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Effects of HA released calcium ion on osteoblast differentiation

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Abstract Hydroxyapatite (HA) is a widely used calcium phosphate implant substitute and has dissolution property. Although HA has been shown a beneficial effect on osteoblast differentiation, the exact mechanism is still unclear. In the present study, we proposed that Ca^{2+} released from HA activated the expression bone associated proteins, OPN and BSP, mediated by L-type calcium channel and calcium/calmodulin-dependent protein kinase (CaMK) 2 which resulted into improved osteoblast differentiation. Results showed that HA elevated ALP expression as well as OPN and BSP expression in MC3T3-E1 cells. The result from western blot of CaMK2α indicated that HA released Ca^{2+} activated CaMK2 through L-type calcium channel. Furthermore, upregulation of OPN and BSP mRNA expression was significantly inhibited when blocking both the L-type calcium channel and CaMK2. These findings suggested that HA accelerated the osteoblast differentiation by releasing Ca^{2+}.

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1 Introduction

Hydroxyapatite (HA) has been widely used for orthopedic and dental implantation because of its biocompatibility. Its properties include lack of toxicity, lack of inflammatory response and the ability to bind to living bone directly [1]. HA accelerates differentiation of osteoblast in vitro [2] and subcutaneous implantation of HA scaffolds seeded with osteoblast have increased bone formation in vivo [3, 4]. These results demonstrate that HA can provide beneficial environment for osteoblast attachment and differentiation.

One of the features related to the use of mineral containing materials is the release of ions. Several studies have shown the effect of ions under osteoblast cell culture systems [5–7]. In particular, the increase of extracellular Ca^{2+} plays a critical role in regulating osteoblast proliferation and differentiation [5, 8, 9]. Enhanced osteoblast differentiation occurred in the presence of additional Ca^{2+} concentration in the cell culture media while to slow down osteoblast differentiation and mineralization, additional phosphorous ion concentration has been suggested [5]. In addition, Ca^{2+} has been shown to promote the expression of bone-associated proteins of osteoblast seeded on Ti and collagen gel [6, 7]. In the case of HA, although it also has the property of dissolvability, it is not well known the effect of synthetic HA-released ions on osteoblast.

Calcium signaling plays a crucial role in many aspects of osteoblast proliferation and differentiation. The increase of extracellular calcium concentration can cause a dramatic increase of intracellular calcium concentration through calcium channels, which results in the activation of numerous targets including calcium/calmodulin (CaM) mediated calcium/calmodulin-dependent protein kinase (CaMK) [8]. Specifically, CaMK2 plays a critical role in osteoblast differentiation by controlling c-fos expression,

an element of AP-1 transcription factor [10]. Therefore, we suggest that this pathway affects osteoblast differentiation by producing bone associated constituents such as bone sialoprotein (BSP) and osteopontin (OPN).

In the present study, we examined the effects of Ca^{2+} from HA to osteoblast differentiation and bone associated proteins production through the 2-dimensional culture system. This study has shown that HA-released Ca^{2+} enhanced the MC3T3-E1 preosteoblast cell differentiation through the L-type calcium channel and CaM–CaMK. The pharmacological blockage of the Ca^{2+} mediated CaM–CaMK pathway resulted into the inhibition of bone associated proteins production. These findings demonstrated that HA mediated Ca^{2+} can play a critical role in osteoblast differentiation.

