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Concentrated Bovine Milk Whey Active Proteins Facilitate Osteogenesis through Activation of the JNK-ATF4 Pathway

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Concentrated fractions of low molecular weight whey proteins (1–30 kDa), that is concentrated bovine milk whey active proteins (CBP), have been found to enhance bone formation in both *in vivo* and clinical studies, but the underlying mechanisms are poorly understood. In this study, we found that CBP promoted osteoblastic differentiation in normal human osteoblasts, and determined the involvement of the c-jun NH_2-terminal kinase (JNK)-activating transcription factor 4 (ATF4) pathway. We observed that alkaline phosphatase activity and mineralization were significantly induced by CBP treatment. In addition, mRNA expression of ATF4 was intensely elevated in CBP-treated osteoblasts, indicating that the late-phase events of differentiation were promoted. We found that CBP activated the phosphorylation of JNK and extracellular signal-regulated kinase (ERK). Furthermore, pathway analyses using the various signaling pathway-specific inhibitors revealed that JNK activation, but not ERK activation, is essential for CBP-induced mineralization and ATF4 expression. Our results indicate that the JNK-mediated ATF4 pathway is required for CBP-promotive osteogenesis.

Key words: whey; osteoblast; JNK; ERK; ATF4

Balance between bone resorption (by osteoclasts) and formation (by osteoblasts) maintains bone homeostasis through a process called bone remodeling. Increases in the rate of bone resorption, which can be caused by increases in bone resorption and/or a dampening of bone formation, can lead to bone disruption and can cause major bone diseases such as osteoporosis.[1)] Appropriate treatment or prevention of osteoporosis is achieved by maximizing peak bone mass in early adulthood and preventing excess bone loss, particularly after menopause.[2)] Thus, along with well-balanced nutrient and energy intake, consumption of foods that promote bone metabolism can confer additional bone health benefits.[3–8)]

Osteoblasts originate in a process that includes multipotent mesenchymal stem cells that undergo proliferation and sequential differentiation from progenitors into proliferating preosteoblasts, bone matrix-producing osteoblasts, and finally osteocytes (bone-lining cells).[9)] The development and maturation of osteoblasts requires several inducing factors, including bone morphogenetic proteins (BMPs) and insulin-like growth factor-I (IGF-I).[10)] These factors initiate mitogen-activated protein kinases (MAPKs) and/or Smad signaling in a specific manner and potentiate the activation and expression of several transcription factors, including runt-related transcription factor 2 (RUNX2), Osterix (OSX), and activating transcription factor 4 (ATF4). These factors play crucial roles in bone development by regulating osteoblastic differentiation in a phase-specific manner.[11,12)] Among these transcription factors, ATF4 has been found to induce osteocalcin (OCN) in late-stage osteoblast differentiation events, whereas RUNX2 and OSX function in the earlier stages of differentiation.[11,12)] Several recent reports indicate that activation of the c-Jun NH_2-terminal kinase (JNK) pathway is required for late-stage osteoblast differentiation events, such as elevation of ATF4 and OCN expression in mouse osteoblast-like cells (MC3T3-E1 cells) and primary osteoblasts.[11,12)]

Bovine milk is widely consumed, and is considered to be a rich source of calcium. Several studies have found that milk whey proteins potentiate bone remodeling.[13–16)] Basic fractions derived from milk whey proteins have been found to promote bone metabolism in both *in vivo* and *in vitro* studies.[15,16)] Its confirmed primary mechanism of action is through inhibition of bone resorption. Recently, concentrated bovine milk whey active proteins (CBP, or growth protein-colostrum), a promoter of bone formation, was identified as an active component of a low molecular weight fraction (1–30 kDa) derived from milk whey proteins.[17,18)] Lee *et al.* found that rats on a CBP diet had a significantly higher bone density than rats fed a standard chow diet.[17)] The femurs of the rats fed the CBP diet were longer and heavier than those of the controls. CBP is thought to potentiate bone strength and growth, but the underlying mechanisms remain unclear. In this study, we found that the involvement of the JNK-ATF4 pathway in the ability of CBP to promote osteogenesis in human primary osteoblasts.

