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ORIGINAL PAPER

Parathyroid hormone-related protein regulates osteoclast inhibitory lectin expression via multiple signaling pathways in osteoblast-like cells

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Abstract Osteoclast inhibitory lectin (OCIL) is a recently identified inhibitor of osteoclast formation. A variety of osteotropic factors regulate OCIL expression in osteoblastic cells, however, little information is available to date concerning how this gene is controlled. Using real-time RT-PCR, we examined the regulation of OCIL expression by PTHrp and the signaling pathways used. We demonstrated in rat osteoblast-like UMR-106 cells, rat calvarial primary osteoblastic cells, and murine MC3T3-E1 cells, PTHrp(1–34) increased OCIL expression. In UMR-106 cells, the increase began and reached maximum later than RANKL induction and OPG suppression. cAMP/PKA signaling activators PTH(1–31), forskolin and dibutyryl cAMP (db-cAMP), and calcium ionophore A23187 all increased OCIL levels. In contrast, PKC activator phorbol-12-myristate-13-acetate reduced OCIL expression in short term but induced OCIL mRNA in long term. PKA inhibitor KT5720, mitogen-activated protein kinase (MAPK) cascade inhibitor PD98059, calmodulin antagonist W-7, and Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) inhibitor KN-62 all significantly blunted PTHrp-stimulated OCIL expression. Moreover, PD98059 blocked the stimulation of OCIL by FSK or db-cAMP but not that by A23187. In primarily cultured osteoblasts, the PTHrp induction of OCIL was blocked by KT5720, W-7, and PD98059 as well. The data established that PTHrp(1–34) regulates OCIL expression in vitro through cAMP/PKA, Ca^{2+}/CaMK II, and MAPK signaling pathways.

Keywords Parathyroid hormone-related protein · Osteoclast inhibitory lectin · Gene expression · Intracellular signaling · Protein kinase

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Introduction

The recent discovery of osteoclast inhibitory lectin (OCIL) as an inhibitor of osteoclastogenesis is of great importance. OCIL was originally identified in osteoblasts, but widely expressed in most tissues, notably in epithelial and mesenchymal cells, as well as in dendritic, macrophage, and lymphocyte populations [1, 2]. Structurally, OCIL belongs to type II transmembrane molecules with a C-type lectin extracellular domain. The tissue distribution of OCIL mRNA and protein overlaps with that of RANKL, the well-known membrane-bound molecule on osteoblast responsible for the formation of osteoclasts [3–5], suggesting that an interaction might exist between these molecules in the skeleton and in extraskeletal tissues.

Previous studies support a role of OCIL in limiting osteoclast formation, although the precise mechanism of OCIL actions remains uncertain. It seems that mOCIL acts directly on macrophage/monocyte cells as evidenced by its inhibitory action on adherent spleen cell cultures, which were depleted of stromal and lymphocytic cells. hOCIL was shown to be able to inhibit bone resorption as well [2], suggesting its potential as a therapy to deal with osteoporosis, although its action and safety when used in vivo needs to be explored.

OPG is also a potent inhibitor of osteoclastogenesis that is secreted by osteoblastic stromal cells [6–8]. Unlike in OPG, which acts as the soluble decoy receptor of RANKL and exerts its effects by binding to and antagonizing RANKL actions, no protein interaction between OCIL and RANKL was revealed. In contrast, OCIL can bind to high-molecular sulfated sugars [9], suggesting that carbohydrate recognition may be important for OCIL engagement to a hitherto unrecognized receptor present on the osteoclast and its progenitor cells.

OCIL mRNA levels were shown to be regulated by a variety of osteotropic factors in osteoblastic cells, including retinoic acid, 1,25-dihydroxyvitamin D_3, prostaglandin E_2 (PGE_2), parathyroid hormone (PTH), interleukin-1α, and interleukin-11 [1, 2]. However, little information is available to date concerning how this gene is controlled. Synthesized by osteoblasts, PTHrp is an important modulator of bone development that acts through auto- or paracrine manner [10–12]. The present study focused on whether PTHrP(1–34) regulates OCIL expression and the signaling pathways used in osteoblast-like cells. We showed that PTHrP(1–34), through multiple signaling pathways, regulates OCIL expression.

Results

PTHrp(1–34) regulates OCIL expression in vitro

Using real-time PCR, we demonstrated that after treatment for 12 h, PTHrp(1–34) induced a dose-dependent increase in steady-state mRNA for OCIL in UMR-106 cells. As shown in Fig. 1, detectable increase was noted at a dose of 10 pM and reached maximum at 10 nM, up to 2.9-fold greater than in control (Fig. 1a). An investigation of the time response of OCIL to 10 nM PTHrp(1–34) treatment showed that the up-regulation began at 6 h, and reached maximal level at 12 h (Fig. 1b). We also examined in the same cell cultures the expression profiles of RANKL and OPG. It indicated that the regulation of RANKL and OPG was much more rapid. At 2 h, RANKL mRNA already increased while OPG decreased. RANKL expression