2 Materials and methods

2.1 Preparation of HA disc

Nano-sized HA was synthesized by a precipitation reaction between calcium hydroxide ($Ca(OH)_2$) and orthophosphoric acid (H_3PO_4) (Sigma, St. Louis, MO, USA) with a Ca/P ratio of 1.67. H_3PO_4 solution (1.2 M) was added drop-wise to $Ca(OH)_2$ (1.0 M) solution under continuous stirring at room temperature, while the pH was kept at 10 by the addition of NH_4OH. The precipitates obtained were further aged for a week. The morphology of HA precipitates was examined using transmission electron microscope (JEM-2000EX, Jeol, Japan) with an accelerating voltage of 200 keV. The crystal structure was studied using a X-ray diffractometer (D/MAX-2500/PC, Rigaku, Japan) using CuKα radiation. After hydrothermal treatments, nano-sized HA powder was compacted into disc-shaped pellet of 12 mm diameter by cold isostatic press at 30,000 psi. The pellets were first heated to 1275°C at heating rate of 10°C/min and then furnace cooled to temperatures 1175°C and held for 10 h.

2.2 Cell culture

Newborn mouse calvaria-derived MC3T3-E1 (subclone 4) preosteoblastic cells (ATCC, USA) were cultured in Minimum Essential Medium Alpha Medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (CAMBREX, Walkersville, MD, USA), 100 U/ml penicillin and streptomycin (Invitrogen) at 37°C in an atmosphere with 5% CO_2. Cells were plated at a density of 5×10^4 cells/cm^2 in cell culture insert (BD Biosciences, NJ, USA) and then were placed above HA in the cell culture insert companion plate. Medium was further supplemented with 50 μg/ml ascorbic acid and 5 mM β-glycerophosphate for differentiation with or without inhibitors. Media and inhibitors were replaced every 2 days for the duration of all experiments.

2.3 Measurement of Ca^{2+} concentration

Ca^{2+} in media was measured by using the Quantichrom Calcium Assay Kit (BioAssay Systems, Hayward, USA) according to manufacturer's direction. HA discs were immersed in MEM alpha medium, which constitutively contains approximately 1.8 mM of Ca^{2+}, for 2 days in CO_2 incubator. Each 5 μl medium was mixed with reagents and then read optical density at 612 nm after 3 min incubation.

2.4 ALP assay

Cell differentiation was assessed by determining alkaline phosphatase (ALP) expression using Quantichrom ALP kit (BioAssay systems). Cells seeded on inserts were washed with PBS and then were added 0.5 ml of 0.5% Triton X-100 for cell lysis. Equal amount of cell lysate and working solution were transferred to 96-well plate and then read at 405 nm wavelength with ELISA.

2.5 Real time PCR

Total RNA was isolated from MC3T3-E1 cells after culture for 10 days using RNAeasy mini kit (Qiagen, Valencia, CA, USA) with DNase treatment as directed by the manufacturer. After quantification, first-strand cDNA was synthesized using 0.5 μg of total RNA in a 20 μl reverse transcriptase reaction mixture (Takara, Otsu, Japan) containing RNase inhibitor. Mouse forward and reverse primers were: 5′-GAGCCTCGTGGCGACACTTA-3′ and 5′-AATTCT GACCCTCGTAGCCTTCATA-3′ for BSP, 5′-CCGGGAG CAGTGTGAGCTTA-3′ and 5′-TAGATGCGTTTGTAGG CGGTC-3′ for OPN and 5′-TCCACGACATACTCAG CAC-3′ and 5′-AACGACCCCTTCATTGAC-3′ for GAPDH. One-step quantitative RT–PCR was performed using the SYBR Green PCR mater mix (Applied Biosystems, Warrington, UK) according to recommended procedures.

2.6 Western blot

MC3T3-E1 cells were washed with chilled PBS and detached by trypsin–EDTA treatment. After centrifugation, cell pellet was resuspended in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 10% glycerol) containing proteinase inhibitors. Protein determinations were performed with the BCA protein assay reagent following the manufacturer's protocols. Protein samples were separated on SDS–PAGE and transferred to nitrocellulose membrane. The membranes were blocked in

5% non fat skim milk in TBS-T (0.1% Tween-20) for 1 h at room temperature followed by incubation with phospho-CaMK2α (Santa Cruz Biotechnology, CA, USA) and β-actin (Sigma, MO, USA) antibodies at 4°C overnight. After washing, the membranes were incubated with HRP conjugated secondary antibodies (Jackson Laboratories, PA, USA) for 1 h. Immunoreactive bands were visualized by chemiluminescence using supersignal bloting kit (Thermo Fisher Scientific, IL, USA).