Materials and Methods

Reagents. BMP-2 was purchased from PeproTech (Rocky Hill, NJ). IGF-I was from R&D Systems (Minneapolis, MN). Anti-phospho-JNK, anti-JNK, anti-phospho-extracellular signal-regulated kinase

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Abbreviations: CBP, concentrated bovine milk whey active proteins; OSX, osterix; OCN, osteocalcin; β-GP, β-glycerophosphate; AsA, ascorbic acid

(ERK), and anti-ERK antibodies were from Cell Signaling Technology (Danvers, MA). Anti-GAPDH was from Merck (Darmstadt, Germany). Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody was from BD Biosciences (San Jose, CA), and anti-mouse IgG antibody was from Invitrogen (Carlsbad, CA). PD98059, bovine chymotrypsin (TLCK-treated, EC 3.4.21.1), α-lactalbumin, and β-lactoglobulin were from Sigma-Aldrich (St. Louis, MO). SP600125 was from Wako Pure Chemical Industries (Osaka, Japan). Bovine whey proteins were from Ingredia Nutritional (Arras, France). CBP was prepared using a proprietary process owned by Seperex Nutritional (Dunedin, New Zealand). Here is an outline of the process: Selected commercial-grade bovine milk was skimmed, pasteurized, ultrafiltered, and spray-dried to produce a high-protein milk protein concentrate. This skimmed milk proteins was subsequently reconstituted and depleted of major non-whey proteins by diafiltration (30-kDa membrane). The permeate was depleted of low molecular weight species by ultrafiltration/diafiltration (1-kDa membrane), followed by blending. Most of the proteins in CBP were within the 1–30 kDa range (Supplemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site). The total protein, lactose, moisture, and fat content of typical finished product were 83.9, 6.0, 5.8, and 3.7% (w/w) respectively. The IGF content of CBP was 0.17 μg/g as compared to 0.18 μg/g in a normal milk source.

Cell cultures. Normal human osteoblast cells (NHOsts, originally obtained from a 13-year-old male subject) were purchased from Cambrex (East Rutherford, NJ). The cells were maintained in Minimum Essential Eagle's Medium, the α modification (α-MEM; Sigma-Aldrich, St. Louis, MO), containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) at 37 °C in a humidified incubator with 5% CO_2. The medium was changed twice per week. Cells were not used beyond passage 12. Osteoblastic induction was done by supplementing the α-MEM medium with 5 mM β-glycerophosphate (β-GP) and 50 μM ascorbic acid (AsA) in the absence and the presence of CBP or BMP-2.

Immunoblotting. NHOsts were lysed in lysis buffer (150 mM NaCl; 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.8; 1% NP-40; 2 mM ethylenediaminetetraacetic acid; 1.5 mM $MgCl_2$; 0.5 mM dithiothreitol; and protease inhibitors). Each of the extracts was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), and proteins were transferred to polyvinylidene fluoride membranes using a semi-dry blotter (Biocraft, Tokyo). After transfer, the membranes were incubated in a blocking solution (5% dried skim milk in Tris-buffered saline containing 0.1% Tween-20) for 1 h to reduce nonspecific binding. They were then exposed to primary antibodies overnight at 4 °C. The blots were washed and exposed to HRP-conjugated secondary antibodies for 1 h. Protein detection was facilitated by means of ECL Plus Western Blotting Detection System (GE Healthcare Biosciences, Piscataway, NJ).

Alkaline phosphatase activity assay. Alkaline phosphatase activity was assayed as described previously.[19] Briefly, assay mixtures containing 0.1 M 2-amino-2-methyl-1-propanol (Sigma-Aldrich), 1 mM $MgCl_2$, 8 mM *p*-nitrophenyl phosphate disodium, and cell homogenates were incubated at 37 °C for 3 min. The reactions were stopped with 0.1 N NaOH, and the absorbance was read at 405 nm. A standard curve was prepared using *p*-nitrophenol. Each value was normalized to the protein concentration.

