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Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-κB☆,☆☆

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Abstract

Signaling pathways involved in oxidative stress-induced inhibition of osteoblast differentiation are not known. We showed in this report that H_2O_2 (0.1–0.2 mM)-induced oxidative stress suppressed the osteoblastic differentiation process of primary rabbit bone marrow stromal cells (BMSC) and calvarial osteoblasts, manifested by a reduction of differentiation markers including alkaline phosphatase (ALP), type I collagen, colony-forming unit-osteoprogenitor (CFU-O) formation, and nuclear phosphorylation of Runx2. H_2O_2 treatment stimulated phospholipase C-γ1 (PLC-γ1), extracellular signal-regulated kinase 1/2 (ERK1/2), and NF-κB signaling but inhibited p38 mitogen-activated protein kinase (MAPK) activation. In the presence of 20 μM PD98059 or 50 μM caffeic acid phenethyl ester (CAPE), specific inhibitor for ERKs or NF-κB, respectively, could significantly reverse the decrease of above-mentioned osteoblastic differentiation markers elicited by H_2O_2 (0.1 mM). Furthermore, PD98059 also suppressed H_2O_2-stimulated NF-κB signaling in this process. These data suggest that ERK and ERK-dependent NF-κB activation is required for oxidative stress-induced inhibition of osteoblastic differentiation in rabbit BMSC and calvarial osteoblasts.

Keywords: Oxidative stress; Osteoblast differentiation; Extracellular signal-regulated kinase; NF-κB; Runx2; Signal transduction

Bone is formed and resorbed continuously, starting in the embryo and continuing throughout adult life. This process occurring in adult bone is called bone remodeling, which is carried out by osteoblasts (bone-forming cells) and osteoclasts (bone-resorption cells). Any loss of osteoblastic activity or an increase in osteoclastic activity would ultimately lead to osteoporosis, characteristics of lower bone mineral densities (BMD), a decrease in bone mass, and makes the bone weaker and more likely to fracture [1].

The differentiation of osteoblast and osteoclast is believed to be particularly important in pathogenesis of osteoporosis. Osteoblasts mature from osteoprogenitors that reside in the bone marrow [2]. A "master" regulator of osteoblast differentiation is the transcription factor Runx2 (core-binding factor 1, Cbfa1) [3]. Runx2 binds to the osteoblast-specific *cis*-acting element 2 (OSE2), which is found in the promoter regions of all the major osteoblast-specific genes (e.g., osteocalcin, type I collagen, and alkaline phosphatase) and controls their expression [4]. Although evidences suggest that signaling pathways including bone morphogenetic protein

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☆☆ *Abbreviations:* ALP, alkaline phosphatase; BMD, bone mineral densities; BMP, bone morphogenetic proteins; BMSC, bone marrow stromal cells; CAPE, caffeic acid phenethyl ester; Cbfa1, core-binding factor 1, CFU-O, colony-forming unit-osteoprogenitor; DAG, diacylglycerol; ERK1/2, extracellular signal-regulated kinase 1/2; ECL, enhanced chemiluminescence; HSF, heat shock factor; IκB, NF-κB inhibitory proteins; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; OSE2, osteoblast-specific *cis*-acting element 2; PI-3K, phosphatidylinositol 3 kinase; PKA, protein kinase A; PLC-γ1, phospholipase C-γ1; ROS, reactive oxygen species.

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(BMP)-Smad signaling [5], mitogen-activated protein kinases (MAPKs) [6–10], phosphatidylinositol 3 kinase (PI-3K) [11] may be involved in osteoblast differentiation, the signaling mechanisms contributing to decreased osteoblastic differentiation in osteoporosis are not well known.

Reactive oxygen species (ROS) such as superoxides and hydrogen peroxide can cause severe damage to DNA, protein, and lipids. High levels of oxidant produced during normal cellular metabolism (e.g., mitochondrial electron transport) or from environmental stimuli (e.g., cytokines, UV radiation) perturb the normal redox balance and shift cells into a state of oxidative stress [12]. Oxidative stress is believed to contribute to etiology of various degenerative diseases such as cerebellar ischemia, atherosclerosis, cancer, and the process of aging [12]. At the cellular level, oxidant injury elicits a wide spectrum of responses ranging from proliferation to growth or differentiation arrest, to senescence, and to cell death by activating numerous signaling pathways, such as PI-3K, nuclear factor-κB (NF-κB), phospholipase C-γ1 (PLC-γ1), p53, heat shock factor (HSF), and MAPKs which may classify into four classes: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNK), p38 MAPK, and ERK5. The magnitude and duration of the stress as well as the cell type involved are important factors in determining which pathways are activated and the particular outcome reflects the balance between these pathways [13].

Several lines of evidence have found a tight association between oxidative stress and pathogenesis of osteoporosis. Marked decrease in plasma antioxidants was found in aged osteoporotic women [14]; there is also a biochemical link between increased oxidative stress and reduced BMD in aged men and women [15]; dietary antioxidant vitamin intake has a beneficial effect on BMD in postmenopausal women [16]; oxidative stress increases differentiation and function of osteoclasts [17].

These observations, combined with recent data indicating that oxidative stress is able to inhibit bone cell differentiation of a preosteoblastic cell line (MC3T3-E1) and of marrow stromal cell line (M2-10B4) [18], prompted us to explore the signaling mechanisms involved in the inhibition of osteoblastic differentiation of bone cells during oxidative stress. We demonstrate that H_2O_2-induced oxidative stress inhibits osteoblastic differentiation of primary rabbit calvarial osteoblast and bone marrow stromal cells (BMSC) via ERK and ERK-dependent NF-κB signaling pathway.

Materials and methods

Reagents. α modified Eagle's medium (αMEM) and fetal bovine serum were from Life Technologies (Gaithersburg, MD). Catalase, U73122, Wortmannin, caffeic acid phenethyl ester (CAPE), β-glycerophosphate, dexamethasone, protein A, α-naphthyl phosphate, and Fast Blue salt were purchased from Sigma–Aldrich (St. Louis, MO). SB203580, PD98059, antibodies specific to phosphorylated p38 MAPK, phosphorylated PLC-γ1, and IκBα were purchased from Cell Signaling Technology (Beverly, MA). Enhanced chemiluminescence (ECL) kit, antibodies specific to BMP-2 type I collagen, p65 NF-κB, phosphorylated IκBα, Runx2, phosphotyrosine, phosphorylated ERK1/2, and actin were purchased form Santa Cruz (Santa Cruz, CA). Polyvinylidene difluoride membranes were from Bio-Rad (Richmond, CA).