2.7 Statistical analysis

Results were expressed as mean ± SD. Data were evaluated for statistical significance using Student's paired *t* test. Experiments were repeated at least three times. In all analyses, a P value of <0.05 was considered statistically significant.

3 Results

Rod-like HA particles were approximately 80 nm in length, revealed during TEM examination (Fig. 1a). The X-ray diffraction analysis of nano-sized HA powder showed no secondary phases (Fig. 1b). As shown in Fig. 1c, the Ca^{2+} release into media was observed in the presence of HA disc. After 2 days, concentration of Ca^{2+} in media in the presence of HA was increased around 1.9 times (3.4 ± 0.118 mM) when compared with that in media under the absence of HA. During this period, there is no significant pH change from the initial pH of 7.4.

To examine the influence of dissolved ions from HA on osteoblast differentiation, MC3T3-E1 cells were cultured on insert with or without HA disc. The differentiation characteristics of the MC3T3-E1 cells were evaluated by the measurement of ALP level as shown in Fig. 2. After 3 weeks, quantification of ALP activity in cells cultured with HA demonstrated approximately 2.4 fold increase when compared with that of cells on plastic culture plate and on insert without HA.

The small groups of extracellular matrix proteins such as OPN and BSP have known to be linked with osteoblast differentiation and mineralization [11–13]. Therefore, we assessed the expression of OPN and BSP level with real time PCR after 10 days of cell culture. The OPN and BSP expression of mRNA level in MC3T3-E1 cells were enhanced significantly as a result from HA (Fig. 3). Quantification of OPN and BSP expression demonstrated an increase of 2.4 fold ± 0.34 and 1.9 fold ± 0.24, respectively, when cultured cells on insert with HA.

Later, we tried to determine the effect of Ca^{2+} released from HA. Increase of extracellular Ca^{2+} have been know to raise intracellular Ca^{2+} through L-type calcium channels

