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## PDE4 inhibitor suppresses PGE<sub>2</sub>-induced osteoclast formation *via* COX-2-mediated p27<sup>KIP1</sup> expression in RAW264.7 cells

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We investigated the effects of phosphodiesterase 3 (PDE3) and PDE4 inhibitors, which are cAMP degrading enzymes, on prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)-induced osteoclast formation. A PDE4 inhibitor decreased PGE<sub>2</sub>-induced osteoclast formation, whereas a PDE3 inhibitor did not, possibly due to the lack of PDE3 expression in RAW 264.7 cells. Cell cycle analysis revealed that the PDE4 inhibitor stimulated PGE<sub>2</sub>-induced p27<sup>KIP1</sup> expression, which leads to increased growth arrest at G<sub>0</sub>/G<sub>1</sub> phase. The PDE4 inhibitor increased cyclooxygenase 2 (COX-2) expression in the presence of PGE<sub>2</sub>. COX-2 overexpression was associated with growth suppression *via* p27<sup>KIP1</sup> expression in RAW 264.7 cells. Taken together, our data demonstrate that the PDE4 inhibitor enhances PGE<sub>2</sub>-induced growth arrest of osteoclast precursors *via* COX-2-mediated p27<sup>KIP1</sup> expression, which in turn negatively regulates osteoclast formation.

### 1. Introduction

Bone remodeling depends on continual resorption of bone by osteoclasts and its replacement by osteoblasts. Imbalances in this process disrupt the maintenance of bone homeostasis, which in turn leads to pathogenic conditions such as osteoporosis, rheumatoid arthritis, lytic bone metastases, or Paget's bone disease (Rodan and Martin 2000).

Osteoclasts differentiate from monocyte-macrophage lineage cells under the tight regulation of osteoblasts (Takahashi et al. 1988; Suda et al. 1999; Wong et al. 1997). Osteoblasts express two cytokines essential for osteoclast differentiation: receptor activator of NF- $\kappa$ B ligand (RANKL, also known as TRANCE, ODF, or OPGL), and macrophage colony-stimulating factor (M-CSF) (Takahashi et al. 1988; Suda et al. 1999; Wong et al. 1997; Yasuda et al. 1998). Osteoclast precursors express RANK and c-Fms, receptors of RANKL and M-CSF, respectively, and differentiate into osteoclasts in the presence of both cytokines. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is an important regulator of many physiological and pathological processes of bone metabolism and is generally believed to modulate bone resorption. PGE<sub>2</sub> stimulates osteoclastic bone resorption by inducing RANKL expression in osteoblasts and by directly enhancing RANKL-induced differentiation of osteoclast precursor cells into osteoclasts (Kobayashi et al. 2005). PGE<sub>2</sub> exerts its effects through specific receptors that consist of four subtypes designated EP1, EP2, EP3, and EP4 (Sugimoto et al. 2007). Recent studies have demonstrated that PGE<sub>2</sub> actions on osteoclasts are mediated *via* EP4 and/or EP2, which increase adenylate cyclase activity, cAMP production, and protein kinase A (PKA) activity.

We previously reported that the inhibition of phosphodiesterase 4 (PDE4), a cAMP-degrading enzyme, enhances PGE<sub>2</sub>-induced RANKL mRNA expression in osteoblasts (Noh et al. 2009). However, PDE4 action on PGE<sub>2</sub>-induced osteoclast formation has not been investigated. Thus, in the present study, we clarified

how PDE4 is involved in PGE<sub>2</sub>-induced osteoclast formation using a PDE4 inhibitor. Understanding the mode of interaction between PGE<sub>2</sub> and PDE4 should have important pharmacological and clinical implications for bone-related diseases.

