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Tunicamycin negatively regulates BMP2-induced osteoblast differentiation through CREBH expression in MC3T3E1 cells

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Tunicamycin, an endoplasmic reticulum (ER) stress inducer, specifically inhibits *N*-glycosylation. The cyclic AMP (cAMP) response element-binding protein H (CREBH) was previously shown to be regulated by UPR-dependent proteolytic cleavage in the liver. On the other hand, the role of CREBH in other tissues is unknown. In the present study, tunicamycin increased the level of CREBH activation (cleavage) as well as mRNA expression in osteoblast cells. Adenoviral (Ad) overexpression of CREBH suppressed BMP2-induced expression of alkaline phosphatase (ALP) and osteocalcin (OC). Interestingly, the BMP2-induced OASIS (structurally similar to CREBH, a positive regulator of osteoblast differentiation) expression was also inhibited by CREBH overexpression. In addition, inhibition of CREBH expression using siRNA reversed the tunicamycin-suppressed ALP and OC expression. These results suggest that CREBH inhibited osteoblast differentiation via suppressing BMP2-induced ALP, OC and OASIS expression in mouse calvarial derived osteoblasts. [BMB reports 2011; 44(11): 735-740]

INTRODUCTION

The endoplasmic reticulum (ER) is a central cellular organelle responsible for the synthesis, folding and post-translational modifications of proteins destined for the secretory pathway. ER stress is caused by an imbalance between the cellular demand for protein synthesis and the capacity of the ER to promote protein maturation and transport can lead to the accumulation of unfolded protein in the ER lumen (1, 2). To avoid these imbalances, eukaryotic cells clear unfolded proteins from the lumen of the ER, through a process known as the unfolded protein response (UPR) (3-5). The glucosamine-containing nucleoside antibiotic, tunicamycin, is an inhibitor of *N*-linked glycosylation and the formation of *N*-glycosidic protein-carbohydrate linkages (6). It specifically inhibits the dolichol pyrophosphate-mediated glycosylation of asparaginyl residues of glycoproteins and induced endoplasmic reticulum (ER) stress (7).

Regulated intra-membrane proteolysis (RIP) is the process by which trans-membrane proteins are cleaved to release cytosolic domains that enter the nucleus to regulate gene transcription (8). Cyclic AMP response element-binding protein H (CREBH) was identified as a RIP-regulated liver-enriched bZIP transcription factor belonging to the cyclic AMP response element binding protein transcription factor (CREB/ATF) family (9). CREBH is regulated by the UPR-dependent proteolytic cleavage and regulates the transcriptional process of genes, such as serum amyloid P-component (SAP) and C-reactive protein (CRP) in response to systemic inflammatory signaling (9-11). However, the role of CREBH in the osteoblast differentiation is still unknown.

Osteoblast differentiation is tightly regulated by various hormones, cytokines and multiple transcription factors (12, 13). Bone morphogenetic proteins (BMPs), which are members of the transforming growth factor β (TGF-β) family, are multifunctional proteins that play important roles in a variety of cellular functions ranging from embryogenesis, cell growth and differentiation to bone development and repair of bone fractures (14, 15). Moreover, they induce the osteogenic trans-differentiation of fibrogenic, myogenic and adipogenic cells both *in vitro* and *in vivo* (16, 17). Several lines of evidence suggest that BMP2 treatment increases osteogenic markers including alkaline phosphatase (ALP) and osteocalcin (OC) expression (18-22).

This study was conducted to examine whether increased expression of CREBH by tunicamycin affects BMP2-induced osteoblast differentiation by regulating osteogenic gene expressions such as ALP and OC in osteoblasts.

