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Parathyroid hormone-related peptide inhibits the expression of bone morphogenetic protein-4 mRNA through a cyclic AMP/protein kinase A pathway in mouse clonal chondrogenic EC cells, ATDC5

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Abstract

The bone morphogenetic proteins (BMPs) play crucial roles in chondrogenic differentiation. Little is known, however, regarding the regulation of BMP gene expression. Here we examined the effect of parathyroid hormone-related peptide (PTHrP) (1–141), a full-length form of PTHrP molecules, on the expression of BMP-4 mRNA in clonal mouse chondrogenic EC cells, ATDC5. In differentiated ATDC5 cells, the expression of BMP-4 mRNA was inhibited by PTHrP (1–141), which stimulated cAMP accumulation and protein kinase A (PKA) activity in these cells. Dibutyryl cAMP, a permeable analog of cAMP, mimicked and H-89, a selective PKA inhibitor, blocked this effect of PTHrP (1–141). Moreover, actinomycin D attenuated the inhibition of BMP-4 mRNA expression by PTHrP (1–141). These results indicate that PTHrP (1–141) transcriptionally inhibits the expression of BMP-4 mRNA through a cAMP/PKA pathway in ATDC5 cells.

Keywords: Chondrogenic differentiation; Bone morphogenetic protein-4; Parathyroid hormone-related peptide; Cyclic AMP; Protein kinase A

1. Introduction

The bone morphogenetic proteins (BMPs) were originally identified based on their osteoinductive activities. When implanted into subcutaneous or muscular tissues of rodents, the BMPs induce ectopic bone formation through sequential processes of endochondral ossification [1,2]. Accumulating evidence indicates that BMP-4 and BMP-6 are expressed in the cartilage of developing bones and that they function as potent stimulators of chondrogenic differentiation in vitro and in vivo [3–7]. In contrast, parathyroid hormone-related peptide (PTHrP) is a potent inhibitor of chondrogenic differentiation of chondrocytes [8–15]. Previous studies showed that targeted overexpression of either PTHrP or PTHrP receptor (R) inhibited chondrogenic differentiation in vitro [9] and in vivo [14,15] and that homozygous mice carrying either PTHrP or PTHrP-R null mutation each exhibited widespread abnormalities of cartilage development [8,10,11]. However, the interrelationship between BMPs and PTHrP during chondrogenic differentiation remains to be elucidated.

Clonal mouse chondrogenic embryonal carcinoma

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(EC) cells, ATDC5, keep track of the multistep chondrogenic differentiation processes in vitro [16–22]. Differentiated ATDC5 cells express PTHrP-R and BMP-4 [21], both of which coordinately modulate the processes of chondrogenic differentiation in these cells. In this study, we indicate that PTHrP (1–141) transcriptionally downregulates the expression of BMP-4 mRNA through a cAMP/protein kinase A (PKA) pathway in ATDC5 cells.

2. Materials and methods

2.1. Materials

Human recombinant PTHrP (1–141) was a kind gift from Dr. T.J. Martin (St. Vincent's Institute of Medical Research, Victoria, Australia), and N^6,2′-*O*-dibutyryl cyclic AMP (dbcAMP), *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), phorbol 12-myristate 13-acetate (PMA), and actinomycin D were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cells and culture conditions

ATDC5 cells were cultured as previously described [17–21]. Briefly, cells were maintained in a log growth phase in DMEM/Ham's F12 hybrid medium (ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA) containing 5% (v/v) fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA), 10 mM human transferrin (Boehringer Mannheim GmbH, Mannheim, Germany), and 30 nM sodium selenite (Sigma Chemical Co.) (maintenance medium) at 37°C in a humidified 5% CO_2/95% air atmosphere. Chondrogenesis and cartilage nodule formation could be induced only in the postconfluent phase when cells were cultured in the maintenance medium supplemented further with 10 mM bovine insulin (Wako Pure Chemical, Osaka, Japan) (differentiation medium). In the present study, we plated ATDC5 cells in 6- or 24-multiwell plastic plates at an initial density of 6×10^4 or 2×10^4 cells/well respectively and cultured these cells for a total of 23 days with medium replacement every other day. Cells were cultured for an initial 21-day period in the differentiation medium in a 5% CO_2/95% air atmosphere and then cells were treated for the indicated time periods with various doses of the test substances in the absence of insulin.