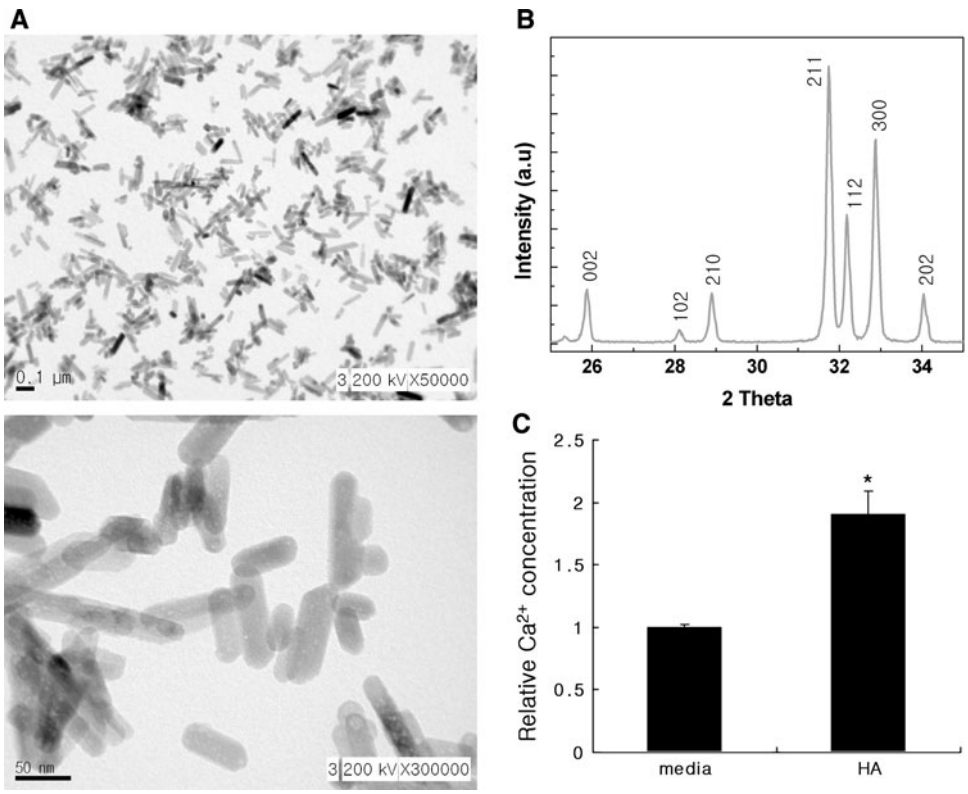

Fig. 1 Transmission electron micrograph (**a**) and X-ray diffraction pattern (**b**) of HA particles. Ca^{2+} released from HA after 2 days in the presence and absence of HA (**c**, * $P < 0.01$)

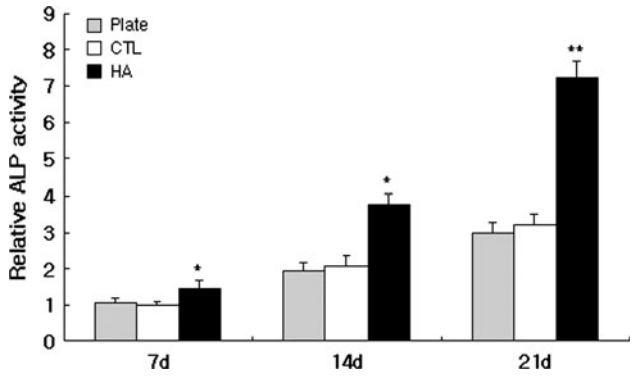

Fig. 2 HA increases ALP activity in MC3T3-E1 cells. At defined time points, ALP activity measured in cells on tissue culture plate (Plate), insert without HA (CTL) and insert with HA (HA). Results were quantified from three individual experiments and expressed as a fold increase relative to cells of CTL at 7 days (* $P < 0.05$; ** $P < 0.01$)

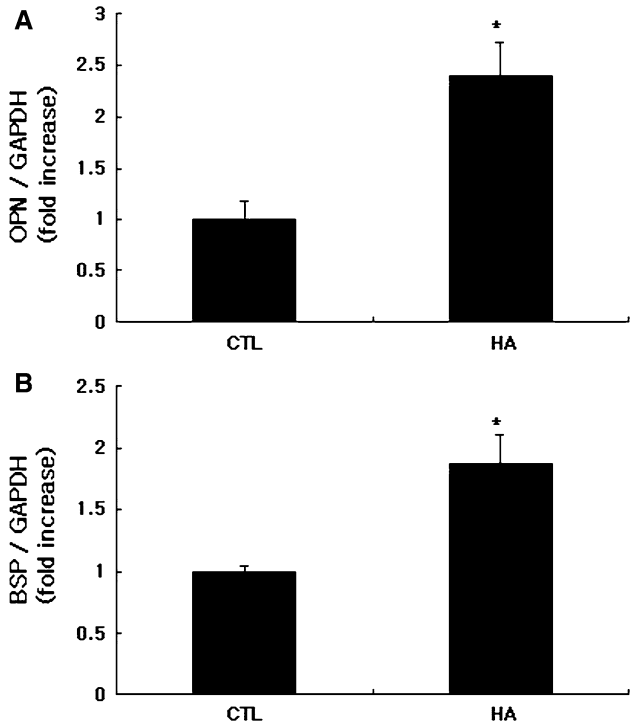

Fig. 3 HA enhances expression of OPN and BSP expression. The MC3T3-E1 cells were harvested 10 days after seeding for RNA extraction. Real-time RT–PCR analyses show the levels of OPN (**a**) and BSP (**b**) expression relative to GAPDH levels. Values from three separate experiments were averaged and expressed as a fold increase over control levels (* $P < 0.05$)

