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Baicalein Stimulates Osteoblast Differentiation Via Coordinating Activation of MAP Kinases and Transcription Factors

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Abstract The identification of anabolic agents that directly stimulate bone formation has recently attracted greater interest. Here, baicalein was identified as a natural compound that stimulates the differentiation of mouse osteoblastic MC3T3-E1 subclone 4 cells. Baicalein induced the activation of NF-κB in the initiation stage of osteoblast differentiation, and it activated the MAP kinase/NF-κB signaling pathway and induced the expression of osteoblast differentiation markers in the early stage. In the late stage, baicalein stimulated the calcium deposition with the activation of MAP kinases and AP-1 family members such as Fra-1 and Fra-2. Another transcription factor, NFATc1, was slightly induced by baicalein in the late stage. Thus, baicalein could stimulate the osteoblast differentiation via the activation of complexly coordinated signaling pathways that include MAP kinases and transcription factors such as NF-κB, AP-1, and NFATc1. J. Cell. Biochem. 104: 1906–1917, 2008.

Key words: baicalein; osteoblast differentiation; MAP kinases; NF-κB; AP-1; NFATc1

Bone mechanically supports the body, protects the vital organs, and produces bone marrow. As a reserve of ions, especially calcium and phosphate, bone participates in the metabolic maintenance of serum homeostasis [Baron, 2003]. To perform these functions, bone is sustained through a dynamic cycle of destruction and rebuilding. The cycling between the removal of old bone from the skeleton by hematopoietically derived osteoclasts and the addition of new bone through differentiation/mineralization by mesenchyme-derived osteoblasts is referred to as bone remodeling [Boyle et al., 2003; Harada and Rodan, 2003]. The proper balance between osteoblastic bone formation and osteoclastic bone resorption maintains skeletal strength and integrity, whereas an imbalance resulting in greater bone resorption than bone formation leads to adult skeletal diseases such as osteoporosis. The development and progress of osteoporosis increase the risk for fractures, particularly in the hip, which can create serious problems. Osteoporosis is associated with many adverse consequences, including substantial skeletal deformity, pain, functional limitation, increased mortality, and severe economic burden [NIH Consensus, 2001].

Much effort has gone into developing drugs for the prevention and treatment of osteoporosis, resulting in several drugs that

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target bone resorption [Rodan and Martin, 2000; Vaananen, 2005]. Although useful, the anti-resorptives are generally not associated with dramatic increases of bone mass. Therefore, the development of anabolic agents that directly stimulate bone formation, which could potentially be combined with anti-resorptives, has recently attracted more interest [Rosen and Bilezikian, 2001; Garces and Garcia, 2006].

Historically, natural compounds and their derivatives have been invaluable as a source of therapeutic agents. A wide variety of natural compounds have beneficial effects on the skeleton with minimal adverse side effects [Whelan et al., 2006; Putnam et al., 2007]. To identify anabolic agents in this study, we evaluated the effects of 222 natural compounds on the differentiation of mouse osteoblastic MC3T3-E1 subclone 4 cells. One of these compounds, baicalein, activated MAP kinases; induced the transcription factors NF-κB, AP-1, and NFATc1; and stimulated osteoblast differentiation.

MATERIALS AND METHODS

Cell Culture

All materials for cell culture were purchased from HyClone (UT). Subclone 4 cells with high differentiation potential [Wang et al., 1999] were purchased from American Type Culture Collection and cultured in the growth medium (GM) [α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 mg/ml of streptomycin] with a change of medium every 3 days in humidified atmosphere of 5% CO_2 at 37°C.

Screening of Natural Compounds Stimulating the Calcium Formation

Cells (2.5×10^3 cells/well) were plated in a 384-well plate and cultured in GM. After the cells reached confluence, cells were cultured in the differentiation medium (DM) [GM with 50 μg/ml of ascorbic acid (Fluka, Germany) and 10 mM of β-glycerophosphate (Sigma, MO)] with a change of medium every 3 days. All 222 natural compounds used in this study were isolated as a single compound form from various medicinal plants used traditionally for treating several diseases or purchased from Sigma based on the chemical structure and its biological activity. Dissolved natural compounds (2 mg/ml in dimethyl sulfoxide, DMSO; Sigma) were then formatted in a 384-well plate and transferred into cells by using QRep 384 Pin Replicator (Genetix, UK) every 3 days when medium was changed. The used concentration of each compound was ~3 μg/ml (that was calculated to ~10 μM in case of baicalein). At the differentiation day 14, cells were washed with calcium and magnesium-free phosphate buffered saline (PBS) twice and fixed with 50 μl of 3.7% formaldehyde in PBS for 3 min. For decalcifying mineralized nodules, 20 μl of 1 N HCl was added to each well and after 24 h, calcium contents in the supernatant was determined by using the *o*-cresolphthalein complexon color development method-based Calcium C kit (Wako Pure Chemicals Industries, Japan) with modification. To confirm the effect of selected compound (baicalein in this study) on the calcium formation, cells were cultured in a 24-well plate and the calcium content was measured in triplicate. Baicalein (Sigma) was dissolved in DMSO and then serially diluted with medium. Used concentration of DMSO was below 0.1% and 0.1% DMSO was used as a control in all experiments.

