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ARTICLES

Genistein Stimulates the Osteoblastic Differentiation via NO/cGMP in Bone Marrow Culture

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Abstract The soybean phytoestrogen, genistein (Gen), has anabolic effects on bone through mechanisms that remain to be elucidated. We examined the role of nitric oxide (NO) and its downstream effector guanylyl cyclase (GC) in mediating the effects of Gen on the proliferation and osteoblastic maturation of primary mouse bone marrow-derived mesenchymal stem cells (BMSCs). Gen ($10^{-8} \sim 10^{-6}$ M) resulted in a dose-dependent increase in cell proliferation as measured by increased [^{3}H]thymidine incorporation, and stimulated osteoblastic maturation as assessed by culture duration-dependent increments in alkaline phosphatase (ALP) activity, calcium deposition into extracellular matrix and *Runx2*/*Cbfa1* gene expression in BMSCs cultures. Gen also resulted in a dose-dependent increase in NO synthase (NOS) activity, NO formation, and cGMP production in BMSCs cultures. The effects of Gen were mimicked by 17β-estradiol (E_2, 10^{-8} M). Concurrent treatment with the estrogen receptor (ER) antagonist ICI182,780 (10^{-7} M) or the NOS inhibitor L-NAME (3×10^{-3} M) diminished the Gen (10^{-6} M)-mediated increase in NOS activity, NO production, and cGMP content. In contrast, a soluble GC inhibitor 1H-[1,2,4]oxadiazolo [4,3,-a]quinoxalin-1-one (ODQ, 10^{-6} M) selectively blocked the Gen (10^{-6} M)-mediated increase in cGMP content but not in NO production and NOS activity. Moreover, inhibition of ER, NOS activity or cGMP blocked Gen-induced proliferation and osteoblastic differentiation of BMSCs and *Runx2*/*Cbfa1* gene expression in culture. Gen has estrogen-like activity and stimulates the proliferation and osteoblastic differentiation of mouse BMSCs at least in part through NO/cGMP pathway. J. Cell. Biochem. 94: 307–316, 2005. © 2004 Wiley-Liss, Inc.

Key words: genistein; phytoestrogen; mesenchymal stem cell; osteoblast; estrogen receptor; nitric oxide; cyclic GMP

Estrogen deficiency is an important component of postmenopausal bone loss [Kanis et al., 1994]. Although hormone replacement therapy (HRT) has been a widely used approach for preventing postmenopausal osteoporosis, HRT not only increases the risk of breast cancer but it also has other undesirable side effects [Recker, 1993; Vihtamaki et al., 1999]. So it is becoming hot to search for alternative therapy of the classical HRT. Soy protein and soy-derived phytoestrogens have received a great deal of attention over the last few years because of their potentially prevention and treatment of postmenopausal osteoporosis without stimulating mammary glands and uteri [Anderson and Garner, 1998; Murkies et al., 1998; Setchell and Lydeking-Olsen, 2003]. In the epidemiological studies, the lower incidence of osteoporosis after menopause in Asian women compared with that in Western women was reported attributable to the traditional soybean-rich Asian diet [Horiuchi et al., 2000; Mei et al., 2001]. This was further supported by the studies carried out on ovariectomy rats and early postmenopausal women, showing that dietary soybean proteins and phytoestrogen genistein (Gen) prevented

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bone loss [Arjmandi et al., 1996; Fanti et al., 1998; Morabito et al., 2002].

Gen, a classic phytoestrogen found predominantly in soy, has a structural similarity to mammalian estrogen and can bind weakly to estrogen receptors (ERs) [Wang et al., 1996; Kuiper et al., 1997; Rickard et al., 2003]. There is compelling evidence that Gen in soybeans stimulates osteoblastic bone formation and inhibits osteoclastic bone resorption in vitro [Gao and Yamaguchi, 1999; Sugimoto and Yamaguchi, 2000; Chen et al., 2003; Heim et al., 2004] and in vivo [Ishimi et al., 1999, 2000]. However, the mechanisms of Gen action on bone cells have not yet been fully understood.

Nitric oxide (NO) was reported to mediate partly the stimulation of 17β-estradiol (E_2) on osteoblast proliferation and differentiation [O'Shaughnessy et al., 2000]. Recent study demonstrates that Gen improves flow-mediated endothelium dependent vasodilatation in ovariectomized rats and healthy postmenopausal women through a NO-dependent mechanism [Squadrito et al., 2000, 2003]. Our previous study also found that asymmetric dimethy-L-arginine (ADMA), an endogenous inhibitor of NO synthase (NOS), impairs osteoblastic differentiation of primary mouse bone marrow-derived mesenchymal stem cells (BMSCs) culture [Xiao et al., 2001a]. In addition, NO has been shown to be an important signaling molecule involved in bone turnover [Grant and El-Fakahany, 2004].

The biological actions of NO are predominantly mediated by activation of guanyly cyclase (GC), which results in increased cGMP level and activation of cGMP-dependent intracellular cascades [Mancini et al., 2000]. NO-cGMP pathway also has been implicated in the regulation of osteoblast growth and differentiation [Mancini et al., 2000; Grant and El-Fakahany, 2004]. Whether the NOS–NO–cGMP pathway is involved in mediating the action of Gen is unknown. Therefore, we examined the role of the NO–GC signaling pathways in mediating the effects of Gen on the growth and osteoblastic differentiation of mouse BMSCs culture.