and then lead to activate CaM which in turn activate CaMKs, especially CaMK2α for osteoblast differentiation. [8, 14]. To elucidate the mechanisms by which HA released Ca^{2+} might accelerate the osteoblast differentiation, we examined the activation of CaMK2α and the effect of selective L-type calcium channel. MC3T3-E1 cells were

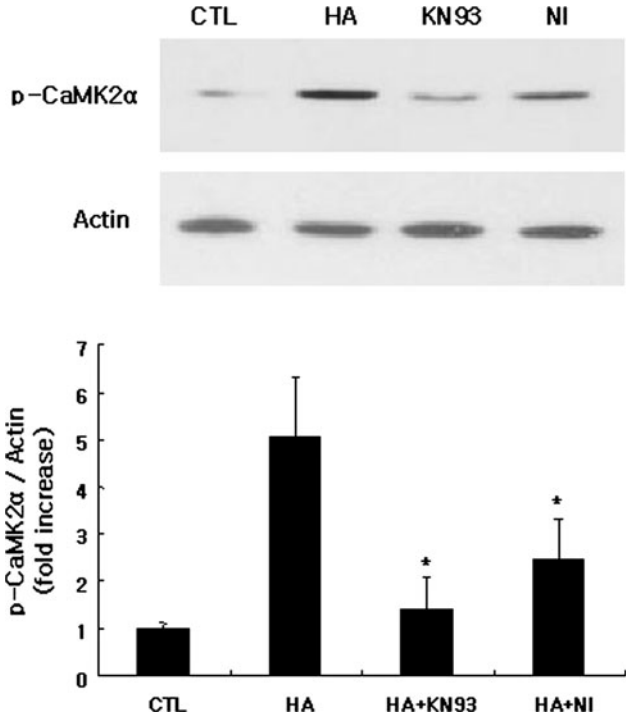

Fig. 4 Effect of calcium channel blocker on HA-induced CaMK2α activation. Cells were incubated with nifedipine (1 μM) or KN93 (10 μM) in the presence of HA. The protein extracts were separated by SDS–PAGE and developed using phosphorylated CaMK2α (Thr 286). β-actin was used as a internal control. Western blot was performed three times independently and optical densities were measured. Values are expressed relative to control and are the means ± SD of three separate experiments (* $P < 0.05$)

cultured with nifedipine (L-type calcium channel inhibitor, 1 μM) or KN93 (CaMK2 antagonist, 10 μM) for 7 days. Protein expression of the p-CaMK2α was enhanced in osteoblast cultured with HA and nifedipine and KN93 almost inhibited the HA-induced phosphorylation of CaMK2α as shown by western blot (Fig. 4).

The role of calcium channel and CaMK2 during OPN and BSP expression was examined using the L-type calcium channel, CaM and CaMK2 inhibitors, nifedipine (1 μM), TFP (10 μM) and KN93 (10 μM), respectively. Total RNA was extracted from MC3T3-E1 cells after culture with or without inhibitors for 10 days. As shown in Fig. 5, the pharmacological inhibition of L-type calcium channel, CaM and CaMK2 resulted in an approximately 50% decrease of mRNA expression of OPN and BSP on control cells. Ca^{2+} released from HA elicited the increase of OPN and BSP expression around 2.5 and 2 times, respectively. However, inhibitors blocked the HA induced up-regulation of OPN and BSP significantly. These data demonstrated that L-type calcium channel and CaM–CaMK2 could play a critical role in osteoblast differentiation, especially when up-regulated by Ca^{2+} from HA.

