SD of five assays. ***P<0.001 relative to pGL3-NF-κB only (One-way ANOVA). N.S. = no significant difference between data within brackets.

We previously reported that basal concentrations of endogenous TNFα potently lower osteoblastic bone formation *in vivo*, reducing maximum achievable peak bone mass, potentially through an NF-κB-dependent mechanism (19). We consequently investigated the actions of 17ß-estradiol and genistein on TNFα-stimulated NF-κB activity in MC3T3 cells. Culture with low-dose TNFα (1 ng/ml) stimulated NF-κB activity ~3 fold (Fig. 3A). This increase was significantly suppressed by physiological doses (10^{-8} and 10^{-7} M) of 17ß-estradiol (Fig. 3A).

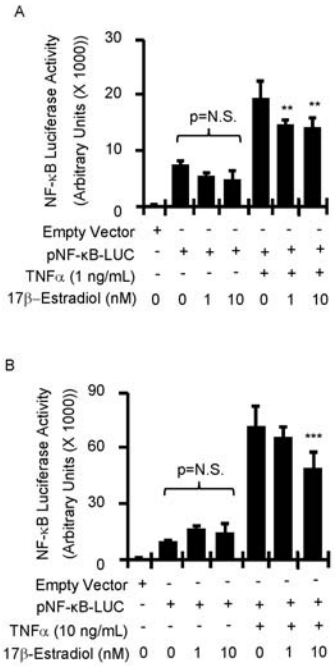

Figure 3. 17ß-estradiol suppresses TNFα-induced NF-κB activation in MC3T3 preosteoblastic cells. MC3T3 cells were transfected with NF-κB reporter plasmid pNF-κB-LUC or empty vector and treated with 17ß-estradiol (10^{-9} or 10^{-8} M), and/or (A) TNFα (1 ng/ml), or (B) TNFα (10 ng/ml). Luciferase activity was determined after 24 h. Data represent mean ± SD of five assays. ***P<0.001 relative to pGL3-NF-κB + TNFα. All TNFα treated wells are significantly (P<0.001) different from pGL3-NF-κB only. N.S. = no significant difference between data within brackets.

TNFα concentrations are known to elevate under inflammatory conditions and in postmenopausal women (24) and may contribute to osteoporosis by upregulating bone resorption and by lowering the compensatory increase in bone formation. To mimic these conditions, we further treated MC3T3 cells with a high dose of TNFα (10 ng/ml) which led to a potent 8-fold increase in NF-κB activity (Fig. 3B). Addition of 17ß-estradiol (10^{-8} M) significantly reduced TNFα-induced NF-κB activation by 32% (Fig. 3B).

We next investigated whether genistein augments or synergizes with low- (1 ng/ml) or high-dose (10 ng/ml) TNFα. Treatment with genistein (10^{-6} or 10^{-5} M) significantly elevated basal NF-κB activity, as did TNFα at 1 ng/ml (Fig. 4A) and 10 ng/ml (Fig. 4B) but failed to significantly amplify TNFα-induced NF-κB activation.

To investigate whether new mRNA transcription was necessary for genistein's stimulatory effects on NF-κB activation suggestive on an indirect action on NF-κB activation we used 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazol (DRB), to inhibit transcription activity. DRB is known to suppress genomic transcription at a dose of 10^{-6} M (25). We thus titrated DRB to identify a dose that does not suppress endogenous luciferase transcription from plasmid DNA, but suppresses normal genomic transcription (Fig 5A). A dose of 10^{-6} M failed to block basal NF-κB-luciferase transcription and was selected for further study. Addition of DRB (10^{-6} M) completely suppressed the capacity of genistein to stimulate NF-κB promoter activity in MC3T3 cells (Fig. 5B).

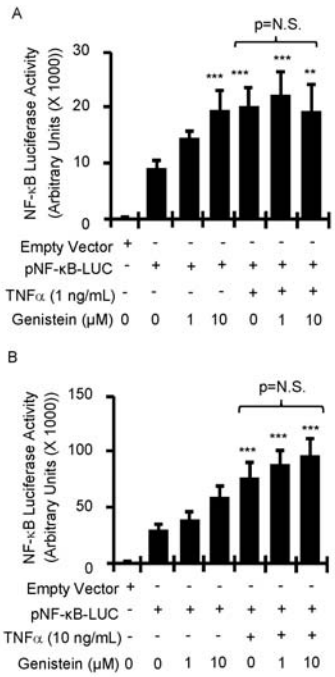

Figure 4. Genistein stimulates NF-κB activity but fails to augment TNFα-stimulated NF-κB activation in MC3T3 preosteoblastic cells. MC3T3 cells were transfected with NF-κB reporter plasmid or empty vector and treated with genistein (10^{-6} or 10^{-5} M), and/or (A) TNFα (1 ng/ml), or (B) TNFα (10 ng/ml). Luciferase activity was determined after 24 h. Data represent mean ± SD of five assays. ***P<0.001 or **P<0.01 relative to pGL3-NF-κB. N.S. = no significant difference between data within brackets.

