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Biochemical Pharmacology 63 (2002) 523–532

Biochemical Pharmacology

Effects of a selective cyclooxygenase-2 inhibitor, celecoxib, on bone resorption and osteoclastogenesis *in vitro*

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Received 22 March 2001; accepted 27 June 2001

Abstract

The effects of an important new anti-inflammatory agent, the selective cyclooxygenase-2 inhibitor celecoxib, on bone resorption and osteoclastogenesis elicited by the inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), the endotoxin lipopolysaccharide (LPS), and the systemic hormones 1α,25-dihydroxyvitamin D_3 and parathyroid hormone were examined *in vitro*. Bone resorption was evaluated by measuring calcium released into the culture medium in a neonatal mouse calvarial bone organ culture. Osteoclastogenesis was evaluated by measuring tartrate-resistant acid phosphatase activity in the cells in cocultures of bone marrow cells and osteoblastic cells and in macrophage-colony-stimulating factor-dependent bone marrow cell cultures. Celecoxib (0.1 μM) completely inhibited the calcium release induced by IL-1β, TNF-α, and LPS. The resorptive effect of 1α,25-dihydroxyvitamin D_3 was inhibited partially by celecoxib. In contrast, celecoxib did not inhibit the calcium release elicited by parathyroid hormone or prostaglandin E_2. Celecoxib (0.1 μM) also markedly inhibited osteoclastogenesis induced by these stimulators of bone resorption except for PGE_2 in the coculture system, whereas it failed to inhibit osteoclastogenesis in macrophage-colony-stimulating factor-dependent bone marrow cell cultures. These results indicate that, under certain conditions, cyclooxygenase-2-dependent prostaglandin synthesis is critical for the bone resorption induced by IL-1β, TNF-α, and LPS, and for the osteoclastogenesis induced by these pro-inflammatory molecules and calciotropic hormones. The prevention of prostaglandin synthesis by inflammatory cytokines in bone cells could contribute to the efficacy of celecoxib in preventing bone loss in rheumatoid arthritis.

Keywords: Celecoxib; Cyclooxygenase-2 inhibitor; Prostaglandin; Calvaria; Bone resorption; Osteoclastogenesis

1. Introduction

Prostaglandins play an important role in bone resorption induced by inflammatory cytokines and growth factors [1]. Prostaglandins are known to be produced from membrane phospholipids by the sequential actions of phospholipase A_2 and cyclooxygenase (COX). Two distinct COX enzymes have been identified: a constitutive form (COX-1) that mediates physiological functions, and an inducible form (COX-2) associated with pathological conditions such as inflammation, pain, and some types of cancer [2].

Celecoxib, 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (Fig. 1), is a highly selective inhibitor of COX-2 and has been shown to be an anti-inflammatory and analgesic agent with gastrointestinal safety superior to that of traditional nonsteroidal anti-inflammatory drugs [3,4]. The inhibitor is commercially available for the treatment of rheumatoid arthritis, which is associated with increased bone resorption as well as focal osteolysis. However, the effect of this compound on bone resorption has not been assessed. The purpose of this study was to examine the effects of celecoxib on bone resorption and osteoclast differentiation elicited by the inflammatory cytokines IL-1β and TNF-α, the endotoxin LPS, and the systemic hormones 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and parathyroid hormone (PTH).

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Abbreviations: COX, cyclooxygenase; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; PTH, parathyroid hormone; PGE_2, prostaglandin E_2; M-CSF, macrophage-colony-stimulating factor; sRANKL, soluble receptor activator of nuclear factor-κB ligand; DMEM, Dulbecco's modified Eagle's medium; α-MEM, alpha modification of Eagle's MEM; FBS, fetal bovine serum; TRAP, tartrate-resistant acid phosphatase.

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PII: S0006-2952(01)00861-9

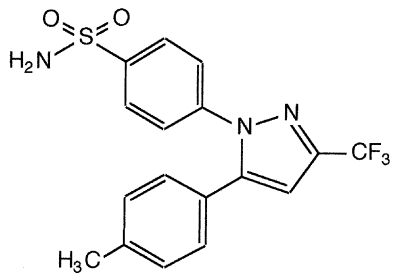

Fig. 1. Chemical structure of celecoxib, 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide.

2. Materials and methods

2.1. Reagents

Celecoxib was provided by the Searle/Monsanto Co. Recombinant human IL-1β was purchased from Biosource International. PTH (bovine fragment 1–34) was from Peninsula Laboratories, Inc. LPS from *Escherichia coli*, serotype 026:B6, and PGE_2 were obtained from Sigma. $1,25(OH)_2D_3$ was a gift from Dr. M.R. Uskokovic (Hoffmann-La Roche). Recombinant murine macrophage-colony-stimulating factor (M-CSF) and murine TNF-α were purchased from R&D Systems. Soluble receptor activator of nuclear factor-κB ligand (sRANKL) was from Pepro Tech EC, Ltd.