Cell Viability and [^{3}H]thymidine Incorporation Assay

Cells suspended in GM were plated in a 96-well plate at the density 1×10^3 cells/well. After 24 h, cells were incubated with serially diluted natural compound for 1 or 3 days. Cell viability was then measured in triplicate by using Cell Counting Kit-8 (Dojindo Molecular Technologies, ML) according to the manufacturer's protocol. Absorbance was measured by using Wallac EnVision microplate reader (PerkinElmer, Finland) and measured absorbance was converted to cell number with the standard curve. The effect of compound on cell viability was double-checked by [^{3}H]thymidine incorporation assay. After 1- or 3-day culture, cells were labeled with 0.5 μCi/ml [^{3}H]thymidine (GE Healthcare Bio-Science, Hong Kong) for 4 h and the incubation was terminated by removal of the medium. Cells were then washed twice with PBS and a scintillation cocktail (Biofluor; PerkinElmer, CT) was added. Each sample was counted in a liquid scintillation counter, 1450 Microbeta Trilux (PerkinElmer). This experiment was performed in triplicate.

Osteoblast Differentiation

Cells (1.5×10^4 cells/well) were plated in a 24-well plate and cultured in GM. After the cells reached confluence, cells were cultured in DM in the absence or presence of natural compound with a change of medium every 3 days.

Alkaline Phosphate (ALP) Staining and Its Activity Assay

At the differentiation day 8, cells were washed with PBS twice, fixed with 3.7% formaldehyde in PBS for 30 s, rinsed with deionized water, and stained under the protection from direct light by using Alkaline Phosphatase kit (Sigma). The images of stained cells were captured under a microscope with DP70 digital camera (Olympus Optical, Japan). For measuring the activity of ALP, cells were washed with PBS twice and sonicated in the lysis buffer [10 mM of Tris–HCl, pH 7.5, 0.5 mM of $MgCl_2$, and 0.1% Triton X-100]. After the centrifugation at 10,000*g* for 20 min at 4°C, the activity of ALP in the supernatant was measured in triplicate by using LabAssay ALP kit (Wako Pure Chemicals Industries). The protein concentration was measured by using BCA Protein Assay kit (Pierce). Significance was determined by Student's *t*-test and differences were considered significant when $P < 0.05$.

Measurement of Calcium Content

Cells were washed with PBS twice, fixed with 3.7% formaldehyde in PBS for 15 min, and decalcified with 300 μl of 1 N HCl for 24 h. The amount of calcium was then measured by using Calcium C kit according to the manufacturer's protocol.

Alizarin Red S Staining

Cells were washed with PBS twice, stained with 40 mM of Alizarin red S solution (pH 4.2) for 10 min at room temperature and washed with deionized water twice. The images of stained cells were captured under a microscope with DP70 digital camera.

von Kossa Staining

Cells were washed with PBS and fixed with 2.5% glutaraldehyde in PBS for 30 min. After washing with deionized water three times, cells were incubated with 5% silver nitrate at room temperature under UV light until calcium turns black. After washing with deionized water three times, the images of stained cells were captured under a microscope with DP70 digital camera.

Evaluation of mRNA Expression Levels

Primers were chosen with an on-line primer design program [Rozen and Skaletsky, 2000] and their sequences were presented in Table I. Total RNA was isolated with TRIzol reagent (Life Technologies, MD) according to manufacturer's protocol. The concentration and purity of total RNA were calculated with absorbance at 260 and 280 nm. First strand cDNA was synthesized with 2 μg of total RNA, 1 μM of oligo-dT_{18} primer and Omniscript Reverse Transcriptase (Qiagen, CA). SYBR green-based quantitative PCR amplification was then performed using Stratagene Mx3000P Real-Time PCR system and Brilliant SYBR Green Master Mix (Stratagene, CA) with first-strand cDNA diluted 1:50 and 20 pmol of primers according to the manufacturer's protocols. The PCR reaction consisted of three segments. The first segment at 95°C for 10 min was for the activation of the polymerase and the second one corresponded to 3-step cycling (40 cycles) at 94°C for 40 s (denaturation), 60°C for 40 s (annealing), and 72°C for 1 min (extension). The third segment was for the generation of PCR product

TABLE I. Primer Sequences Used in This Study

Target gene	Forward (5′–3′)	Reverse (5′–3′)
ALP	GCTGATCATTCCCACGTTTT	CTGGGCCTGGTAGTTGTTGT
OCN	AAGCAGGAGGGCAATAAGGT	TTTGTAGGCGGTCTTCAAGC
COLI	ACGTCCTGGTGAAGTTGGTC	CAGGGAAGCCTCTTTCTCCT
OPN	CGATGATGATGACGATGGAG	TGGCATCAGGATACTGTTCATC
DMP	CGGCTGGTGGACTCTCTAAG	TGTCTGCCTCATCCTCACTG
BSP	GGGAGGCAGTGACTCTTCAG	TCTGCATCTCCAGCCTTCTT
c-Jun	TCCCCTATCGACATGGAGTC	TGAGTTGGCACCCACTGTTA
JunD	CGACCAGTACGCAGTTCCTC	AACTGCTCAGGTTGGCGTAG
c-Fos	CCAGTCAAGAGCATCAGCAA	AAGTAGTGCAGCCCGGAGTA
Fra-1	AGAGCTGCAGAAGCAGAAGG	CAAGTACGGGTCCTGGAGAA
Fra-2	ATCCACGCTCACATCCCTAC	GTTTCTCTCCCTCCGGATTC
NFATc1	GGGTCAGTGTGACCGAAGAT	GGAAGTCAGAAGTGGGTGGA
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