### 2. Investigations and results

PGE<sub>2</sub> enhances RANKL-induced osteoclast formation in cultures of mouse bone marrow macrophages and RAW264.7 cells (Wani et al. 1999; Kobayashi et al. 2005). Because we previously found that PGE<sub>2</sub>-induced osteoclast formation is augmented by a PDE3 inhibitor (i.e., milrinone) and a PDE4 inhibitor (i.e., rolipram) indirectly *via* RANKL in osteoblasts (Noh et al. 2009), we examined the direct effects of milrinone and rolipram on osteoclast precursors. In accordance with a previous study, PGE<sub>2</sub> enhanced RANKL-induced TRAP-positive osteoclast formation in RAW264.7 cells. Rolipram inhibited RANKL-induced osteoclast formation in the presence of PGE<sub>2</sub>, whereas milrinone had no effect (Fig. 1A). The inhibitory effect of rolipram on osteoclast formation in the presence of PGE<sub>2</sub> was concentration-dependent, with a maximal effect at 10  $\mu$ M (Fig. 1B). To assess which stage was critical for inhibition of osteoclastogenesis by PGE<sub>2</sub> and rolipram, we split the culture period into days 0–1.5 and days 1.5–4. Osteoclastogenesis was inhibited when PGE<sub>2</sub> and rolipram were added to the culture medium at earlier (days 0–1.5) or later stages (days 1.5–4) of differentiation, and complete inhibition was shown by the entire period treatment (days 0–4; Fig. 1C). These results suggest that PGE<sub>2</sub> and rolipram affect molecules that act in both early and late periods of osteoclast differentiation in the presence of M-CSF and RANKL. To understand the mechanism by which a PDE4 inhibitor, but not a PDE3 inhibitor, affects osteoclast formation in the presence of PGE<sub>2</sub>, we investigated the expression patterns of PDE3 and PDE4 in RAW264.7 cells. Two PDE3 isoforms (3A and 3B)