Cell culture and treatment. Rabbit calvarial osteoblasts and BMSC were isolated from newborn and 3-month-old rabbit, respectively, and cultivated in αMEM supplemented with 10% fetal bovine serum. Cultures were trypsinized upon confluence and propagated to passage 2 before being subcultured into 24-well plates or 100-mm petri dishes or on glass slides for further experiment.

Cells were treated with different concentrations of H_2O_2, catalase (scavenger of H_2O_2), and specific inhibitors of the test signaling pathways including U73122 (5 μM) to inhibit PLC-γ1, Wortmannin (100 nM) to inhibit PI-3K, caffeic acid phenethyl ester (CAPE, 50 μM) to inhibit NF-κB, SB203580 (10 μM) to inhibit p38 MAPK, and PD98059 (20 μM) to inhibit ERKs for various times in the normal or differentiation medium (αMEM containing 10 mM β-glycerophosphate and 10^{-8} M dexamethasone,). The effect dose of these inhibitors was obtained from the literature.

Cell viability analysis. BMSC subcultured into 24-well plates in differentiation medium were treated with different concentrations (0.1–0.5 mM) of H_2O_2. After 1, 4 or 12 days, cell viability was determined by counting the viable cell number with a hemocytometer after staining with trypan blue.

Alkaline phosphatase (ALP) staining and formation of colony-forming unit-osteoprogenitor (CFU-O). Cells subcultured on glass slides in differentiation medium were treated with or without H_2O_2, catalase, and inhibitors once confluent. After 4 days, the cell cultures were fixed with neutral formaldehyde and subjected to modified Gomori's ALP staining. The ratio of ALP-positive cells to total cells was quantified under a microscope.

BMSC subcultured into 24-well plates (5×10^4 cells/well) in differentiation medium were treated with or without H_2O_2, catalase, and inhibitors for 12 days (the culture supernatant was replaced with fresh differentiation medium and reagent every 3 days). CFU-O in BMSC culture was assessed by modified Gomori's ALP staining. Total colonies found to have more than 32 segregate cells were recognized and calculated as positive bone ALP staining.

Determination of ALP activity. Calvarial osteoblasts and BMSC subcultured into 24-well plates were treated with or without H_2O_2, catalase, and inhibitors once confluent for 4 days. To quantify ALP activities, a semiquantitative method using α-naphthyl phosphate as the substrate and Fast Blue salt as the diazonium salt was used. Briefly, cells were washed three times with ice-cold Tris-buffered saline, pH 7.4, and scraped immediately upon addition of ice-cold 50 mM Tris-buffered saline, and the collected lysates were sonicated for 20 s at 4 °C. Protein levels were determined by BCA assay and used to normalize ALP activity. The kinase assay was performed in assay buffer (10 mM $MgCl_2$ and 0.1 M alkaline buffer, pH 10.3) containing 10 mM *p*-nitrophenylphosphate in alkaline buffer (3.71 mg/ml assay buffer) as the substrate. Tubes were incubated in a 37 °C water bath and timed. The reaction was stopped by the addition of 0.3 N NaOH. Reaction mixtures were transferred into cuvettes and absorbance was read at OD_{405}. The relative ALP activity is defined as micromolar of *p*-nitrophenol phosphate hydrolyzed per minute per micrograms of total protein (units).

Western blot. Cells subcultured into 100 mm dishes were treated with H_2O_2, catalase, and specific inhibitors once confluent for various times. Stimulated cells (1×10^7) were washed with cold PBS and lysed in Laemmli buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% bromophenol blue) for 5 min at 95 °C. Cell lysates were analyzed by SDS–PAGE and transferred

electrophoretically to polyvinylidene difluoride membrane. Blots were probed with specific antibodies and immunoreactive proteins were revealed by ECL kit.

Preparation of nuclear extract and determination of Runx2 phosphorylation. Stimulated cells were lysed with ice-cold buffer containing 10 mM Tris, pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, and 0.7% Nonidet P-40 on ice for 10 min, and centrifuged at 500*g* for 5 min. The nuclear pellets were further lysed with buffer containing 40 mM Tris, pH 7.9, 350 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.2 mM EGTA, 20% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μM dithiothreitol, 2 μg/ml leupeptin, and 1 μg/ml aprotinin on ice for 20 min and harvested by centrifugation at 12,000*g*, 4 °C, for 10 min. The nuclear extracts were then incubated with anti-Runx2 antibody for 1 h at 4 °C. After incubation, the immune complexes were precipitated with protein A. The immunoprecipitate (20 μg) was mixed with Laemmli buffer for 5 min at 95 °C. The mixtures were subjected to Western blot assay. Total Runx2 on the blot was recognized by anti-Runx2 antibody and the phosphorylated Runx2 on the blot was further recognized by specific anti-phosphotyrosine antibody. Runx2 activity was reflected on the phosphorylated Runx2 visualized with ECL agents.

Statistical analysis. Each experiment was repeated a minimum of three times. Statistical analyses were performed by Student's *t* test. Data are presented as means ± SD.

Results

Low doses of H_2O_2 inhibit osteoblastic differentiation of BMSC

Cellular responses elicited by H_2O_2 depend upon the severity of the damage, which is further influenced by the cell type and the magnitude of the dose of the exposure [13]. In our experiments, BMSC underwent severe cell death after high dose of H_2O_2 (0.5 mM) treatment for 1, 4 or 12 days as determined by the trypan blue dye-exclusion method. For low doses (0.1 or 0.2 mM) of H_2O_2, however, the cell viability of BMSC did not significantly affect compared with the controls (Fig. 1A).