RESULTS

Tunicamycin, an ER stress inducer, suppressed BMP2-induced osteogenic gene expressions in MC3T3E1 cells

Tunicamycin is a strong ER stress inducer. The cell viability of MC3T3E1 cells were not affected significantly by tunicamycin at levels up to 2 μg/ml, whereas 5 and 20 μg/ml were toxic to MC3T3E1 cells at 24 h (Fig. 1A), and ER stress related genes, such as Bip and CHOP, were up-regulated by 2 μg/ml of tunicamycin (Fig. 1B). Under this condition, tunicamycin inhibited the BMP2-induced mRNA expression of osteogenic genes such

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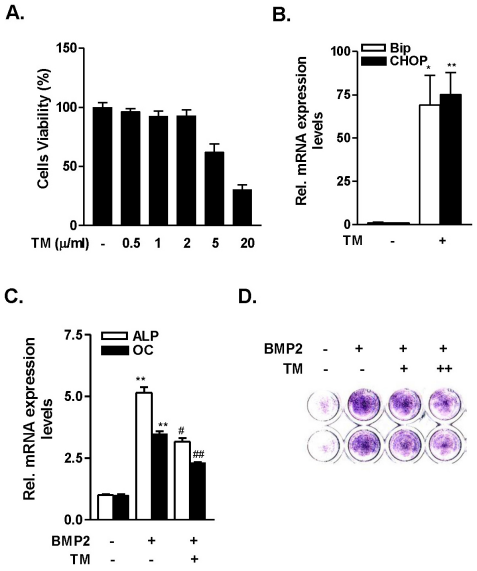

Fig. 1. Tunicamycin inhibited BMP2-induced ALP and OC expression. (A) Effect of tunicamycin on the cell viability of osteoblast cells. The viability of the MC3T3E1 cells after 24 h treatment with various concentrations of tunicamycin were analyzed using an EZ-Cytox Cell Viability Assay Kit, as described in the Materials and Methods section (TM; tunicamycin). (B, C) Expression levels of the ER stress markers and osteogenic markers by tunicamycin. MC3T3E1 cells were treated with 2 μg/ml of tunicamycin for 24 h (B) and tunicamycin (2 μg/ml) for 24 h after 200 ng/ml of BMP2 treatment for 48 h (C). The cells were harvested for total RNA isolation, and real-time PCR were carried out using the specific primers. *P < 0.05, and **P < 0.01 compared to the untreated control, respectively. #P < 0.05, and ##P < 0.01 compared to the BMP2 only treated group, respectively. (D) Alkaline phosphatase (ALP) staining. Cells were treated with tunicamycin (+ = 0.5 μg/ml, ++ = 2 μg/ml) for 24 h after being treated with or without BMP2 (200 ng/ml) for 4 day. The cells were stained with a BCIP®/NBT solution for ALP staining.

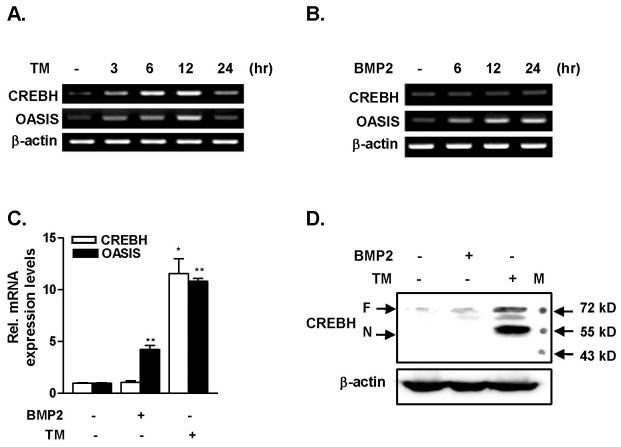

Fig. 2. Tunicamycin but not BMP2 increased CREBH expression. A-C, Changes in CREBH and OASIS expression by tunicamycin or BMP2 treatment. MC3T3E1 cells were treated with tunicamycin (2 μg/ml) (A) or BMP2 (200 ng/ml) (B) for the indicated times, and primary calvarial cells were treated with tunicamycin (2 μg/ml) or BMP2 (200 ng/ml) for 12 h (C). The cells were harvested for total RNA isolation. RT-PCR (A, B) and real-time PCR (C) were carried out using CREBH, OASIS and β-actin primers. *P < 0.05, **P < 0.01 compared to the untreated control, respectively. (D) Western blot analysis of CREBH expression. Cells were treated with BMP2 (200 ng/ml) or tunicamycin (2 μg/ml) for 24 h, and total proteins were extracted for Western blot analysis using the indicated antibodies (F, full length; N, nuclear form; M, size marker).

as ALP and OC (Fig. 1C). BMP2-induced ALP staining levels were also suppressed by tunicamycin (Fig. 1D). Overall, these results suggest that tunicamycin inhibits BMP2-induced osteoblast differentiation by suppressing ALP and OC expression.

