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Phytoestrogen Genistein Stimulates the Production of Osteoprotegerin by Human Trabecular Osteoblasts

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Abstract The anti-resorptive effects of estrogen on bone metabolism are thought to be mediated through modulation of paracrine factors produced by osteoblastic lineage cells that act on osteoclastic lineage cells. Receptor activator of nuclear factor-κB ligand (RANKL) is the essential factor for osteoclast formation and activation and enhances bone resorption. By contrast, osteoprotegerin (OPG), which is produced by osteoblastic lineage cells acts as a decoy receptor that neutralizes RANKL and prevents bone loss. Recently, 17β-estradiol was found to stimulate OPG mRNA levels and protein secretion in a human osteoblastic cell line through activation of the estrogen receptor (ER)-α. In this study, we assessed the effects of the phytoestrogen genistein on OPG mRNA steady state levels (by semiquantitative RT-PCR and Northern analysis) and protein production (by ELISA) in primary human trabecular osteoblasts (hOB) obtained from healthy donors. Genistein increased OPG mRNA levels and protein secretion by hOB cells by up to two- to six-fold in a dose- ($P < 0.0001$) and time-dependent ($P < 0.0001$) fashion with a maximum effect at 10^{-7} M. Co-treatment with the pure ER antagonist ICI 182,780 completely abrogated the stimulatory effects of genistein on OPG protein secretion, indicating that these effects were specific and directly mediated through the ER. Pre-treatment with genistein partially prevented the inhibitory effects of the glucocorticoid dexamethasone on OPG mRNA and protein production. The stimulation of OPG mRNA levels by genistein was not affected by the protein synthesis inhibitor, cycloheximide and was shown to be due to enhancement of OPG gene transcription. In conclusion, our data suggest that the phytoestrogen genistein is capable of upregulating the production of OPG by human osteoblasts. Thus, dietary sources of phytoestrogens may help to prevent bone resorption and bone loss by enhanced osteoblastic production of OPG. J. Cell. Biochem. 84: 725–735, 2002. © 2002 Wiley-Liss, Inc.

Key words: anti-estrogens; estrogen; estrogen receptor; genistein; osteoblast; osteoprotegerin; RANK ligand

Estrogen replacement has been the mainstay of therapy for the prevention and treatment of osteoporosis in postmenopausal women. However, long-term compliance with, and adherence to, estrogen therapy is limited, and there are concerns regarding its safety [Eastell, 1998]. Phytoestrogens are a heterogeneous group of non-steroidal plant-derived compounds that exhibit estrogen-like effects at various reproductive and non-reproductive estrogen target tissues, including bone [Fitzpatrick, 1999]. The isoflavone genistein, the major phytoestrogen contained in soybeans and soy-based products, has been demonstrated to act as an anti-resorptive and bone-sparing agent [Anderson and Garner, 1998]. In rodents, genistein inhibited osteoclast activity and survival in vitro [Gao and Yamaguchi, 2000] and prevented trabecular bone loss following ovariectomy in vivo [Ishimi et al., 1999]. In addition, genistein also decreased biochemical markers of bone turnover in pre- and post-menopausal women [Wangen et al., 2000]. While the public and scientific interest in genistein and other phytoestrogens is growing,

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their mechanism(s) of action on bone cells has largely remained unclear.

Estrogens regulate the function of osteoblasts and osteoclasts through activation of high affinity estrogen receptors (ER). Recent studies have suggested that the anti-resorptive effects of estrogens are largely mediated through direct effects on osteoclasts and by osteoblast-derived paracrine factors that modulate osteoclast formation and activation [Spelsberg et al., 1999]. Osteoprotegerin (OPG), a member of the tumor necrosis factor (TNF) receptor (TNFR) superfamily, is secreted by osteoblastic lineage cells [Simonet et al., 1997; Yasuda et al., 1998], and acts as a decoy receptor for receptor activator of nuclear factor-κB ligand (RANKL) [Lacey et al., 1998]. RANKL represents the essential osteoblast-derived factor required for osteoclast formation and activation, and induces bone loss, whereas, OPG blocks these effects and prevents bone resorption in various physiological and pathophysiological situations [Teitelbaum, 2000]. The expression of RANKL and OPG by osteoblastic lineage cells is modulated by a variety of osteotropic steroid and peptide hormones as well as cytokines [Teitelbaum, 2000].

We have demonstrated that 17β-estradiol enhanced the expression of OPG mRNA and protein by a human fetal osteoblastic lineage cell line (hFOB/ER-9), which had been stably transfected with the ER-α in a dose- and time-dependent fashion [Hofbauer et al., 1999a]. These results were recently confirmed by Saika et al. [2001] using the murine stromal cell line ST-2, and were found to be mediated through transactivation of the ER-α. By contrast, ovariectomy resulted in decreased OPG production and increased RANKL production [Asami et al., 1999; Lindberg et al., 1999]. Here we report that the phytoestrogen genistein stimulates OPG mRNA levels and protein secretion by human trabecular osteoblasts, which predominantly express ER-α.

MATERIALS AND METHODS

Materials

Cell culture medium and supplements were purchased from GIBCO-BRL (Karlsruhe, Germany). Culture flasks and dishes were obtained from Nunc (Roskilde, Denmark). Unless otherwise stated, all other chemicals were purchased from Sigma (Munich, Germany). The random primer labeling kit (Decaprime II) was from Ambion (Austin, TX) and [α-^{32}P]-dCTP was from DuPont-NEN (Boston, MA). The human β-actin cDNA insert and the ExpressHyb hybridization solution were purchased from Clontech (Palo Alto, CA). Genistein was purchased from Carl Roth Inc (Karlsruhe, Germany).