The osteoprecursor cells in bone marrow stromal cultures can spontaneously differentiate into osteoblasts, with the expression of ALP. To determine whether H_2O_2-induced oxidative stress inhibits osteoblastic differentiation of bone cells, we first tested the effects of

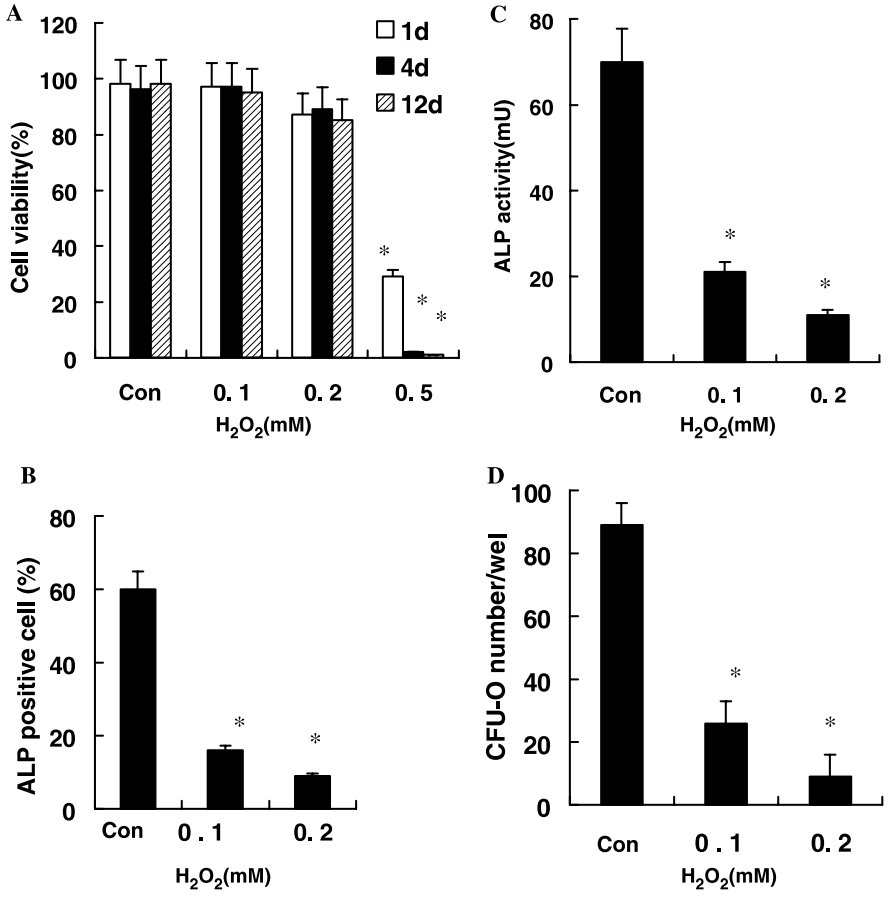

Fig. 1. Low doses of H_2O_2 inhibit osteoblastic differentiation of BMSC while high dose of H_2O_2 induces cell death. (A) BMSC isolated from 3-month-old rabbit were treated with 0.1, 0.2 or 0.5 mM H_2O_2 for 1, 4 or 12 days and cell viability was detected by the trypan blue dye-exclusion method. (B) Low doses (0.1 and 0.2 mM) of H_2O_2 were added in confluenced BMSC cultured in differentiation medium (αMEM containing 10 mM β-glycerophosphate and 10^{-8} M dexamethasone) for 4 days. The expression of ALP was measured by ALP staining and the ratio of ALP positive cells to total cells was counted under a microscope. (C) BMSC treated as described in (B) were subjected to ALP activity assay. (D) BMSC subcultured into 24-well plates (5×10^4 cells/well) were treated with 0.1 or 0.2 mM H_2O_2 in differentiation medium for 12 days and CFU-O numbers were detected by ALP staining. Con, control; *significant differences compared to controls ($P < 0.001$). Data are means ± SD for three independent experiments.

H_2O_2 on ALP activity during the differentiation of BMSC. Low doses (0.1 or 0.2 mM) of H_2O_2 were added to confluenced BMSC cultured in differentiation medium for 4 days. The expression of ALP was measured histochemically by ALP staining and the ratio of ALP positive cells was counted. We found that H_2O_2 inhibited ALP activity in BMSC. On plates in the presence of 0.1 and 0.2 mM H_2O_2, ALP positive cells were significantly ($P < 0.001$) less than in controls, suggesting that oxidative stress reduced the expression of early marker of osteoblastic differentiation (Fig. 1B). Furthermore, we used a quantitative ALP assay to confirm the results obtained from ALP staining. Protein levels were measured by BCA assay and were used to normalize ALP activity. Consistent with histochemical data, 0.1 and 0.2 mM of H_2O_2 significantly reduced the expression of ALP activity in BMSC (Fig. 1C).

We next tested the effects of H_2O_2-induced oxidative stress on CFU-O formation. BMSC were treated with 0.1 or 0.2 mM H_2O_2 for 12 days, CFU-O numbers were then detected by ALP staining as described in Materials and methods. Results showed that CFU-O numbers on plates treated with H_2O_2 were dramatically ($P < 0.001$) decreased compared with the control (Fig. 1D).

These results suggest that high dose of H_2O_2 induces cell death while low doses of H_2O_2 suppress ALP expression and CFU-O formation during the osteoblastic differentiation of BMSC.

Oxidative stress suppressed nuclear Runx2 phosphorylation and expression of type I collagen during BMSC differentiation

It is known that BMP-2 is a factor capable of initiating osteoblastogenesis from uncommitted progenitors by stimulating the transcription of the gene encoding Runx2 and the expression and activation of Runx2 play a key role during osteoblast differentiation and skeletogenesis [3]. To elucidate the effects of oxidative stress on this osteoblastic differentiation-related signaling, we studied whether the expression of BMP-2, Runx2, and type I collagen and activation of Runx2 are affected by oxidative stress during osteoblastic differentiation of BMSC. It was found that 0.1 or 0.2 mM of H_2O_2 treatment for 24 h did not change the protein levels of BMP-2, total Runx2, but inhibited the expression of type I collagen and activation of Runx2, as demonstrated by a dramatic decrease of phosphorylated Runx2 in nuclear fraction extracts (Fig. 2). Pretreatment of 500 U/ml catalase, a scavenger of H_2O_2, reversed H_2O_2-induced inhibition of type I collagen expression and nuclear Runx2 phosphorylation completely, but had no effect on BMP-2 and nuclear Runx2 levels (Fig. 2). These results demonstrate that H_2O_2-induced oxidative stress inhibits nuclear Runx2 phosphorylation and expression of type I collagen, but has no effect on expression of BMP-2 and Runx2 during osteoblastic differentiation of BMSC.