MATERIALS AND METHODS

Reagents

Alpha minimum essential medium (α-MEM), phenol red-free α-MEM, Dulbecco's modified eagle medium (DMEM), phenol red-free DMEM, Hanks' balanced salt solution (HBSS), fetal bovine serum (FBS), penicillin–streptomycin solution, sodium dodecyl sulfate (SDS), and Trizol reagent were obtained from Gibco BRL (Grand Island, NY). Gen, 17β-estrodial (E_2), N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{G}-monomethyl-L-arginine (L-NMMA), 3-isobutyl-1-methyl-xanthine (IBMX), trypsin, ascorbic acid, β-glycerophosphate, *p*-nitrophenyl phosphate (*p*-NPP), *p*-nitrophenol (*p*-NP), diethanolamine, dextran-coated charcoal, dimethyl sulfoxide (Me_2SO), and calcium kit were purchased from Sigma Chemical Co. (St. Louis, MO). ICI182,780 and 1H-[1,2,4]oxadiazolo [4,3,-a]quinoxalin-1-one (ODQ) were purchased from Tocris Cookson, Inc., Ltd. (Avonmouth Bristol BS11 8TA, UK). Tissue culture plastic wares were purchased from Corning-Costar Co. (Corning-Costar, NY). Nitrate/nitrite colorimetric assay kit was purchased from Jingmei Biotech Co., Ltd. (Shenzhen, China). Nitric oxide synthase (NOS) assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Cyclic GMP EIA kit was purchased from R&D Systems, Inc. (Minneapolis, MN). Luciferase assay system was purchased from Promega (Madison, WI) molecular biology reagents and enzymes were purchased from Boehringer Mannheim (Indianapolis, IN). iQ™ SYBR® Green Supermix and Bio-Rad reagent for protein assay were obtained from Bio-Rad Laboratories (Hercules, CA). [^{3}H]thymidine was purchased from Shanghai Institute of Nuclear Research (Shanghai, China). All other chemicals were of analytical grade and from Shanghai Biotech Co., Ltd. (Shanghai, China).

Cell Culture

Bone marrow cells were obtained from 8- to 10-week-old female mice of the Kunming strain (the Experimental Animal Center of Xiangya Medical College, Central South University, Changsha, China) as previously described [Qu et al., 1998; Xiao et al., 2001a]. Briefly, the femurs and tibias were dissected, the ends of the bones were cut, and the marrow was flushed out with 2 ml of ice-cold α-MEM containing 10% v/v FBS by using a needle and syringe. A suspension of single bone marrow cells was obtained by repeated aspiration of the cell preparation through a 22-gauge needle, and then cells were counted with a hemocytometer. Cells were

seeded into 12-well plates or 60 mm plates at a density of 1×10^7 cells/ml and cultured for 5 days in α-MEM supplemented with 15% v/v FBS, 100 U/ml of sodium penicillin G, and 100 μg/ml of streptomycin sulfate and kept in a humidified incubator with 5% CO_2 and 95% air at a temperature of 37°C. On day 5, all nonadherent cells were removed with the first medium change and then the adherent cells (representing BMSCs) were grown for additional periods of up to 12 days in the differentiation medium [phenol red-free α-MEM containing 10% v/v FBS (dextran-coated charcoal stripped, DCS) supplemented with 5×10^{-3} M β-glycerophosphate and 25 μg/ml of ascorbic acid for inducing osteoblastic differentiation of BMSCs]. In the subsequent experiments, the beginning day of culture in the differentiation medium was defined as day 0. The treatments were respectively added every 2 days and the medium was replaced every 4 days thereafter.

C2C12, a muti-pluripotent cell line, was maintained in DMEM supplemented with 10% v/v FBS with medium changed every 3 days.

[^{3}H]thymidine Incorporation Assay

Bone marrow cells were seeded into 12-well plates at a density of 1×10^7 cells/ml (0.25×10^7 cells/cm^2) and cultured for 5 day in α-MEM supplemented with 15% v/v FBS. For 24 h prior to experiment, cultures were placed in 0.1% v/v FBS and phenol red-free α-MEM. After 24 h, the cells were placed in phenol red-free α-MEM containing 7.5% v/v FBS (DCS) and treated with the reagents as indicated for 48 h. The cells were labeled with 370 KBq of [^{3}H]thymidine for the last 24 h of incubation, and then were rinsed with phosphate buffered saline (PBS, 3×5 min) and 10% trichloroacetic acid (1×30 min). Finally, the cells were dissolved in 200 μl of 0.2 N NaOH and left overnight at 4°C. Radioactivity was determined by scintillation counting.

Alkaline Phosphatase (ALP) Activity

Cellular ALP activity was assayed according to our previous study [Xiao et al., 2001a]. At the completion of the incubation period, cells were harvested after removing the media, washing twice with PBS, and treating for 10 min with 0.25% trypsin to achieve cell detachment. Enzyme activity was determined colorimetrically after incubated at 37°C for 30 min with *p*-NPP as the substrate at pH 10.3 and reading the optical density at 400 nm and total protein content was measured with a Bio-Rad protein assay kit. ALP activity is expressed as nmol/min/μg protein.

Quantitation of Calcium Deposition

Calcification of BMSCs was assessed by a modification of the Wada procedure [Xiao et al., 2001a]. The cultures were decalcified with 0.6N HCl for 24 h. The calcium content was determined by measuring the concentrations of calcium in the HCl supernatant by the *o*-cresolphthalein complexone method. After decalcification, the cultures were washed with PBS and solubilized with 0.1N NaOH/0.1% SDS. Total protein content was measured with a Bio-Rad protein assay kit. The calcium content of the cell layer was normalized to the protein content.

Measurement of NO Production

Nitrite production in conditioned media (CM) was measured as indirect measurement of NO production using nitrate/nitrite colorimetric assay kit by a modified Griess assay [Green et al., 1982; Xiao et al., 2001a]. Briefly, 100 μl of CM or nitrite standards (0–100 μM) were mixed with 100 μl of Griess reagent. Absorbance was then measured at 530 nm against a blank prepared with 100 μl of distilled water, and the release of NO into the culture medium was expressed as nitrite concentration, which was determined from a standard curve. Total protein content was measured with a Bio-Rad assay kit. The results were expressed as μM/μg protein.