Cell Culture

Bone specimens were obtained from the iliac crest of 6 patients (4 women, 2 men; 35.2 ± 6.1 years) undergoing corrective surgery after traumatic fracture. None of the patients had signs or symptoms of bone or joint disease. The study was approved by the Institutional Review Board of the University of Goettingen, and written informed consent was obtained from all patients. We obtained RNA from primary first-passage osteoblastic cells (hOB) from cultures of trabecular bone explants as previously described [Siggelkow et al., 1999]. These hOB cells have been shown to represent the phenotype of the mature osteoblast, were grown at 37°C, maintained in phenol red-free minimal essential medium (MEM) supplemented with 10% charcoal-stripped fetal calf serum (cs-FCS) from Allgäu Bio Tech Service (Goerisried, Germany), and were cultured in serum-free MEM supplemented with 0.125% (w/v) bovine serum albumin (BSA) for 4 days prior to RNA isolation.

Immunocytochemistry

For detection of estrogen receptor-α (ER-α) and progesterone receptor (PR) protein expression, hOB cells were fixed in acetone (−20°C, 20 min) on poly-L-lysine-coated slides as previously described [Bevitt et al., 1997]. After washing in Tris-buffered saline (TBS; 0.05 M Tris/HCl, pH 7.6), cells were blocked for 2 h at room temperature with rabbit serum to prevent non-specific binding of antibodies. A monoclonal mouse anti-human ER-α antibody (clone 6F11, Novocastra, Newcastle, UK) and a monoclonal mouse anti-human PR antibody (clone 1 A6) were diluted at 1:100 in TBS/0.1% BSA and were used as a primary antibody for 2 h at 37°C in a humidified chamber. Slides were washed three times in TBS, and specific antibody binding was subsequently assessed by application of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG antibodies (DAKO, Hamburg, Germany) diluted at 1:500 in TBS/0.1% BSA for 30 min at room temperature. Slides were washed as described above, mounted in

Aquamount (DAKO), and assessed by fluorescence microscopy. An isotype control of the primary antibody was used as a negative control.

RT-PCR Analysis

Total RNA was prepared using the RNeasy total RNA extraction kit from Qiagen (Hilden, Germany). The cDNA was synthesized from 1 μg of total RNA in a total volume of 40 μl containing 3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 10 mM DTT, dCTP, dGTP, dATP, and dTTP each at 0.4 mM, RNAse inhibitor (40 U), M-MLV reverse transcriptase (400 U), and poly-dT15 primer (80 pM) from Roche Molecular Biochemicals (Mannheim, Germany). Reaction times were 1 h at 38°C and 10 min at 72°C. Aliquots of the total cDNA were amplified in each PCR reaction in a 15 μl reaction mixture containing 20 pmol of 5′ and 3′ primer each, 50 mM KCl (pH 8.3), 10 mM Tris-HCl (pH 9.0), 1.5 nM $MgCl_2$ and dCTP, dGTP, dATP, and dTTP each at 0.02 mM and Taq polymerase (0.5 U) (Roche Molecular Biochemicals). Each cDNA sample was run in triplicate for each PCR reaction. Competitive RT-PCR was performed using exogenous DNA competitors ("mimics") as internal control [Köhler, 1995] that were synthesized with the PCR mimic construction kit from Clontech. PCR reactions were carried out in 15 μl reactions at a cycle number ensuring a linear amplification profile (L7, 2 min at 94°C, 20 cylces (of 1 min at 94°C, 1 min at 54°C, 2 min at 72°C), 10 min at 72°C; OPG, 2 min at 94°C, 26 cycles (of 30 s at 94°C, 60 s at 54°C, 90 s at 72°C), 7 min at 72°C; ER-α and ER-α, each 30 s at 95°C, 32 cycles (of 30 s at 95°C, 15 s at 56°C, 1 min at 72°C), 3 min at 72°C). The oligonucleotides for L7 (sense: 5′-AGATGTACAGAACTGAAATTC-3′; antisense: 5′-ATTTACCAAGAGATCGAGCAA-3′); OPG (sense: 5′-GAACCCCAGAGCGAAATACA-3′; antisense: 5′-CGCTGTTTTCACAGAGGTCA-3′); ER-α (sense: 5′-AATTCAGATAATCGACG-CCAG-3′; antisense: 5′-GTGTTTCAACATTC-TC-CCTCCTC-3′); ER-α (sense: 5′-TAGTGGT-CCATCGCCAGTTAT-3′; antisense: 5′-GGGA-GC-CACACTTCACCAT-3′) and PR (sense: 5′-ATGGAAGGGCAGCATAACTATTTAT-3′; antisense: 5′-CTCATCTCTTCAAACTGGCTT-TGACT-3′) were synthesized at MWG (Ebersberg, Germany).

PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining under UV light. In all experiments, the expression of each gene was quantified as target to mimic ratio and normalized to the ribosomal house-keeping gene L7. To ensure specificity of the PCR products, the amplification product was sequenced with the Abi Prism system from Perkin Elmer (Weiterstadt, Germany).

Northern Blot Analysis

Total RNA (10 μg) was separated on a denaturing agarose gel containing 2.2 M of formaldehyde and transferred onto a nylon membrane. Hybridization with a human full-length OPG cDNA and a human β-actin cDNA were carried out as previously described [Hofbauer et al., 1999b]. Band intensity was quantified by densitometry. All experiments were carried out at least three times, and representative blots are shown.

Nuclear Run-On Assay

Two micrograms of full-length OPG cDNA were denatured and fractionated in an agarose/formaldehyde gel under denaturing conditions and transferred to a nylon membrane. The hOB cells (2×10^8 cells) were treated either with vehicle or genistein (10^{-8} M) for 48 h. Then cellular nuclei were prepared according to the method of Dignam et al. [1983]. Nuclear RNA was radiolabeled using [^{32}P]-dCTP (15 μl/reaction) and extracted with the Qiagen RNeasy kit from Qiagen. The two lanes on the nylon membrane were then cut and separately hybridized each with radiolabeled RNA (10^7 cpm/μg) as described [Hofbauer et al., 1999b]. The membrane strips were exposed to an autoradiography film.