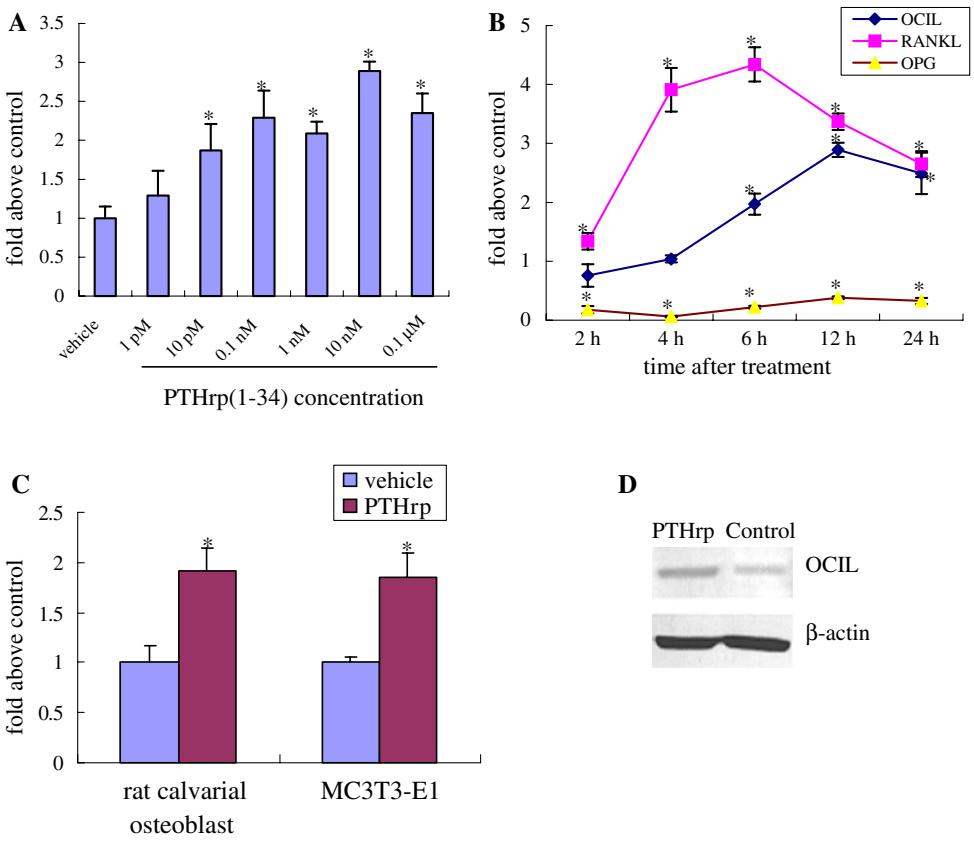

Fig. 1 Effects of PTHrp(1–34) on OCIL expression. UMR-106 cells were treated with either various concentrations of PTHrp(1–34) for 12 h (**a**) or 10 nM PTHrp(1–34) for the time indicated (**b**). Time courses of PTHrp(1–34) on RANKL and OPG mRNA levels were also examined in UMR-106 (**b**). OCIL mRNA levels were also examined in rat calvarial primary osteoblasts and murine MC3T3-E1 cells (**c**) and OCIL protein levels were evaluated in MC3T3-E1 (**d**) at 24 h after 10 nM PTHrp(1–34) treatment. OCIL, RANKL, or OPG mRNA levels in the cells treated only with vehicle (water) at the corresponding time points (control) were set as 1. *Significant vs. control, $P < 0.05$

reached maximal level (4.3-fold) at 6 h. In contrast, OPG changed even faster, with maximal inhibition of 92% at 4 h (Fig. 1b).

To study whether OCIL is a ubiquitous PTHrp target gene, we tested two other PTHrp-responsive osteoblastic cell preparations: rat calvarial primary osteoblastic cells and mouse MC3T3-E1 cells (Fig. 1c). PTHrp(1–34) stimulated OCIL expression in both cells.

In addition, we carried out Western blotting analysis and demonstrated an up-regulation of OCIL, at the protein level, by PTHrp(1–34) in MC3T3-E1 osteoblastic cells (Fig. 1d).

PTH, PTH analogs, and signaling activators regulate OCIL expression

PTH(1–34) of 10 nM time-dependently regulated OCIL expression in UMR-106 cells. It increased OCIL mRNA at any time between 6 and 24 h, with the highest induction of 2.8-fold at 24 h (Fig. 2a).

Like PTH, PTHrp(1–34) activates both the cAMP/PKA and PLC/PKC pathways [13, 14]. We next analyzed the effects of signal-selective analogs of PTH and PTHrp (1–34). Specifically, 10 nM PTH(1–31), which activates the cAMP/PKA pathway, stimulated OCIL expression in a time-dependent manner (Fig. 2b). In contrast, 10 nM PTH(3–34), which does not stimulate the cAMP/PKA pathway but stimulates PKC signaling and increases intracellular Ca^{2+} instead, only slightly stimulated OCIL expression at 6 h after treatment (1.4-fold vs. control) (Fig. 2c).

We also applied two compounds that increase intracellular cAMP: 2 μM forskolin and 200 μM db-cAMP both stimulated OCIL expression in a time-dependent manner, with the maximal effect of 4.2- and 4.5-fold, respectively, at 24 h (Fig. 3a).

We next investigated whether PKC or Ca^{2+} signaling pathway also plays a role in the regulation of OCIL. At early time points, 100 nM PMA, a potent activator of PKC, slightly inhibited OCIL expression, with maximal inhibition of 50% at 6 h. At 24 h, PMA switched its effect to slight stimulation (Fig. 3b). In contrast, 5 μM calcium ionophore A23187 dramatically stimulated OCIL expression at any time point (2–24 h) examined, with maximal 5.1-fold increase at 6 h (Fig. 3c).