2.2. Organ culture of neonatal calvaria and bone resorption assay

The neonatal mouse calvarial bone organ culture system has been widely used to assess bone resorption induced by a variety of agents [5–8] and described in detail [5]. Briefly, calvaria were dissected aseptically from 4- to 6-day-old CD-1 mice (Harlan S.D.), and were cultured free-floating in roller tubes containing 2 mL of DMEM supplemented with 15% (v/v) heat-inactivated horse serum. 100 U/mL of penicillin G and 100 μg/mL of streptomycin (penicillin/streptomycin) were also added to the medium. The cultures were gassed with a mixture of 50% O_2, 5% CO_2, and 45% N_2 and incubated at 37° for either 72, 96, or 120 hr. Unless otherwise specified, these were regassed every 24 hr, and culture medium was changed after 24 hr. Bone resorption was evaluated from the release of calcium into the culture medium at 24, 48, 72, 96, and/or 120 hr of culture. The concentration of calcium in 0.08 mL aliquots of the medium was determined by fluorescent titration with a Calcette automatic calcium analyzer (Precision Systems, Inc.). The experiments were performed at least two times.

2.3. Primary osteoblastic cells

Primary osteoblastic cells were isolated from mouse calvaria according to the method of Takahashi *et al.* [9] with a slight modification. Briefly, calvaria were dissected from 1-day-old CD-1 mice and washed with α-MEM. These were digested consecutively with 0.1% (w/v) collagenase (Wako Pure Chemicals Co.) and 0.2% (w/v) dispase (Godo Shusei) in α-MEM for 5 min (population 1) and 4×10 min (populations 2–5) at 37° with mild shaking. Released cells from populations 2–5 were pooled and cultured in 100-mm dishes at 37° in a humidified 5% CO_2 atmosphere in α-MEM supplemented with 10% (v/v) FBS and penicillin/streptomycin (α-MEM + FBS). After confluence, cells were resuspended with 0.2% (w/v) collagenase in α-MEM and trypsin/EDTA solution (GIBCO BRL) and subcultured. These were then harvested and stored at −80° until used.

2.4. Bone marrow cells

Tibiae and femora were aseptically dissected from 5- to 7-day-old CD-1 mice. The bone ends were cut off, and the marrow was forced out in α-MEM + FBS. The marrow suspension was filtered through a fine meshed sieve to remove bone particles and agitated by gentle pipetting to obtain a single cell suspension. The bone marrow cells were washed with α-MEM + FBS and resuspended to be used for either coculture experiments or M-CSF-dependent bone marrow cell culture experiments.

2.5. Coculture of bone marrow cells and osteoblastic cells

Bone marrow cells (1×10^5) were cocultured with primary osteoblastic cells (1×10^4) in 0.2 mL of α-MEM + FBS in the wells of 96-well plates as previously described [10]. Cultures were maintained for 6 days with or without treatments. On day 3, the medium in each well was replaced with the respective fresh medium. The experiments were performed three times.

2.6. M-CSF-dependent bone marrow cell culture

The bone marrow cells were preincubated for 24 hr in α-MEM + FBS in the presence of M-CSF (5 ng/mL) at a density of 2.5×10^6 cells/mL in 100-mm dishes. After the preincubation, nonadherent cells were collected and resuspended in α-MEM + FBS containing 20 ng/mL of M-CSF. This procedure depletes stromal cells in bone marrow cell cultures. M-CSF was added to replace the M-CSF that would be provided by osteoblastic cells or stromal cells in the coculture model. The cells were plated at 1×10^5 cells per well in 96-well plates and cultured for 4 days with or without treatments. The method has been described previously [11]. The experiments were performed two times.

2.7. Assay for osteoclastogenesis

After the indicated days of culture, the activity of TRAP, a marker enzyme of osteoclasts, was assayed in the cells in

each well as an indicator for osteoclast differentiation. The cells were fixed in 10% (v/v) formalin for 10 min and washed in 95% (v/v) ethanol. Unless otherwise specified, these were then incubated in 0.1 mL of phosphatase substrate solution (3.7 mM *p*-nitrophenyl phosphate in 50 mM citrate buffer, pH 4.6) in the presence of 10 mM sodium tartrate at 25° for 30 min. After the incubation, the solution was removed from each well and reacted with 0.1 mL of 0.1 N NaOH. Absorbance at 410 nm in the reaction mixture was measured. After the assay for TRAP activity, the cells were stained for TRAP with 0.1 mg/mL of naphthol AS-MX phosphate and 0.6 mg/mL of fast red violet LB salt in 0.1 M sodium acetate buffer (pH 5.0) containing 50 mM sodium tartrate as described before [12]. The TRAP activity in the cells in each well correlated with the number of TRAP-positive ($TRAP^+$) cells in the well (data not shown).