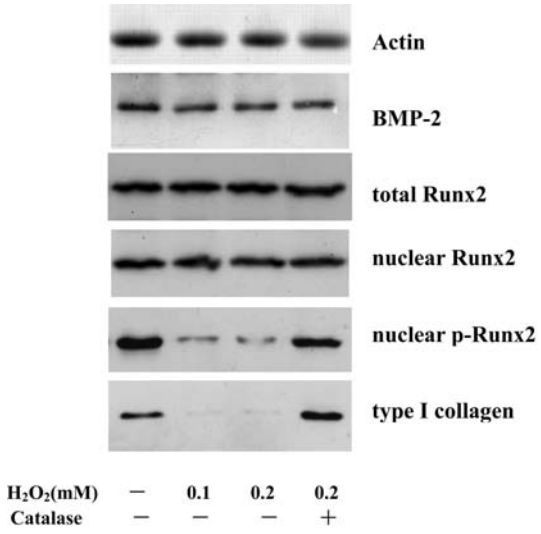

Fig. 2. H_2O_2 suppresses nuclear Runx2 phosphorylation and expression of type I collagen, during BMSC differentiation. Confluenced BMSC were incubated with or without 500 U/ml catalase for 30 min and then treated with 0.1 or 0.2 mM H_2O_2 for 24 h. The expression of BMP-2, type I collagen, and Runx2 was analyzed by Western blot. Phosphorylation of Runx2 in nuclear extracts was determined as described in Materials and methods. Actin was used as loading controls. p-Runx2, phosphorylated Runx2.

Signaling pathways involved in H_2O_2-induced oxidative stress in BMSC

PLC-γ1 plays an important role in the regulation of cell proliferation and differentiation by generation of the second messenger, diacylglycerol (DAG), and inositol 1,4,5-trisphosphate. Recently, an anti-apoptotic role of PLC- γ1 activation in oxidative stress has been reported in our laboratory [19] and others [20]. NF-κB transcription factors are involved in regulating large numbers of genes related to immune function, inflammation, apoptosis, cell proliferation, and differentiation [21], and oxidative stress is known to be an activator of NF-κB [22]. Activation of NF-κB occurs via phosphorylation of the inhibitory IκB proteins, followed by proteasome-mediated degradation of IκB, resulting in the release and nuclear translocation of active NF-κB [23]. ERK1/2 and p38 MAPK are members of MAPKs and are known to be involved in both oxidative stress and osteoblast differentiation [7,8,24]. To determine whether these signaling pathways are also affected by oxidative stress during osteoblastic differentiation of rabbit BMSC, cells were treated with 0.1 mM H_2O_2 for 0.3, 2 or 12 h and the cell lysates were subjected to Western blot analysis with anti-phosphorylated PLC-γ1, IκBα, p38 MAPK or ERK1/2 antibody, and anti-IκBα, p65 NF-κB antibody. We found that phosphorylation of PLC-γ1 and ERK1/2

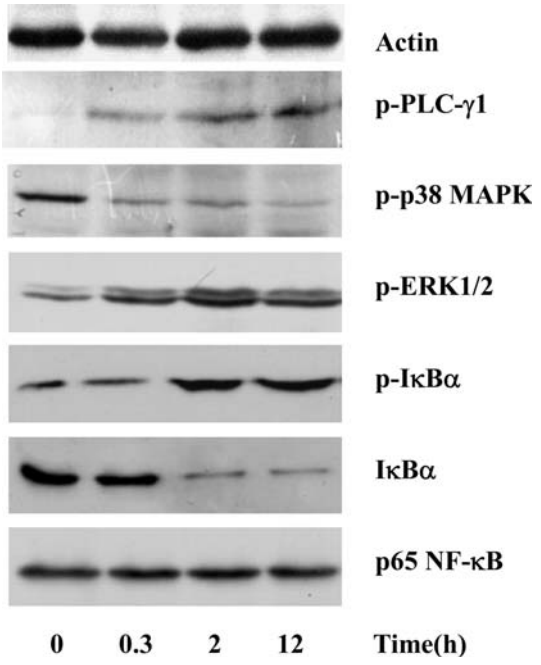

Fig. 3. Signaling pathways involved in H_2O_2-induced oxidative stress in BMSC. BMSC were treated with 0.1 mM H_2O_2 for 0.3, 2 or 12 h and the cell lysates were subjected to Western blot analysis with anti-phosphorylated PLC-γ1, IκBα, p38 MAPK or ERK1/2 antibody, and anti-IκBα, p65 NF-κB antibody. p-PLC-γ1, p-IκBα, p-p38 MAPK or p-ERK1/2, phosphorylated PLC-γ1, IκBα, p38 MAPK or ERK1/2, respectively.

increased markedly by 0.1 mM H_2O_2 from 0.3 to 12 h; IκBα activation began to rise after treatment for 2 h, accompanied by reduction in levels of IκBα protein; p38 MAPK was notably inhibited by H_2O_2 treatment; and the protein level of p65 NF-κB in BMSC did not change by H_2O_2 stimulation (Fig. 3). Our results demonstrate that PLC-γ1, ERK1/2, and NF-κB signaling pathways are stimulated while p38 MAPK is inhibited by H_2O_2-induced oxidative stress during rabbit BMSC differentiation.

ERKs and NF-κB are required for oxidative stress-induced inhibition of osteoblastic differentiation in BMSC

To determine signaling pathways involved in oxidative stress-induced inhibition of osteoblastic differentiation, specific inhibitors for PLC-γ1, PI-3K, p38 MAPK, ERKs, and NF-κB were added in BMSC followed by exposure to 0.1 mM H_2O_2 or not for 4 or 12 days. The osteoblastic differentiation of BMSC was detected by ALP-staining, ALP activity assay, and CFU-O formation. It is found that 20 μM PD98059, 50 μM CAPE, and 500 U/ml catalase, the specific inhibitor for ERK, NF-κB, and H_2O_2, respectively, suppressed H_2O_2-induced decrease of ALP positive cell, ALP activity, and CFU-O number significantly ($P < 0.001$) during osteoblastic differentiation of BMSC (Figs. 4A, B and C). As much as 5 μM U73122, 100 nM Wortmannin, and 10 μM SB203580, the specific inhibitor for PLC-γ1, PI-3K, and p38 MAPK, respectively, however, had no notable effect on the decrease of ALP positive cells, ALP activity, and CFU-O number elicited by H_2O_2 (Figs. 4A, B, and C). We also found that SB203580 reduced these differentiation markers significantly ($P < 0.001$) while in the absence of H_2O_2, but catalase or other inhibitors had no statistical effect. These results suggest that activity of ERK and NF-κB is required for oxidative stress-induced inhibition of osteoblastic differentiation of BMSC.