OPG Protein Measurement

Conditioned medium was harvested from cultured cells and centrifuged to remove debris. Samples were stored at −80°C until used. OPG were determined in triplicate measurements using a highly sensitive, commercial sandwich enzyme immunoassay from Immunodiagnostik (Bensheim, Germany). Measurements were performed in samples diluted from 1:10 to 1:100 according to the manufacturer's instructions. In this assay, two highly specific antibodies against human OPG are used. As a capture antibody, a monoclonal IgG antibody is used that had been raised by immunizing a murine hybridoma cell line with recombinant human OPG (rhOPH). The detection antibody is

a biotin-labeled polyclonal anti-human OPG antibody derived from a goat after immunization with rhOPG. The lower limit of detection of this assay is 4 pg/ml. The intra-assay CV is between 8 and 10%, and the inter-assay CV is between 12 and 15%.

Statistical Analysis

Unless otherwise stated, all experiments were reproduced at least three times using primary first-passage cell cultures from healthy young donors. Values are expressed as the mean $\pm$ SEM of triplicate measurements and data obtained from representative experiments are shown. Student's paired *t*-test was used to evaluate differences between the sample of interest and its respective control. For analysis of time courses and dose responses, multiple measurement ANOVA followed by Newman-Keuls post-test analysis was performed. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

The hOB cells displayed a characteristic pattern of gene expression and protein production of various osteoblastic differentiation markers (type I collagen, alkaline phosphatase, and osteocalcin) that were developmentally regulated over time in culture and were stimulated by differentiating agents such as dexamethasone and vitamin D (data not shown), indicating that they represent the mature osteoblastic phenotype [Siggelkow et al., 1999]. To assess their estrogen responsiveness, we first characterized the hOB cells for ER-α, ER-α, and PR gene expression. RT-PCR analysis of hOB cells revealed marked ER-α and PR mRNA levels, whereas, ER-α mRNA levels were low and inconsistently present (data not shown). As shown in Figure 1A,B, both ER-α and PR mRNA levels were found to be stable over three weeks in culture (which was the time frame used for all subsequent studies), and increased by two- to three-fold, thereafter. Immunohistochemical analysis confirmed the presence of ER-α and PR protein by Day 16 (Fig. 1C), whereas, ER-β protein could not be detected. ER-α mRNA levels were found to be up-regulated by 17β-estradiol (10^{-8} M) and genistein (10^{-7} M) for 72 h by up to 2.9- and 3.2-fold, respectively ($P < 0.0001$) (Fig. 1D). To further test whether the ER-α is functionally active in hOB cells, we analyzed gene expression of the PR, a known estrogen target gene [Harris et al., 1995], following treatment with estrogens. Both genistein and 17β-estradiol increased PR gene expression in a dose- and time-dependent manner by three-fold, respectively ($P < 0.0001$ by ANOVA) (Fig. 2). There were no qualitative or quantitative differences between hOB cells obtained from female or male donors.

To assess the estrogenic action of phytoestrogens on OPG mRNA expression in osteoblastic cells, time courses, and dose responses with genistein were performed in primary first-passage hOB cells along with negative controls (ethanol vehicle) and positive controls (17β-estradiol). For time course experiments, the hOB cells were treated with genistein at a concentration of 10^{-7} M for 0, 6, 12, 24, 48, and 72 h. Genistein increased OPG mRNA levels (as assessed by RT-PCR) and OPG protein secretion by hOB in a time-dependent fashion (Fig. 3). The maximum effect occurred after 48 to 72 h and was 6.3- to 7.2-fold ($P < 0.0001$ for protein values). Of note, the increase of OPG gene expression and protein secretion by genistein was comparable to that induced by 17β-estradiol (Fig. 3). In order to characterize the stimulatory effects of genistein on OPG gene expression and protein secretion, a dose response experiment with concentrations ranging from 10^{-11} to 10^{-6} M or vehicle (ethanol) for a constant exposure time of 72 h was performed. Genistein increased OPG mRNA steady state levels (as assessed by semiquantitative RT-PCR) and protein secretion (as assessed by ELISA measurement of conditioned medium) ($P < 0.0001$ by ANOVA for protein) in a dose-dependent manner (Fig. 4). At the most effective dose of genistein (10^{-7} M for 72 h), the induction of OPG mRNA levels and protein concentrations was 2.6- and 3.5-fold, respectively. These stimulatory effects of genistein were also similar to those of 17β-estradiol (Fig. 4).

To test whether genistein-induced induction of OPG mRNA required de novo protein synthesis, we treated first-passage hOB cells either with vehicle, genistein (10^{-7} M), the protein synthesis inhibitor, cycloheximide (10 μg/ml), or a combination of genistein (10^{-7} M) and cycloheximide (10 μg/ml). As shown in Figure 5, cycloheximide failed to abrogate the stimulatory effect of genistein on OPG mRNA levels. This suggests that no newly synthesized protein is required for the induction of OPG mRNA