NOS Activity Measurement

After treatment of 8 days, the supernatant was discarded and the cell layer was washed twice with PBS. The cells were detached on ice with a tuber scraper. Then, cells were disrupted (3×7 s) with ultrasonic disrupter in water bath at 0°C. The specimen was collected and centrifuged at 15,000*g* at 4°C for 10 min. The NOS activity was determined in the supernatant using NOS colorimetric assay kit. Briefly, 50 μl of supernatant were mixed with assay reagent containing L-arginine and NOS in the specimen catalyzed L-arginine to L-citrulline and NO. NOS activity was indirectly determined by measuring NO production in colorimetric assay. Absorbance was obtained at 530 nm against a blank prepared with distilled water to deter-

mine the concentration of nitrite, which is NO metabolite. NOS activity was calculated with the formula according to the kit reference. Total NOS (tNOS) and inducible NOS (iNOS) activity were separately measured by using Ca^{2+}-containing or Ca^{2+}-free assay buffer. Total protein content was measured with a Bio-Rad assay kit. The NOS activity was normalized to the protein content and expressed as percentage of control level.

Measurement of the Accumulation of cGMP

Bone marrow stromal cells, after treated for 8 days with Gen ($10^{-8} \sim 10^{-6}$ M) in the presence or absence of L-NAME (3×10^{-3} M) or ICI182,780 (10^{-7} M) or ODQ (10^{-6} M), were washed with phenol red-free α-MEM and then incubated with phenol red-free α-MEM supplemented with 0.5×10^{-3} M of IBMX, a diesterase inhibitor, at 37°C for 15 min. The reagents were then added and the cells were then incubated for another 1 h, and the amount of cGMP in the media was measured with the EIA kit.

Real-Time RT-PCR

For quantitative real-time RT-PCR, 2.0 μg total RNA was isolated from the BMSCs cultured in 60-mm plates by the single-step method using Trizol reagent [Xiao et al., 2004]. PCR reactions contained 100 μg template (cDNA or RNA), 300 μM of each forward and reverse primer, and 1× iQ™ SYBR® Green Supermix in 50 μl. Samples were amplified for 40 cycles in an iCycler iQ™ real-time PCR detection system with an initial melt at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR product accumulation was monitored at multiple points during each cycle by measuring the increase in fluorescence caused by the binding of SybrGreen I to dsDNA. The threshold cycle (C_t) of tested-gene product from the indicated genotype was normalized to the C_t for cyclophilin A. The primers sequences used to amplify the genes in Table III are available upon request.

Assay of Runx2/Cbfa1 Promoter Activity

Effect of Gen on p1.4Cbfa1-Luc transcription was analyzed in stably transfected C2C12 cells [Xiao et al., 2001b]. The cells were plated at a density of 1×10^4 cells/well in 24-well plates and grown in DMEM containing 10% v/v FBS for 24 h, then cultures were placed in phenol red-free DMEM supplied with 0.1% v/v FBS. After an additional 24 h, the cells were placed in phenol red-free DMEM supplemented with 5% v/v FBS (DCS) and treated with Gen ($10^{-8} \sim 10^{-6}$ M) in the presence and absence of L-NMMA (1×10^{-3} M) or ICI182,780 (10^{-7} M) or ODQ (10^{-6} M). After 48 h treatment, the luciferase activity was measured using the Luciferse assay system (Promega) on a monolight 2010 luminometer (PharMingen, San-Diego, CA).

Statistical Analysis

The data were expressed as the mean ± SD. and statistically analyzed by one-way ANOVA. Values considered to be statistically significant were $P < 0.05$.

RESULTS

Effect of Gen on DNA Synthesis, ALP Activity, and Calcium Deposition in Mouse BMSCs Cultures

We had shown in our previous study that in this mouse BMSCs culture model [Xiao et al., 2001a] ALP activity and calcium deposition increased in a time-dependent way and reached the highest level after 8 and 12 days incubation, respectively. Thus, we determined these two indices at the corresponding time point. In the presence of [^{3}H]thymidine, treatment with Gen 10^{-8}–10^{-6} M for 24 h dose-dependently increased the [^{3}H]thymidine incorporation into DNA. The same dose-dependent reactions were also found in ALP activity and calcium deposition, respectively after 8- and 12-days incubation (Table I). The stimulations were statistically significant at 10^{-7} M. Gen (10^{-6} M) was found to give the maximal response when compared with control (Table I). This concentration was then used for the proceeding experiments. The increases induced by Gen (10^{-6} M) on [^{3}H]thymidine incorporation, ALP activity and calcium deposition were inhibited by coincubation with a pure ER antagonist ICI182,780 (10^{-7} M), or a NOS inhibitor L-NAME (3 or 6×10^{-3} M), or a soluble guanylyl cyclase (GC) inhibitor ODQ (10^{-6} M), although these inhibitors alone had no effect on these indices (Table II). E_2 (10^{-8} M), similar to Gen (10^{-6} M), also increased the [^{3}H]thymidine incorporation, ALP activity, and calcium deposition respectively after treated for indicated days, which were also abolished by either ICI-182,780 (10^{-7} M) or L-NAME (3 or 6×10^{-3} M), or ODQ (10^{-6} M) (Table II).

