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cAMP enhances BMP2-signaling through PKA and MKP1-dependent mechanisms

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ABSTRACT

Recent studies suggest that the elevation of intracellular cyclic adenosine monophosphate (cAMP) and the activation of the protein kinase A regulate BMP-induced osteogenesis. However, the precise mechanisms underlying the enhancing effect of cAMP on BMP2 signaling were not completely revealed. In this study we investigated the effect of elevated cAMP level and PKA activation on the BMP2-induced osteoblastic differentiation in pluripotent C2C12 cells. Alkaline phosphatase activity and its mRNA were consistently induced by BMP2 treatment. The pretreatment of C2C12 cells with Forskolin, a cAMP generating agent, dbcAMP, an analogue of cAMP, or IBMX (3-isobutyl 1-methyl xanthine), and a nonspecific inhibitor of phosphodiesterases elicited further activation of alkaline phosphatase. Furthermore, elevated intracellular cAMP level increased BMP2-induced MKP1. On the other hand, BMP2-induced Erk phosphorylation (p44/p42) and cell proliferation were suppressed in the presence of cAMP. Thus, cAMP might enhance BMP2-induced osteoblastic differentiation by a MKP1-Erk-dependent mechanism.

Bone morphogenetic proteins (BMPs) superfamily regulates the proliferation, differentiation, and apoptosis of various types of cells and organs not only in embryonic development but also in postnatal physiological function [1]. Genetic disruptions of BMPs have resulted in various skeletal and extraskeletal developmental abnormalities [2]. At the cellular level, BMPs bind to two major types of membrane-bound serine/threonine kinase receptors, type-I and type-II receptors. The classic BMP signaling pathway operates by activation of the Smad family of transcription factors, and there is evidence that it can also act through a Smad-independent p38 MAPK signaling pathway [3]. There have also been reports suggesting that other pathways, such as ERK, c-Jun N-terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3-K), and Wnt may substitute for, activate, or modulate BMP signaling [4,5]. Various BMPs, including BMP2, BMP4, and BMP7, induce the differentiation of multipotential mesenchymal cells (e.g., C3H10T1/2 cells) into both osteochondrogenic lineage cells and osteoblast precursor cells. BMP2 specifically converts the differentiation pathway of C2C12 myoblasts into that of osteoblast lineage cells [6].

Several evidences indicate that osteoblasts, chondrocytes, myocytes, and adipocytes are all derived from common progenitor cells called undifferentiated mesenchymal cells [7–9]. During the process of their differentiation, progenitor cells acquire specific phenotypes depending upon the differentiated cell types under the control of respective regulatory factors. The differentiation process of osteoblasts can be divided into at least two stages. One is the commitment of undifferentiated mesenchymal cells into osteoblast progenitors. The other is the maturation of osteoblast progenitors into osteoblasts which express the various phenotypes of bone-forming cells; production of a large amount of extracellular matrix proteins including type I collagen and osteocalcin, high levels of alkaline phosphatase (ALP) activity, and responsiveness to calcitropic hormones such as parathyroid hormone (PTH) (for reviews see [10,11]).

The cyclic monophosphate nucleotides (cAMP and cGMP) are found ubiquitously in mammalian cells and act as second messenger transducers to effect the intracellular action of a variety of hormones, cytokines, and neurotransmitters. In turn, these nucleotides also modulate the signal transduction processes regulated by a range of cytokines and growth factors. The study reported by Isogai et al. [12] regarding the effects of PTH on osteogenic differentiation showed that continuous exposure to PTH stimulated osteogenic differentiation in immature osteoblasts and that the cAMP pathway was the key element of this mechanism. In another report, Tintut et al. [13] studied the role of the cAMP-signaling pathway in vascular calcification and showed that the cAMP-enhancing agents stimulated osteoblast-like differentiation of calcifying vascular cells. These findings substantiate the notion that the cAMP-signaling pathway is important in osteoblast recruitment from osteoprogenitor cells.

Recent studies demonstrate that the inhibition of phosphodiesterase (PDE), enzymes involved in the degradation of cAMP, by

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pentoxifyline and rolipram enhance BMP4-induced osteogenic differentiation of mesenchymal cells [14]. Although these effects might be linked to the increase in cAMP levels induced by PDE inhibitors, little is known about the precise mechanisms by which cAMP signaling contribute to the osteoblastic cell differentiation.

In this study, we have investigated the effect of different cAMP generating agents on osteoblastic cell differentiation using C2C12 cells, a murine pluripotent mesenchymal cell line that is able to differentiate in osteoblasts when treated with BMP2. C2C12 myoblast/osteoblast transdifferentiation induced by BMP2, in response to intracellular elevation of cAMP, was assessed by measuring the alkaline phosphatase (ALP) activity, Osteocalcin, Osterix, and Runx2 mRNA. In addition, we evaluated the signaling pathways involved in this effect.