2.3. RNA extraction and Northern analysis

Total RNA was prepared from the cultures as previously described [17–21]. For Northern hybridization, total RNA (20 μg) was denatured, separated by 1% agarose gel electrophoresis, and transferred on Nytran membranes (Schleicher and Schuell, Dassel, Germany). Hybridization probes were prepared by the random-primer method with a BcaBEST labeling kit (Takara, Ohtsu, Japan). A 0.6-kb fragment of BMP-4 mRNA was used as the hybridization probe. After hybridization, the membranes were exposed to BioMax films (Eastman Kodak, Rochester, NY, USA) at −80°C with Cronex lightening plus intensifying screens (DuPont, Boston, MA, USA). The quantitation of hybridization signals was performed by scanning densitometry with a Model 301 Densitometer (Fuji X-Ray, Tokyo, Japan).

2.4. Measurement of cAMP accumulation

Effects of PTHrP (1–141) on cAMP accumulation were assessed as described previously [23]. In brief, cells were cultured for 21 days in 24-multiwell plates under the conditions described above. Before experiments, cells were rinsed once with buffer containing Hanks' medium, 20 mM HEPES (pH 7.5; 22°C), and 0.1% (w/v) heat-inactivated bovine serum albumin, then incubated for 30 min at room temperature in the above buffer further containing 0.5 mM 3-isobutyl-1-methylxanthine (Sigma Chemical Co.) and PTHrP (1–141) (10 nM) or its vehicle. The reaction was terminated by the addition of ice-cold HCl (final HCl concentration, 0.1 N). The samples were boiled (100°C; 2.5 min) to extract cAMP and diluted with distilled water and then, the cAMP content in both cells and medium was measured by a radioimmunoassay.

2.5. Measurement of PKA activity

Effects of PTHrP (1–141) on PKA activity were assessed by the methods of Tamanini et al. [24] and Lee et al. [25]. In brief, cells were cultured for 21 days in 24-multiwell plates under the conditions described

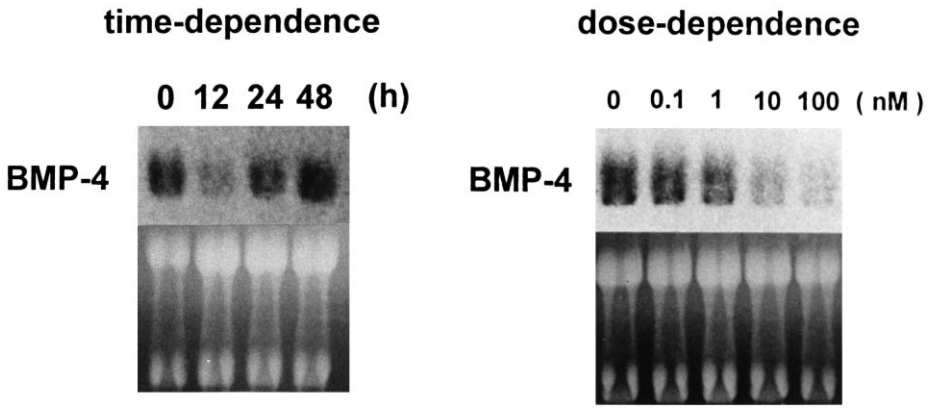

Fig. 1. Effect of exogenously administered PTHrP (1–141) on BMP-4 gene expression in differentiated ATDC5 cells. ATDC5 cells cultured on plastic plates for 21 days were exposed to 10 nM of PTHrP (1–141) for the indicated time periods (time dependence), and to either vehicle or PTHrP (1–141) (0, 0.1, 1, 10 and 100 nM) for an additional 12 h (dose dependence). Northern analysis was performed using total RNA (20 μg per lane). The integrity and equal loading of RNA were confirmed by ethidium bromide staining. The results shown are representative of three independent experiments.

above and then exposed to PTHrP (1–141) (10 nM) or their vehicle for 3 h at 37°C. Cells were rinsed with PBS, extracted with extraction buffer (5 mM EDTA, 50 mM Tris, pH 7.5), and homogenized. Kinase activity in 10 μg of extracted protein was assessed after incubation for 20 min at 30°C in the buffer containing 10 mM HEPES at pH 7.0, 10 mM $MgCl_2$, 100 mM ATP, 100 mM dithiothreitol, 0.1 mM [γ-^{32}P]ATP (30 mCi/nmol), and 100 mM kemptide, followed by termination of the reaction by the addition of 100 μl of ice-cold 60% acetic acid. The reaction mixture was applied to a Dowex 1X8-100 anion exchanger column. Phosphorylated kemptide was then eluted with 0.8 ml of 30% acetic acid and the radioactivity was quantitated by a liquid scintillation counter.