TABLE I. Dose-Dependent Effect of Genistein (Gen) on the [^{3}H]Thymidine Incorporation, Alkaline Phosphatase (ALP) Activity and, Calcium Deposition of Mouse Bone Marrow-Derived Mesenchymal Stem Cells (BMSCs)

Group	[^{3}H]thymidine incorporation (% control)	ALP activity (ηM/min/μg protein)	Calcium deposition (μg/mg protein)
Control	98 ± 3	1.70 ± 0.11	224 ± 9
Gen (M)			
10^{-8}	105 ± 12	1.79 ± 0.10	247 ± 24
10^{-7}	121 ± 20^b	1.92 ± 0.13^b	267 ± 22^b
10^{-6}	177 ± 11^c	2.10 ± 0.18^b	279 ± 12^c
10^{-5}	116 ± 20^b	1.99 ± 0.14^b	247 ± 9

n = 6, mean ± SD, $^bP < 0.05$, $^cP < 0.01$ versus control (Me_2SO).

Effect of Gen on NO Production and NOS Activity in Mouse BMSCs Cultures

The NO metabolite level in the CM was elevated in a dose-dependent manner after treated with Gen ($10^{-8} \sim 10^{-6}$ M) for 8 days, the increment in NO production was small but significant. The tNOS activity but not iNOS activity was stimulated by Gen (10^{-8}–10^{-6} M), while only 10^{-6} M Gen gave the statistically significant response ($P < 0.05$ vs. control). No apparent response was found in iNOS activity. E_2 (10^{-8} M) showed the similar effect as Gen (10^{-6} M) on NO release and NOS activity of BMSCs. The increases in NO release and NOS activity caused by Gen (10^{-6} M) and E_2 (10^{-8} M) were abolished by ICI182,780 (10^{-7} M) or L-NAME (3×10^{-3} M), but not by ODQ (10^{-6} M), a downstream effect inhibitor of NOS/NO cascade (data not shown).

Effect of Gen on cGMP Content in the CM of Mouse BMSCs Cultures

The cGMP content in the CM was dose-dependently elevated by Gen ($10^{-8} \sim 10^{-6}$ M) after treatment for 1 h (Fig. 1). Gen (10^{-6} M) gave the greatest response when compared with control ($P < 0.01$). The increase caused by Gen (10^{-6} M) was diminished when coincubated with ICI182,780 (10^{-7} M) or L-NAME (3×10^{-3} M) or ODQ (10^{-6} M); however, these antagonists alone did not affect the cGMP production (Fig. 1). Positive control E_2 (10^{-8} M) also increased the cGMP content in the CM, which was attenuated by ICI182,780 (10^{-7} M) (Fig. 1).

Effect of Gen on the mRNA Expression of Runx2/Cbfa1

The real-time RT-PCR analysis was performed to evaluate the effect of Gen on the

TABLE II. Inhibition of ICI182,780, or L-NAME or ODQ on the Gen (10^{-6} M)-Induced Stimulation on the Proliferation and Osteoblastic Differentiation of Mouse BMSCs

Group	[^{3}H]thymidine incorporation (% control)	ALP activity (ηM/min/μg protein)	Calcium deposition (μg/mg protein)
Control	98 ± 3	1.70 ± 0.11	224 ± 9
Gen	177 ± 11^b	2.10 ± 0.18^b	279 ± 12^b
ICI182,780	97 ± 9	1.60 ± 0.12	219 ± 12
Gen + ICI182,780	154 ± 6^c	1.74 ± 0.07^c	234 ± 10^c
L-NAME*	98 ± 9	1.50 ± 0.21	210 ± 10
Gen + L-NAME*	124 ± 13^c	1.70 ± 0.15^c	219 ± 15^c
ODQ	102 ± 14	1.67 ± 0.17	224 ± 8
Gen + ODQ	123 ± 12^c	1.73 ± 0.13^c	230 ± 10^c
E_2	221 ± 31^b	2.50 ± 0.35^b	363 ± 22^b
E_2 + ICI182,780	124 ± 11^d	1.70 ± 0.21^d	248 ± 23^d
E_2 + L-NAME*	114 ± 39^d	1.84 ± 0.17^d	263 ± 20^d
E_2 + ODQ	164 ± 28^d	1.97 ± 0.21^d	253 ± 22^d

n = 6, mean ± SD, $^bP < 0.01$ versus control (Me_2SO). $^cP < 0.05$ versus Gen. $^dP < 0.05$ versus E_2.
*The concentration of L-NAME used in assay of the [^{3}H]thymidine incorporation was 6×10^{-3} M, and in assay of the ALP activity and the calcium deposition was 3×10^{-3} M.

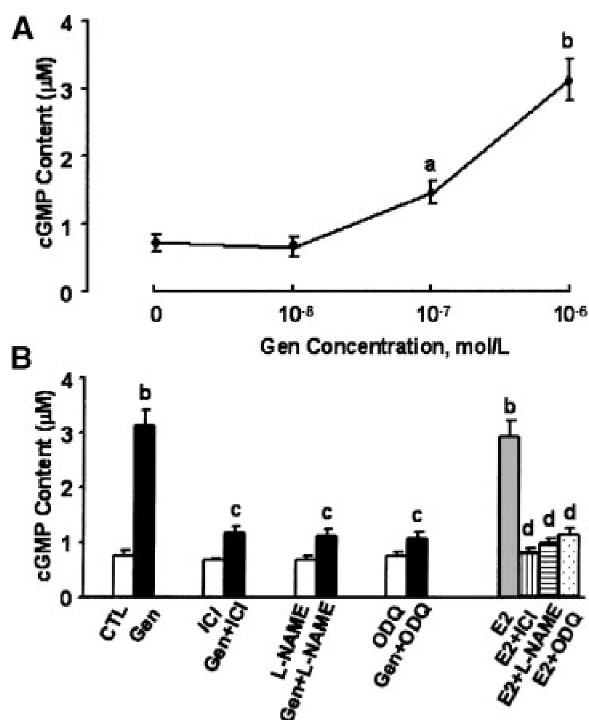

Fig. 1. Effect of genistein (Gen) on cGMP content in the conditioned media (CM) of mouse bone marrow-derived mesenchymal stem cells (BMSCs). **A**: Dose-dependent effects of Gen on cGMP content in the CM of mouse BMSC cultures. **B**: Inhibition of ICI or L-NAME or ODQ on the Gen (10^{-6} M)-induced increases on cGMP content in the CM of mouse BMSC cultures. BMSCs were treated with Gen (10^{-6} M) in the presence or absence of ICI (10^{-7} M) or L-NAME (3×10^{-3} M) or ODQ (10^{-6} M) for 8 days. The amount of cGMP in the media was measured with the EIA kit as described in "Materials and Methods." E_2 as a positive control. n = 6, mean ± SD. $^aP < 0.05$, $^bP < 0.01$ versus control (Me_2SO). $^cP < 0.05$ versus Gen. $^dP < 0.05$ versus E_2.