Inhibition of ERK and NF-κB suppressed H_2O_2-induced decrease of nuclear Runx2 phosphorylation and type I collagen expression during BMSC differentiation

It is not known whether the activity of ERKs and NF-κB is involved in the inhibition of nuclear Runx2 phosphorylation and type I collagen expression induced by H_2O_2. Confluenced BMSC were treated with 0.1 mM H_2O_2 for 24 h in differentiation medium with or without PD98059 or CAPE. Cell lysates or nuclear extract were subjected to western analysis with anti-BMP-2, Runx2, and type I collagen antibody or determination of Runx2 phosphorylation as described in Materials and methods. H_2O_2-induced inhibition of nuclear Runx2 phosphorylation and type I collagen expression were reversed in the presence of 20 μM PD98059 or 50 μM CAPE (Fig. 5). Protein levels of BMP-2 and Runx2, however, were not affected by PD98059 or CAPE treatment. It is demonstrated that ERKs and NF-κB mediate H_2O_2-induced inhibition of nuclear Runx2 phosphorylation and type I collagen expression during BMSC differentiation.

Stimulation of NF-κB signaling by H_2O_2 is ERK-dependent during BMSC differentiation

PI-3K and PLC-γ1 may regulate the activation of p38 MAPK, ERKs, and NF-κB, and there have been many crosstalks between these signaling pathways [25,26]. To determine the upstream regulators of p38 MAPK, ERKs, and NF-κB signaling in H_2O_2-induced oxidative stress during BMSC differentiation, cells were treated with 0.1 mM H_2O_2 for 6 h with or without specific inhibitors and the activation of p38 MAPK, ERKs, and NF-κB was detected by Western blot analysis with anti-phosphorylated p38 MAPK, ERKs, and IκBα antibody, respectively. We found that inhibition of PI-3K or PLC-γ1 by Wortmannin or U73122 did not exhibit any dramatic effect on H_2O_2-induced ERK and IκBα phosphorylation or p38 MAPK inhibition. Inhibition of p38 MAPK by SB203580 did not affect H_2O_2-induced ERK and IκBα activation (Fig. 6). Inhibition of ERKs by PD98059 did not change H_2O_2-induced p38 MAPK inhibition, but interestingly suppressed H_2O_2-induced phosphorylation and degradation of IκBα completely (Fig. 6). At the same time,