Multiple signaling pathways are involved in PTHrp(1–34) regulation of OCIL in UMR-106 cells

To further determine the involvement of PKA pathway in PTHrp(1–34) regulation of OCIL in UMR-106 cells, selective adenylate cyclase inhibitor KT5720 was used. As shown in Fig. 4, KT5720 blocked the PTHrp-stimulated OCIL mRNA expression by 60% and 61%, respectively, at 6 and 12 h. Moreover, selective PKA inhibitor H-89 blocked OCIL expression induced by db-cAMP. These

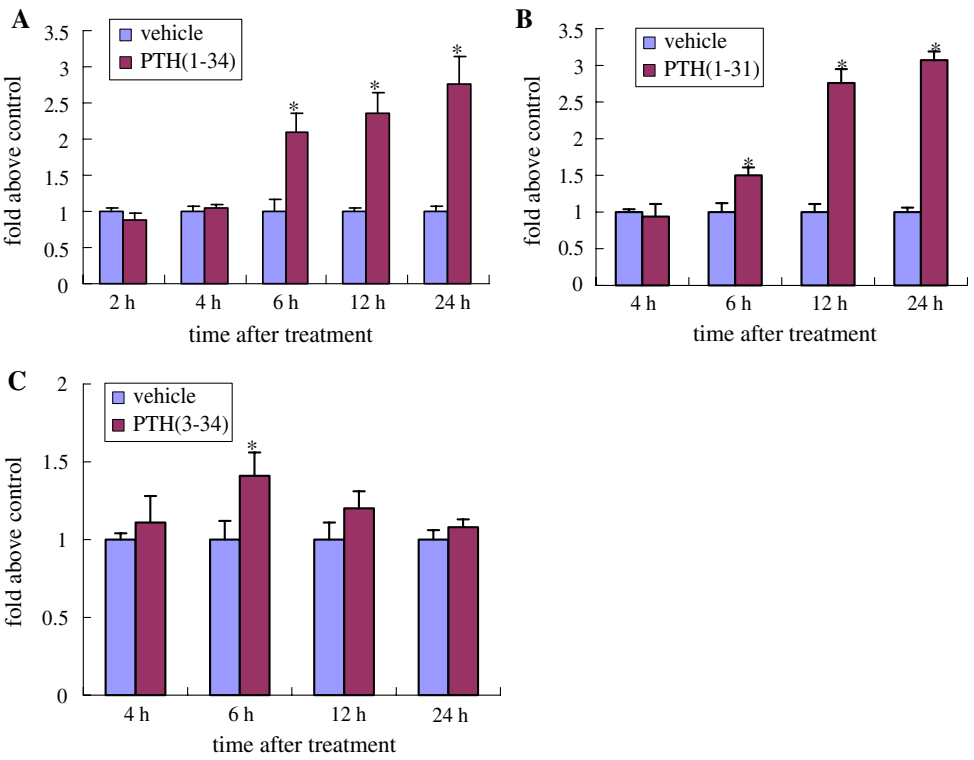

Fig. 2 Real-time PCR analysis of OCIL mRNA levels from UMR-106 cells treated for the indicated times with 10 nM PTH(1–34) (**a**) and its analogs, i.e., 10 nM PTH(1–31) (**b**), or 10 nM PTH(3–34) (**c**). The OCIL mRNA levels in the cells treated only with vehicle (water) at the corresponding time points (control) were set as 1. *Significant vs. control, $P < 0.05$

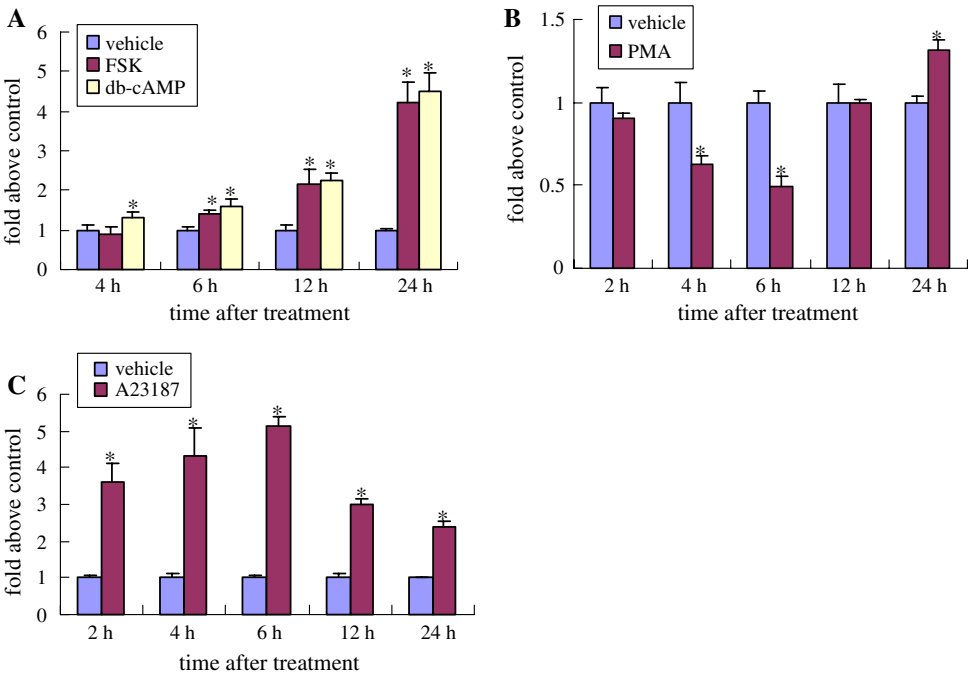

Fig. 3 Real-time PCR analysis of OCIL mRNA from UMR-106 cells treated for the indicated times with signaling activators, i.e., 2 μM forskolin, or 200 μM db-cAMP (**a**), or 100 nM PMA (**b**), or 5 μM A23187 (**c**). The OCIL mRNA levels in the cells treated only with vehicle (DMSO) at the corresponding time points were set as 1. *Significant vs. vehicle, $P < 0.05$