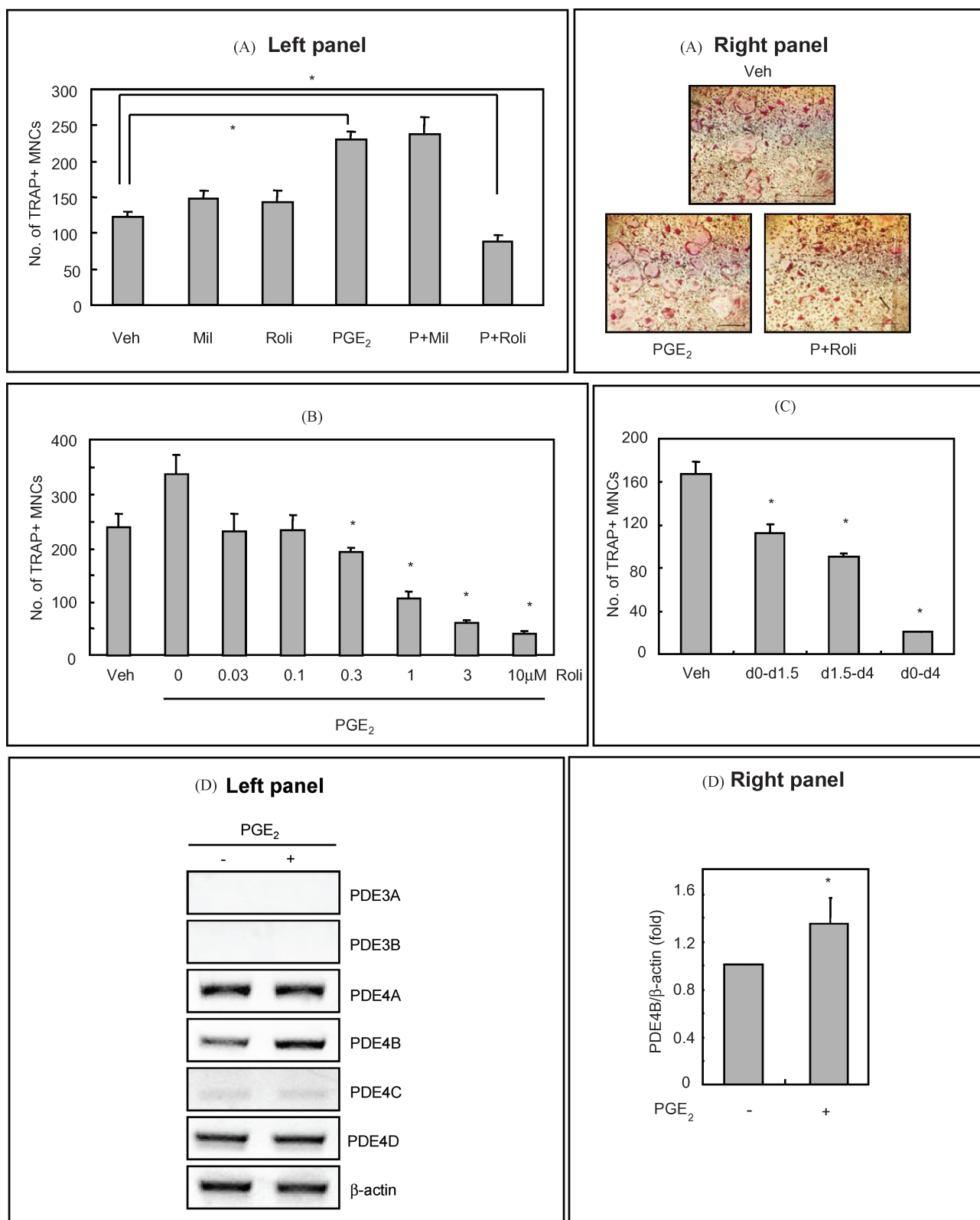


Fig. 1: The effects of PDE3 and PDE4 inhibitors on PGE<sub>2</sub>-induced osteoclast formation (A-C) RAW264.7 cells were cultured with or without 100 ng/ml RANKL for 4 days. Cells were then fixed and stained for TRAP. TRAP-positive (+) multinucleated cells were counted. All data are expressed as means + S.D. of triplicate cultures. (A) Milrinone (PDE3 inhibitor; 10 µM) or rolipram (PDE4 inhibitor; 10 µM) was added in the absence or presence of PGE<sub>2</sub> (100 nM) for 4 days. (B) Indicated rolipram concentrations were added in the absence or presence of PGE<sub>2</sub> (100 nM) for 4 days or (C) 10 µM rolipram was added in the presence of PGE<sub>2</sub> for the indicated time intervals. (D) RAW264.7 cells were cultured in the absence or presence of 100 nM PGE<sub>2</sub> for 3 h. PDE3 and PDE4 isotype mRNA expression was determined by RT-PCR (left panel). Band intensity of PDE4B was determined using image J software and expressed as a percentage relative to the mRNA level in cells before adding PGE<sub>2</sub> (right panel). \* *p* < 0.05 compared to vehicle. Scale bar, 200 µM

and four PDE4 isoforms (4A–D) have been identified (Houslay et al. 1997). In RT-PCR analysis, PDE4A, 4B, and 4D mRNA were detectable under basal conditions (Fig. 1D, left panel). Furthermore, treatment with PGE<sub>2</sub> produced a major

increase in PDE4B mRNA in RAW264.7 cells (Fig. 1D). Conversely, PDE3A, 3B, and 4C mRNA were not detected, which explains the lack of milrinone responsiveness in RAW264.7 cells (Fig. 1D, left panel).