2.6. Statistical analysis

Statistical significance was assessed by one-way analysis of variance and unpaired Student's *t*-test.

3. Results

3.1. PTHrP (1–141) downregulates the expression of BMP-4 mRNA in differentiated ATDC5 cells

In the presence of insulin, ATDC5 cells form cartilaginous nodules through a cellular condensation stage. The process proceeded in an orderly and synchronous manner as evidenced by the expression of phenotypic marker genes with cartilage characteristics as previously reported [17,21]. We recently showed that BMP-4 was stimulatory and PTHrP (1–141) was inhibitory to chondrogenic differentiation in differentiated ATDC5 cells [21]. These observations raise the possibility that PTHrP (1–141) may regulate BMP-4 gene expression. As shown in Fig. 1, the steady-state levels of BMP-4 transcript were indeed downregulated by PTHrP (1–141) treatment: PTHrP (1–141) (10 nM) markedly downregulated the BMP-4 transcript and maximal inhibition occurred 12 h after PTHrP (1–141) treatment.

Table 1
Effects of PTHrP (1–141) on cAMP accumulation and PKA activity in differentiated ATDC5 cells

	Control	PTHrP 10 nM
cAMP accumulation (pmol/μg DNA/30 min)	0.095 ± 0.0063	11.1 ± 0.68*
PKA activity (% of control)	100 ± 7.5	121 ± 3.0*

ATDC5 cells cultured on plastic plates for 21 days were exposed to PTHrP (1–141) (10 nM) or its vehicle for indicated time periods. cAMP accumulation and PKA activity were assessed as described in Section 2. Data are shown as the mean ± S.D. of six separate determinations. Experiments were performed three times with similar results.
*$P < 0.01$, statistically significant difference from the corresponding control groups.

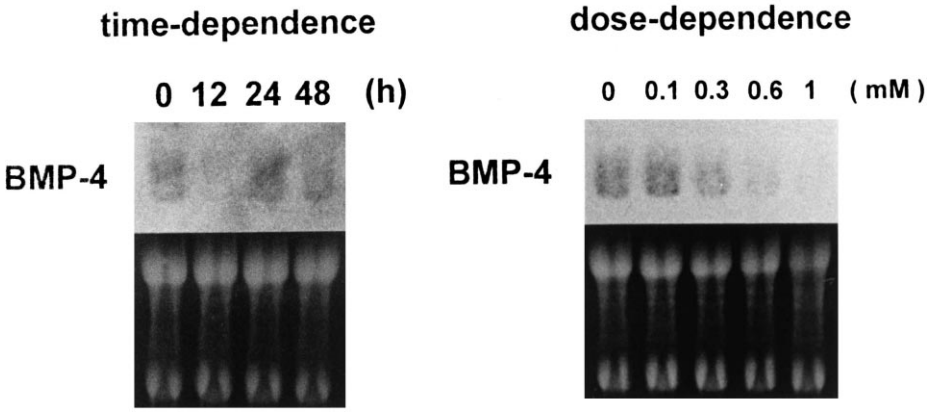

Fig. 2. Effect of dbcAMP on BMP-4 gene expression in differentiated ATDC5 cells. ATDC5 cells cultured on plastic plates for 21 days were exposed to 0.4 mM of dbcAMP for the indicated time periods (time dependence), and to either vehicle or dbcAMP (0.1, 0.3, 0.6, and 1 mM) for an additional 12 h (dose dependence). Northern analysis was performed using total RNA (20 μg per lane). The integrity and equal loading of RNA were confirmed by ethidium bromide staining. The results shown are representative of three independent experiments.

3.2. PTHrP (1–141) stimulates cAMP accumulation and PKA activity in differentiated ATDC5 cells

PTHrP is a known stimulator of intracellular production of cAMP via its binding to PTH/PTHrP cell surface receptors. In differentiated ATDC5 cells, 1 mM of dbcAMP stimulated PKA activity (1.7-fold stimulation over the control, data not shown) and 10 nM of PTHrP (1–141) also caused significant stimulation of cAMP accumulation and PKA activity (117- and 1.2-fold stimulation over the controls, respectively) (Table 1).