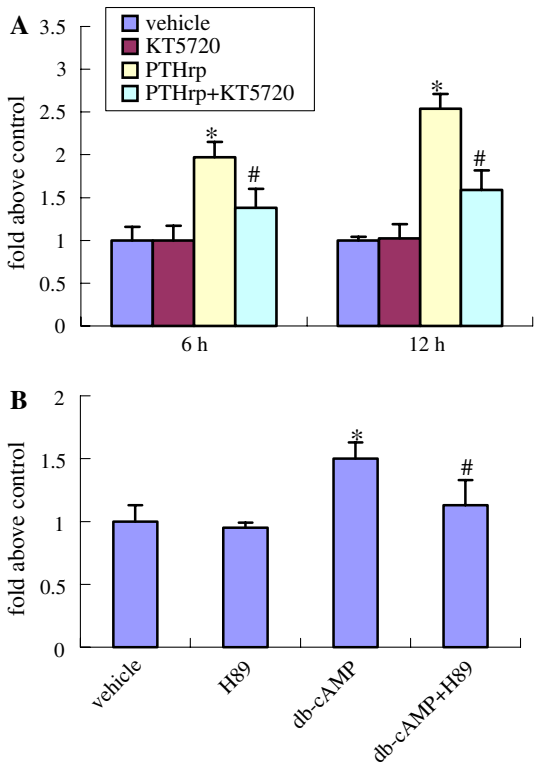

Fig. 4 Real-time PCR analysis of OCIL mRNA levels from UMR-106 cells treated with PKA signaling inhibitors. Cells were pretreated with KT5720 (1 μM) or DMSO for 1 h, incubated in the presence or absence of 10 nM PTHrp(1–34) for the indicated times, and subjected to RNA studies (**a**). In an independent experiment, cells were pretreated with H89 (5 μM) or DMSO for 1 h, followed by 200 μM db-cAMP treatment for an additional 6 h (**b**). The OCIL mRNA levels in the cells treated only with vehicle (DMSO) at the corresponding time points (control) were set as 1. *Significant vs. control, $P < 0.05$; #Significant vs. PTHrp(1–34) treatment (**a**) or vs. db-cAMP treatment (**b**), $P < 0.05$

data confirmed that PKA activation is involved in the up-regulation of OCIL by PTHrp(1–34). In contrast, KT5720 had no any effect on basal OCIL expression.

We then examined whether PLC is involved in PTHrp(1–34) regulation of OCIL. Selective PLC inhibitor U73122 was used to treat the cells. U73122 blocked the PTHrp-stimulated OCIL mRNA expression by about 80% at 6 h, although it did not significantly reverse OCIL induction at 12 h (Fig. 5a). The data suggested that at least one pathway downstream of PLC might have a stimulatory effect on OCIL expression, which was consistent with the previous finding that calcium ionophore A23187 dramatically elevated OCIL expression.

Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) is a ubiquitously expressed ser/thr protein kinase that is activated by Ca^{2+} and calmodulin and has been implicated in the regulation of cell cycle and transcription [15]. We observed in this work that calmodulin antagonist W-7 (50 μM) and selective Ca^{2+}/CaMK II inhibitor KN-62 (10 μM) blunted PTHrp-induced OCIL expression by 46% and 60% at 6 h, and by 61% and 42% at 12 h, respectively (Fig. 5b). In addition, W-7 significantly abolished stimulation of OCIL by calcium ionophore A23187 at 2 h (Fig. 5c). In contrast, these agents did not reduce the OCIL levels in control. These data demonstrate the involvement of Ca^{2+}/CaMK II activation in PTHrp(1–34) induction of OCIL.

On the contrary, the preincubation of cells with selective PKC inhibitor chelerythrine (5 μM) followed by PTHrp (1–34) treatment led to an insignificant increase of OCIL versus PTHrp(1–34) alone ($P > 0.05$). Chelerythrine alone