temperature dissociation curves (also called "melting curves") at 95°C for 1 min, 55°C for 30 s, 95°C for 30 s. All reactions were run in triplicate and analyzed by the $2^{-\Delta\Delta CT}$ method [Livak and Schmittgen, 2001]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control gene. Significance was determined by Student's *t*-test with GAPDH-normalized $2^{-\Delta\Delta CT}$ values, and differences were considered significant when $P < 0.05$.

Transient Transfection and Dual Luciferase Reporter Assay

Cells were plated in a 96-well plate at a density of 5×10^3 cells/well. After 90–95% confluent, cells were transfected with Lipofectamine 2000 reagent (Invitrogen, CA) according to the manufacturer's protocol. Each transfection contained 200 ng/well of NF-κB, AP-1, or NFATc1-firefly luciferase reporter plasmid (BD Bioscience Clontech, CA) plus 20 ng/well of pGL4.73-*Renilla* luciferase (Promega, WI). Five hours after transfection, cells were treated with baicalein in DM. After 24 h, the transfected cells were lysed and both the firefly and *Renilla* luciferase activities were determined using the Dual-Luciferase Reporter Assay kit (Promega). The firefly luciferase activity was normalized with the *Renilla* luciferase activity. The data were presented to the relative ratios of normalized luciferase activity to the NF-κB control. DMSO was used as a vehicle control. Significance was determined by Student's *t*-test, and differences were considered significant when $P < 0.01$.

Western Blot Analysis

Cells were homogenized in ice-cold buffer [10 mM of Tris–HCl (pH 7.5), 150 mM of NaCl, 0.05% (v/v) Tween 20, 1 mM of PMSF and one protease inhibitor cocktail tablet (Roche) in 10 ml] and centrifuged at 10,000*g* for 15 min at 4°C. The supernatant was used as cytoplasmic protein fraction and nuclear proteins were extracted by using NucBuster Protein Extraction kit (Novagen, Germany). The protein concentration was determined by using BCA protein assay kit. Proteins were mixed with sample buffer [100 mM of Tris–HCl, 2% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 2% glycerol, and 0.01% bromophenol blue, pH 7.6], incubated at 95°C for 15 min, and loaded onto 10% polyacrylamide gels. Electrophoresis was performed using the Mini Protean 3 Cell (Bio-Rad, CA). Proteins separated on the gels were transferred onto nitrocellulose membrane (Whatman, Germany) and in order to ascertain the loading amount of proteins and the efficiency of transfer, the transferred membrane was stained with Ponceau S staining solution. The stained membrane was washed and incubated in blocking buffer [10 mM of Tris–HCl, pH 7.5, 150 mM of NaCl, 0.1% Tween 20, and 3% nonfat dry milk] for 2 h. The membrane was then incubated with 1:1,000 diluted primary antibody (Santa Cruz Biotechnology, CA) for 2 h and after washing with blocking buffer for 15 min three times, it was probed with 1:2,000 diluted secondary antibody for 1 h. The membrane was washed with blocking buffer for 15 min three times and developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Chemiluminescent signal was detected with LAS-3000 Luminescent image analyzer (Fuji Photo Film Co., Japan).

RESULTS

Baicalein Was Identified to Stimulate Osteoblast Differentiation

The effect of 222 natural compounds on the calcium deposition was evaluated in MC3T3-E1 subclone 4 cells at day 14 of differentiation (Fig. 1). More calcium was deposited in cells cultured in DM than in GM. Seven natural compounds stimulated calcium deposition at a level more than fourfold that observed in DM. One of these compounds was identified as baicalein (indicated by an arrow in Fig. 1). Another of the anabolic compounds that stimulated calcium deposition was luteolin, which has recently been reported to stimulate osteoblast differentiation [Choi, 2007].

Baicalein Increases ALP Activity and Stimulates Mineralization

Before confirmation of the stimulating effect of baicalein on osteoblast differentiation, its effect on cell viability was examined. As shown in Figure 2, baicalein slightly attenuated the cell growth rate but did not decrease thymidine incorporation at the concentrations used in this study ($\leq$10 μM). The anabolic activity of baicalein in the early stage of osteoblast differentiation was evaluated by staining for alkaline phosphatase (ALP), which is an early phase marker of osteoblast differentiation

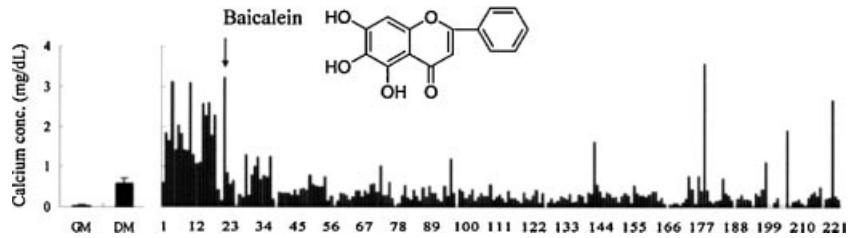

Fig. 1. Screening of natural compounds with a potential to stimulate the calcium formation in the differentiation of MC3T3-E1 subclone 4 cells. Confluent cells were differentiated and the calcium content was then measured at day 14 of differentiation. The calcium contents in cells cultured in GM or DM were used as a negative or positive control, and natural compounds were added in cells cultured in DM. One of compounds stimulating the calcium formation, baicalein was indicated as arrow and its chemical structure was presented.