expression of mRNA for Runx2/Cbfa1 isoforms in BMSCs cultures at day 12. When compared to control cultures, the Gen-treated (10^{-6} M) groups had a three-fold increase in Runx2/Cbfa1 mRNA message, both Runx2/Cbfa1 type I and type II isoform had equal effects. *Osteocalcin*, the downstream gene of Runx2/Cbfa1, was also significantly increased in the Gen-treated (10^{-6} M) groups. Again, 3×10^{-3} M L-NAME or 10^{-7} M ICI182,780 reversed the upregulation of *Runx2*/*Cbfa1* and *Osteocalcin* gene by Gen when it was added with 10^{-6} M Gen into the culture medium. In contrast, ODQ (10^{-6} M) only partially abolish Gen-induced increments. L-NAME or ICI182,780 or ODQ alone had no effect on the gene expression (Table III). E_2 (10^{-8} M) showed the similar effect as Gen (10^{-6} M) on Runx2/Cbfa1 and Osteocalcin expression, which was abolished by ICI182,780 (10^{-7} M) (Table III).

Stimulation of Gen on Runx2/Cbfa1 Promoter Activity

To examine the effect of Gen on Runx2/Cbfa1 promoter, we stably transfected mouse Runx2/Cbfa1 P1 promoter/luciferase reporter constructs (p1.4Cbfa1-Luc) into undifferentiated, mesenchymal-derived, pluripotent C2C12 cells. Gen ($10^{-8} \sim 10^{-6}$ M) caused dose-dependent increases in p1.4Cbfa1-Luc expression levels as compared with control (Gen 10^{-6} M vs. control, $P < 0.01$). The increase induced by Gen (10^{-6} M) was abolished in the presence of L-NMMA (1×10^{-3} M) or ICI182,780 (10^{-7} M) or ODQ (10^{-6} M); however, treatment with L-NMMA or ICI182,780 or ODQ (10^{-6} M) alone had no effect (Fig. 2).

DISCUSSION

Our findings provide the first evidence that Gen also acts on mouse BMSCs to promote cell proliferation and osteoblastic differentiation

TABLE III. Inhibition of ICI182,780, or L-NAME or ODQ on the Gen (10^{-6} M)-Induced Runx2 Expression of Mouse BMSCs by Real-Time PCR

Group	Runx2	Runx2-I	Runx2-II	Osteocalcin
Control	1.00 ± 0.15	1.00 ± 0.26	1.00 ± 0.17	1.00 ± 0.27
Gen	3.18 ± 0.17^b	3.24 ± 0.25^b	3.08 ± 0.15^b	6.38 ± 0.31^b
ICI182,780	1.05 ± 0.15	1.03 ± 0.24	0.98 ± 0.16	1.04 ± 0.28
Gen + ICI182,780	1.16 ± 0.18^c	1.15 ± 0.27^c	1.17 ± 0.17^c	1.75 ± 0.27^c
L-NAME*	1.18 ± 0.19	1.13 ± 0.28	1.22 ± 0.18	1.02 ± 0.28
Gen + L-NAME*	1.18 ± 0.13^c	1.11 ± 0.23^c	1.14 ± 0.13^c	1.17 ± 0.33^c
ODQ	1.08 ± 0.14	0.96 ± 0.25	1.15 ± 0.15	1.02 ± 0.25
Gen + ODQ	2.51 ± 0.16^c	2.29 ± 0.21^c	2.44 ± 0.11^c	4.65 ± 0.32^c
E_2	2.92 ± 0.14^b	2.26 ± 0.25^b	2.18 ± 0.18^b	6.19 ± 0.28^b
E_2 + ICI182,780	1.01 ± 0.16^d	1.05 ± 0.23^d	1.23 ± 0.16^d	1.67 ± 0.26^d

n = 4, mean ± SD, $^bP < 0.01$ versus control (Me_2SO). $^cP < 0.05$ versus Gen. $^dP < 0.05$ versus E_2.
*The concentration of L-NAME was 3×10^{-3} M.