Mineralization assay. Mineralization was assessed by von Kossa staining of cell cultures[20] or by calcium measurement in the cultures, as described previously.[19] Briefly, NHOst cells were fixed with 95% ethanol and stained for 5 min with 3% $AgNO_3$ by the von Kossa method. At the same time, they were fixed with ice-cold 70% ethanol and stained with Alizarin Red (Sigma-Aldrich) to measure intercellular calcium. Briefly, the ethanol was removed and the cells were rinsed twice with deionized water. They were then stained with 40 mM Alizarin Red S in deionized water (adjusted to pH 4.2) for 10 min at room temperature. For quantitation, the cells stained with Alizarin Red were destained for 15 min with 10% (w/v) cetylpyridinium chloride (Wako Pure Chemical Industries, Osaka, Japan) in 10 mM sodium phosphate (pH 7.0). The extracted stain was transferred to a 96-well plate, and the absorbance was measured at 562 nm using a 1420 ARVO series multilabel counter (Perkin-Elmer Japan, Kanagawa, Japan).

RNA isolation and real-time PCR. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Mississauga, Canada), following the manufacturer's instructions. RNA was stored in RNase-free water at −80 °C until reverse transcription. First-strand cDNA synthesis was performed using a PrimeScript® II High Fidelity RT-PCR Kit (Takara, Shiga, Japan) following the manufacturer's instructions. For expression studies, the target transcripts were amplified and expression levels were measured using the Applied Biosystems 7500 Real Time PCR system (Applied Biosystems, Foster City, CA) with the following TaqMan® Gene Expression Assays: *Atf4* (assay ID Hs00909569_g1), and *18S* (assay ID Hs99999901_s1). *18S* was used as the housekeeping gene for quantity normalization, and all data are presented as fold change against control (*i.e.*, the untreated group in each case).

Treatment of CBP with chymotrypsin. Enzymatic digestion was carried out as described previously.[21] Briefly, the CBP substrate was hydrolyzed with chymotrypsin at 50 °C at pH 8.0 for 2–6 h. Following hydrolysis, chymotrypsin was inactivated by heating at 80 °C for 20 min, and the hydrolysates were cooled and stored at −20 °C for further analysis.

Statistical analyses. Data were expressed as mean ± SD for at least three independent experiments. Statistical analyses were done using by Tukey-Kramer test.

Results

CBP promoted bone mineralization by activating alkaline phosphatase in NHOsts

Recent reports indicate that CBP, comprised of low molecular weight milk whey proteins, contributes to constitutive bone remodeling.[17,18] It had a molecular weight range of 1–30 kDa, while most of the typical milk whey proteins lay in a molecular weight range of 46–58 kDa (Fig. 1). In order to clarify the effects of CBP on osteoblast differentiation in NHOsts, we investigated CBP-induced bone formation, which is principally involved in the differentiation of progenitor cells into mature osteoblasts in bone. Osteogenesis occurs through successive stages of differentiation. During the early phases of differentiation, alkaline phosphatase activity in the osteoblasts is elevated. Hence we validated the catalytic activity of alkaline phosphatase in NHOst cells treated with β-GP/AsA to induce differentiation in the absence and the presence of various concentrations of CBP. As shown in Fig. 2A, after 7 d of exposure to CBP, alkaline phosphatase activity in the NHOsts increased in

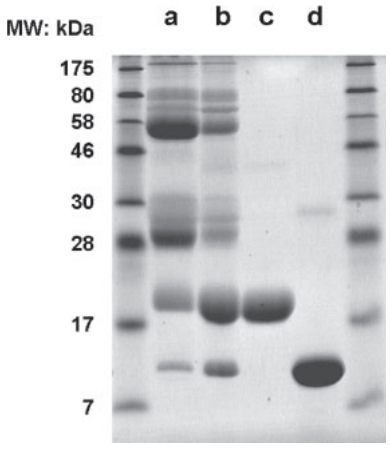

Fig. 1. CBP Protein Profiles after SDS–PAGE Separation.
Coomassie Brilliant Blue staining of protein samples (2 μg) from whey proteins (a), CBP (b), β-lactoglobulin standard (c), and α-lactalbumin standard (d).