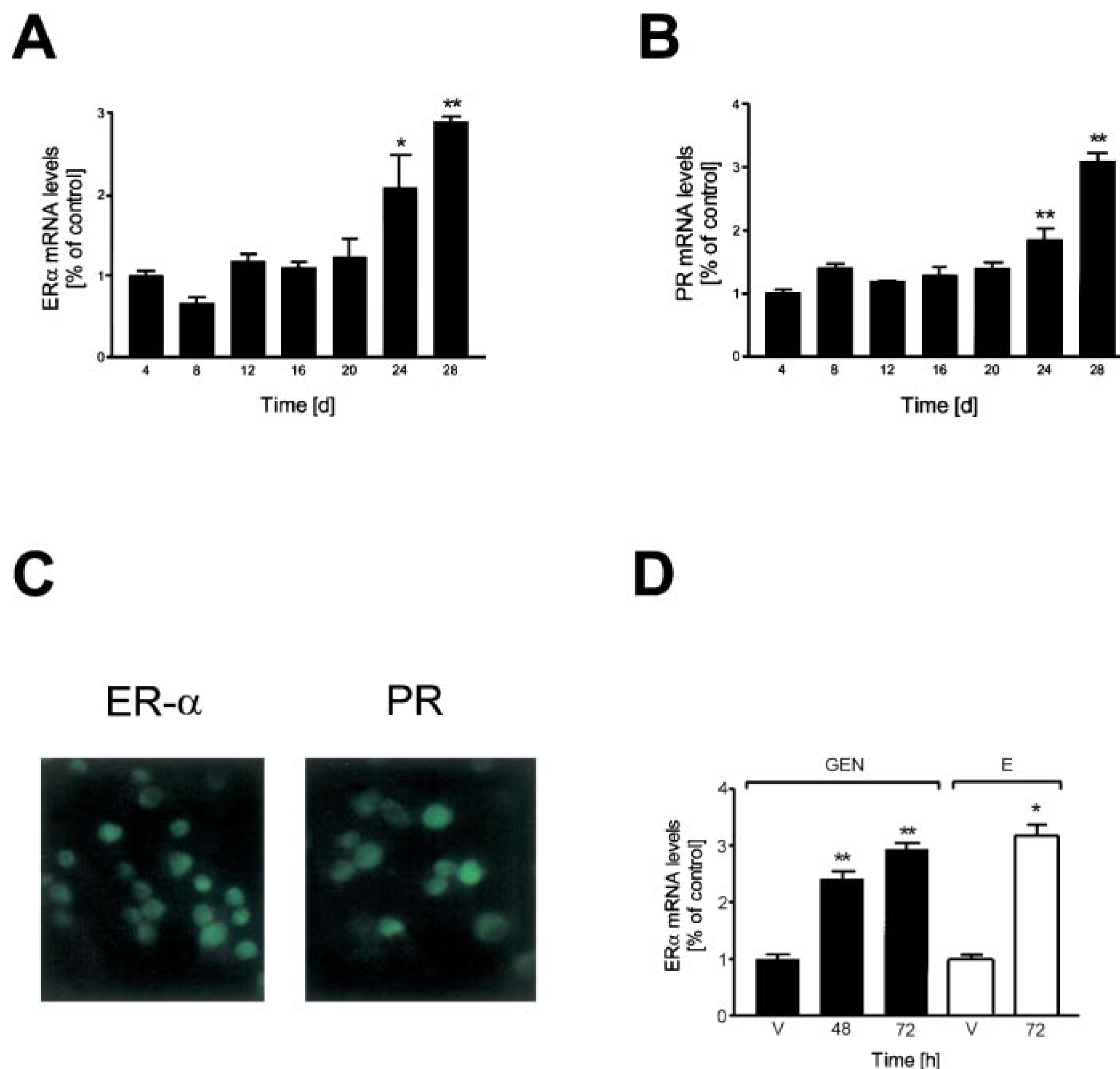

Fig. 1. The hOB cells are estrogen-responsive osteoblastic lineage cells. Semi-quantitative RT-PCR analysis for (**A**) estrogen receptor (ER)-α and (**B**) PR gene expression by hOB cells over time (in days) in culture. **C**: Immunohistochemical detection of ER-α and PR protein after 16 days of culture, respectively. An isotype control of primary antibody was used as negative control. **D**: RT-PCR demonstrating upregulation of ER-α gene expression by ER ligands 17β-estradiol (E, 10^{-8} M) and genistein (GEN, 10^{-7} M). Data in A, B, and D represent the mean ± SEM in triplicate measurement of steroid hormone receptor/L7 ratios normalized to day four (A and B) or the vehicle control (V, ethanol) (D). $P < 0.0001$ by ANOVA for A, B, and genistein in D. Post-test Newman-Keuls analysis: *$P < 0.01$ and **$P < 0.001$ for individual values compared to the respective control at Day 4 (A, B) or vehicle (D). *$P < 0.01$ by Student's paired *t*-test for 17β-estradiol (D).

expression by genistein. Next, we analyzed the effects of genistein treatment on OPG gene transcription by hOB cells directly by using a nuclear run-on assay. Compared to vehicle treatment, genistein markedly increased OPG mRNA expression (Fig. 5). Together, these results indicate that genistein acts mainly at the transcriptional level to induce OPG production.

To determine whether the stimulatory effects of genistein on OPG production are directly and specifically mediated by the ER, we employed a combination of genistein (10^{-7} for 72 h) and the pure ER antagonist ICI 182,780 (for the last 48 h prior to RNA isolation). The genistein-induced increase of OPG mRNA levels and protein secretion were dose-dependently abrogated by co-treatment with ICI 182,780 (by 68% for OPG protein at a concentration of 10^{-6} M for 48 h; $P < 0.01$) (Fig. 6). Treatment with ICI 182,780 alone inhibited OPG mRNA levels and protein production only weakly (by 14 and 16%, respectively; $P > 0.05$ for protein). We also evaluated whether genistein may counteract the