Tunicamycin increased CREBH expression and cleavage

As reported previously, tunicamycin increased CREBH expression with induction of ER stress in liver cells (11). BMP2 also induced mild ER stress and osteoblast differentiation by increasing the expression of old astrocyte specifically induced substance (OASIS), which is structurally similar to CREBH (23). This study examined whether CREBH and OASIS expressions were regulated by tunicamycin in MC3T3E1 cells. Tunicamycin significantly increased CREBH and OASIS expression up to 12 h (Fig. 2A). RT-PCR was performed to determine if CREBH expression was also regulated by BMP2 treatment. As shown in Fig. 2B, CREBH expression was not changed, whereas OASIS expression was increased by BMP2. To further confirm the expression of CREBH, primary calvarial cells were treated with BMP2 or tunicamycin. Tunicamycin increased CREBH and OASIS expression in primary calvarial cells. However, BMP2 did not induce CREBH expression but increased OASIS expression (Fig. 2C). Western blot analysis also showed that tunicamycin increased the level of CREBH protein expression and the cleaved form (nuclear or active form) of CREBH was also increased by tunicamycin, whereas the level of CREBH expression was not changed by BMP2 (Fig. 2D). These results suggest that tunicamycin increased CREBH and OASIS expression. In contrast, CREBH was not regulated by BMP2, even though it is structurally similar to OASIS.

CREBH suppressed BMP2-stimulated ALP, OC and OASIS expression

To determine if ALP and OC expressions are regulated by CREBH, the nuclear form of adenoviral CREBH (Ad-CREBH) or Ad-siCREBH was used to induce overexpression or inhibit CREBH expression, respectively. Results of real-time PCR showed that the overexpression of CREBH by Ad-CREBH decreased BMP2-induced ALP and OC expression in a dose-dependent manner. Interestingly, BMP2-induced OASIS expression was also suppressed by Ad-CREBH (Fig. 3A). BMP2-induced ALP staining and OC production levels were decreased by Ad-CREBH in a dose dependent manner (Fig. 3B and C). To further determine the effects of CREBH on osteogenic gene expression, CREBH expression was suppressed using siRNA

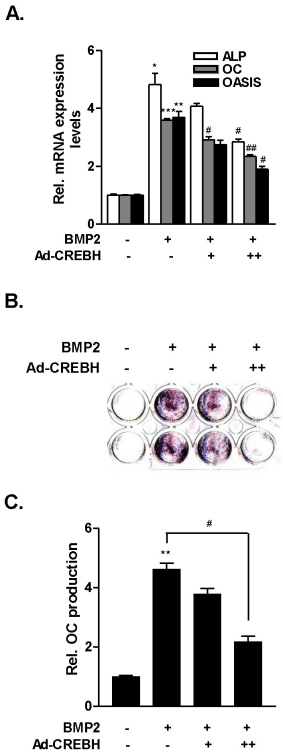

Fig. 3. CREBH inhibited BMP2-induced ALP and OC expressions. (A) Expression of ALP, OC and OASIS by CREBH overexpression. Cells were infected with the nuclear (active) form of Ad-CREBH (+ = 20 MOI, ++ = 100 MOI) for 24 h after being treated with or without BMP2 (200 ng/ml) for 4 days. Real-time PCR were carried out using ALP, OC, OASIS and β-actin primers after total RNA isolation. *P < 0.05, **P < 0.01, and ***P < 0.01 compared to the untreated control, respectively. $^{\#}$P < 0.05, and $^{\#\#}$P < 0.01 compared to the BMP2 only treated group, respectively. (B, C) ALP staining and OC production. Cells were treated with Ad-CREBH (+ = 20 MOI, ++ = 100 MOI) for 24 h after treatment with or without BMP2 (200 ng/ml) for 4 day. The cells were stained with a BCIP®/NBT solution for ALP staining (B) and OC in the culture medium was measured using an OC specific ELISA kit (C). **P < 0.01 compared to the untreated control and $^{\#}$P < 0.05 compared to the indicated group.