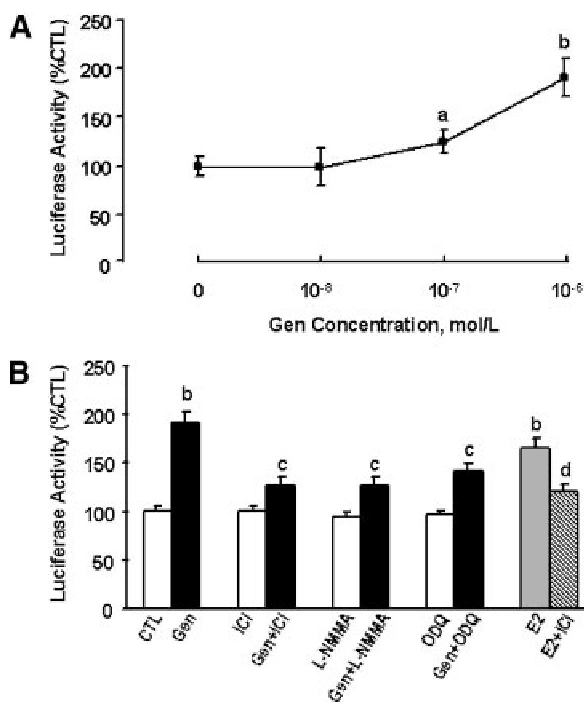

Fig. 2. Effect of Gen on Runx2/Cbfa1 P1 promoter activity. **A:** Dose-dependent effects of Gen on Runx2/Cbfa1 P1 promoter activity. **B:** Inhibition of ICI or L-NMMA or ODQ on the Gen (10^{-6} M)-induced increases in Runx2/Cbfa1 P1 promoter activity. C2C12 cells stably transfected with the 1.4 kb Runx2/Cbfa1 P1 promoter-Luciferase construct (p1.4Cbfa1-Luc) and treated with Gen in the presence or absence of ICI (10^{-7} M) or L-NMMA (1×10^{-3} M) or ODQ (10^{-6} M) for 48 h. E_2 as a positive control. n = 6, mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ versus control (Me_2SO). $^{c}P < 0.05$ versus Gen. $^{d}P < 0.05$ versus E_2.

through activation of ER-dependent NO/cGMP pathway (Table II). The effective dose range for the actions of Gen in this assay system was between 10^{-8} and 10^{-6} M, levels attainable by dietary supplementation of soybean-rich food [Yamamoto et al., 2001; Chen et al., 2003]. These findings in BMSC cultures were in a line with previous studies showing a stimulatory effect of Gen and daidzein, another isoflavone, on the osteoblastic cell line MC3T3-E1 [Yamaguchi and Sugimoto, 2000; Choi et al., 2001; Kannoa et al., 2004] and Gen effects on human bone marrow stromal cells [Heim et al., 2004]. Moreover, our findings indicate that the bone anabolic effects of isoflavones likely involve recruitment and differentiation of osteoblastic precursors that results in increased osteoblast-mediated bone formation and increased bone mass in a dose-dependent manner [Choi et al., 2001; Kannoa et al., 2004].

We also provide evidence that ER mediates, at least in part, Gen-stimulated proliferation and osteoblastic differentiation in mouse BMSCs. Not only was Gen effects on mouse BMSCs growth and differentiation similar to that observed with 17β-estrodial, but these effects were significantly inhibited by the antiestrogen ICI182,780. A role of ER in mediating Gen effects on osteoblasts has been previously reported [Wang et al., 1996]. Indeed, Gen can bind to ERβ and ERα, but possesses higher affinity for ERβ than for ERα [Kuiper et al., 1997]. However, Gen is a much weaker ER agonist and is non-selective for ERβ and ERα [Rickard et al., 2003]. Since the anabolic skeletal effects of estrogen are thought to be mediated by ERβ rather than ERα [Vidal et al., 2000], the actions of Gen on bone may differ from those of estrogen treatment. In contrast to the anabolic effects of lower does, Gen at higher doses (10^{-5} M) was not as efficient (Table I), findings consistent with the reported adverse effects on bone cells of high dose Gen [Anderson et al., 1998]. The effects of high dose Gen may be mediated by ER-independent effects. In this regard, it was reported that Gen has biphasic effect on the growth of the breast cancer cells, at concentrations that are sufficient to elicit transcriptional activity in transfection experiments ($\leq 10^{-6}$ M), Gen stimulates the proliferation of breast cancer cells in an ER-dependent way; while at concentrations more than 10^{-5} M, Gen becomes cytotoxic. Although Gen is known to have actions to inhibit tyrosine kinase and topoisomerases [Yoon et al., 1998], the concentrations required for these actions are much greater than those used in the current studies. Nevertheless, it is likely that Gen is also acting through estrogen independent pathways, since BMSC proliferation is not completely blocked by either ER antagonist (Table II).

We also have evidence that the NO/cGMP pathway is downstream of Gen-dependent ER activation. In accord with our previous study, which found that ADMA impaired osteoblastic differentiation of mouse BMSCs culture [Xiao et al., 2001a], the positive actions of Gen on mouse BMSCs growth and differentiation were blocked by L-NAME. We then further determined NO production in the CM and the NOS activity of the cells after Gen treatment. Gen promoted the NO production in the CM and the activity of calcium-dependent NOS isoform but not that of calcium-independent NOS isoform. Our observations coincided with that of others, which reported that estrogen stimulates NOS

activity in osteoblastic cells by activation of eNOS mRNA expression [Armour and Ralston, 1998]. In contrast to the dramatic effect of Gen as evidenced by the effect of NO inhibitors to block cGMP production (Fig. 2), we observed only modest, but significant effects of Gen to stimulate NO production in the CM of BMSC. The reasons for this discrepancy may be the sensitivity and limitation of experimental condition in our current study.

The Gen-induced increases in NO release, NOS activity, and extracellular cGMP level were all diminished by ICI182,780, which suggested that Gen stimulated NO and cGMP production through ER(s). It has been reported that estrogen, by binding to ERα, leads to rapid activation of the eNOS, which is the principal mechanism of NO release in estrogen-induced endothelium-dependent vasodilatation. This action of estrogen is independent from gene transcription and was proposed to be associated with the activation of MAPK, tyrosine kinase-dependent pathway and phosphatidylinositol-3-OH kinase (PI3K)/Akt pathway [Chen et al., 1999; Simoncini et al., 2000]. Further studies are needed to determine the NO dependent pathway in the effect of Gen by binding to ER.