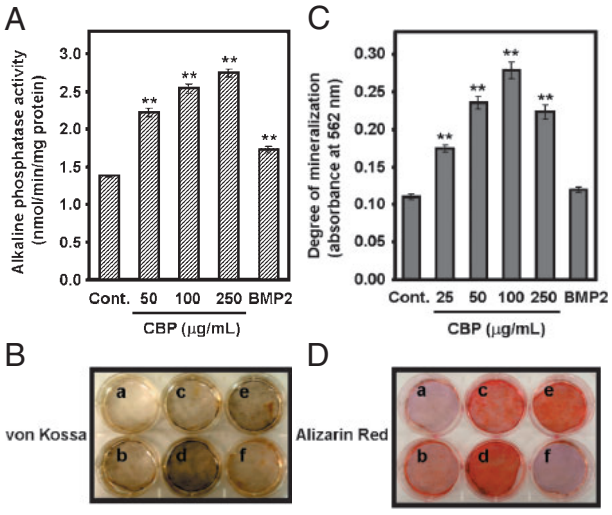

Fig. 2. CBP Enhances Alkaline Phosphatase Activation and Osteoblastic Mineralization in NHOst Cultures.

Osteoblastic induction was performed by supplementing α-MEM medium with 5 mM β-GP and 50 μM AsA in the absence and the presence of CBP or BMP-2. A, The catalytic activity of alkaline phosphatase was quantified in NHOsts following 7 d of osteoblastic induction. B–D, Mineralization levels were quantified in NHOsts after 21 d of osteoblastic induction by von Kossa staining or Alizarin Red staining. Culture plates are labeled as follows: a, control; b–e, 25, 50, 100, and 250 μg/mL CBP, respectively; f, 100 ng/mL BMP-2. Results were validated using SP600125 and PD98059. Values represent mean ± SD for triplicate determinations ($^{**}p < 0.01$ control by Tukey-Kramer test).

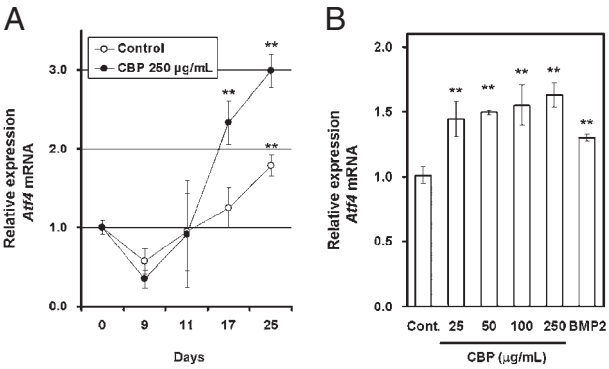

Fig. 3. CBP Upregulated the Expression of ATF4 during Osteoblastic Differentiation.

Osteoblastic differentiation was induced by supplementing the α-MEM medium with 5 mM β-GP and 50 μM AsA in the absence and the presence of CBP (25, 50, 100, or 250 μg/mL) or BMP-2 (100 ng/mL). *Atf4* expression was quantified by real-time PCR after the indicated induction times (A) or after 17-d of induction (B). Values represent mean ± SD triplicate determinations ($^{**}p < 0.01$ control by Tukey-Kramer test).

a dose-dependent manner. Bone matrix maturation proceeds through the complex regulation of a series of bone metabolism related-proteins. This depends strictly on the osteoblast differentiation stages.[11,12] Even if the expression of a single marker protein at the early stage can be induced, final progress to osteogenesis remains unsure if the expression levels of the other marker proteins do not change. Hence evaluation of mineralization at a late phase of osteoblast differentiation is considered to be more valuable in assessing the potency of food factors in bone metabolism than an early stage-marker such as alkaline phosphatase. To determine whether CBPs potentiate the differentiation of NHOsts at the later stages, we assessed mineralization levels in NHOsts by von Kossa staining or Alizarin red staining of the cultures. As shown in Fig. 2B–D, after 21 d of exposure to a combination of CBP and β-GP/AsA, the mineralization levels in NHOsts increased in a dose-dependent manner, despite a slight decline at the maximum concentration of CBP (250 μg/mL). The process appeared to reach a plateau. BMP-2 (100 ng/mL), a well-known regulatory factor that induces osteoblast differentiation, was used as positive control.

CBP-mediated upregulation of ATF4 expression was associated with late-stage osteogenesis

In osteogenesis, ATF4 functions as a transcription factor for OCN at the later stages of osteoblastic differentiation.[11,12] We investigated to determine whether induction of the ATF4 expression is enhanced by the CBP during NHOst differentiation. Analysis of the mRNA levels in the CBP-treated and untreated cells indicated that the expression of *Atf4* mRNA was elevated between 17 and 25 d. This elevation was significantly higher in the CBP-treated cells (Fig. 3A). CBP significantly induced *Atf4* mRNA expression in a dose-dependent manner, reaching maximum values at 250 μg/mL, as compared to the untreated cultures (Fig. 3B).