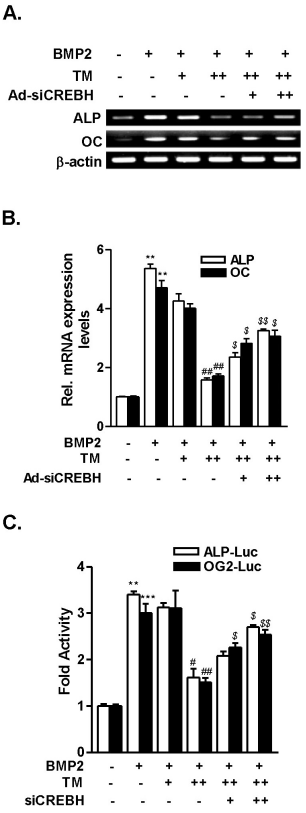

Fig. 4. Inhibition of CREBH reversed tunicamycin-suppressed ALP and OC expressions. (A, B) Changes in ALP and OC expression. Cells were treated with tunicamycin (+ = 0.5 μg/ml, ++ = 2 μg/ml) for 24 h in the presence or absence of Ad-siCREBH (+ = 20 MOI, ++ = 100 MOI) after treatment with or without BMP2 (200 ng/ml) for 4 days. RT-PCR (A) and Real-time PCR (B) were carried out using ALP, OC, OASIS and β-actin primers after total RNA isolation. **P < 0.01 compared to the untreated control, and $^{\#\#}$P < 0.01 compared to BMP2 only treated group, respectively. $^{\$}$P < 0.05 and $^{\$\$}$P < 0.01 compared to BMP2 with TM (++), not siCREBH, treated group, respectively. (C) Luciferase activities of ALP-Luc and OG2-Luc. The MC3T3E1 cells were transfected with 200 ng of ALP-Luc or OG2-Luc reporter plasmid and 100 ng of pCMV-β-galactosidase as an internal control with or without siCREBH (+ = 100 ng/well, ++ = 200 ng/well). Twelve hours after transfection, the cells were treated with or without BMP2 (200 ng/ml) for 24 h, and treated with or without TM (+ = 0.5 μg/ml, ++ = 2 μg/ml) for an additional 24 h. The luciferase assay was performed, and the results are expressed as the fold activity relative to the control. **P < 0.01, and ***P < 0.001 compared to the untreated control. $^{\#}$P < 0.05 and $^{\#\#}$P < 0.01 compared to BMP2 only treated group, respectively. $^{\$}$P < 0.05 and $^{\$\$}$P < 0.01 compared to BMP2 with TM (++), not siCREBH, treated group, respectively.

constructs. The inhibition of CREBH expression reversed the suppressive effects of tunicamycin on BMP2-induced ALP and OC expression (Fig. 4A and B). Finally, to further confirm the transcriptional regulation of ALP and OC by CREBH, a transient transfection study was performed using ALP promoter (ALP-Luc) and OC promoter (OG2-Luc) constructs. The BMP2-induced ALP-Luc and OG2-Luc activity were suppressed by tunicamycin. However, these suppressed activities were released by siCREBH in a dose-dependent manner (Fig. 4C). Over all, these results suggest that the BMP2-induced ALP and OC expression were blocked by CREBH in MC3T3E1 cells. Moreover, BMP2-induced expression of the osteogenic positive regulator OASIS was also decreased by CREBH.