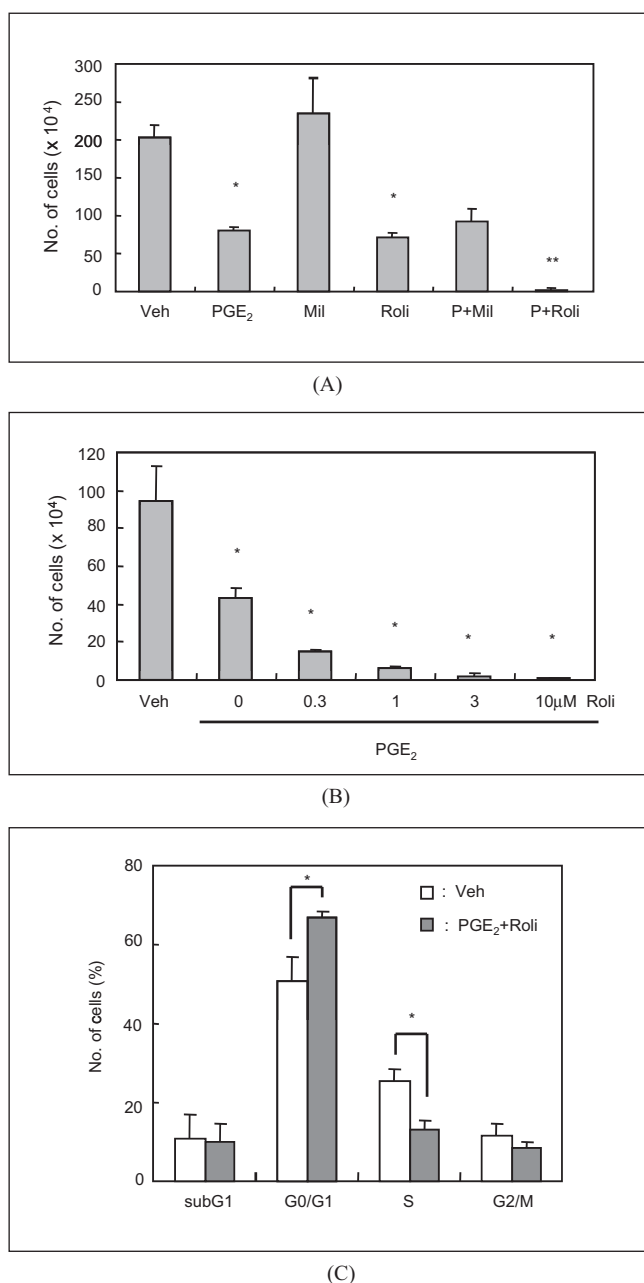


Fig. 2: The effect of PDE4 inhibitor on cell growth in the presence of PGE<sub>2</sub> in RAW264.7 cells. Cells were cultured with milrinone (10 μM) or rolipram (10 μM) in the absence or presence of PGE<sub>2</sub> (100 nM) for 3 days. \* $p < 0.05$  compared to vehicle, \*\* $p < 0.05$  compared to PGE<sub>2</sub>. Indicated rolipram concentrations were added in the presence of PGE<sub>2</sub> (100 nM) for 3 days. \* $p < 0.05$  compared to vehicle. Cells were treated with PGE<sub>2</sub> plus rolipram for 36 h and cell cycle stage was determined by FACS analysis. \* $p < 0.05$ . Data are expressed as means + S.D. of triplicate cultures.

In the process of osteoclast formation, osteoclast precursors undergo a transient proliferation phase (Okahashi et al. 2001; Sankar et al. 2004; Kwak et al. 2005) and then differentiate into osteoclasts in response to M-CSF and RANKL. Because rolipram decreased osteoclast formation at an early stage in the presence of PGE<sub>2</sub> (Fig. 1C), we explored the possibility that rolipram with PGE<sub>2</sub> suppresses the growth of osteoclast precursors. RAW264.7 cells were exposed to rolipram for 3 days in the presence of PGE<sub>2</sub> and the cell growth was measured by trypan blue exclusion assay. We found that milrinone did not potentiate PGE<sub>2</sub>-induced growth suppression (Fig. 2A). In contrast, rolipram enhanced the growth suppression induced by PGE<sub>2</sub>. RAW264.7 cells displayed concentration-related growth suppression in response to increasing concentrations of rolipram (Fig. 2B).

Cell population growth depends on the balance of proliferation and death in that population. The effects of PGE<sub>2</sub> and rolipram on cell proliferation were investigated by flow cytometry in propidium iodide (PI)-stained cells after 36 h (Fig. 2C). Rolipram with PGE<sub>2</sub> caused a significant accumulation of cells in the G<sub>0</sub>/G<sub>1</sub> phase and an accompanying decrease in the S phase compared to vehicle-treated cells. At this time point we did not find any changes in the sub-G<sub>1</sub> phase, an index of apoptotic DNA fragmentation. Thus, growth inhibition by PGE<sub>2</sub> and rolipram resulted from cell cycle arrest.

Cyclins and cyclin-dependent kinases (CDKs) cooperate to promote progression from the G<sub>1</sub> phase to the S phase. Cyclin-dependent kinase inhibitors (CKIs) interfere with cell cycle progression to cause phase-specific cell cycle arrest (Elledge and Harper 1994). Because cyclin D1 and CDK inhibitor p27<sup>KIP1</sup> regulate the proliferation of osteoclast precursors (Zhou et al. 2006), we first determined whether rolipram affects the expression of these G<sub>1</sub>-related proteins in cells treated with PGE<sub>2</sub>. Under normal culture conditions, up-regulation of cyclin D1 and down-regulation of p27<sup>KIP1</sup> were observed in osteoclast precursors (Fig. 3A, left panel and 3B, left panel). We found no consistent difference in cyclin D1 expression following PGE<sub>2</sub> and rolipram treatment (Fig. 3A, right panel). However, for both PGE<sub>2</sub> and rolipram, p27<sup>KIP1</sup> expression was higher 3 h after treatment and persisted for 48 h after treatment (Figs. 3B, right panel and 3C). Rolipram increased p27<sup>KIP1</sup> expression in a concentration-dependent manner in the presence of PGE<sub>2</sub> (Fig. 3D). These results imply that the combination of PGE<sub>2</sub> and rolipram induces cell cycle arrest by inducing p27<sup>KIP1</sup>, which normally inhibits progression in the G<sub>1</sub>-S phases of the cell cycle.