Finally, we showed that Gen activation of ER/NO resulted in upregulation of *Runx2/Cbfa1* gene expression. It is known that Runx2/Cbfa1, an osteoblastic-specific transcription factor, plays a key role in osteoblast recruitment and differentiation as well as postnatal bone formation [Ducy et al., 1997, 1999]. In our study, we found that Gen stimulated Runx2/Cbfa1 P1 promoter activity (Fig. 2) and enhanced both Runx2/Cbfa1 type I and type II isoforms mRNA expression (Table III), which is consistent with similar studies in human primary bone marrow stromal cell cultures [Heim et al., 2004]. Heim et al. found that Gen acted similarly to E_2 during the early osteogenic phase by upregulation of the osteoblast-determining Cbfa1 and downregulation of the adipogenic regulator PPARγ, while Gen also upregulated OPG:RANKL ratio and TGFβ mRNA expression during late differentiation via an ER-dependent mechanism. As shown in Table III, we also observed the increment of Runx2/Cbfa1 expression by Gen and E_2 was diminished by ICI182,780, an antiestrogen, and L-NAME, an NOS inhibitor, indicating that Gen affected expression of the gene at least in part through the same way as Gen promoted the proliferation and differentiation of mouse BMSCs, although ODQ did not completely but significantly block the effect of Gen on Runx2/Cbfa1 expression (Fig. 2 and Table III), suggesting Gen was acting through both cGMP-dependent and -independent pathways. The precise signal transduction events mediating these effects has not been investigated in our study. E_2 has been shown to upregulate Runx2/Cbfa1 activity via activation protein-1 (AP-1) binding site [Tou et al., 2001]. Moreover, cGMP, by activating G-kinase, modulated c-fos expression, which is a part of AP-1 transcription factor complex. Further studies will be needed to understand the mechanism whereby E_2 upregulates Runx2/Cbfa1 activity.

In conclusion, Gen can stimulate proliferation and osteoblastic differentiation of mouse BMSCs through mechanisms involving the ER–NO–cGMP pathway. Activation of this pathway may mediate the anabolic effects of Gen on bone and serve as an important mechanism for this isoflavone effectiveness as an antiosteoporotic treatment.

REFERENCES

Anderson JJ, Garner SC. 1998. Phytoestrogens and bone. Baillieres Clin Endocrinol Metab 12:543–557.

Anderson JJ, Ambrose WW, Garner SC. 1998. Biphasic effects of genistein on bone tissue in the ovariectomized, lactating rat model. Proc Soc Exp Biol Med 217:345–350.

Arjmandi BH, Alekel L, Hollis BW, Amin D, Stacewicz-Sapuntzakis M, Guo P, Kukreja SC. 1996. Dietary soybean protein prevents bone loss in an overiectomized rat model of osteoporosis. J Nutr 126:1161–1167.

Armour KE, Ralston SH. 1998. Estrogen upregulates endothelial constitutive nitric oxide synthase expression in human osteoblast-like cells. Endocrinology 139:799–802.

Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn ME, Shaul PW. 1999. Estrogen reporter alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. J Clin Invest 103:401–406.

Chen X, Garner SC, Quarles LD, Anderson JJ. 2003. Effects of genistein on expression of bone markers during MC3T3-E1 osteoblastic cell differentiation. J Nutr Biochem 14:342–349.

Choi EM, Suh KS, Kim YS, Choue RW, Koo SJ. 2001. Soybean ethanol extract increases the function of osteoblastic MC3T3-E1 cells. Phytochemistry 56:733–739.

Ducy P, Zhang R, Geoffrey V, Ridall AL, Karsenty G. 1997. Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell 89:747–764.

Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, Amling M, Karsenty G. 1999. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Genes Dev 13:1025–1036.

Fanti P, Monier-Faugere MC, Geng Z, Schmidt J, Morris PE, Cohen D, Malluche HH. 1998. The phytoestrogen genistein reduces bone loss in short-term ovariectomized rats. Osteoporos Int 8:274–281.

Gao YH, Yamaguchi M. 1999. Inhibitory effect of genistein on osteoclast-like cell formation in mouse marrow cultures. Biochem Pharmacol 58:767–772.

Grant MK, El-Fakahany EE. 2004. Therapeutic interventions targeting the nitric oxide system: Current and potential uses in obstetrics, bone disease, and erectile dysfunction. Life Sci 74:1701–1721.

Green LC, Wagner DA, Glogowski J, et al. 1982. Analysis of nitrate, nitrite, and (15N) nitrate in biological fluids. Anal Biochem 126:131–138.

Heim M, Frank O, Kampmann G, Sochocky N, Pennimpede T, Fuchs P, Hunziker W, Weber P, Martin I, Bendik I. 2004. The phytoestrogen genistein enhances osteogenesis and represses adipogenic differentiation of human primary bone marrow stromal cells. Endocrinology 145: 848–859.

Horiuchi T, Onouchi T, Takahashi M, Ito H, Orimo H. 2000. Effect of soy protein on bone metabolism in postmenopausal Japanese women. Osteoporos Int 11: 721–724.

Ishimi Y, Miyaura C, Ohmura M, Onoe Y, Sato T, Uchiyama Y, Ito M, Wang X, Suda T, Ikegami S. 1999. Selective effects of genistein, a soy bean isoflavone, on B-lyphopoiesis and bone loss caused by estrogen deficiency. Endocrinology 140:1893–1900.

Ishimi Y, Arai N, Wang X, Wu J, Umegaki K, Miyaura C, Takeda A, Ikegami S. 2000. Difference in effective dosage of genistein on bone and uterus in ovariectomized mice. Biochem Biophys Res Commun 274:697–701.

Kanis JA, Melton LJ III, Christiansen C, Johnston CC, Khaltaev N. 1994. The diagnosis of osteoporosis. J Bone Miner Res 9:1137–1141.