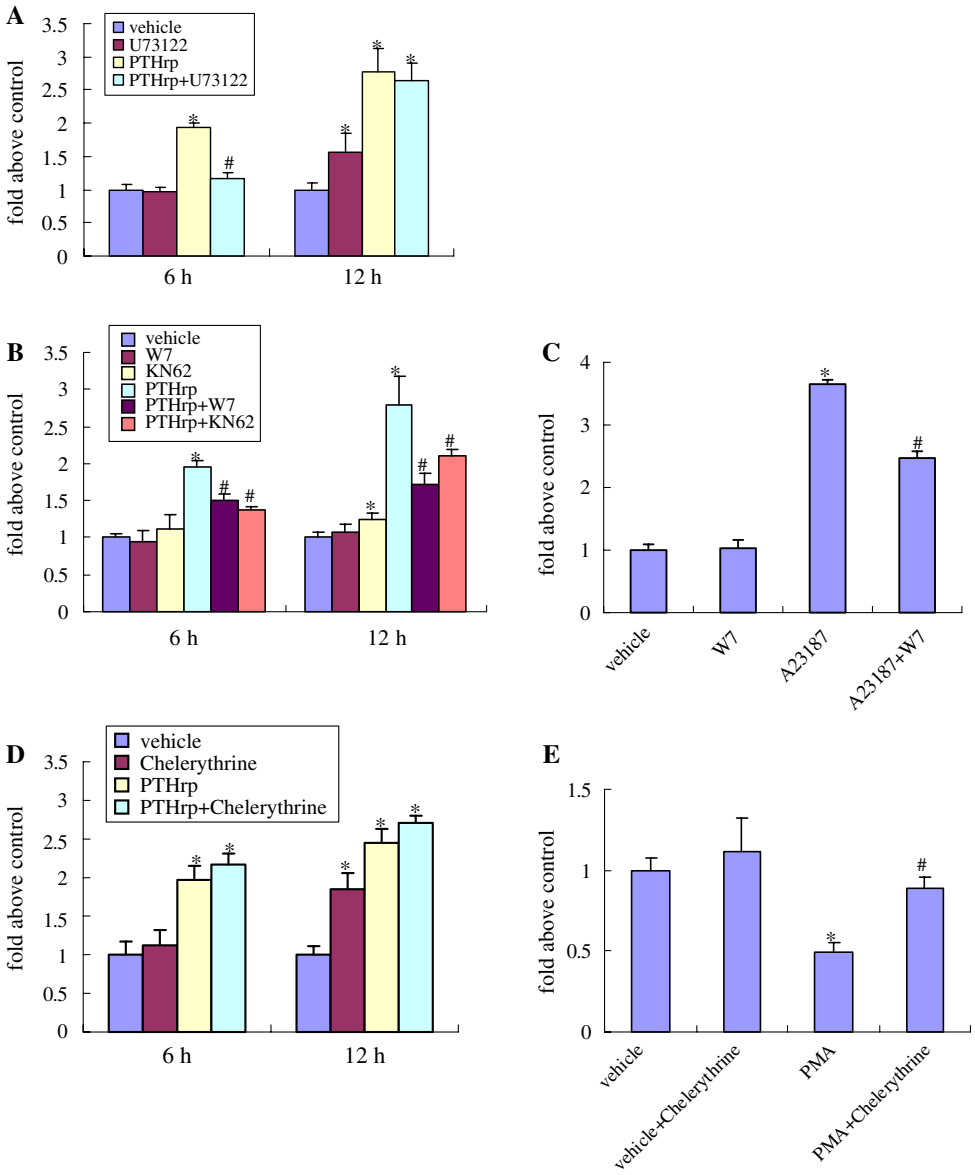

Fig. 5 Real-time PCR analysis of OCIL mRNA from UMR-106 cells treated with PLC, PKC, or Ca^{2+}/Calmodulin signaling inhibitors. UMR-106 cells were pretreated with or without 1.5 μM U73122 (**a**), 50 μM W-7 or 10 μM KN-62 (**b**), or 5 μM chelerythrine (**d**) for 1 h, incubated in the presence or absence of 10 nM PTHrp(1–34) for the indicated times, and subjected to OCIL mRNA analysis. In an independent experiment, UMR-106 cells were pretreated with W-7 (50 μM) or vehicle (DMSO) for 1 h, followed by the treatment with 5 μM A23187 for an additional 2 h (**c**). In another experiment, cells were pretreated with or without chelerythrine (5 μM) for 1 h, followed by the treatment with 100 nM PMA or DMSO for an additional 6 h (**e**). The OCIL mRNA levels in the cells treated only with vehicle (water in (**a**) or DMSO in other experiments) at the corresponding time points were set as 1. *Significant vs. control, $P < 0.05$; #Significant vs. PTHrp(1–34), PMA, or A23187 treatment, $P < 0.05$

even increased OCIL level by 1.8-fold at 12 h (Fig. 5d). Moreover, the disruption of OCIL expression by PMA at 6 h could be almost completely rescued by chelerythrine (Fig. 5e). Collectively, it implies that PKC might probably exert an inhibitory effect on OCIL expression.

Moreover, we examined the effects of the signaling inhibitors on OCIL induction by PTH(3–34). As chelerythrine significantly increased PTHrp(3–34)-induced OCIL expression, W-7 dramatically reduced OCIL induction (Fig. 6).

The mitogen-activated protein kinase (MAPK) signaling cascade has recently received attention as essential downstream targets of G protein-coupled receptor signaling [16]. In this work, PD98059, a highly selective inhibitor of MAPK kinase (MAPKK, MEK) activation and MAPK cascade, was effective in blocking in the cells the PTHrp-induced production of OCIL mRNA, by 60% at 6 h and by 79% at 12 h, respectively. Nevertheless, it failed to show significant inhibitory effect in control cells (Fig. 7a).

There exist interactions among different signaling pathways

To further understand the molecular mechanism underlying the OCIL induction, we subsequently investigated whether the OCIL induction triggered by PKA activators and calcium ionophore could be reversed by MAPK signaling inhibitor in UMR106 cell. At 6 h, PD98059 pretreatment attenuated the induction of OCIL by PKA activator forskolin or db-cAMP, albeit to different degrees (Fig. 7b). In

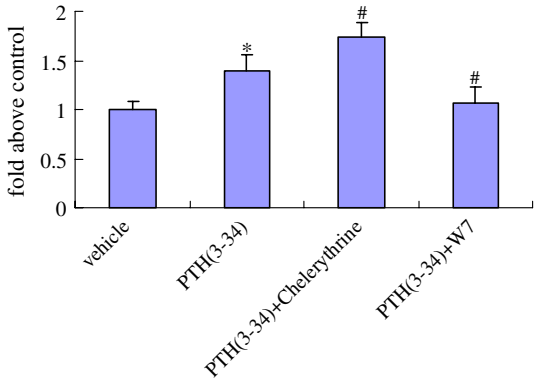

Fig. 6 Real-time PCR analysis of signaling pathways involved in PTH(3–34) induction of OCIL. UMR-106 cells were pretreated with or without 5 μM chelerythrine or 50 μM W-7, followed by the treatment with or without 10 nM PTHrp(3–34) for an additional 6 h. *Significant vs. control, $P < 0.05$; #Significant vs. PTHrp(3–34) treatment, $P < 0.05$

contrast, it did not affect the OCIL levels induced by A23187 (Fig. 7c). This suggested that MAPK activation is mediated through PKA signaling.

PTHrp(1–34) regulates OCIL expression in primary osteoblastic cells

As shown in Fig. 8, expression of OCIL in primarily cultured osteoblasts was assessed. PTHrp(1–34) stimulated OCIL expression by about 2-fold after 6 h treatment. This effect was inhibited by KT5720, by W-7 and to the greatest degree, by PD98059. In contrast, inhibition of PKC by chelerythrine showed a significant additive effect on PTHrp-induced OCIL expression ($P < 0.05$).