DISCUSSION

In this study, the role of CREBH in osteoblast differentiation was demonstrated for the first time. CREBH inhibited BMP2-stimulated osteogenic gene expression, such as ALP and OC. Moreover, CREBH suppressed BMP2-induced OASIS expre-

ssion even though it was structurally similar to CREBH.

Under the inflammation state, ER stress induced cleavage of CREBH to release an *N*-terminal fragment (active or nucleus form) that moved to the nucleus and regulated transcription of target gene in the liver (11). From a bone regeneration standpoint, inflammation is a major contributor to bone loss. Rheumatoid arthritis (RA) is a typical example of such a disease that is associated with osteoporosis (24, 25). Indeed, even tiny increases in inflammatory activity in humans can increase the risk of fracture (26). In the present study, tunicamycin (massive ER stress inducer) induced CREBH expression and cleavage, and suppressed osteogenic gene expression such as ALP and OC. These finding suggest that ER stress negatively regulates osteoblast differentiation through CREBH expression.

Recently, the role of UPR transducers, such as PERK and IRE1, during osteoblast differentiation was reported. BMP2 was shown to activate PERK and IRE1, and these UPR transducers regulated osteogenic gene expression via ATF4 or Xbp1 (21, 27). Murakami et al evaluated the effects of another UPR transducer, OASIS, and found that OASIS (-/-) mice exhibited severe osteopenia, which involved a decrease in type I collagen (Col1a1) in the bone matrix, and they confirmed that the BMP2-activated OASIS regulated Col1a1 transcription via direct binding to unfolded protein response element (UPRE)-like sequence (23). Moreover, they suggested that the BMP2 can induce mild ER stress because BMP2 increased the ER stress marker genes, such as Bip and CHOP. In the present study, tunicamycin was shown to regulate CREBH and OASIS expression with increased ER stress marker genes. On the other hand, CREBH was not regulated by BMP2, even though it was structurally similar to OASIS. Moreover, overexpressed CREBH suppressed BMP2-induced OASIS expression. These results suggest that BMP2-induced ER stress was weaker than tunicamycin-induced ER stress. Based on these finding, we believe that the BMP2-induced ER stress may be a physiological condition that stimulates the osteogenic process. Nevertheless, tunicamycin-induced ER stress is non-physiological (pathological) condition, such as inflammation, which suppresses the osteogenic process in osteoblast cells. Further studies will be needed to determine the precise mechanism for the difference between BMP2-induced ER stress and tunicamycin- or TNFα-induced ER stress in regards to the regulation of CREBH and OASIS in osteoblasts.

In summary, BMP2-induced mild ER stress enhanced osteoblast differentiation via OASIS. However, tunicamycin-induced ER stress suppressed osteoblast differentiation through the CREBH-dependent regulation of OASIS and osteogenic gene expression, such as ALP and OC.

MATERIALS AND METHODS

Reagents

Recombinant human BMP2 peptide was obtained from R&D (Minneapolis, MN). CREBH antibody was provided by Dr. H.S. Choi (Chonnam National University). Tunicamycin was obtained from MP Biomedicals (Ilkirch, France).

Plasmids and adenoviruses

The luciferase reporters driven by the osteocalcin promoter (OG2-Luc) and alkaline phosphatase promoter (ALP-Luc) were previously described (28). Expression constructs of CREBH, siCREBH, adenovirus encoding for CREBH, and Ad-siCREBH were provided by Dr. H.S. Choi (Chonnam National University).

Cell culture, transient transfection assays and viral infection

MC3T3E1 (mouse calvarial osteoblast) cells were cultured in α-minimal essential medium (α-MEM; GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; GIBCO-BRL) and antibiotics in a humidified atmosphere containing 5% CO_2 at 37°C. Transient transfections were performed as previously described (29). For viral infection, the cells were treated with the indicated viruses at the designated multiplicity of infection (MOI) under serum-free conditions. After 4 h, an equivalent volume of medium containing 10% FBS was added, and the cells were incubated for an additional 24-48 h.