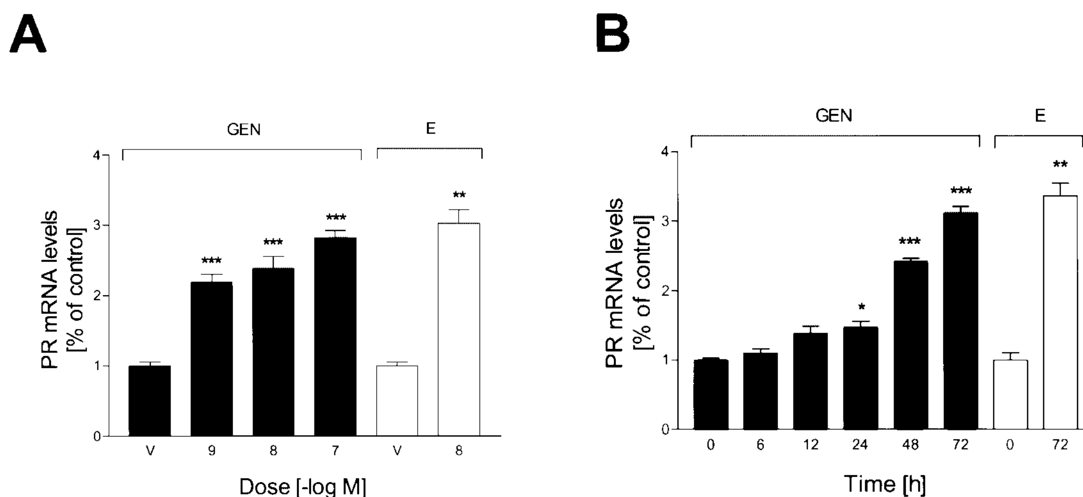

Fig. 2. Genistein and 17β-estradiol upregulate PR gene expression. **A**: Dose response: The hOB cells were treated with various concentrations of genistein (GEN) and 17β-estradiol (**E**) (numbers indicate the dose in log M) for 72 h. **B**: Time course: The hOB cells were treated with genistein (GEN) at 10^{-7} M or 17β-estradiol (E) at 10^{-8} M for various time points (in h). Semi-quantitative RT-PCR was performed and the data represent the mean ± SEM of triplicates of the PR/L7 ratios normalized to the vehicle control (V, ethanol) (A) or the control at 0 h (**B**), $P < 0.0001$ by ANOVA for GEN. Post-test Newman-Keuls analysis: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for individual values compared to the respective control at 0 h. $^{**}P < 0.01$ by Student's paired *t*-test for E.

inhibitory effects of glucocorticoids on OPG mRNA and protein production [Hofbauer et al., 1999b]. To test this, hOB cells were treated with either vehicle, genistein (at various concentrations for 72 h) and dexamethasone (10^{-8} M for the last 24 h prior to RNA isolation). Genistein completely and dose-dependently prevented the inhibitory effects of dexamethasone on OPG mRNA and protein production (Fig. 7).

DISCUSSION

Estrogens have major beneficial effects on bone [Eastell, 1998]. The primary mechanism of estrogens is to decrease bone resorption, which

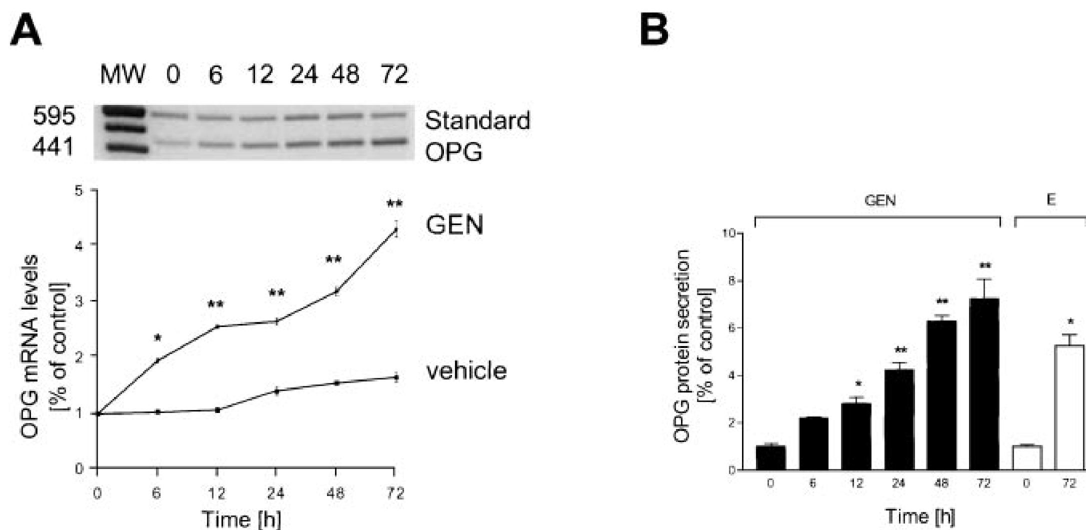

Fig. 3. Genistein stimulates OPG mRNA levels and protein secretion in a time-dependent manner. **A**: RT-PCR analysis of OPG mRNA levels from hOB that were cultured for the time indicated (in h) in the presence of genistein (GEN, 10^{-7} M) and 17β-estradiol (E, 10^{-8} M) (**B**) OPG protein secretion was measured by ELISA from conditioned medium harvested from the hOB treated as described in (A). Data represent the mean ± SEM of triplicates of OPG/L7 ratios (A) or OPG protein concentrations (B). $P < 0.0001$ by ANOVA for GEN. Post-test Newman-Keuls analysis: $^{*}P < 0.01$ and $^{**}P < 0.001$ for individual values compared to the respective control (A) or 0 h (**D**). $^{*}P < 0.01$ by Student's paired *t*-test for E.