(Fig. 3A), and measuring its activity (Fig. 3B) at day 8 of differentiation. The expression and activity of ALP were slightly increased in cells cultured in DM compared with those cultured in GM. However, interestingly, both the expression and activity of ALP were dramatically increased with the addition of baicalein in DM. Baicalein (10 μM) significantly increased ALP activity, to a level twofold that of the control.

The anabolic activity of baicalein in the late stage of osteoblast differentiation was evaluated by measuring the concentration of deposited calcium and visualizing the mineralization. The amount of newly accumulated calcium was measured at days 11 and 17 of differentiation (Fig. 4A). At day 11, 10 μM baicalein had strongly stimulated calcium deposition, to a level 14-fold the control level. At the day 17, although the calcium level in the control was about 80% compared to that in cells cultured with 10 μM of baicalein, baicalein induced the calcium deposition in a dose-dependent manner. In addition, considering that there was no difference in the calcium level in cells cultured in 10 μM baicalein between days 11 and 17, the experimental concentration of calcium at saturation in differentiated osteoblasts cultured in a 24-well plate was approximately 4 mg/dl. At day 17 of differentiation, the mineral (especially calcium) deposition was visualized by both Alizarin red S staining and von Kossa staining methods (Fig. 4B). Baicalein induced mineralization in a dose-dependent manner.

Baicalein Increases the Expression of Osteoblast Differentiation Markers

To elucidate the mechanism of action for baicalein in the process of osteoblast differentiation, the effect of baicalein on the mRNA expression levels of osteoblast differentiation markers and the activations of MAP kinases and transcription factors were examined. As measured by quantitative real-time PCR, the mRNA levels of ALP; osteocalcin (OCN); type I collagen (COLI); small integrin-binding ligand, *N*-glycosylated proteins, including osteopontin (OPN); dentin matrix protein (DMP); and bone sialoprotein (BSP) were all increased at day 8 of differentiation with baicalein treatment

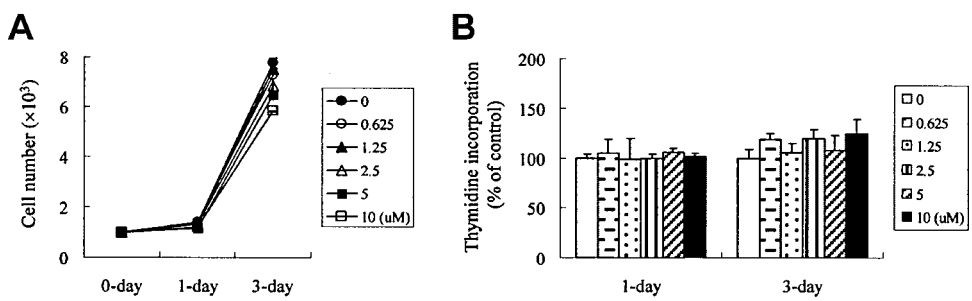

Fig. 2. Effect of baicalein on the cell viability. Cells (1,000 cells/well) were cultured in a 96-well plate for 1 day, and then treated with baicalein. After incubation for 1 or 3 days, the number of cells (**A**) and the level of [^{3}H] thymidine incorporation (**B**) were evaluated as described in ''Materials and Methods Section.''

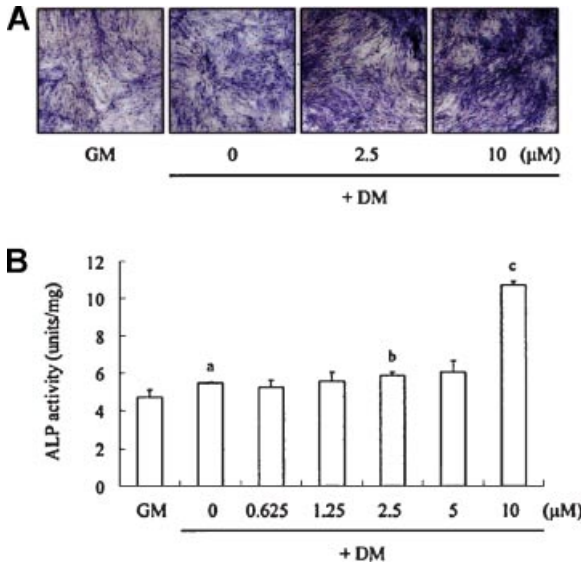

Fig. 3. Effect of baicalein on the ALP protein level (**A**) and its activity (**B**) at day 8 of differentiation. ALP activity (U/mg) was represented to the mean ± SD of triplicate cultures. a, $P < 0.05$ (ALP activity in cells cultured in GM vs. that in DM); b, $P < 0.05$; and c, $P < 0.001$ (ALP activity in cells cultured in DM in the absence of baicalein vs. that in presence of baicalein). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