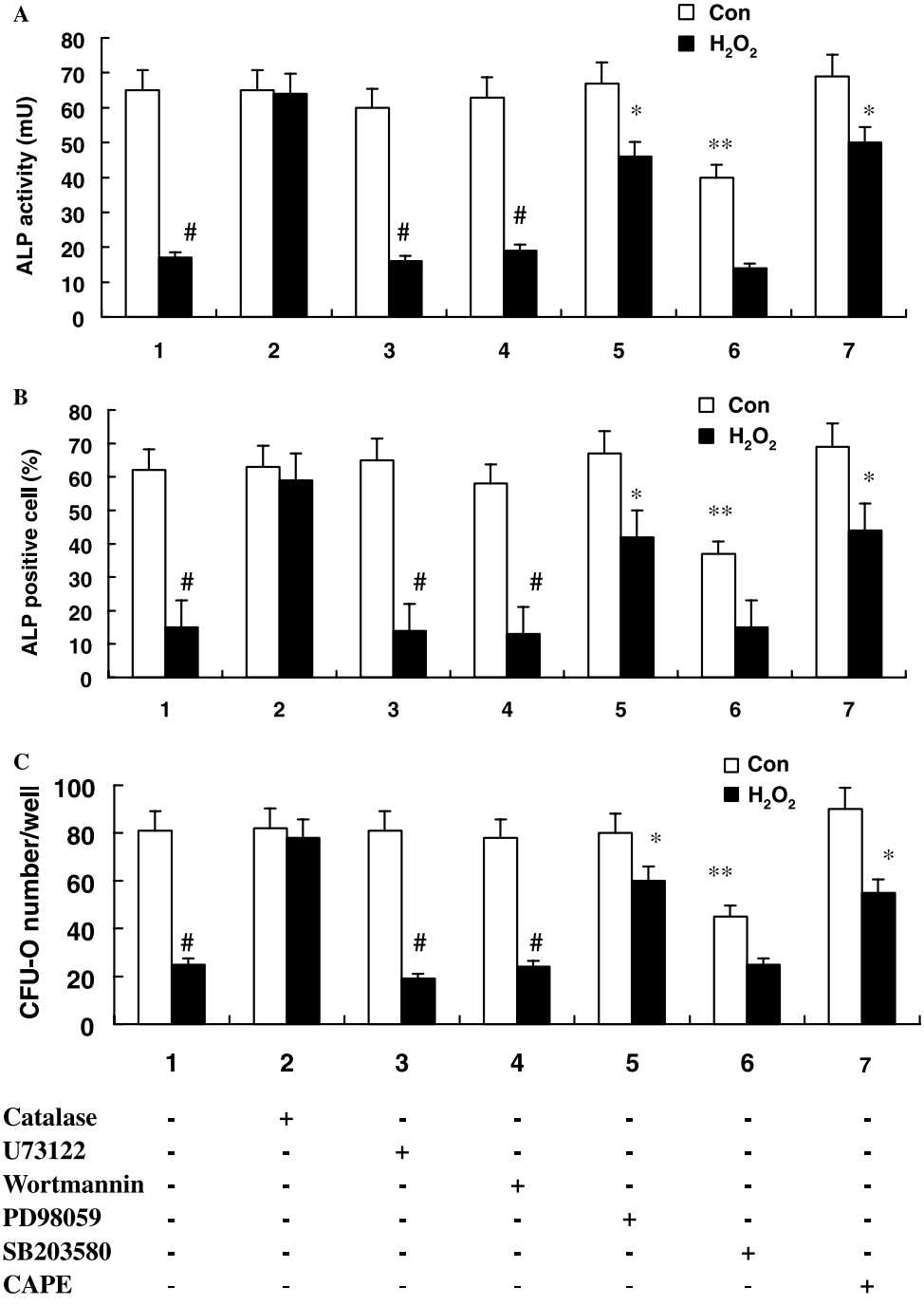

Fig. 4. ERKs and NF-κB are required for H_2O_2-induced inhibition of osteoblastic differentiation in BMSC. (A) Confluenced BMSC were incubated with or without 0.1 mM H_2O_2 for 4 days in differentiation medium in the presence or absence of 500 U/ml catalase, or 5 μM U73122, or 100 nM Wortmannin, or 10 μM SB203580, or 20 μM PD98059, or 50 μM CAPE. The expression of ALP was measured by ALP activity assay. (B) The ratio of ALP positive cells to total cells was counted under a microscope after ALP staining. (C) Adherent BMSC (24-well plates, 5×10^4 cells/well) were treated as (A) for 12 days and CFU-O numbers were detected by ALP staining. Con, control; *significant differences compared to H_2O_2-treated group of columns 1, 3, 4, and 6 ($P < 0.001$); #significant differences compared to its control group ($P < 0.001$); **significant differences compared control group of column 1. Data are means ± SD for three independent experiments.

pretreatment with H_2O_2 scavenger, catalase, reversed H_2O_2-induced ERK and IκBα phosphorylation or p38 MAPK inhibition. Taking these together, it is shown that ERK is required for H_2O_2 stimulation of NF-κB signaling in rabbit BMSC.

ERK-dependent NF-κB activation is required for H_2O_2-induced inhibition of calvarial osteoblast differentiation

We used rabbit BMSC to demonstrate that H_2O_2-induced oxidative stress interfered with the differentia-

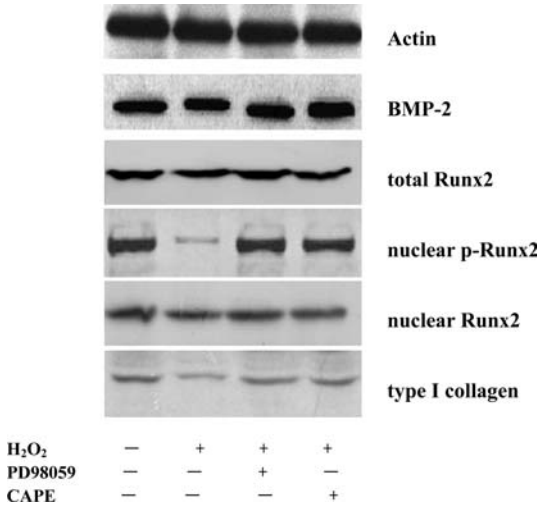

Fig. 5. Inhibition of ERKs and NF-κB suppressed H_2O_2-induced inhibition of nuclear Runx2 phosphorylation and type I collagen expression during BMSC differentiation. Confluenced BMSC were treated with 0.1 mM H_2O_2 for 24 h in differentiation medium with or without 20 μM PD98059 or 50 μM CAPE. Cell lysate or nuclear extract was subjected to Western blot analysis with anti-BMP-2, Runx2, and type I collagen antibody or determination of Runx2 phosphorylation.

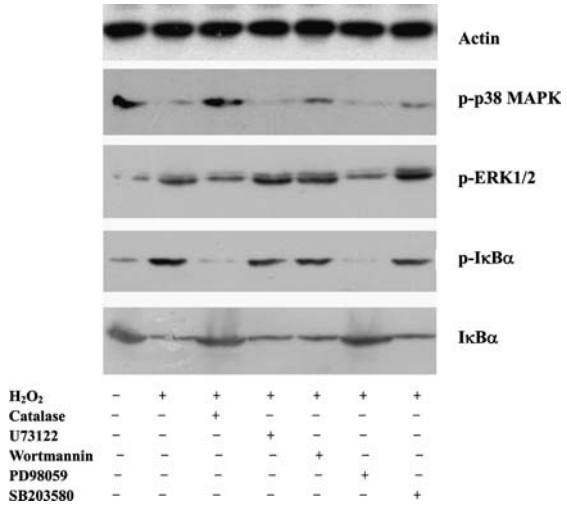

Fig. 6. Stimulation of NF-κB signaling by H_2O_2 is ERK-dependent during BMSC differentiation. Cells were treated with 0.1 mM H_2O_2 for 12 h in differentiation medium with or without 500 U/ml catalase, 5 μM U73122, 100 nM Wortmannin, 10 μM SB203580, or 20 μM PD98059, and the activation of p38 MAPK, ERK1/2, NF-κB was detected by Western blot analysis with anti-phosphorylated p38 MAPK, ERK1/2, and IκBα antibody, respectively.

tion process of osteoblasts, manifested by the lower expression of ALP, type I collagen, and less CFU-O formation, and that ERKs and ERK-dependent NF-κB activation mediated inhibition of Runx2 phosphorylation may contribute to this process. We then studied the effect of H_2O_2 on the differentiation of primary rabbit calvarial osteoblasts and the role of ERK, NF-κB, and Runx2 in this process to further support our conclusion drawn from studies using BMSC. Similarly, calvarial osteoblasts were treated with 0.1 mM H_2O_2 for the indicated times in differentiation medium with or without catalase, specific inhibitor for ERKs or NF-κB, and then ALP activity, activation of p38 MAPK, ERK1/2, IκBα, Runx2, and expression of BMP-2, type I collagen or Runx2 were detected as described previously. Results showed that p38 MAPK specific inhibitor SB203580 reduced ALP activity significantly ($P < 0.001$) in the absence of H_2O_2 stimulation, but catalase or other inhibitors had no significant effect (Fig. 7A). As much as 0.1 mM of H_2O_2 inhibited expression of ALP activity (Fig. 7A) and type I collagen; suppressed p38 MAPK and nuclear Runx2 phosphorylation; and activated ERK1/2 and IκBα ; but did not exhibit any dramatic effect on BMP-2 and total Runx2 expression during calvarial osteoblast differentiation (Figs. 7B and C). Scavenging of H_2O_2 by 500 U/ml catalase or inhibition of ERKs and NF-κB by 20 μM PD98059 or 50 μM CAPE reversed H_2O_2-induced inhibition of ALP activity (Fig. 7A), type I collagen expression, and nuclear Runx2 phosphorylation but had no effect on BMP-2 and total Runx2 expression (Fig. 7B). Catalase and PD98059 also inhibited H_2O_2-induced phosphorylation and degradation of IκBα (Fig. 7C). Taken together, all these data suggest that H_2O_2-induced oxidative stress inhibits rabbit calvarial osteoblast differentiation as identified by the lower expression of type I collagen, ALP activity, and nuclear Runx2 phosphorylation, and that this process is mediated by ERK and ERK-dependent NF-κB activation.