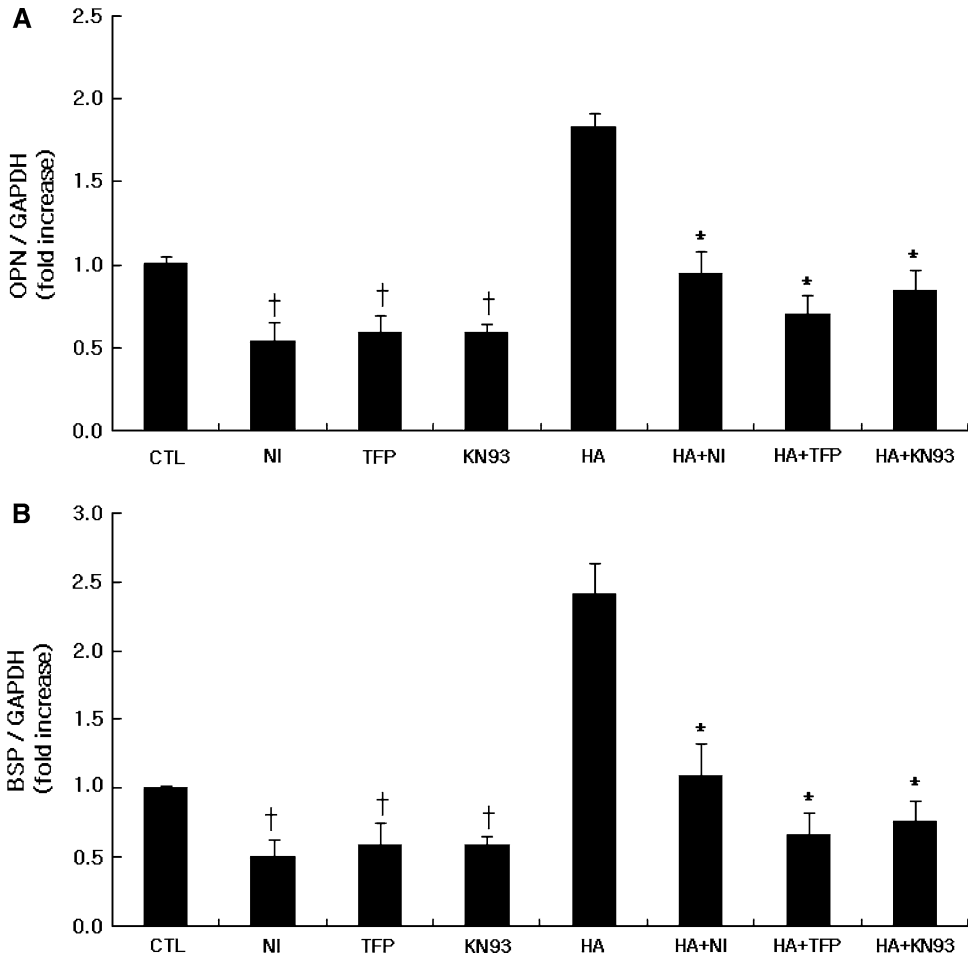

Fig. 5 Effect of L-type calcium channel and CaM–CaMK2 blockers on OPN and BSP expression during osteoblast differentiation. The MC3T3-E1 cells were cultured with nifedipine (1 μM), TFP (10 μM) and KN93 (10 μM) and then harvested 10 days after seeding for RNA extraction. Real-time RT–PCR analyses showed the levels of OPN (**a**) and BSP (**b**) expression relative to GAPDH levels. Data from three separate experiments were averaged and then are expressed as a fold increase over control levels († $P < 0.05$ vs. CTL; * $P < 0.05$ vs. HA)

4 Discussion

HA is one of widely used substitutes for bone implant and has dissolution behavior. Although a number of investigations have shown that calcium phosphates including HA have a beneficial effect on osteoblast proliferation and/or differentiation, the exact mechanism is not well understood [2, 3, 15]. Understanding the changes of osteoblast mechanism mediated by HA will provide a basis for improvement of HA for control of osteoblast. Unlike other researches, we seeded preosteoblast cells, MC3T3-E1, on cell culture inserts for focusing on HA released ion, especially Ca^{2+}. This could exclude the effect of surface topology and absorbed serum proteins to HA. Our results demonstrated that osteoblast differentiation was enhanced by Ca^{2+} released from HA through L-type calcium channel and CaMK2 which regulated the production of OPN and BSP.