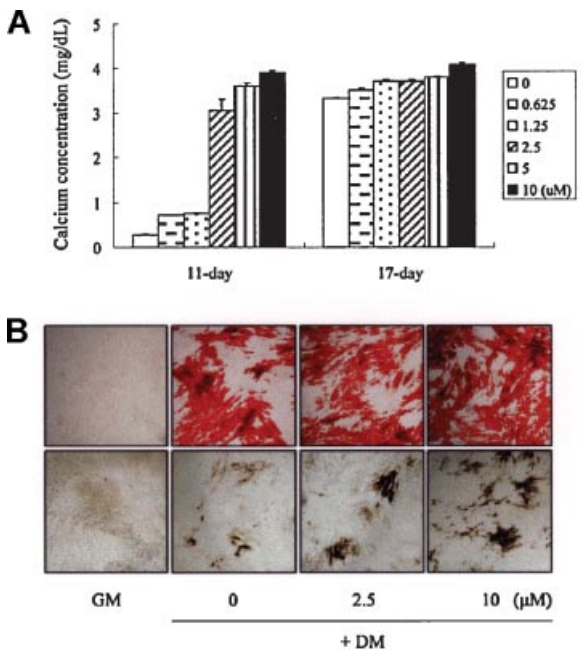

Fig. 4. Effect of baicalein on the calcium formation. **A**: At days 11 and 17 of differentiation, the level of deposited calcium was measured as described in ''Materials and Methods Section.'' Values represent the mean ± SD of triplicate cultures. **B**: At day 17, calcified mineralization was visualized by two staining methods, Alizarin red S staining (**upper**) and von Kossa staining (**bottom**; original magnification, 40). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Fig. 5). At day 17 of differentiation with baicalein, the mRNA levels of ALP and COLI were slightly reduced, whereas those of OCN, OPN, and DMP were dramatically increased, in a dose-dependent manner. The mRNA levels of OPN and DMP at 10 μM baicalein were approximately 18-fold those of the control. BSP was slightly induced by baicalein at low doses.

Baicalein Increases Activation of NF-κB in Initiation Stage of Osteoblast Differentiation

The effect of baicalein on the activation of transcription factors (NF-κB, AP-1 and NFATc1) in the initiation stage of osteoblast differentiation was further evaluated by luciferase activity assay (Fig. 6A). When compared to the normalized level of NF-κB luciferase activity, baicalein significantly induced the activation of NF-κB, but not AP-1 and NFATc1.

Effect of Baicalein on Activation of MAP Kinases and Translocation of NF-κB

In the experiment to evaluate the effect of baicalein on the activations of MAP kinases and NF-κB, baicalein increased the phosphorylation of three MAP kinases (JNK, ERK, and p38) at day 8 of differentiation (the early stage of osteoblast differentiation; Fig. 6B); both JNK and ERK were activated strongly, whereas p38 was slightly activated by baicalein. At low concentrations (≤5 μM), baicalein induced the translocation of NF-κB p65 subunit into nucleus, but it was dramatically decreased at 10 μM baicalein. At day 17 of differentiation (the late stage of osteoblast differentiation), baicalein dose-dependently increased ERK activation and decreased JNK activation. Kinase p38 was slightly activated at 1.25–2.5 μM baicalein. The amount of translocated NF-κB p65 subunit was decreased by baicalein in a dose-dependent manner.

Effect of Baicalein on Expression and Activation of AP-1 and NFATc1

Baicalein induced the activation of NF-κB during the initiation stage, but not other stages, of osteoblast differentiation, suggesting that NF-κB may be a major transcription factor in the initiation of baicalein-induced differentiation. To determine whether baicalein activates additional transcription factors in the early and late stages of differentiation, the effect of baicalein on the mRNA levels of AP-1 family members (c-Jun, JunD, c-Fos, Fra-1, and Fra-2)

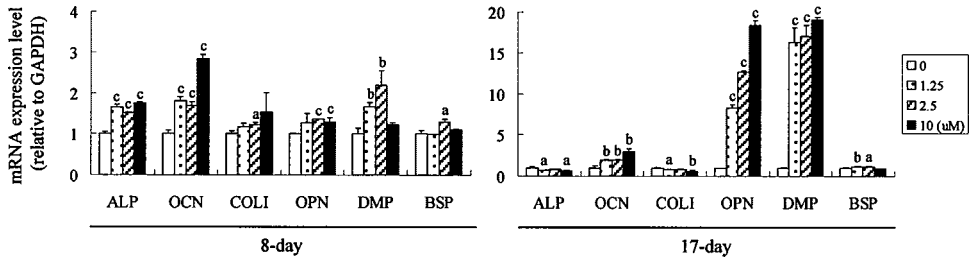

Fig. 5. Effect of baicalein on the mRNA expression levels of osteoblast differentiation-related genes. The mRNA expression levels of them were evaluated by real-time PCR. a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$ (compared to control).