PGE<sub>2</sub> induces COX-2 expression (Hinz et al. 2000). Thus, we investigated COX-2-mediated growth arrest by PGE<sub>2</sub> and rolipram in RAW264.7 cells. We first confirmed that rolipram increased COX-2 expression in the presence of PGE<sub>2</sub> in RAW264.7 cells (Fig. 4A). Next, we determined the effects of COX-2 overexpression on proliferation of RAW264.7 cells. Cells were transiently transfected with the mouse COX-2 gene and COX-2 overexpression was associated with a significant extent of growth arrest as indicated by a decrease in proliferation (Fig. 4B). Furthermore, the expression level of p27<sup>KIP1</sup> in COX-2-transfected cells was significantly higher than mock-transfected counterparts, which correlates well with the growth arrest in COX-2-transfected cells (Fig. 4C). These data suggest that overexpression of COX-2 induces growth arrest in osteoclast precursors. Taken together, our results indicate that PDE4 inhibitor with PGE<sub>2</sub> induces growth arrest via COX-2-mediated p27<sup>KIP1</sup> expression in RAW264.7 cells, which in turn negatively regulates osteoclast formation.

### 3. Discussion

Cell proliferation and differentiation are coordinated processes in the development of osteoclasts. Osteoclast precursors grown in co-culture with osteoblasts actively proliferate for the first 4 days, after which they withdraw from the cell cycle by day 5 (Tanaka et al. 1993). When osteoclast precursors are treated with M-CSF and RANKL, they initially proliferate for 2–3 days before differentiating (Mizoguchi et al. 2009). Thus, cell-cycle progression and subsequent withdrawal in osteoclast precursors are required for their differentiation.

The growth of osteoclast precursors strongly influences the level of osteoclastogenesis. For a given cell culture duration, there is an optimal cell number, with reduced the development of osteoclasts at both lower and higher densities (Gardner 2007). In this study, PGE<sub>2</sub> reduced overall cell number in osteoclast