3.3. dbcAMP downregulates the expression of BMP-4 mRNA in differentiated ATDC5 cells

In differentiated ATDC5 cells, PTHrP (1–141) caused significant stimulation of cAMP accumulation and PKA activity. We next examined the possibility if a cAMP/PKA pathway mediated the inhibition of BMP-4 expression by PTHrP (1–141). As shown in Fig. 2, direct stimulation of PKA activity by 0.4 mM of dbcAMP, a cell membrane-permeable analog of cAMP, resulted in transient and marked inhibition of BMP-4 gene expression in differentiated ATDC5 cells, compared with the effect of PTHrP (1–141) treatment. Additionally, retreatment with 0.4 mM of dbcAMP or 10 nM of PTHrP (1–141) 12 h after the initial treatment resulted in continued inhibition of BMP-4 gene expression for the subsequent 12 h (data not shown).

3.4. H-89 blocks the inhibitory action by PTHrP (1–141) on BMP-4 mRNA expression

To confirm that the inhibitory action of PTHrP (1–141) is mediated via a cAMP/PKA pathway, we

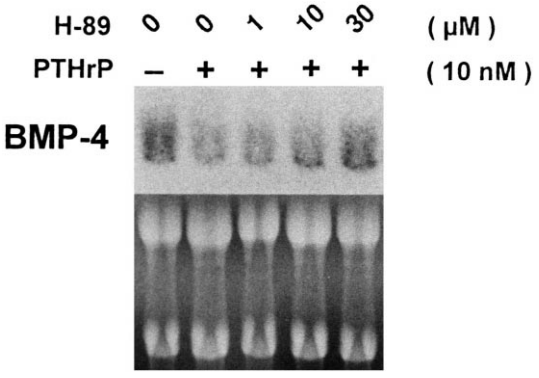

Fig. 3. Blocking of the inhibitory effect of PTHrP (1–141) on BMP-4 gene expression by H-89, a PKA inhibitor, in differentiated ATDC5 cells. ATDC5 cells cultured on plastic plates for 21 days were treated for an additional 12 h with a submaximal dose of PTHrP (1–141) (10 nM), either alone or in the presence of varying doses of H-89 (1, 10, and 30 μM). Northern analysis was performed using total RNA (20 μg per lane). The integrity and equal loading of RNA were confirmed by ethidium bromide staining. The results shown are representative of two independent experiments.

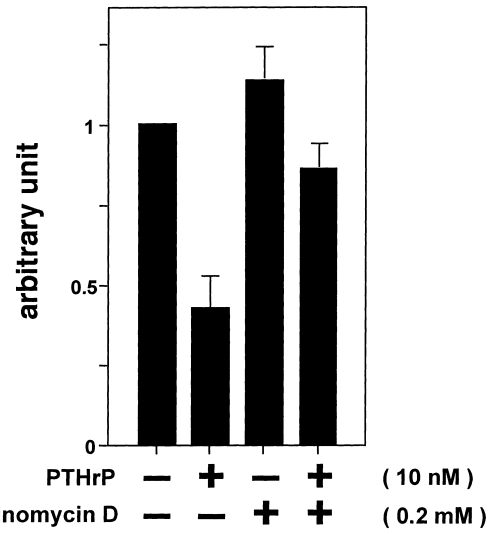

Fig. 4. Effect of actinomycin D on BMP-4 mRNA decrease by PTHrP (1–141) in differentiated ATDC5 cells. ATDC5 cells were cultured on 6-multiwell plastic plates for 21 days. The medium was replaced and cells were cultured in the presence or absence of actinomycin D (0.2 mM), PTHrP (1–141) (10 nM), or combinations as indicated. Actinomycin D was added 15 min prior to addition of PTHrP (1–141). Northern analysis was performed using total RNA (20 μg per lane) extracted 12 h after PTHrP (1–141) treatment. Data are shown as the mean ± S.D. of three separate determinations.

examined the effect of H-89, a selective PKA inhibitor, on the inhibitory action of PTHrP (1–141) on BMP-4 gene expression. As shown in Fig. 3, the action of PTHrP (1–141) was blocked by H-89 in a dose-dependent manner. Treatment with H-89 alone did not affect the steady-state levels of BMP-4 transcript (data not shown).

3.5. *The inhibitory action by PTHrP (1–141) on the expression of BMP-4 in differentiated ATDC5 cells is attenuated by actinomycin D*

cAMP/PKA signaling directly regulates the gene transcripts via the cAMP responsive element binding protein and the cAMP responsive element modulator which bind cAMP responsive elements in the promoter region of cAMP responsive genes. We hypothesized that PTHrP (1–141) transcriptionally regulated the levels of BMP-4 gene transcript in differentiated ATDC5 cells. In Fig. 4, it is shown that pretreatment of ATDC5 cells with actinomycin D, a transcriptional inhibitor, significantly attenuated the effect of PTHrP (1–141) on the inhibition of BMP-4 gene expression.