and of NFATc1 was examined. At day 8 of differentiation, the levels of c-Jun, c-Fos, Fra-1, and NFATc1 mRNA were slightly increased by baicalein (Fig. 7A). Interestingly, the levels of c-Jun and Fra-2 mRNA were significantly increased with baicalein at 10 μM but not at lower doses. At day 17 of differentiation, the levels of c-Fos, Fra-1, Fra-2, and NFATc1 mRNA were significantly increased by baicalein (Fig. 7B). The baicalein-induced mRNA levels of c-Fos, Fra-1, Fra-2, and NFATc1 were greater at day 17 than at day 8, suggesting that AP-1 family members and NFATc1 may be involved in the late stage of baicalein-induced osteoblast differentiation.

Baicalein induced the translocation of Fra-1, Fra-2, and NFATc1, but not c-Fos, into nucleus at day 17 of differentiation (Fig. 7C). Baicalein

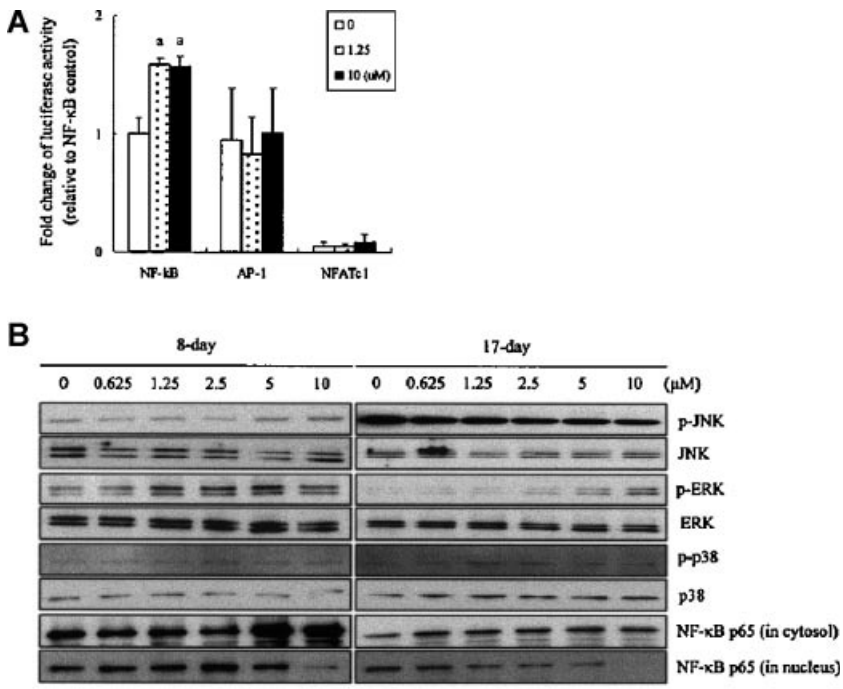

Fig. 6. Effect of baicalein on the activation of transcription factors and MAP kinases. **A**: The effect of baicalein on the activation of transcription factors in the initiation stage of osteoblast differentiation was evaluated by the luciferase activity assay. Cells were seeded in a 96-well plate, transfected with NF-κB, AP-1, or NFATc1-firefly luciferase reporter plasmid plus pGL4.73-*Renilla* luciferase, and after 5 h, baicalein was treated. After 24 h, the activities of both luciferases were measured. The firefly luciferase activity was normalized with the *Renilla* luciferase activity. The data were presented to the relative ratios of normalized luciferase activity to the NF-κB control. DMSO was used as a vehicle control. a, $P < 0.01$ (compared to control). **B**: The effect of baicalein on the activation of MAP kinases and NF-κB at the differentiation days 8 and 17 was evaluated by Western blot analysis. The activation of MAP kinases was evaluated by measuring the protein levels of both unphosphorylated and phosphorylated forms and NF-κB activation was evaluated by measuring the protein level of its p65 subunit in the cytosolic and nucleus fractions.