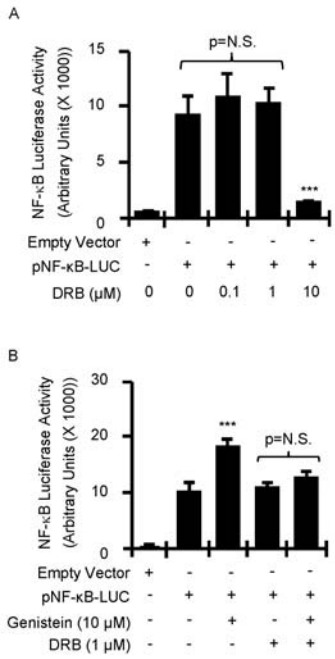

Figure 5. Suppression of transcription inhibits the capacity of genistein to stimulate NF-κB activation in MC3T3 preosteoblastic cells. MC3T3 cells were transfected with NF-κB promoter plasmid or empty vector control and treated with: (A) A dose range of DRB (10^{-7} to 10^{-5} M) to establish a dose that does not suppress basal luciferase transcription. (B) Cells were treated with vehicle or genistein (10^{-5} M) in the presence or absence of DRB (1 μM) dose determined to not suppress luciferase-specific transcription. Luciferase activity was quantitated 24 h later. Data represent mean ± SD of five assays. ***P <0.001 relative to pGL3-NF-κB. N.S. = no significant difference between data within brackets.

Taken together, our data suggest that while 17ß-estradiol potently suppresses osteoblastic NF-κB activation, genistein promotes NF-κB induction, but through an indirect mechanism involving transcriptional regulation of a NF-κB inducing factor or factors.

Discussion

The estrogen 17ß-estradiol, and the phytoestrogen genistein, stimulate bone formation and suppress bone resorption *in vivo* (26). Genistein can stimulate protein synthesis through activation of aminoacyl-tRNA synthetase in osteoclastic cells (15) and stimulate apoptosis of mature osteoclasts through a Ca^{2+} signaling mechanism (17). Furthermore, the suppressive effect of genistein on rat osteoclasts involves, in part, the inhibition of protein kinase activity and the activation of protein tyrosine phosphatase activity in osteoclasts (18). However, the molecular mechanisms by which genistein regulates osteoblast formation and activity is presently unknown.

This study was conducted to determine if the actions of 17ß-estradiol and genistein are mediated through suppression of NF-κB, a signal transduction pathway known to be critical to osteoclastic differentiation and activity (27,28), and recently reported to antagonize osteoblastic differentiation *in vitro* through suppressive actions on Smad signaling (19,20).

Interestingly, our data reveal differential actions of 17ß-estradiol and genistein on basal and TNFα-induced NF-κB activity in MC3T3 preosteoblastic cells. Although both 17ß-estradiol and genistein promoted enhanced differentiation and mineralization of MC3T3 cells in culture, 17ß-estradiol failed to impact basal NF-κB activity in these cells, while genistein elicited a dose-dependent increase in NF-κB activity. Furthermore, 17ß-estradiol had a significant inhibitory effect on TNFα-induced NF-κB activity, while genistein failed to augment or suppress the stimulation of NF-κB activity induced by TNFα.

The reason for these differential effects of 17ß-estradiol and genistein are likely complex and remain to be elucidated. However, one possible explanation is that genistein has a higher affinity for estrogen receptor (ER)ß than for ERα (29). Consequently, different downstream signals mediated by ERα and ß may account for some of the differential actions observed in this system.

The specific mechanisms that regulate NF-κB activation are well studied and involve both canonical and alternative NF-κB pathways (22). The mechanism by which genistein induces NF-κB activity in osteoblasts however, remains unknown and may directly target the canonical pathway, the alternative pathway, both pathways, or it may regulate NF-κB indirectly via other signaling molecules. The inability of genistein to amplify the NF-κB-inducing capacity of TNFα at a sub-saturating concentration (1 ng/ml) favors an indirect action of genistein on NF-κB. This is further supported by our data demonstrating that the stimulatory effect of genistein is mediated through a transcriptional mechanism, as suppression of new transcription by DRB prevents induction of NF-κB by genistein.

The capacity of genistein to stimulate rather than suppress NF-κB activity in MC3T3 cells was also unexpected in light of previous studies in a macrophage cell line where genistein was reported to inhibit LPS induced NF-κB activation (21). Different patterns of ER expression, cell types, and modes of stimulation, may all provide possible explanations for these differences.

Although physiological concentrations of 17ß-estradiol only repressed NF-κB activation by 10 ng/ml of TNFα by a modest ~35%, the data suggest that the deficiency in 17ß-estradiol associated with menopause, may significantly contribute to the decline in bone mass due to the loss of this mitigating signal on osteoblastic NF-κB activity. Furthermore, enhanced NF-κB activation in osteoblasts driven by higher TNFα concentrations in postmenopausal conditions may lead to a diminished compensatory increase in bone formation in response to elevated bone resorption, thus uncoupling the system and setting the stage for development of osteoporosis.

While 17ß-estradiol may stimulate bone anabolism in part, by antagonizing TNFα-induced NF-κB activation, genistein not only failed to prevent cytokine-induced NF-κB activation, but directly promoted NF-κB activation in MC3T3 cells. How genistein augments osteoblastic differentiation remains unknown, however our data suggest important mechanistic differences in the mechanisms by which 17ß-estradiol and genistein promote bone formation.

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