Materials and methods

Reagents and antibodies. Forskolin, dibutyryl cyclic adenosine monophosphate (dbcAMP), IBMX, H89, and GF-109203X (GFX) were purchased from Sigma–Aldrich (St. Louis, MO, USA). U0126, PD-98059 and SB203580 were obtained from Calbiochem–Novabiochem Corp. (San Diego, CA, USA). MKP-1 (C-19) Polyclonal antibody was obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Polyclonal anti-p38, anti-ERK1/2, anti-pERK1/2, anti-pERK5, anti-pp38, anti-pSmad1/5/8 were obtained from Cell signaling (Cell signaling Technology, MA, USA).

Cell cultures. C2C12, C3H10T1/2, and MC3T3-E1 clone 4 were purchased from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (C2C12) or alpha-minimal essential medium (MC3T3 and

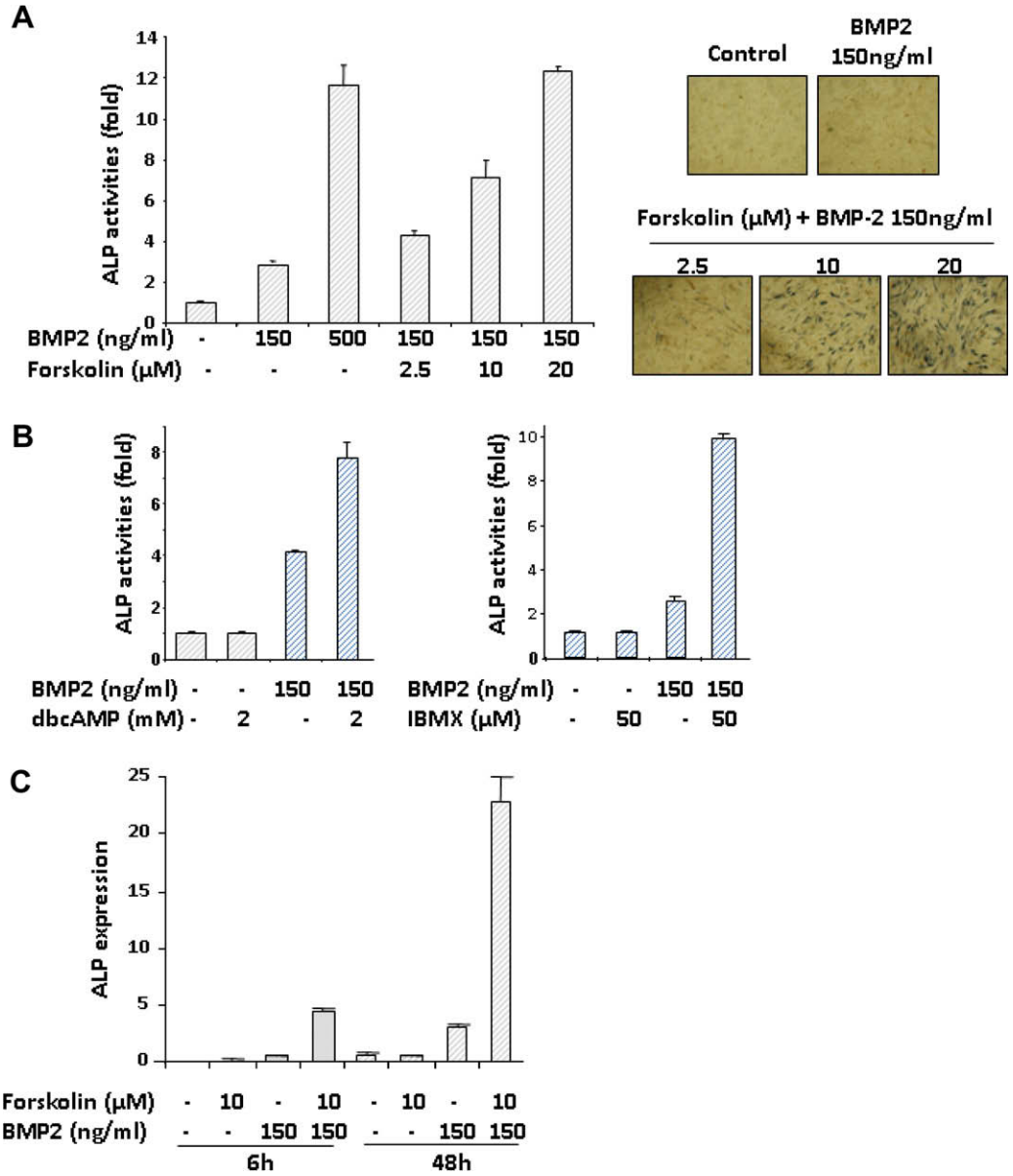

Fig. 1. Effects of cAMP on BMP-induced osteoblast differentiation in C2C12 cells. (A) Cells were treated with BMP2, or BMP2 after 1h pretreatment with forskolin as indicated. After 5 days, the cells were used to measure ALP activities or fixed and stained for ALP as described in Materials and methods. (B) Cells were pretreated with BMP2 alone or pretreated with dbcAMP or IBMX before BMP2 stimulation. After 5 days, the cells were used to measure ALP activities. (C) C2C12 cells were stimulated for 6 and 48 h as indicated, total RNA was extracted and expression of Alkaline phosphatase (ALP) was analyzed using real-time RT-PCR, normalized to S18 expression and presented as the level relative to unstimulated cells (−).