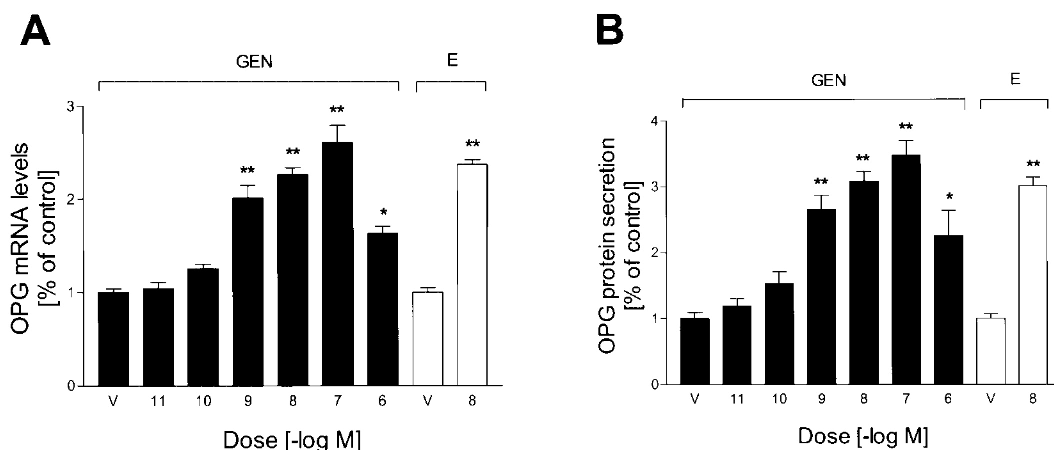

Fig. 4. Genistein stimulates OPG mRNA levels and protein secretion by hOB in a dose-dependent manner. **A**: RT-PCR analysis of OPG mRNA levels isolated from hOB that were cultured for 72 h in the presence of various concentrations of genistein (GEN) and 17β-estradiol (**E**) (numbers indicate the dose in log M). The values indicate the mean ± SEM of OPG/L7 ratios normalized to the vehicle control (V, ethanol). **B**: OPG protein secretion was measured by ELISA from conditioned medium harvested from the hOB treated as described in A. Data represent the mean ± SEM of triplicates (percent of control). $P < 0.0001$ by ANOVA for GEN; Post-test Newman-Keuls analysis: $^{*}P < 0.01$ and $^{**}P < 0.001$ for individual values compared to the respective control. $^{**}P < 0.001$ by Student's paired *t*-test for E.

has been attributed to direct inhibitory effect on osteoclasts, the suppression of bone-resorbing cytokines, and the stimulation of anti-resorptive factors [Spelsberg et al., 1999]. However, because of the concomitant side-effects of exogenous estrogen administration, alternatives to the conventional estrogen replacement therapy are desirable [Anderson and Garner, 1998; Fitzpatrick, 1999]. Phytoestrogens are a heterogeneous, plant-derived group of ER ligands [Anderson and Garner, 1998; Fitzpatrick, 1999]. Because of their content in food and the

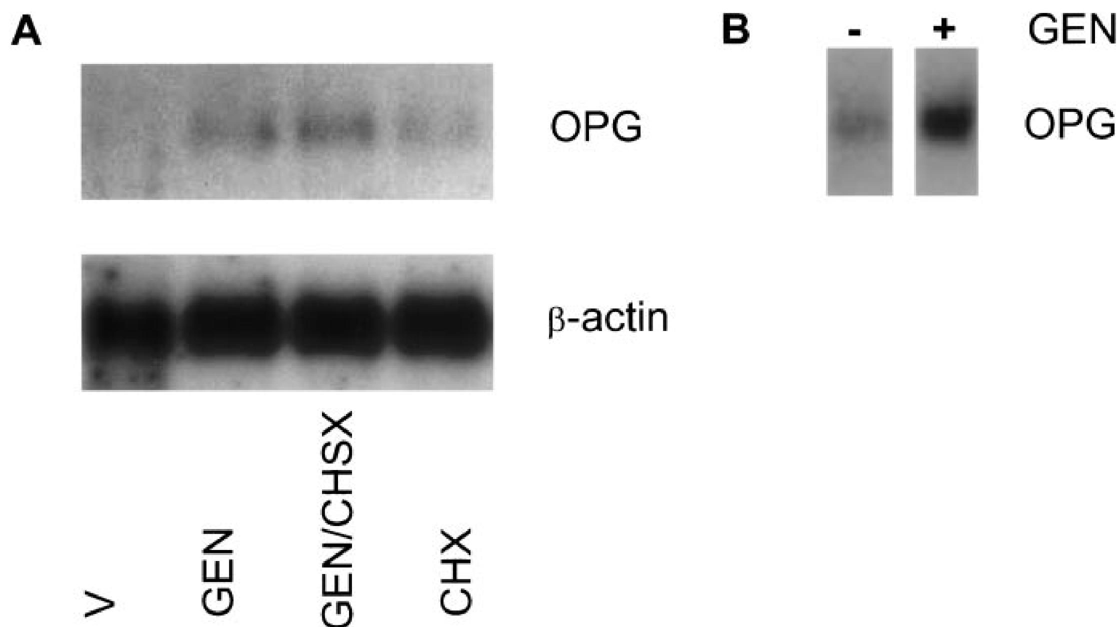

Fig. 5. Molecular mechanism of genistein-induced OPG expression. **A**: Inhibition of de novo protein synthesis by cycloheximide. The hOB cells were treated for 48 h with vehicle, genistein (10^{-7} M), cycloheximide (10 μg/ml), or genistein (10^{-7} M) and cycloheximide (10 μg/ml). Ten micrograms of total RNA were then assessed for OPG mRNA (2.9 kb) and β-actin mRNA (2.0 kb) levels by Northern hybridization. **B**: Effects of genistein on OPG gene transcription assessed by nuclear run-on assay. Two microgarms of OPG cDNA were assessed by Southern blot analysis using radiolabeled OPG mRNA (200,000 cpm) from vehicle-treated (−) or genistein-treated (+) hOB cells as a probe.