CBP activated MAPK signaling pathways in NHOsts

The indispensability of MAPK signaling for osteogenesis is well documented in osteoblasts. In addition, while differentiation-inducing factors activate MAPK and/or Smad signaling, each pathway regulating specific interactions, it remains unclear whether whey proteins, including CBP, activate MAPK signaling pathways in osteoblasts. Hence we examined the involvement and activation of ERK, a major MAPK pathway enzyme, and JNK in CBP-treated NHOsts. As shown in Fig. 4A, the addition of 100 μg/mL, CBP markedly increased the phosphorylation of JNK and ERK at 1–3 h, though phosphorylation was attenuated after 6 h. JNK and ERK phosphorylation was also detectable after crude whey protein treatment (data not shown). Furthermore, we found that the increased phosphorylation of JNK and ERK 1 h after CBP treatment was dose-dependent, and characteristically peaked at 250 μg/mL (Fig. 4B).

CBP-activated JNK, but not ERK, was involved in osteoblastic mineralization and the induction of ATF4 mRNA expression

We examined to determine whether CBP-mediated activation of JNK and/or ERK signaling pathways is involved in osteoblast mineralization and the regulation of ATF4 expression. Validation was conducted using the following inhibitors: SP600125, a selective and reversible inhibitor of JNKs, and PD98059, an inhibitor of MAPK kinases in the ERK pathway. In this analysis, JNK and ERK phosphorylation was inhibited by 10–25 μM SP600125 and 10–50 μM PD98059 (Supplemental Fig. 2). As shown in Fig. 5A, the addition of 10 μM SP600125 suppressed CBP-mediated osteoblastic mineralization to approximately the levels of the untreated cultures. In contrast, PD980159 did not affect CBP-induced osteoblastic mineralization. Furthermore,

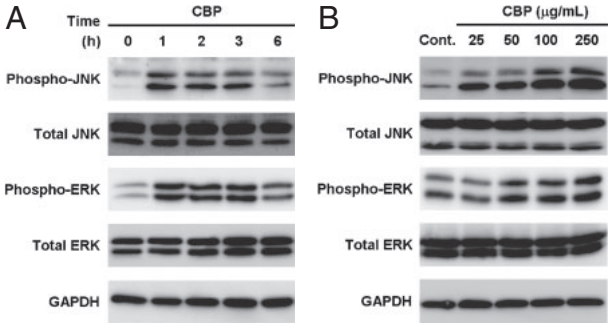

Fig. 4. CBP Induced the Phosphorylation of JNK and ERK.

NHOst cultures were serum-starved for 24 h and then treated with CBP (100 μg/mL) for the indicated times or with CBP at the indicated concentrations for 1 h. Whole-cell lysates were examined by immunoblotting with antibodies for phospho-JNK, JNK, phospho-ERK, ERK, and GAPDH.

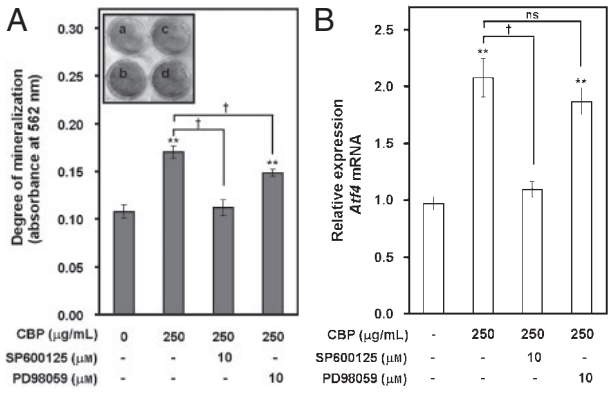

Fig. 5. CBP-Stimulated Mineralization and ATF4 Expression Were Induced through the JNK Signaling Pathway.