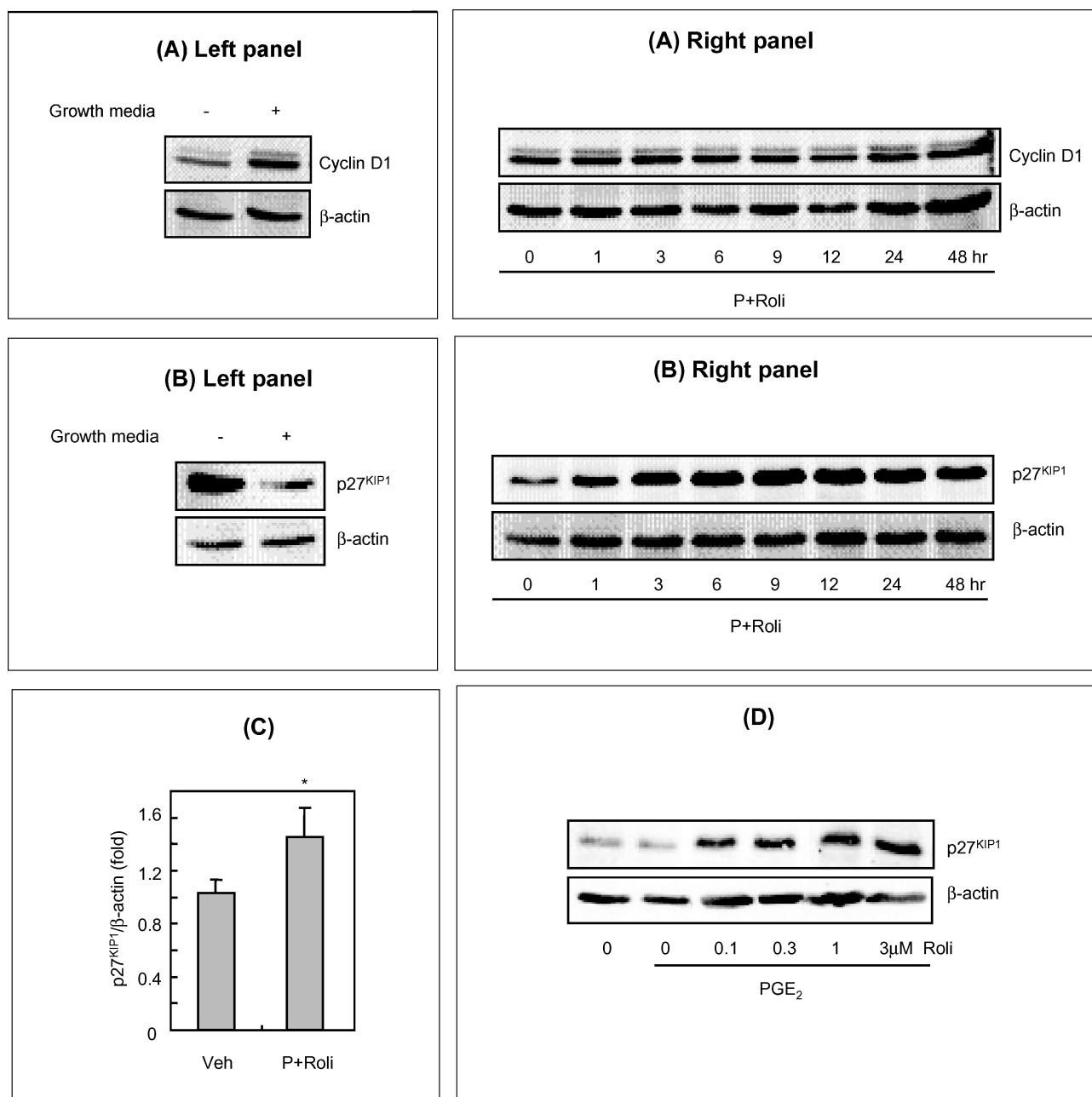


Fig. 3: The effect of PDE4 inhibitor on p27<sup>KIP1</sup> expression in the presence of PGE<sub>2</sub> RAW264.7 cells were cultured without (A and B, left panel) or with rolipram (10 μM) plus PGE<sub>2</sub> (100 nM) for the indicated time intervals and the protein level of cyclin D1 (A, right panel) or p27<sup>KIP1</sup> (B, right panel–D) was determined using an immunoblot analysis with anti-cyclin D1 or p27<sup>KIP1</sup> antibodies. (C) The band intensity of p27<sup>KIP1</sup> after rolipram treatment (10 μM) plus PGE<sub>2</sub> (100 nM) for 3 h is indicated by fold induction. Data are expressed as means + S.D. of triplicates. Concentration-dependent effect of rolipram on p27<sup>KIP1</sup> in the presence of PGE<sub>2</sub> is shown in (D)

precursor cultures, which was strengthened by a PDE4 inhibitor (Fig. 2A). These effects were due to growth arrest of precursors in the G<sub>0</sub>/G<sub>1</sub> phase by the induction of p27<sup>KIP1</sup> (Figs. 2 and 3). Although PGE<sub>2</sub> overcame the hindered cell growth on osteoclast formation, co-treatment of PGE<sub>2</sub> and rolipram failed to do so (Fig. 1A).

Some studies have reported increased cell growth and decreased apoptosis in cells with spontaneous COX-2 up-regulation and in cells engineered to overexpress COX-2 (Cao and Prescott 2002; Han and Wu 2005). In contrast, other studies have reported inhibition of cell growth by forced COX-2 overexpression in many types of cells (Zahner et al. 2002; Xu et al. 2006). Moreover, the importance of the CDK inhibitor p27<sup>KIP1</sup> has been demonstrated in COX-2-dependent cell cycle arrest (Zahner et al. 2002). *via* One factor involved in the differential effects of COX-2 may be the cell or tissue type. In RAW264.7 cells, COX-2 was induced by PGE<sub>2</sub> and the PDE4 inhibitor (Fig. 4A)

COX-2 is associated with their anti-proliferative effects *via* up-regulation of p27<sup>KIP1</sup> (Fig. 4). Paradoxically, COX-2-dependent PGE<sub>2</sub> induction in osteoclast precursors is required for their differentiation into osteoclasts (Han et al 2005). Thus, the balance between the decrease in cell number and the direct osteoclastogenic effects of COX-2 on osteoclast precursors might determine the response.

Although PGE<sub>2</sub> stimulates osteoclastic bone resorption *in vitro*, intermittent PGE<sub>2</sub> administration increases bone mass *in vivo* (Jee and Ma 1997). Detailed analysis of the effects of PGE<sub>2</sub> showed that it does so mainly by increasing bone formation and accelerating osteoclast apoptosis. Recently, the potential use of PDE4 inhibitors for treating osteoporosis has been suggested because rolipram, a PDE4 inhibitor, prevents bone loss in osteopenia models (Yao et al. 2007). These anti-osteoporotic effects were achieved by maintaining elevated bone formation and decreasing bone turnover. Given our data indicating