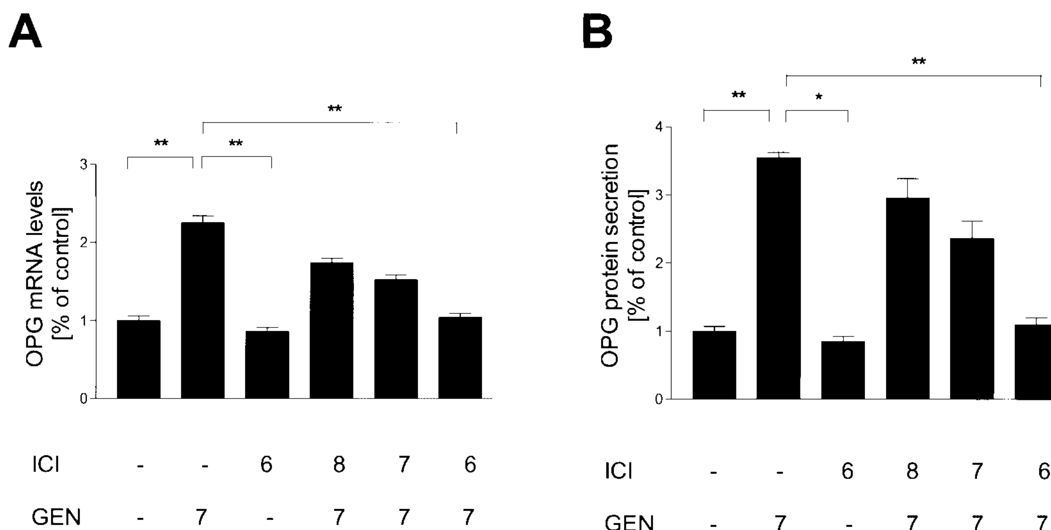

Fig. 6. The estrogen receptor antagonist ICI 182,780 blocks genistein-induced OPG mRNA expression and protein secretion. **A**: RT-PCR analysis of OPG mRNA levels from hOB following treatment with either ethanol vehicle (V), genistein (GEN), ICI 182,780 (ICI), or combinations of both GEN and ICI for 72 h as described in the Result section. The numbers indicate the dose in log M. Data represent the mean ± SEM of triplicates of the OPG/L7 ratio normalized to 100%. **B**: Conditioned medium was harvested from hOB treated with a combination of genistein and ICI 182,780 as described in (A). OPG protein secretion was determined by ELISA and the data are presented as the mean ± SEM of triplicates normalized to 100%. $^{*}P < 0.01$, $^{**}P < 0.001$ by Student's paired *t*-test.

possibility of increasing phytoestrogen uptake by a diet rich in soy products, these substances may be conveniently delivered to a large population [Anderson and Garner, 1998; Fitzpatrick, 1999]. Genistein and other phytoestrogens have been shown to decrease biochemical markers of bone resorption and to mitigate bone loss in animal models of estrogen deficiency [Fanti et al., 1998; Ishimi et al., 1999; Gao and Yamaguchi, 2000] and postmenopausal women

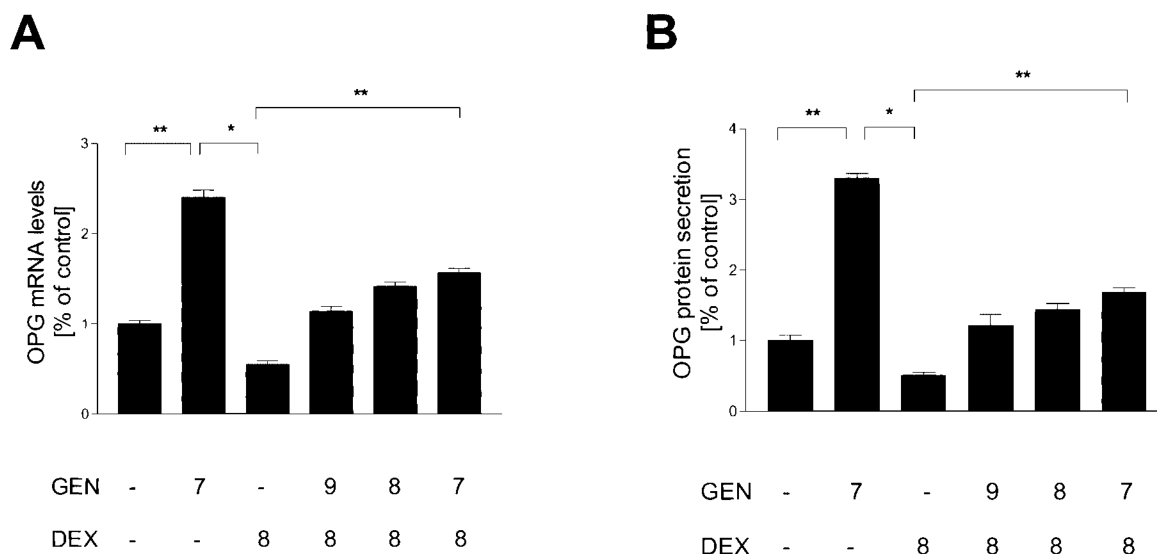

Fig. 7. Genistein prevents dexamethasone-induced inhibition of osteoblastic OPG mRNA expression and protein production. **A**: RT-PCR analysis of OPG mRNA levels from hOB following treatment with either ethanol vehicle (V), genistein (GEN), dexamethasone (DEX), or combinations of both GEN and DEX for 72 h as described in the Result section. The numbers indicate the dose in log M. Data represent the mean ± SEM of triplicates of the OPG/L7 ratio normalized to 100%. **B**: Conditioned medium was harvested from hOB treated with a combination of genistein and dexamethasone as described in (A). OPG protein secretion was determined by ELISA and the data are presented as the mean ± SEM of triplicates normalized to 100%. $^{*}P < 0.01$, $^{**}P < 0.001$ by Student's paired *t*-test.