Discussion

PTH was reported to stimulate OCIL expression, though the signaling cascades involved remain unknown [1]. PTHrP(1–34) and PTH(1–34) are known to affect osteoblast metabolism through the common type-1 PTH/PTHrP receptor [17]. Accordingly, we have provided evidence herein demonstrating that PTHrP(1–34) stimulates OCIL mRNA expression. This effect is not restricted to UMR-106 cells because PTHrP(1–34) up-regulated OCIL mRNA in rat calvarial primary osteoblasts and murine MC3T3-E1 cells as well.

A variety of osteotropic factors that increased RANKL levels were believed to induce OCIL expression. In contrast, OPG expression was decreased by these factors [1, 2]. This raises the hypothesis that OCIL functions as a tonic inhibitor of RANKL actions under physiological conditions in bone. In this work, we demonstrated that the regulation of RANKL and OPG by PTHrp(1–34) preceded that of OCIL. Similar results were obtained when we used PGE_2 and PTH(1–34) to treat UMR-106 cells (Zheng, unpublished). These data tend to support the above hypothesis. PTHrp(1–34), PTH(1–34), and PGE_2 as well, only modestly induced OCIL expression by about 3-fold in all

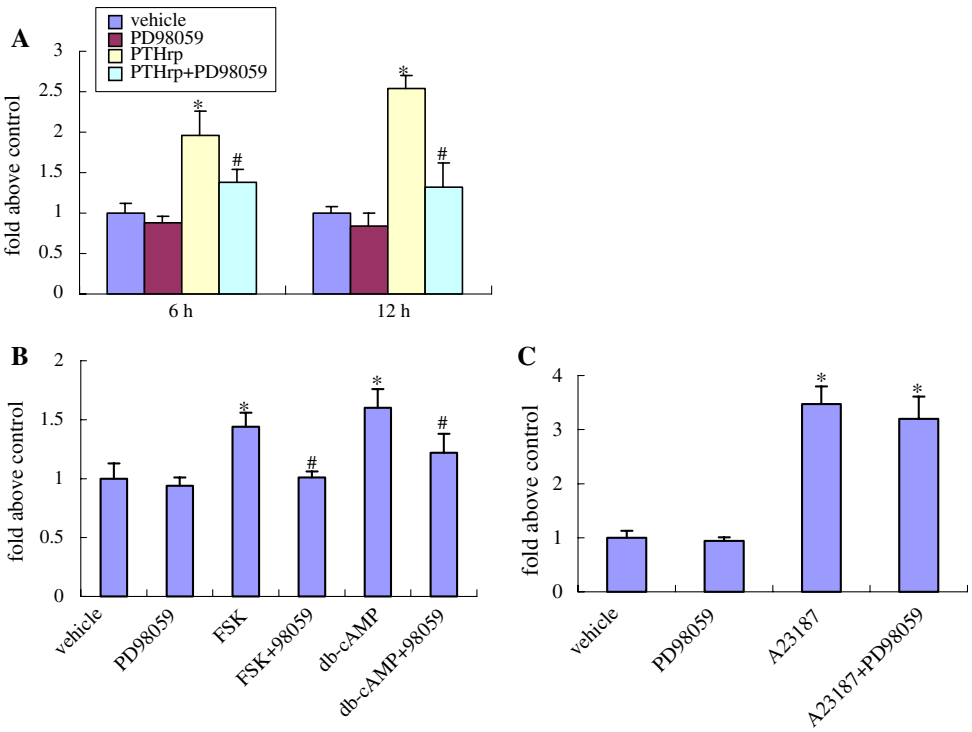

Fig. 7 Real-time PCR analysis of OCIL mRNA levels from UMR-106 cells treated with MAPK signaling inhibitor. UMR-106 cells were pretreated with PD98059 (50 μM) or vehicle (DMSO) for 1 h, incubated in the presence or absence of 10 nM PTHrp(1–34) for the times as indicated (**a**), or incubated with 2 μM forskolin, or 200 μM db-cAMP (**b**) for an additional 6 h, or 5 μM A23187 (**c**) for an additional 2 h, and subjected to OCIL mRNA analysis. The OCIL mRNA levels in the cells treated only with vehicle (DMSO) at the corresponding time points (control) were set as 1. *Significant vs. control, $P < 0.05$; #Significant *vs.* PTHrp(1–34) (**a**) or the corresponding activator treatment (**b**), $P < 0.05$

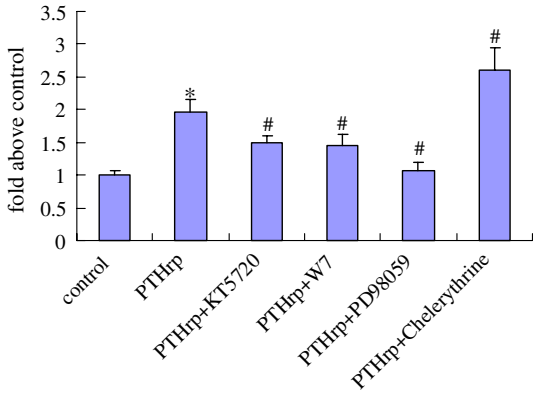

Fig. 8 Real-time PCR analysis of signaling pathways involved in PTHrp(1–34) induction of OCIL in rat primary osteoblasts. The cells were pretreated with or without KT5720, W-7, PD98059, or chelerythrine for 1 h, incubated in the presence or absence of 10 nM PTHrp(1–34) for 6 h. *Significant vs. control, $P < 0.05$; #Significant vs. PTHrp(1–34) treatment, $P < 0.05$

repeated experiments. The modest increase of OCIL might explain why the OCIL induction could only somewhat blunt but not "stop" the osteoclast formation induced by these agents. Thus, OCIL induction by osteotropic factors including PTHrp(1–34) might be interpreted as a "self-balance" mechanism of osteoblastic cells. However, this mechanism seems indispensable in limiting excessive bone resorption since knockdown of OCIL expression using siRNA in osteoblastic cells tend to enhance osteoclast formation (Zheng, unpublished).