Kannoa S, Hirano S, Kayamab F. 2004. Effects of phytoestrogens and environmental estrogens on osteoblastic differentiation in MC3T3-E1 cells. Toxicology 196: 137–145.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endorinology 138: 863–870.

Mancini L, Moradi-Bidhendi N, Becherini L, Martineti V, MacIntyre I. 2000. The biphasic effects of nitric oxide in primary rat osteoblasts are cGMP dependent. Biochem Biophys Res Commun 274:477–481.

Mei J, Yeung SS, Kung AW. 2001. High dietary phytoestrogen intake is associated with higher bone mineral density in postmenopausal but not premenopausal women. J Clin Endocrinol Metab 86:5217–5221.

Morabito N, Crisafulli A, Vergara C, Gaudio A, Lasco A, Frisina N, D'Anna R, Corrado F, Pizzoleo MA, Cincotta M, Altavilla D, Ientile R, Squadrito F. 2002. Effects of genistein and hormone-replacement therapy on bone loss in early postmenopausal women: A randomized double-blind placebo-controlled study. J Bone Miner Res 17: 1904–1912.

Murkies AL, Wilcox G, Davis SR. 1998. Clinical review 92: Phytoestrogens. J Clin Endocrinol Metab 83:297–303.

O'Shaughnessy MC, Polak JM, Afzal F, Hukkanen MV, Huang P, MacIntyre I, Buttery LD. 2000. Nitric oxide mediates 17β-estradiol-stimulated human and rodent osteoblast proliferation and differentiation. Biochem Biophys Res Commun 277:604–610.

Qu Q, Perala-Heape M, Kapanen A, Dahllund J, Salo J, Vaananen HK, Harkonen P. 1998. Estrogen enhances differentiation of osteoblasts in mouse bone marrow culture. Bone 400:201–209.

Recker RR. 1993. Current therapy for osteoporosis. J Clin Endocrinol Metab 76:14–16.

Rickard DJ, Monroe DG, Ruesink TJ, Khosla S, Riggs BL, Spelsberg TC. 2003. Phytoestrogen genistein acts as an estrogen agonist on human osteoblastic cells through estrogen receptors α and β. J Cell Biochem 89: 633–646.

Setchell KD, Lydeking-Olsen E. 2003. Dietary phytoestrogens and their effect on bone: Evidence from in vitro and in vivo, human observational, and dietary intervention studies. Am J Clin Nutr 78:593S–609S.

Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK. 2000. Interaction of estrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 407:538–541.

Squadrito F, Altavilla D, Squadrito G, Saitta A, Cucinotta D, Minutoli L, Deodato B, Ferlito M, Campo GM, Bova A, Caputi AP. 2000. Genistein supplementation and estrogen replacement therapy improve endothelial dysfunction induced by ovariectomy in rats. Cardiovasc Res 45: 454–462.

Squadrito F, Altavilla D, Crisafulli A, Saitta A, Cucinotta D, Morabito N, D'Anna R, Corrado F, Ruggeri P, Frisina N, Squadrito G. 2003. Effect of genistein on endothelial function in postmenopausal women: A randomized, double-blind, controlled study. Am J Med 114:470–476.

Sugimoto E, Yamaguchi M. 2000. Anabolic effect of genistein in osteoblastic MC3T3-E1. Int J Mol Med 5: 515–520.

Tou LQ, Quibria N, Alexander JM. 2001. Regulation of human *Cbfa1* gene transcription in osteoblasts by eslective estrogen receptor modulators (SERMs). Mol Cell Endocrinol 183:71–79.

Vidal O, Lindberg MK, Hollberg K, Baylink DJ, Andersson G, Lubahn DB, Mohan S, Gustafsson JA, Ohlsson C. 2000. Estrogen receptor specificity in the regulation of skeletal growth and maturation in male mice. Proc Natl Acad Sci USA 97:5474–5479.

Vihtamaki T, Savilahti R, Tuimala R. 1999. Why do postmenopausal women discontinue hormone replacement therapy? Maturitas 33:99–105.

Wang TT, Sathyamoorthy N, Phang JM. 1996. Molecular effects of genistein on estrogen receptor mediated pathways. Carcinogenesis 17:271–275.

Xiao ZS, Quarles LD, Chen QQ, Yu YH, Qu XP, Jiang CH, Deng HW, Li YJ, Zhou HH. 2001a. Effect of asymmetric dimethylarginine on osteoblastic differentiation. Kidney Int 60:1699–1704.

Xiao ZS, Liu SG, Hinson TK, Quarles LD. 2001b. Characterization of the upstream mouse Cbfa1/Runx2 promoter. J Cell Biochem 82:647–659.

Xiao ZS, Hjelmeland AB, Quarles LD. 2004. Selective deficiency of the "bone-related" Runx2-II unexpectedly preserves osteoblast-mediated skeletogenesis. J Biol Chem 279:20307–20313.

Yamaguchi M, Sugimoto E. 2000. Stimulatory effect of genstein and daidzein on protein synthesis in osteoblastic

MC3T3-E1 cells: Activation of aminoacyl-tRNA synthetase. Mol Cell Biochem 214:97–102.

Yamamoto S, Sobue T, Sasaki S, Kobayashi M, Arai Y, Uehara M, Adlercreutz H, Watanabe S, Takahashi T, Iitoi Y, Iwase Y, Akabane M, Tsugane S. 2001. Validity and reproducibility of a self-administered food frequency questionnaire to assess isoflavone intake in a Japanese population in comparison with dietary records and blood and urine isoflavones. J Nutr 131:2741–2747.

Yoon HK, Chen K, Baylink DJ, Lau KH. 1998. Differential effects of two protein tyrosine kinase inhibitors, tyrphostin and genistein, on human bone cell proliferation as compared with differentiation. Calcif Tissue Int 63: 243–249.