[Fanti et al., 1998; Wangen et al., 2000]. However, the mechanisms of phytoestrogen action on bone cells are only partially understood. The great variability of phytoestrogen content in food and lack of pure phytoestrogen preparations have been major obstacles in evaluating the effects of phytoestrogens on bone metabolism [Kurzer and Xu, 1997]. RANKL and OPG are the essential regulators of osteoclast biology and bone resorption [Simonet et al., 1997; Lacey et al., 1998; Yasuda et al., 1998], and are modulated by various cytokines and hormones known to affect bone metabolism [Teitelbaum, 2000]. In this study, we report that the phytoestrogen genistein stimulates the production of OPG by primary human untransformed osteoblastic cells (hOB).

The hOB cells represent a primary osteoblastic lineage cell population that were used in the first or second passage and that had been derived from healthy young donors undergoing surgery for traumatic fractures [Siggelkow et al., 1999]. The hOB cells display the mature osteoblastic phenotype when cultured under appropriate conditions [Siggelkow et al., 1999] and mainly express the endogenous ER-α and the ER target gene, PR [Harris et al., 1995; MacNamara and Loughrey, 1998], as assessed by RT-PCR and immunohistochemistry. Both ER-α and PR gene expression were stable over a period of 2–3 weeks in culture, and were upregulated, thereafter, which is consistent with previous studies [Arts et al., 1997; MacNamara and Loughrey, 1998]. Therefore, all experiments were performed within the 2-week-period of stable expression of ER-α and PR. Of note, PR was upregulated by genistein, which was similar to the effect of 17β-estradiol as previously reported for the hFOB/ER-9 cells [Harris et al., 1995]. These data indicate that the hOB cells represent an estrogen-responsive human cell model of mature osteoblastic lineage cells, which is suitable to study the effects of ER ligands on osteoblastic gene expression. The predominant expression of ER-α (as compared to ER-β) in osteoblastic lineage cells confirms previous reports [Arts et al., 1997; Lim et al., 1999] and was shown to account for the stimulatory effects of 17β-estradiol on OPG production in human and murine osteoblastic lineage cells [Saika et al., 2001]. Of course, our studies do not allow to exclude an important role for ER-β in regulating bone metabolism [Onoe et al., 1997].

The stimulation of OPG by genistein was substantial (two- to six-fold), occurred in a dose- and time-dependent manner, was present at the mRNA level and protein, and was similar to that observed with 17β-estradiol. Genistein-stimulated OPG production was completely blocked by the pure antiestrogen ICI 182,780 in a dose-dependent manner, indicating that the induction of OPG by genistein is specifically mediated through the ER. Our studies also indicate that genistein induces OPG production mainly through a transcriptional mechanism because de novo protein synthesis was not required and genistein stimulated OPG transcription as assessed by a nuclear run-on assay. These data are in accordance with the study by Saika et al. [2001] demonstrating transcriptional activation of OPG by 17β-estradiol in ST-2 cells stably transfected with the ER-α and confirm other observations demonstrating transcriptional mechanisms as the primary mode of action of OPG regulation [Hofbauer et al., 1999b; Wan et al., 2001]. Our study also provides first evidence that genistein (by inducing OPG) may abrogate the inhibitory effects of the glucocorticoid dexamethasone, one of the most potent inhibitor of OPG production [Hofbauer et al., 1999b]. This suggests that stimulatory and inhibitory factors compete for the regulation of OPG, and further underlines the role of OPG (and RANKL) as the essential downstream effector molecules of bone resorption and bone metabolism [Teitelbaum, 2000]. Together, our data indicate that the increase of OPG production following genistein treatment meet the criteria for a specific and physiologically relevant response. By contrast, RANKL was not consistently expressed by the hOB cells and was not modulated by genistein (data not shown). RANKL expression has been shown to decrease during human osteoblastic differentiation, which further supports that the hOB represent mature, differentiated human osteoblasts [Gori et al., 2000]. Thus, similar to 17β-estradiol [Hofbauer et al., 1999a; Saika et al., 2001], the primary effect of genistein on decreasing the RANKL-to-OPG ratio in the bone marrow microenvironment is to enhance OPG production.

A recent study indicated that the relative binding affinity of genistein (purchased from the same commercial source) to the ER-α is 4% (compared to that of 17β-estradiol) and 87% to ER-β (compared to that of 17β-estradiol),

respectively. However, based on a transactivation assay using a luciferase reporter gene construct, transcriptional activity of genistein was 2.0-fold higher (than that of 17β-estradiol) at the ER-α and 1.8-fold higher (than that of 17β-estradiol) at the ER-β [Kuiper et al., 1998]. Moreover, the dose range for effects of genistein in these assay systems were between 10^{-9} and 10^{-7} M [Kuiper et al., 1998], which was similar to the effective genistein dose in our study. These studies indicate that, once such genistein concentrations are reached in compartments of estrogen-responsive cells such as osteoblasts, biologically relevant effects will ensue.

Our findings provide the first evidence that genistein acts on human osteoblastic cells to increase the secretion of OPG, a potent inhibitor of bone resorption. Thus, a local increase of OPG levels in the bone microenvironment may be an important component of the paracrine mechanisms by which genistein reduces bone resorption. If so, this would have obvious implications for the treatment of women with postmenopausal osteoporosis. In conclusion, we found that the phytoestrogen genistein, which is contained in various soy products may enhance OPG production in human osteoblastic cells in vitro, which may contribute to the bone-sparing effects of a diet rich in phytoestrogens.

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