Osteoblastic induction was performed by supplementing α-MEM medium with 5 mM β-GP and 50 μM AsA in the absence and the presence of CBP (250 μg/mL), SP600125 (10 μM), and/or PD98059 (10 μM). A, Mineralization levels were quantified in NHOsts after 17 d of osteoblastic induction by Alizarin Red staining of the cultures (inset). Culture plates are labeled as follows: a, control; b, CBP (250 μg/mL); c, CBP (250 μg/mL) and SP600125 (10 μM); d, CBP (250 μg/mL) and PD98059 (10 μM). B, *Atf4* expression was quantified after 17 d of incubation by real-time PCR. Values represent mean ± SD triplicate determinations ($^{**}p < 0.01$ control; $^{\dagger}p < 0.01$ CBP treatment alone; ns, not significant by Tukey-Kramer test).

SP600125 (10 μM) completely inhibited the expression of *Atf4* mRNA following CBP treatment, while PD98059 (10 μM) had only a minor inhibitory effect on *Atf4* mRNA expression (Fig. 5B).

Chymotryptic hydrolysates of CBP also activated the MAPK signaling pathways in NHOsts

In humans, ingested milk products such as casein and whey proteins are enzymatically digested in the gastrointestinal system, releasing peptides of various sizes that become available for absorption into the circulation. Besides these main milk proteins, small amounts of peptides are found naturally in milk. They show certain biological activities, including antimicrobial properties.[13] CBP has a heterogeneous composition, including both small amounts of peptides and low molecular milk whey proteins (1–30 kDa), such as α-lactalbumin and β-lactoglobulin (Fig. 1). Hence we examined to determine whether the conformations of whey proteins in CBP are essential for its osteogenic activities. We used the

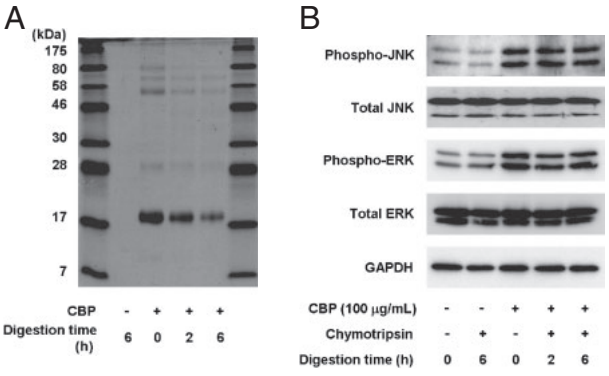

Fig. 6. CBP Hydrolysates Produced by Chymotrypsin Proteolysis Induced the Phosphorylation of JNK and ERK.

A, Coomassie Brilliant Blue staining of protein samples (2 μg) from chymotrypsin alone, intact CBP, 2-h CBP chymotryptic digest, and 6-h CBP chymotryptic digest. B, NHOst cultures were starved for 24 h and were then treated with 100 μg of chymotrypsin alone, intact CBP, 2-h CBP chymotryptic digest, or 6-h CBP chymotryptic digest for 1 h. Whole-cell lysates were examined by immunoblotting with antibodies for phospho-JNK, JNK, phospho-ERK, ERK, and GAPDH.

enzymatic proteolysis of CBP by chymotrypsin. In electrophoretic analysis using Coomassie Brilliant Blue staining, most of the whey proteins in CBP were hydrolyzed after a 6 h chymotryptic digest of at least (Fig. 6A). As shown in Fig. 6B, the CBP hydrolysates produced by chymotrypsin proteolysis also induced phosphorylation of JNK and ERK and induction by naïve CBP.

Discussion

Previous *in vitro*, *in vivo*, and clinical studies illustrate that concentrated low-molecular-weight whey proteins (1–30 kDa) such as CBP can be beneficial for bone growth and the prevention of skeletal diseases,[17,18] but little is known about the underlying mechanisms. In this study, we found that CBP promoted the development and differentiation of osteoblasts using normal human primary cultures. In addition, we defined the participation of the JNK-ATF4 pathway in osteoblastic mineralization following CBP treatment.