Ca^{2+} is a ubiquitous intracellular messenger responsible for controlling various cellular processes including mitosis, cell death, movement and differentiation. Many studies examined the mechanisms of Ca^{2+} in osteoblast function. The elevation of extracellular Ca^{2+} can raise intracellular Ca^{2+} through L-type and non-L-type calcium channels and calcium sensing receptors [8, 16, 17]. The increased level of extracellular Ca^{2+} induced both chemotaxis and proliferation of primary osteoblasts and M3T3-E1 cells which induced by calcium sensing receptors [9, 17, 18]. Various effects of upregulation of intracellular Ca^{2+} can be mediated by the CaM-dependent protein kinases which include CaMK 1, 2 and 4. Although CaMKs have been most extensively studied in neuronal tissue, they are also expressed in other tissues and are believed to affect a variety of processes [14]. CaMK1, especially ß isoform, has been known to regulate proliferation of calvarial osteoblast [19]. On the other hand, CaMK2α is involved in osteoblast differentiation by controlling c-fos expression [10]. In this experiment, Ca^{2+} existed 2 mM in normal media. HA increased Ca^{2+} concentration to around 3.4 mM which promotes activation of CaMK2α. In addition, our results showed that elevated Ca^{2+} in media released from HA improved the ALP release as well as OPN and BSP production in MC3T3-E1 cells. TFP, KN 93 and nifedipine almost blocked the up-regulation of OPN and BSP production mediated by HA, although inhibitors deteriorated the differentiation ability of control osteoblast after exposure to ascorbic acid and ß-glycerophosphate.

Therefore, we suggest that HA ameliorate osteoblast differentiation by releasing Ca^{2+} which activates the CaM–CaMK2 pathway. Moreover, HA-released Ca^{2+} may turn on CaMK2α activity mainly through L-type calcium channels because CaMK2α activity was regulated by nifedipine in our experiment.

Upregulation of bone-related proteins, such as the non-collagenous SIBLING proteins, is a requirement of maintaining the osteoblast phenotype. Alteration of these proteins can have a significant effect on osteoblast function and consequently matrix mineralization [11]. BSP and OPN have been known to contain an arginine–glycine–aspartic acid motif that mediates cell binding through direct interactions with a number of transmembrane integrin pairs [20]. Therefore, both BSP and OPN have been implicated as key proteins for mediating cellular adhesion at bone implant interfaces and collagen matrix of developing bone which result in cellular differentiation and mineralization [11, 21, 22]. Previous experiment showed that these proteins expression was enhanced in MG63 cells on titanium surfaces implanted with Ca^{2+} compared with cells on control titanium [6]. Similarly, our results demonstrated that BSP and OPN expression was increased in the presence of HA and was regulated in transcription level through L-type calcium channel and CaM–CaMK2. In support of our results, CaMK2α has been involved in the expression of c-fos, AP-1 transactivation, and AP-1 DNA binding activity [10]. Moreover, the AP-1 element has shown to play a role in BSP and OPN transcription [23, 24].

In summary we try to elucidate how HA gave beneficial effects for the process of osteoblast differentiation. Our data showed that HA-released Ca^{2+} improved the MC3T3-E1 cell differentiation by increasing BSP and OPN expression through L-type calcium channel which triggered CaM–CaMK2 pathway. These results suggest that property of dissolvability of HA induced more suitable environment in differentiating osteoblast.

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