Discussion

The signaling pathways involved in oxidative stress-induced inhibition of osteoblastic differentiation have not been reported previously. We showed in this report that H_2O_2-induced oxidative stress suppressed the differentiation process of osteoblasts in rabbit primary BMSC and calvarial osteoblast, manifested by a reduction of differentiation markers including ALP, type I collagen, CFU-O formation, and Runx2 activation. H_2O_2 treatment stimulated PLC-γ1, ERK1/2, and NF-κB signaling but inhibited p38 MAPK activation. Only in the presence of specific inhibitors for ERK or NF-κB could significantly reverse the reduction of above-mentioned differentiation markers by oxidative stress during osteoblastic differentiation of BMSC and calvarial osteoblast. Furthermore, ERK-specific inhibitor also suppressed H_2O_2-stimulated NF-κB signaling in this process. These data suggest that ERK and ERK-dependent NF-κB activation is required for oxidative stress-induced inhibition of osteoblastic differentiation in BMSC and calvarial osteoblasts.

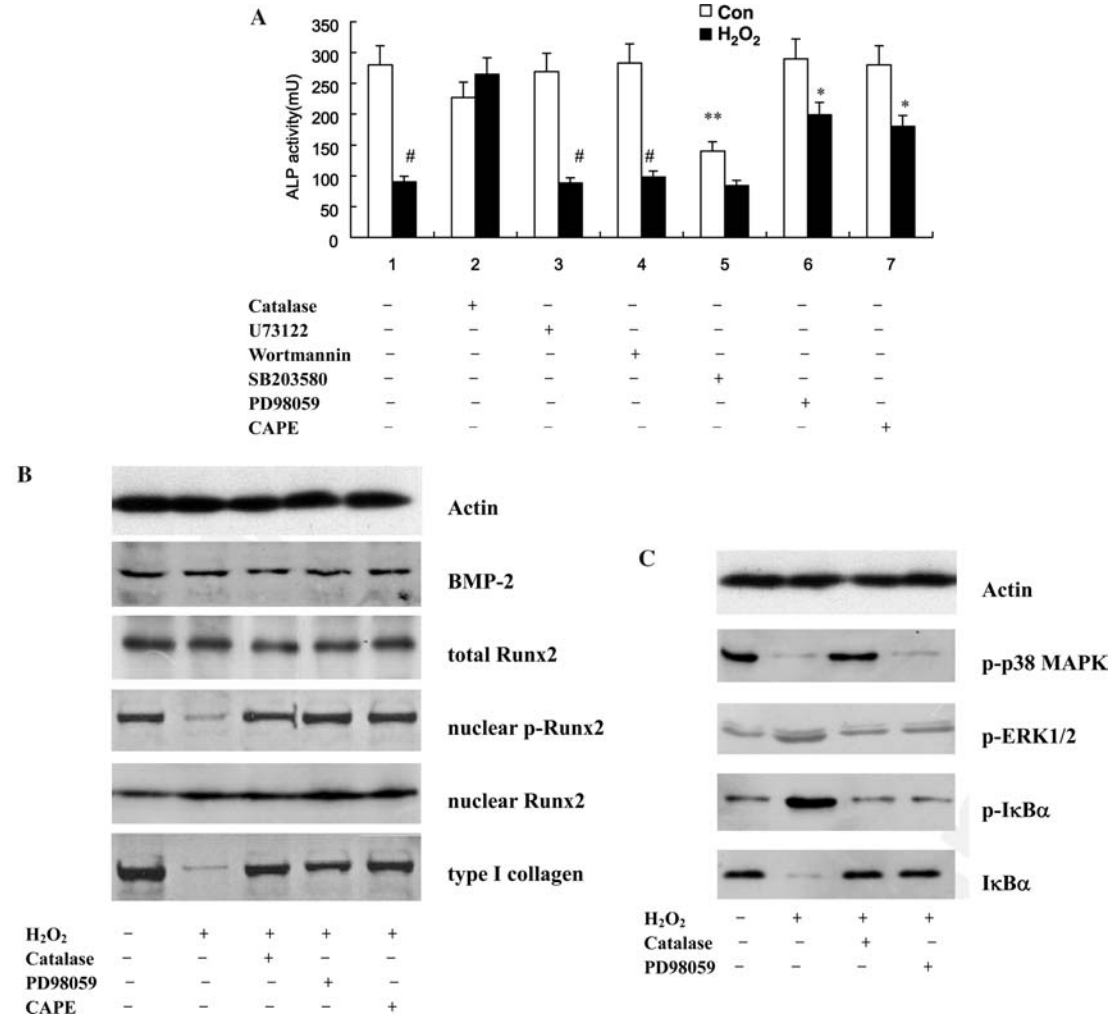

Fig. 7. ERK-dependent NF-κB activation is required for H_2O_2-induced inhibition of rabbit calvarial osteoblastic differentiation. (A) Calvarial osteoblasts isolated from newborn rabbit were treated with 0.1 mM H_2O_2 or not for 4 days in differentiation medium with or without 500 U/ml catalase, 20 μM PD98059, 50 μM CAPE, 5 μM U73122, 100 nM Wortmannin or 10 μM SB203580. ALP activity was detected as described previously. (B) Cells treated with 0.1 mM H_2O_2 and specific inhibitors for 24 h, nuclear phosphorylation of Runx2 and expression of BMP-2, type I collagen or Runx2 were detected as described previously. (C) Cells treated with 0.1 mM H_2O_2 and specific inhibitors for 12 h, phosphorylation of p38 MAPK, ERK1/2, and IκBα was detected by Western blot analysis. Actin was used as loading controls. *Significant differences compared to H_2O_2-treated group of columns 1, 3, 4, and 5 ($P < 0.001$); #significant differences compared to its control group ($P < 0.001$); **significant differences compared control group of column 1. Data are means ± SD for three independent experiments.