We found that CBP-mediated potentiation of alkaline phosphatase activity can act as an early-phase marker of osteoblast differentiation, while CBP-mediated strengthening of osteoblastic mineralization is a late-phase differentiation event (Fig. 2). On the other hand, the positive data on CBP in mineralization assays were linked directly to evaluate the effects of CBP on osteogenesis as compared to assessment by the use of early stage-markers, since mineralization in osteogenesis occurs at the terminal step of osteoblast differentiation. Subsequently, real-time PCR analyses revealed that CBP treatment in NHOsts induced *Atf4* mRNA expression (Fig. 3). Several stimulators of osteoblastic differentiation activate MAPK pathways, including the JNK and ERK pathways, suggesting that these pathways play key roles in the regulation of osteoblast differentiation.[10] We also observed that CBP and crude whey proteins stimulated MAP kinases ERK and JNK, acting as novel stimulators of osteoblastic differentiation. This suggests that one or both pathways

are involved in the induction of osteoblastic differentiation by low-molecular-weight whey proteins. The involvement of these pathways in the CBP response was elevated by the use of specific JNK and ERK inhibitors (SP600125 and PD98059 respectively). We found that JNK is mainly responsible for the regulating CBP-induced expression of ATF4 and mineralization. BMP-2, a stimulator of osteogenesis, can activate JNK and p38 following activation of the Smad pathway. A previous study found that complete inhibition of JNK by SP600125 resulted in slight reductions in alkaline phosphatase activity and a significant decline in BMP-2-induced OCN, suggesting that JNK plays a crucial role in regulating late osteoblastic differentiation.[12] Recent evidence indicates that the protein expression level of JNK2α2 (p54^{JNK2}) among four isoforms, which consist of JNK1 and JNK2 caused by differential splicing and exon use, was distinctively increased in the late differentiation period.[11] In addition, in JNK2 knockdown osteoblasts, matrix mineralization was found to decline almost to zero, indicating that the increased cellular content of p54^{JNK2} contributes to the differentiation of osteoblastic cells. In this study, we found that CBP significantly induced both JNK and ERK activation for short durations after single stimulation with CBP (Fig. 4), indicating that CBP induces the phosphorylation of JNK and ERK as a downstream event by stimulating osteoblastic differentiation. Furthermore, it is possible that repeated doses of CBP contribute to the activation of JNKs, including p54^{JNK2}, at the terminal osteoblast differentiation stage, resulting in the promotion of osteogenesis. Indeed, both matrix mineralization and ATF4 expression were strongly inhibited by SP600125, a JNK inhibitor (Fig. 5). Conversely, we observed that the effects of PD98059 on CBP-induced matrix mineralization and ATF4 expression in NHOsts were only moderate. This difference between SP600125 and PD98059 treatments suggests that the JNK and ERK pathways regulate distinct phases of osteoblastic differentiation. Previous reports have proposed that the role of the ERK signaling pathway varies according to the osteoblast differentiation stage.[11,22] Hence we speculate that induction of the ERK pathway in NHOst cells exposed to CBP is more responsible for the earlier stages of osteoblastic differentiation. This warrants further investigation. Taken together, our data indicate that ERK and JNK are implicated in CBP-induced osteoblastic differentiation and have distinct effects on ATF4 expression and mineralization.

In a fraction of low-molecular-weight milk whey proteins (1–30 kDa), α-lactalbumin and β-lactoglobulin are most of the proteins.[13] Componential analysis by electrophoresis revealed that CBP also contained α-lactalbumin and β-lactoglobulin (Fig. 1). Hence we investigated to determine whether α-lactalbumin and β-lactoglobulin alone can promote osteoblastic differentiation. Standard preparations of these proteins did not promote significant JNK phosphorylation (data not shown), indicating that multiple actions, including interacting effects with the other proteins, might be attributable to CBP-induced osteogenesis. In contrast, the CBP hydrolysates produced by chymotrypsin proteolysis also significantly induced JNK phosphorylation (Fig. 6B), indicating that the composition of the whey proteins in CBP is dispensable for their osteogenic activities. In humans, ingested CBP is hydrolyzed through gastrointestinal digestion into peptide forms prior to absorption and availability to circulation, like other milk-derived proteins. Thus these results also indicate that the effects of CBP on osteogenesis are not affected by the gastrointestinal digestive system, although further investigation is needed in biological models, taking into account multiple enzymatic conditions.

In this study, we elucidated the underlying mechanisms that regulate the ability of CBP to modulate osteogenesis in human primary osteoblasts. They indicate that CBP promotes late-stage osteoblastic differentiation through the JNK-ATF4 signaling pathway. They provide a contribution to knowledge of the prophylactic efficacy of whey proteins against bone diseases.

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