Cell viability assay

The cell viability was determined using an EZ-Cytox Cell Viability Assay Kit (Dojindo, Tokyo, Japan) according to the manufacturer's instructions. Briefly, cells were seeded into a 96 well plate, and an Ex-Cytox solution was added to each well of the plate. After incubation for 3 h in a CO_2 incubator at 37°C, the absorbance of each well was measured at 420 nm using a microplate reader (Bio-Tek Instruments, Winooski, Vt, USA) with a reference wavelength of 650 nm.

PCR analysis

The total RNA was isolated from the cultures using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR was performed using 0.8 μg of total RNA. Each reaction consisted of initial denaturation at 94°C for 1 min followed by three-step cycling: denaturation at 94°C for 30 sec, annealing at a temperature optimized for each primer pair for 30 sec, and extension at 72°C for 30 sec. After the required number of cycles (25-30 cycles), the reactions underwent a final extension at 72°C for 5 min. The PCR primer sequences were as follows: CREBH, (F) 5′-CTGGGGTAGAACAGGAACCA-3′ and (R) 5′-GCCAGCCTGGTCTACAAAGAG-3′; OASIS, (F) 5′-CCTTGTGCCTGTCAAGATGGAG-3′ (R) 5′-GCAGCAGCCATGGCAGAGGAG-3′; ALP, (F) 5′-GATCATTCCCACGTTTTCAC-3′ and (R) 5′-TGCGGGCTTGTGGGACCTGC-3′; OC, (F) 5′-CTCCTGAGAGTCTGACAAAGCCTT-3′ and (R) 5′-GCTGTGACATCCATTACTTGC-3′; β-actin, (F) 5′-TTCTTTGCAGCTCCTTCGTTGCCG-3′ and (R) 5′-TGGATGGCTACGTACATGGCTGGG-3′. For real-time PCR analysis, 1 μg of the total RNA was reverse-transcribed to cDNA using oligo $(dT)_{15}$ primer and Maxime RT PreMix Kit (iNtRoN Biotechnology, Daejeon, South Korea). The SensiMix*Plus* SYBR (Quantace, London, UK) was utilized for real-time PCR on the Roter-Gene™ 6000 (Corbett Life Science,

Mortlake, Sydney, Australia) using gene- specific primers under following conditions. 95°C for 10 min, followed by 45 cycles at 95°C for 15 sec and at 60°C for 20 sec. b-actin was used as a control, and all reactions were performed in triplicate. Quantification of relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. The PCR primer sequences were as follows: Bip, (F) 5′-GAGCCGTCTGATTGGCGATGC-3′ and (R) 5′-TTCCAAGTG CGTCCCGATGAGG-3′; CHOP, (F) 5′-GAGCTGGAAGCCTGG TATGAG-3′ and (R) 5′-T GTGCGTGTGACCTCTGTTGG′; ALP, (F) 5′-ATCTTTGGTCTGGCTCCCATG-3′ and (R) 5′-TTTCCCGTT CACCGTCCAC-3′; OC, (F) 5′-GCAATAAGGTAGTGAACAGAC TCC-3′ and (R) 5′-GTTTGTAGGCGGTCTTCAAGC′; β-actin, (F) 5′-ACCCACACTGTGCCCATCTAC-3′ and (R) 5′-GCCATCTCCT GCTCGAAGTC-3′. The CREBH and OASIS primer sequences used for real-time PCR were same as those used for RT-PCR primers.

Alkaline phosphatase staining and osteocalcin production assay

For ALP staining, the cultured cells were fixed with 70% ethanol, rinsed three times with deionized water, and treated with a BCIP®/NBT solution (Sigma Aldrich, St. Louis, MO) for 15 min. The level of OC secreted into the culture medium was determined using a mouse osteocalcin ELISA kit (Biomedical Technologies Inc., Stoughton, MA) according to the manufacturer's instructions.

Statistical analysis

All experiments were repeated at least three times and statistical analysis was performed using a Student's *t*-test or analysis of variance analyses followed by a Duncan's multiple comparison tests. P values < 0.05 were considered significant. The results are expressed as the mean $\pm$ SEM of triplicate independently.

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