The following evidences presented in this work suggest that PTHrp(1–34) regulation of OCIL involves cAMP/PKA signaling: (1) PTH(1–31), a ligand of the type-1 PTH/PTHrP receptor that activates only PKA increased OCIL mRNA; (2) PKA signaling activators forskolin and db-cAMP mimicked PTHrP(1–34) effects on OCIL; (3) selective adenylate cyclase inhibitor KT5720 blocked the effects of PTHrP(1–34).

Interestingly, PKC activation also regulates OCIL expression. However, it does not elicit stimulation, but inhibition instead. We observed that PMA, which is known to stimulate PKC in short term but inhibit PKC in long term, reduced OCIL expression at 4 and 6 h, but slightly increased OCIL level at 24 h. The inhibition at 6 h could be completely abolished by selective PKC inhibitor chelerythrine, and the pretreatment of cells with chelerythrine led to an insignificant increase versus PTHrp(1–34) alone.

PTH(3–34) cannot stimulate PKA activation due to the loss of two N-terminal residues. But it is still capable of stimulating PKC and increasing intracellular Ca^{2+} concentration. Our work using PTH(3–34) confirmed that it slightly stimulated OCIL expression. The fact that PKC pathway inhibits rather than stimulates OCIL expression suggests that the induction of OCIL in response to PTH(3–34) is mediated through Ca^{2+}-dependent signaling. This was further demonstrated by the experiments using signaling inhibitors.

PLC inhibitor U73122 blocked the PTHrp-stimulated OCIL mRNA expression at 6 h, suggesting that at least one pathway downstream of PLC promotes OCIL expression. This was consistent with the finding that calcium ionophore A23187 substantially increase OCIL mRNA levels. Quinn et al. reported that UMR-106 cells contain CaMK II [18]. We demonstrated that calmodulin antagonist W-7 and CaMK II inhibitor KN-62 both disrupted PTHrp(1–34)-stimulated OCIL production. Therefore, we postulate that phosphorylation event(s) through PTHrp(1–34) activation of CaMK II is (are) an important component of the PTHrp-signaling process for OCIL expression.

In recent years there is the growing evidence that PTH and PTHrp also activate the MAPK cascade through both PKA and PKC in a G protein-dependent manner to regulate cell growth, differentiation, apoptosis, and gene expression [19–22]. In this work, we observed that MAPK cascade inhibitor PD98059 blocked in UMR-106 cells the production of OCIL mRNA induced by PTHrp(1–34). In the subsequent cross-talk studies we proved that MAPK activation might be mediated by cAMP/PKA signaling since PD98059 attenuated the induction of OCIL by forskolin and db-cAMP, but not that by A23187.

We see no effect of PTHrp(1–34) on OCIL at the early stage (2–4 h), though A23187 and db-cAMP both stimulated OCIL expression before 6 h after treatment. The finding that PKC activation inhibited OCIL expression gives the explanation. PTHrp(1–34) effects on OCIL include two aspects: one is the stimulatory effect mediated through cAMP/PKA, Ca^{2+}/CaMK II, and MAPK signaling, the other inhibitory effect via PKC pathway. These opposite actions neutralized each other at the early stage, resulting in no observable net change of OCIL. At later stage, the stimulatory effect overweighed the inhibitory effect, leading to a net effect of OCIL induction. We demonstrated that the above mentioned signaling pathways also mediate the PTHrp induction of OCIL expression in primary osteoblasts.

In conclusion, our work demonstrates that PTHrP(1–34) up-regulates OCIL expression through activation of PKA, MAPK, and Ca^{2+}/CaMK II transduction pathways. Maybe the cloning and analysis of OCIL promoter sequence, which is now undertaken in our lab, will help to further clarify the mechanism of OCIL regulation.

Materials and methods

Reagents

Synthetic human PTHrP(1–34), PTH(1–31), PTH(1–34), and bovine PTH(3–34) were obtained from Phoenix

(St Joseph, MO). Forskolin, db-cAMP, and calcium ionophore A23187 were from Biomol (Plymouth Meeting, PA). Phorbol 12-myristate 13-acetate (PMA), H-89, PD98059, U73122, KN-62, and W-7 were from Alexis (San Diego, CA). KT5720 and chelerythrine were from Calibiochem (San Diego, CA). TRI reagent was from MRC (Cincinnati, OH). SYBR Green PCR Mix was from Toyobo (Japan).

Cell culture

Stock cultures of UMR-106 cells were maintained and passaged every 3–4 days when reaching confluence in DMEM/F12 media containing 10% fetal bovine serum (FBS). About 2×10^5 cells were plated in T-25 flasks in 5 ml DMEM/F12 containing 10% FBS. When cells reached 70-80% confluence, medium was replaced with serum-free DMEM/F12 supplemented with 0.1% BSA and the cells were incubated overnight before the addition of appropriate agents.

Rat calvarial primary osteoblastic cells were obtained from neonatal rat calvariae by sequential digestions with collagenase and trypsin using previously described procedures [23]. Cells were cultured in α-MEM containing 10% FBS until confluence at day 3. Then the cells were passaged and the medium was switched to differentiation medium (α-MEM medium containing 10% FBS, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid). MC3T3-E1 cells were maintained in DMEM plus 10% FBS. After confluence, cells were passaged and cultured in the differentiation medium described above. Before treatments, both types of cells were serum starved in DMEM for 1 day. Treatments of cells were done at 70–80% confluence.

In vitro treatments with agents

To study the regulation of OCIL expression by PTHrp (1–34), UMR-106, MC3T3-E1, or rat calvarial primary osteoblastic cells were incubated with PTHrp(1–34) at indicated doses for indicated time intervals followed by RNA studies. For time dependence studies, 10 nM PTHrp(1–34) was added to UMR-106 cultures for indicated times.