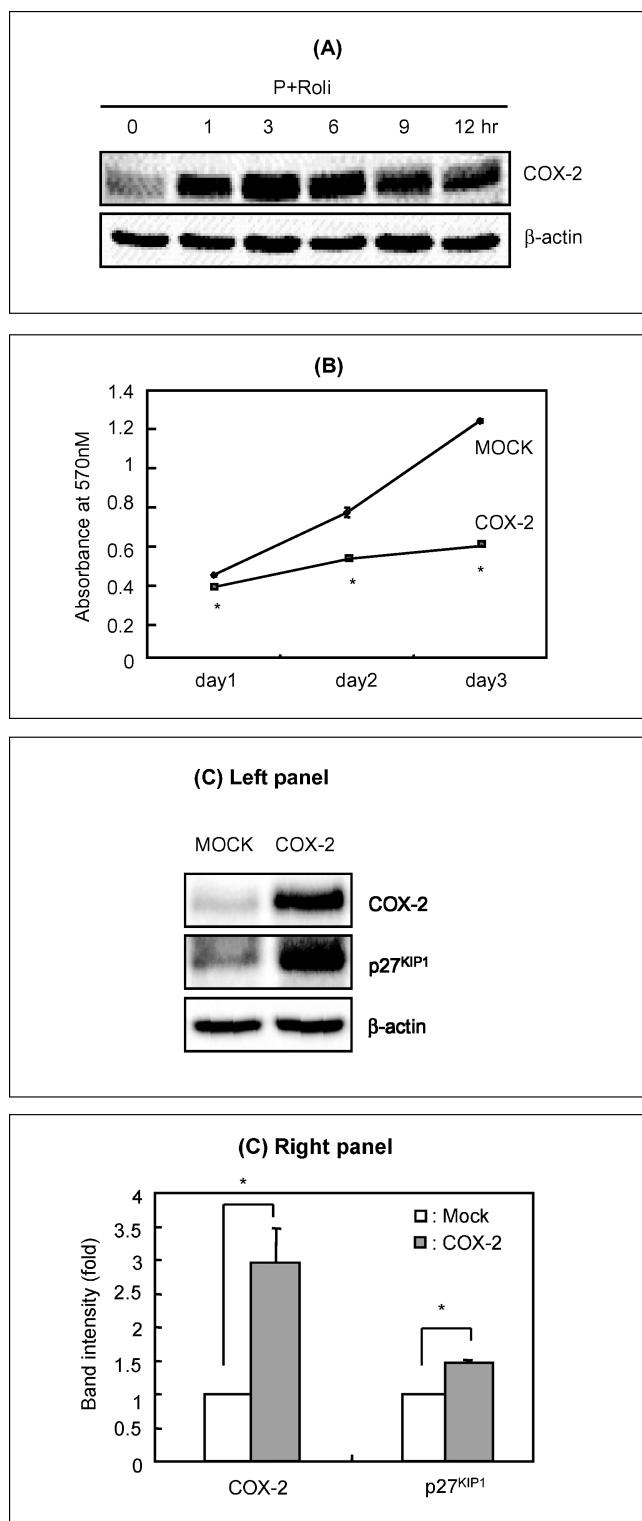


Fig. 4: Involvement of COX-2 on growth suppression induced by PGE<sub>2</sub> and the PDE4 inhibitor RAW264.7 cells were treated with PGE<sub>2</sub> (100 nM) and rolipram (10  $\mu$ M) for the indicated time intervals. COX-2 expression was measured by immunoblot analysis. (B and C) RAW264.7 cells were transiently transfected with mock or COX-2 plasmids. The growth of transfected cells was determined by MTT assay (B) or the protein level of transfected cells was determined by immunoblot analysis (C, left panel) and indicated by fold induction (C, right panel). Data are expressed as means + S.D. of triplicates. \**p* < 0.05

synergistic inhibitory effects on osteoclast formation, combining PGE<sub>2</sub> with a PDE4 inhibitor would have a greater effect than PGE<sub>2</sub> or rolipram alone for the treatment of bone loss, although further study is necessary to elucidate this.

In conclusion, our study demonstrates that the PDE4 inhibitor rolipram enhances PGE<sub>2</sub>-induced growth arrest of osteoclast precursors *via* COX-2-mediated p27<sup>KIP1</sup>, which in turn negatively regulates osteoclast formation.

## 4. Experimental

### 4.1. Reagents

Recombinant macrophage colony-stimulating factor (M-CSF) was purchased from R&D Systems (Minneapolis, MN). Recombinant human soluble RANKL was from PeproTech EC (London, UK). Antibodies against cyclin D1 and p27<sup>KIP1</sup> were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against COX-2 was purchased from CAYMAN chemical company (MI, USA). All other reagents were from Sigma-Aldrich (St. Louis, MO).