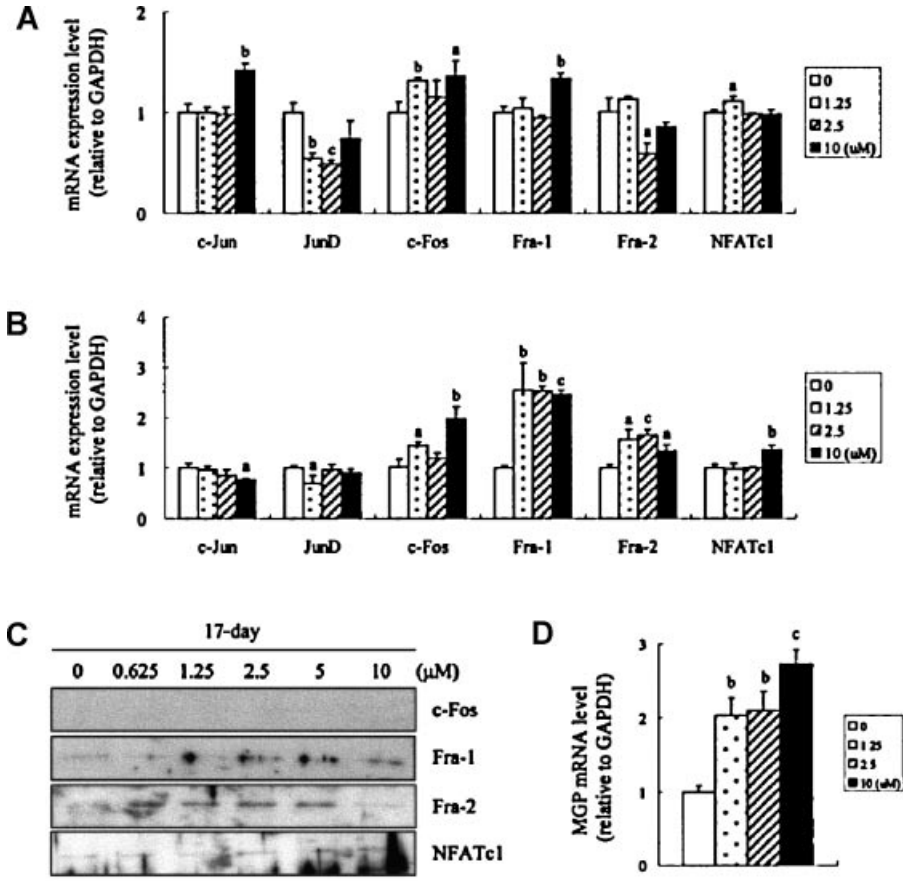

Fig. 7. Effect of baicalein on the mRNA/protein levels of AP-1 family members and NFATc1, and the mRNA expression level of MGP. The mRNA expression levels of AP-1 family members and NFATc1 were evaluated by real-time PCR at day 8 (**A**) and day 17 (**B**). At day 17 of differentiation, the activations of AP-1 and NFATc1 were evaluated by measuring their protein levels in the nucleus fractions (**C**). At day 17, the mRNA expression level of MGP was evaluated by real-time PCR (**D**). a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$ (compared to control).

increased the mRNA level of matrix Gla protein (MGP), a Fra-1-regulated gene, in a dose-dependent manner and with an expression pattern that correlated with the level of Fra-1 in the nucleus (Fig. 7D).

DISCUSSION

Baicalein is a flavonoid present in the root of *Scutellaria baicalensis* Georgi, a traditional Chinese herbal medicine. It has numerous pharmacological activities, including anti-inflammatory [Hong et al., 2002; Chen et al., 2004], anti-allergic [Kimata et al., 2000], anti-oxidative [Shao et al., 2002; Hwang et al., 2005], anti-viral [Ono and Nakane, 1990], and anti-genotoxic activities [Lee et al., 2000]. Various activities of baicalein and its potential to modulate ERK activation have been described in previous studies [Nakahata et al., 2003; Chen et al., 2006]; however, the effect of baicalein on bone metabolism has not yet been reported.

In the present study, baicalein did not affect the viability of MC3T3-E1 subclone 4 cells but did stimulate osteoblast differentiation/mineralization and increased mRNA levels of biomarkers. Baicalein significantly increased the mRNA levels of all of the examined biomarkers in the early stage of differentiation, and significantly increased the mRNA levels of OCN, OPN, and DMP in a dose-dependent manner in the late stage.

The induction of osteoblast differentiation-related genes by baicalein could result from the coordinated activation of MAP kinases and transcription factor signaling pathways. The activation of p38 has been reported to be critical for the control of ALP expression during the

differentiation of MC3T3-E1 cells [Suzuki et al., 2002], and 1, 25$(OH)_2$D3-stimulated ALP activity has been shown to be directly related to the ERK pathway in primary human osteoblasts [Chae et al., 2002]. Flow-induction of OPN expression was also mediated through the ERK pathway, and COLI expression was regulated by both the ERK and JNK pathways [Wu et al., 2006]. In a study on vascular smooth muscle cells, injury-induced OPN expression was also dependent on the ERK pathway [Moses et al., 2001]. Furthermore, uridine triphosphate-induced OPN expression has been shown to involve a coordinated regulation of the protein kinase C (PKC)-activated NF-κB and ERK-activated AP-1 signal pathways [Renault et al., 2005]. The induction of AP-1 was also required for okadaic acid-stimulated OPN gene regulation [Kim et al., 2002].

The maximal level of OPN expression occurs when osteoblasts reach full differentiation. OPN promoter activity is controlled by regulatory elements in a skeletal tissue-specific manner and in response to AP-1-induced activation [Gerstenfeld et al., 1995]. Other small integrin-binding ligand, *N*-glycosylated proteins have also been shown to be regulated by the activation of MAP kinase(s) and transcription factors, including AP-1. JunB was shown to be phosphorylated by p38 during MC3T3-E1 cell differentiation and to subsequently bind to the AP-1 binding site in the promoter of DMP, thus activating its expression [Narayanan et al., 2004]. Furthermore, the p38 signaling pathway has been suggested to play a role in the regulation of BSP expression in the mineralization stage of MC3T3-E1 cells, by regulating the expression of PKC isoforms [Lampasso et al., 2006].