To examine the involvement of signaling pathways in PTHrp(1–34) regulation of OCIL, specific signaling activators or vehicles were added to the UMR-106 cultures followed by incubation for indicated times before RNA extraction.

For inhibitor studies, specific inhibitors that block cAMP/PKA, PLC, PKC, MAPK, or Ca^{2+}-calmodulin signaling or vehicles were added to the serum-free media for 1 h, respectively. Cultures were then treated with or without PTHrp(1–34) or signaling activators for an additional 6 or 12 h.

To evaluate whether there is any interaction among different pathways, specific inhibitor that blocks MAPK signaling was added to the serum-free media for 1 h. Cultures were subsequently treated with or without cAMP/PKA activator or calcium ionophore. After an additional 6 h, RNA extraction was done.

Forskolin, A23187, PMA, H-89, KT5720, PD98059, U73122, KN-62, and W-7 were dissolved in dimethylsulfoxide; peptides, db-cAMP, and chelerythrine in distilled water. Dimethylsulfoxide had no effect on OCIL mRNA levels.

In each experiment, the control received vehicle solvent only and incubated for the same time intervals as those treated with the agents described above before RNA studies. All cultures were maintained in a humidified 5% CO_2 atmosphere at 37°C.

RNA extraction and real-time reverse transcription-polymerase chain reaction

Cells were harvested at the indicated time points after treatments. Total RNA was isolated using TRI reagent according to the manufacturer's recommended protocol. For reverse transcriptase reaction, 2 μg total RNA was converted to cDNA by M-MLV reverse transcriptase using random hexamer primers in a 25 μl reaction system. Then 1.5 μl was used for PCR. Real-time PCR amplifications were done on Lightcycler (Roche) apparatus using gene-specific primers and SYBR Green real-time PCR kit. The primers used for the PCR are summarized in Table 1. Since there are several OCIL-related genes for both rat OCIL and murine OCIL, the primers for OCIL were strictly designed to be specific and checked online (www.ncbi.nlm.nih.gov/blast) to exclude the possibility to amplify the OCIL-related genes. PCR amplification reactions were performed in 20 μl containing primers at 0.5 μM and dNTPs (0.2 mM each) along with SYBR Green PCR mix. Aliquots of purified recombinant plasmids harboring respective amplified genes were diluted appropriate fold to generate relative standard curves to which sample cDNA was compared [24].

For OCIL, RANKL, and OPG amplification, the thermal cycling conditions were as follows: an initial denaturation at 95°C for 3 min, followed by 40 cycles of 10 s of denaturing at 95°C, 10 s of annealing at 58°C, and 10 s of extension at 72°C. β-actin or 18S ribosomal RNA (18S were used in the studies of transcriptional and translational regulators and signaling inhibitors, and β-actin in other experiments) was amplified as internal control and the temperature cycling was the same as above described for OCIL. OCIL, RANKL, or OPG amount was normalized to that of β-actin or 18S in the RT sample. Real-time PCR efficiencies were calculated using the formula: Efficiency

Table 1 The primers used for real-time PCR studies

Gene name	Primer sequence	Size of PCR product (bp)
Rat OCIL	Forward: 5′-CTGAGTATAACAACTCGGTTTC-3′	190
	Reverse: 5′-GTGTTCCTCATGACTGTTAG-3′	
Rat RANKL	Forward: 5′- CCGTGCAAAGGGAATTACAAC-3′	134
	Reverse: 5′- GATGGTGAGGTGAGCAAACG -3′	
Rat OPG	Forward: 5′-TCAGAAAGGAAATGCAACAC-3′	130
	Reverse: 5′-CGGTATAATCTTGGTAGGCAC -3′	
Rat β-actin	Forward: 5′-GAACCCTAAGGCCAACCGTG-3′	104
	Reverse: 5′-AGGCATACAGGGACAACACAGC-3′	
Rat 18S	Forward: 5′-TCGAACGTCTGCCCTATCAAC-3′	105
	Reverse: 5′-TTTCTCAGGCTCCCTCTCCG-3′	
Murine OCIL	Forward: 5′-TCCCTACCTATGCTTAGTCC-3′	117
	Reverse: 5′-GTAGCAGTAAAGCTTAGCAC-3′	
Murine β-actin	Forward: 5′-TCAGGTTACTGGTTCGGTCTG-3′	109
	Reverse: 5′-ACCAGAGGCATACAGGGACAG-3′	

$(E) = [10^{(1/\text{slope})}] - 1$. The efficiencies of reactions for all the amplified genes were more than 90%.

To confirm the identity of the PCR products, the amplified fragments were sequenced by Shanghai Shenggong biotech company.

Protein extraction and western blotting

After treatments, MC3T3-E1 cells were washed twice with PBS and lysed in lysis buffer [50 mmol/l Tris–Cl (pH 8.0), 150 mmol/l NaCl, 0.02% NaN_3, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1% Triton X-100] and centrifuged for 5 min at 4°C. The supernatant was then frozen in aliquots at −70°C. For protein analysis, 50 μg protein was resolved by SDS-PAGE on a 15% gel, transferred to a nitrocellulose membrane. The membrane was blocked overnight with 5% nonfat milk and then incubated for 3 h with an anti-OCIL monoclonal antibody (Acris GmbH, Germany) and then for 1 h with horseradish peroxidase-conjugated goat anti-murine immunoglobulin G (Santa Cruz Biotechnology, USA). Immunoreactive bands were visualized using a Western blot lightning kit (Tiangen biotech company).

Statistical analysis

Statistical analysis was performed by "*t*" test or one-way analysis of variance (ANOVA) and Student–Newman–Keuls test, when ANOVA demonstrated significances. A P value of <0.05 was regarded as significant.

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