### 4.2. Osteoclasts formation

RAW264.7 cells suspended in DMEM containing 10% FBS were seeded at  $2 \times 10^3$ /well in 96-well plates. Cells were cultured for 4 days with 100 ng/ml RANKL. Cells were washed with phosphate-buffered saline (PBS), fixed with 10% formaldehyde for 5 min, and permeabilized with 0.1% Triton X-100 for 10 s. The fixed cells were stained for tartrate-resistant acid phosphatase (TRAP) as described previously (Park and Yim 2007). The number of TRAP-positive cells with more than 3 nuclei was counted under a light microscope.

### 4.3. RT-PCR analysis

Two micrograms of total RNA prepared using Easy-Blue (iNtRON Biotechnology, Inc.) were reverse-transcribed with RevertAid<sup>TM</sup> first strand cDNA synthesis Kit (Fermentas, EU) and amplified by PCR. The sequences of primers used are as follows: PDE3A, 5'-gctgtgaaagcagtatagc-3' (forward), 5'-gaaaatgtctgagacgagtc-3' (reverse); PDE3B, 5'-tgctcaggagacgctgtg-3' (forward), 5'-gatccacacctgaaacagtcg-3' (reverse); PDE4A, 5'-ggaactcacacctgtg-3' (forward) and 5'-gttctgtgtaaacaggtcc-3'; PDE4B, 5'-tgaaatcctgctgcat-3' (forward) and 5'-tccacagaagctgtgtgct-3' (reverse); PDE4C, 5'-tggtatcagataggattcc-3' (forward) and 5'-ctctgtgtaaaccttgctg-3' (reverse); PDE4D, 5'-cggaactcgctctgatg-3' (forward) and 5'-acagaggcgtgtgctg-3' (reverse);  $\beta$ -actin, 5'-tgtgatggtgggaatgggtcag-3' (forward), 5'-tttgatgacagcagcattcc-3'. The PCR program was as follows: 32 (all mouse PDEs) or 22 ( $\beta$ -actin) cycles, after an initial denaturation step at 94 °C for 3 min, then denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s (all mouse PDEs) or at 55 °C for 30 s ( $\beta$ -actin), and extension at 72 °C for 60 s, with a final extension at 72 °C for 10 min. The PCR products were separated on 1.2–2.0% agarose gels and stained with ethidium bromide.

### 4.4. Fluorescence-Activated Cell Sorting (FACS) analysis

RAW264.7 cells were cultured with or without PGE<sub>2</sub> and rolipram for 36 h, and were fixed in absolute ethanol at 4 °C for at least 3 h. Before analysis, cells were incubated for 30 min in PBS containing propidium iodide (PI) and 1  $\mu$ g/ml RNase A. The cell cycle moiety of Raw264.7 cells was analyzed on FACSscan (Becton Dickinson). The cell cycle was analyzed by using the CELLQUEST program.

### 4.5. Immunoblot analysis

Total cell lysates were isolated, separated by SDS-PAGE, and transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5 % nonfat-milk in TBS-T, and then immunostained with anti-phospho p38 (1:1000), anti-p27<sup>KIP1</sup> (1:1000), anti-cyclin D1 (1:2000) or with anti-COX-2 antibody (1:4000) followed by secondary horseradish peroxidase-conjugated antibody (1:5000). The membranes were developed using an enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK).

### 4.6. Cell proliferation with MTT or trypan blue exclusion assay

RAW264.7 cells were seeded in 96-well culture plates, and the cell viability was assessed by a trypan blue exclusion or (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay. For MTT assay, cells were treated with 5 mg/ml MTT for 4 h at 37 °C. After this, the medium was removed and

the cells were dissolved with dimethyl sulfoxide. The formazan reduction product was measured by reading absorbance at 570 nm in a plate reader. For trypan blue exclusion assay, cells were stained with 0.2% TB (Sigma, Spain) for 2 min and the proportion of unstained, living cells was calculated by counting on a hemocytometer the number of cells able to exclude the dye.

#### 4.7. Overexpression of COX-2

Mouse full-length COX-2 cDNA in pcDNA6 (8 µg) were transfected into RAW264.7 cells ( $5 \times 10^5$ ) using Lipofectamine™ 2000 CD reagent (Invitrogen, CA, USA) according to the manufacturer's instructions, and transfected cells were selected with blasticidin (Sigma-Aldrich, U.S.A.). Four days after transfection, cells were used for western blotting or MTT assay.

#### 4.8. Statistical analysis

Data are presented as the mean ± SD from at least triplicate experiments. Statistical analysis was performed by one-way analysis of variance followed by the Student's t-test. A *p* value < 0.05 was considered statistically significant.

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