In the early and late stages of osteoblast differentiation, baicalein induced the activation of ERK. ERK is essential for the proliferation, differentiation, adhesion, spreading, migration, and integrin expression of osteoblasts [Hipskind and Bilbe, 1998; Lai et al., 2001]. Baicalein-induced activation of JNK was observed in the early stage, and the activation of p38 (at 1.25–2.5 μM baicalein) was observed in the early and late stages. The activation of MAP kinases results in the activation of transcription factors such as NF-κB and/or AP-1.

Transcription factors may function independently or cooperatively. The NF-κB-dependent AP-1 regulation suggests that these transcription factors are cooperatively regulated by MAP kinase signaling cascades. The coactivation of both factors has been reported to synergistically enhance the transcription of genes in response to a variety of stimuli [Verma et al., 1995; Fujioka et al., 2004]. For example, the p65 subunit of NF-κB can act as an accessory protein for the serum response factor, consequently regulating the expression of genes, including AP-1 family members, through serum responsive elements in their promoters [Franzoso et al., 1996]. Here, baicalein induced the activation of NF-κB during the initiation of differentiation; in the early stage, it induced NF-κB activation at low doses and increased the mRNA levels of AP-1 members at high doses. In the late stage of osteoblast differentiation, the level of AP-1 mRNA was increased by baicalein, as was the level of AP-1 protein in the nucleus. Therefore, the induction of AP-1 via the activation of NF-κB in the initiation and early stages could control the early- and late-stage expression of several osteoblast-specific genes (OCN, COLI, OPN, and DMP) through AP-1 binding sites in their promoters [Stein et al., 1996; Narayanan et al., 2004; Sakata et al., 2004].

AP-1 is a dimeric transcription factor formed by Jun proteins (c-Jun, JunB, and JunD) and Fos proteins (c-Fos, Fra-1, Fra-2, FosB, and ΔFosB). AP-1 family members are required for bone formation and remodeling [Wagner, 2002]. In the present study, baicalein increased the mRNA levels of Fra-1 and Fra-2, and increased their protein levels in the nucleus during the late stage of differentiation. Our results are consistent with previous results showing increased expression of Fra-1 and Fra-2 during differentiation [McCabe et al., 1995]. Fra-1, an important regulator of bone mass, affects bone matrix production and the maintenance of osteoblast activity [Jochum et al., 2000; Eferl et al., 2004]. Specifically, MGP mRNA has been shown to be regulated by Fra-1 [Farzaneh-Far et al., 2001; Eferl et al., 2004]. In the present study, up-regulation of MGP mRNA by baicalein was also observed in the late stage of osteoblast differentiation, and its mRNA transcription pattern was correlated with the level of Fra-1 protein in the nucleus. Fra-2 is an important factor in the late stage of osteoblast differentiation. When osteoblasts were treated with anti-sense RNA against Fra-2, large nodule formations and OCN expression were suppressed [McCabe et al., 1996].

Together with the activation of MAP kinases and transcription factors such as NF-κB and AP-1, the activation of calcineurin/NFAT signaling is also critical for osteoblast differentiation. NFAT proteins are present in a hyperphosphorylated form in the cytoplasm, and its dephosphorylation by the protein phosphatase calcineurin unmasks conserved nuclear localization sequences. This event allows NFAT to enter the nucleus and activate gene expression in concert with binding partners such as members of the AP-1 family [Boise et al., 1993]. Recently, two research groups reported that NFATc1 expression resulted in osteoblast differentiation [Koga et al., 2005; Winslow et al., 2006]. In the present study, we showed that 10 μM baicalein slightly induced the mRNA level of NFATc1 and its protein level in the nucleus in the late stage of differentiation, suggesting a role for NFATc1 in the late stage of baicalein-induced osteoblast differentiation.

In conclusion, baicalein stimulated osteoblast differentiation through the complexly coordinated MAP kinase/NF-κB/AP-1 signaling pathways. Baicalein can trigger osteoblast differentiation through the activation of NF-κB and subsequently accelerate the process of differentiation by inducing the activation of MAP kinases and NF-κB. During the late stage of differentiation, baicalein induced mineralization by inducing the activation of MAP kinases and other transcription factors such as AP-1 and NFATc1. These events could lead to the induction of genes for establishing the mineralization process (Fig. 8). Further studies are required to elucidate a functional role for baicalein in the processes leading to osteogenesis and/or osteoblast differentiation.

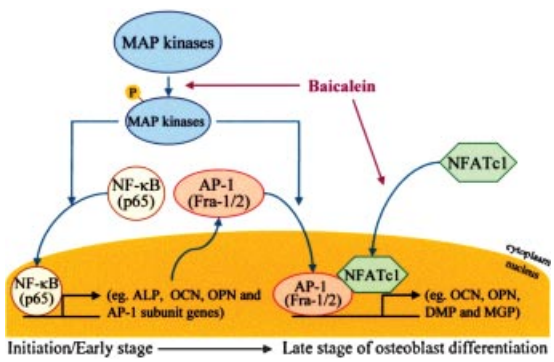

Fig. 8. Suggested mode of action of baicalein to stimulate the osteoblast differentiation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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