4. Discussion

The BMP family plays a vital role in skeletal development, and several BMP family genes are expressed at sites of bone formation with a unique spatiotemporal pattern, suggesting that the coordinated expression of these molecules is crucial for the cascade of events underlying chondrogenic differentiation. Previous studies showed that BMP receptor type IA is essential for proper progression of the chondrogenic differentiation program in vitro and in vivo [26,27]. Furthermore, recent in vivo studies indicated that activation of BMP signaling by either overexpression of the BMP family in chondrocytes or deletion of a BMP antagonist resulted in deformities of bone rudiments by the acceleration of the progression of chondrogenic differentiation [28,29]. These lines of evidence raise the possibility that BMP signals may be maintained at an appropriate level during chondrogenic differentiation. In ATDC5 cells, the expression of BMP-6 mRNA was differentiation stage-related, and BMP-6 acts as a potent stimulator of chondrogenic differentiation [21]. We previously indicated that acquisition of PTH/PTHrP responsiveness is closely associated with chondrogenic differentiation in ATDC5 cells [22], and that BMP-6 and PTHrP signals coordinately regulate the rate of chondrogenic differentiation via downregulation of the transcript levels of the BMP-6 gene by PTHrP in these cells [21]. In contrast, both BMP-4 and BMP type IA receptor in ATDC5 cells are constitutively expressed during chondrogenic differentiation (data not shown), and BMP-4 has the same abilities to stimulate chondrogenic differentiation as BMP-6 [21], suggesting that the amplitude of BMP-4 expression in ATDC5 cells is regulated during chondrogenic differentiation. Indeed, our recent observations that BMP-4 and BMP-6 markedly downregulated the transcript levels of BMP-4 in ATDC5 cells indicate that the stimulatory effects of BMP-4 on chondrogenic differentiation in ATDC5 cells are under the regulatory control by the negative feedback mechanism [21]. Moreover, in the present study, we revealed that PTHrP (1–141) transcriptionally down-

regulates the expression of BMP-4 mRNA. Our data thus favor the notion that the stimulatory effects of BMP-4 on chondrogenic differentiation in ATDC5 cells are under the negative control of both BMPs and PTHrP signals, which maintained the amplitude of endogenous BMP-4 signal relatively constant in both differentiation stage-related and differentiation stage-unrelated manners.

We next characterized the participation of the intracellular signal transduction pathways of PTHrP (1–141) in the inhibition of BMP-4 gene expression. Previous studies indicated that the PTH and PTHrP signals via PTH/PTHrP common cell surface receptors are transduced by the pathways of PKA and protein kinase C (PKC) [30–34]. In differentiated ATDC5 cells, PTHrP (1–141) stimulates both cAMP accumulation and PKA activity (Table 1) and direct stimulation of PKA activity by dbcAMP mimicked the effects of PTHrP (1–141) on the inhibition of BMP-4 gene expression in these cells (Fig. 2). Furthermore, H-89, a PKA inhibitor, blocked the inhibitory action of BMP-4 gene expression by PTHrP (1–141). Additionally, PMA, an activator of PKC, had no significant effects on BMP-4 gene expression in ATDC5 cells (data not shown). These results suggest that the inhibitory action of PTHrP (1–141) on BMP-4 gene expression is mediated via a cAMP/PKA signal transduction pathway. Kurihara et al. [35] and Feng et al. [36] isolated a murine genomic clone of BMP-4 gene and analyzed the promoter region of this gene. Although cAMP responsive elements cannot be identified in potential regulatory response regions in the 5′-flanking region of the mouse BMP-4 gene, our observation in the present study that actinomycin D, a transcriptional inhibitor, attenuated the inhibitory action of PTHrP on BMP-4 gene expression suggests that PTHrP transcriptionally regulates BMP-4 gene expression. Verification of the repressor activity of PTHrP (1–141) and PKA on BMP-4 gene expression needs further study.

In conclusion, the amplitude of the BMP-4 signal in chondrogenic ATDC5 cells may be regulated at an appropriate level. The transcript level of BMP-4 is negatively modulated by the PTHrP signal via a cAMP/PKA pathway.

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