It has previously been shown in vitro and in rodents that free radicals are involved in osteoclastogenesis and in bone resorption [17], H_2O_2 stimulates bone resorption in mouse calvariae [27]. Oxidative stress may increase bone resorption through activation of NF-κB which play an important role in osteoclastogenesis [27,28]. Accordingly, the effect and molecular control of oxidative stress on osteoblast differentiation and function are understood much less than those of osteoclasts. Mody et al. [18] have shown that H_2O_2 or xantine/xanthine oxidase (XXO)-induced oxidative stress is able to inhibit bone cell differentiation of a preosteoblastic cell line (MC3T3-E1) and of a marrow stromal cell line (M2-10B4) that undergoes osteoblastic differentiation. Recent data showed that extracorporeal shock wave (ESW) induced superoxide but not H_2O_2 enhanced osteogenic cell growth and maturation [29]. Our results from primary rabbit BMSC and calvarial osteoblasts are consistent with Mody's but the dose of H_2O_2 in our experiments (0.1 mM) is much lower than that in Mody's (1 mM). The discrepancy between these results may be caused by the different cell type, different source, dose, and duration of oxidative stimulus.

The roles of ERKs and p38 MAPK in the differentiation of osteoblasts are disputable. Although several studies of mouse C2C12 cells indicate that p38 MAPK is required for BMP-2-induced expression of Runx2, ALP, and osteocalcin [6,30], a similar study gives opposite results [7]. Studies using MC3T3-E1 cells suggest that activation of p38 is critical for ALP expression induced by

fetal calf serum, or pentoxifylline [8,9]. Lately, a study using MC3T3-E1, mouse primary calvarial osteoblasts, and BMSC indicates that p38 MAPK, but not ERKs, is necessary for osteoblast differentiation [31]. Although we demonstrate that p38 MAPK plays a positive role during rabbit calvarial osteoblast and BMSC differentiation, and that oxidative stress inhibits p38 MAPK as well as osteoblastic differentiation of primary rabbit calvarial osteoblasts and BMSC, whether p38 MAPK acts as a target of oxidative stress in this process remains for further confirmation. There are several studies showing that ERK is essential for the early stages of osteoblast differentiation and is involved in the stimulation of osteoblast-related gene expression by extracellular matrix–integrin receptor interaction, BMP-2, growth factors as well as mechanostressing [10,29,32,33]. Furthermore, it is shown that ERKs may phosphorylate Runx2 and therefore affect osteoblast differentiation [33]. But some other reports suggest that ERK plays a negative role in BMP-2 and growth factor-induced osteoblast differentiation [31,34]. In our experiments ERK is activated during H_2O_2-induced oxidative stress and inhibition of ERKs by PD98059 suppressed H_2O_2-induced reduction of type I collagen, ALP activity, CFU-O formation, and nuclear Runx2 phosphorylation during primary rabbit calvarial osteoblasts and BMSC differentiation. Based upon our results, ERKs may act as a target of oxidative stress in H_2O_2-induced inhibition of osteoblast differentiation.

How does oxidative stress-stimulated ERK participate in H_2O_2-induced suppression of osteoblast differentiation? ERK signaling pathways have been implicated in NF-κB activation induced by mechanical deformation or oxidative stress through phosphorylation of IκB in rat osteosarcoma ROS 17/2.8 cells and HeLa cells [35,36]. NF-κB is involved in the response to various stimuli. In bone, its major role was emphasized by the phenotype of NF-κB knockout mice exhibiting an osteopetrosis mainly due to an impairment in osteoclastogenesis and osteoclastic function [28]. At the same time, several reports point to the negative regulation of osteoblast differentiation by NF-κB in MC3T3 cells [37] and human osteosarcoma cell line Saos-2 [38]. Although a recent report showed that ROS did not mediate NF-κB activation [39], our results show that ERKs mediate oxidative stress phosphorylation of IκBα and activation of NF-κB, which is also essential for the inhibition of rabbit BMSC and calvarial osteoblast differentiation elicited by H_2O_2. Taking these together, it is demonstrated that NF-κB may be the downstream of ERK signaling participating in H_2O_2-induced suppression of osteoblast differentiation. Considering the pluripotent of ERK signaling, other ERK-regulated transcription factors may also be involved in this process in addition to NF-κB.

Several questions still remain regarding the mechanism of ERKs and NF-κB regulation of osteoblast differentiation in oxidative stress. First, the recovery of osteoblast differentiation parameters by PD98059 was similar to but not stronger than that by CAPE as shown in Fig. 4 and Fig. 7A. It implies that the role of NF-κB in H_2O_2-induced suppression of osteoblast differentiation may have both ERK-dependent and ERK-independent mechanisms. Second, activation of NF-κB has been shown to be sufficient to drive RANTES expression thereafter inducing ERK phosphorylation and activation in human hepatic stellate cell [40]. How is the possibility that ERK lies downstream of NF-κB in our system? Whether a feedback regulative mechanism occurs in this process. Ongoing studies in our laboratory are exploring this interesting possibility.

While the signaling mechanisms of NF-κB-mediated inhibition of osteoblast differentiation are unclear, both ERKs and NF-κB activation are essential for oxidative stress-induced inhibition of ALP activity, type I collagen expression, and nuclear Runx2 phosphorylation during BMSC and calvarial osteoblast differentiation based upon our results. The importance of Runx2 for osteoblasts has been highlighted by he evidence that knockout of the Runx2 gene in mice prevents osteoblast development [3]. Our data suggest that Runx2 phosphorylation may be the downstream target of ERKs and NF-κB activation in oxidative stress-induced inhibition of rabbit BMSC and calvarial osteoblasts differentiation.

It has been well documented that posttranslational modification and/or protein-protein interactions may regulate Runx2 and activation of Runx2 via phosphorylation by MAPK or protein kinase A (PKA) pathway may be crucial for this factor to be transcriptionally active, as reviewed recently by Franceschi and Xiao [41]. However, regulation of Runx2 activity is complicated by recent data which demonstrate that Runx2 is negatively regulated by the phosphorylation of two conserved serines (S104 and S451) [42]. Furthermore, other mechanisms such as Runx2 release and nuclear translocation may also be involved in the process. Although the results of nuclear Runx2 tyrosine phosphorylation are consistent with osteoblastic differentiation markers (ALP, type I collagen, and CFU-O formation) in our experiments, the role of this nuclear phosphorylation in overall Runx2 activation remains to be fixed.

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