2.8. Statistical analysis

The data were subjected to analysis of variance followed by Fisher's least significant difference (LSD) test. $P < 0.05$ was considered to be a significant difference.

3. Results

3.1. Effect of celecoxib on bone resorption

Celecoxib (0.01–0.1 μM) concentration-dependently inhibited calcium release from neonatal mouse calvarial bones stimulated by IL-1β (10 ng/mL) in 72-hr cultures (Fig. 2). Fig. 3 shows the time course and reversibility of the effect of celecoxib on IL-1β-stimulated bone resorption. IL-1β caused a time-dependent increase in calcium release during a 72-hr culture. This was abolished by treatment with celecoxib (0.1 μM). When celecoxib was removed after 72 hr and the bones were cultured for an additional 48 hr in fresh medium without celecoxib, their response to IL-1β recovered, although it was less than in the bones that had never been treated with celecoxib. Celecoxib (0.1 μM) also completely inhibited the calcium release induced by TNF-α (10 ng/mL) (Fig. 4) or by LPS (1 μg/mL) (Fig. 5). Fig. 6 shows the effect of celecoxib on 1,25-$(OH)_2D_3$ (10 nM)-stimulated bone resorption. The resorptive effect of this hormone was inhibited partially by celecoxib. In contrast, celecoxib did not inhibit the calcium release elicited by PTH (3 nM) (Fig. 7A). As shown in Fig. 7B, the resorptive effect of PTH was not affected even when calvaria were pretreated with celecoxib for 24 hr before adding PTH. Fig. 8 shows the effects of celecoxib on the bone resorption stimulated by PGE_2. Celecoxib did not affect the calcium release elicited by PGE_2 at either a low (0.125 μM) (A) or a high (0.5 μM) (B) PGE_2 concentration.

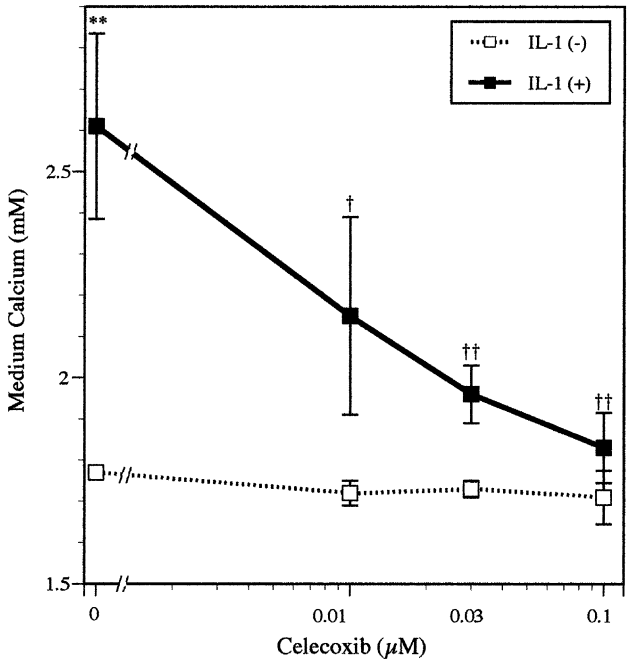

Fig. 2. Concentration-dependence of the effect of celecoxib on IL-1β (10 ng/mL)-stimulated bone resorption in neonatal mouse calvaria assessed as described in Section 2. Each point represents the mean ± SEM of responses from three calvaria. (**) $P < 0.01$: significant increase compared with the control (no celecoxib, no IL-1β); (†) $P < 0.05$ and (††) $P < 0.01$: significant inhibition compared with treatment without celecoxib.

3.2. Effect of celecoxib on osteoclastogenesis

All of the stimulators of bone resorption tested in the calvarial cultures also increased TRAP activity in cocultures of bone marrow cells and osteoblastic cells, which reflected formation of $TRAP^+$ cells (Figs. 9 and 10). Celecoxib (0.1 μM) markedly inhibited the increase in TRAP activity and the formation of $TRAP^+$ cells induced by all of these stimulators except for PGE_2 (Figs. 9 and 10). As shown in Fig. 11, the inhibitory effect of celecoxib on TRAP activity elicited by IL-1β was concentration-dependent. Fig. 12 shows the effect of celecoxib on osteoclastogenesis in bone marrow cells cultured in the absence of osteoblastic cells or stromal cells. Cells were cultured in the presence of M-CSF and either the stimulators or sRANKL with or without celecoxib. Among the agents tested, only TNF-α and sRANKL-induced osteoclastogenesis in this model, and these responses were not affected by celecoxib (0.1 μM).

4. Discussion

COX-2 inhibitors are compounds that selectively and potently inhibit COX-2, while preserving COX-1 activity. Thus, they have anti-inflammatory and analgesic properties with little or no gastrointestinal side-effects [2]. Although much attention has been focussed on their effects

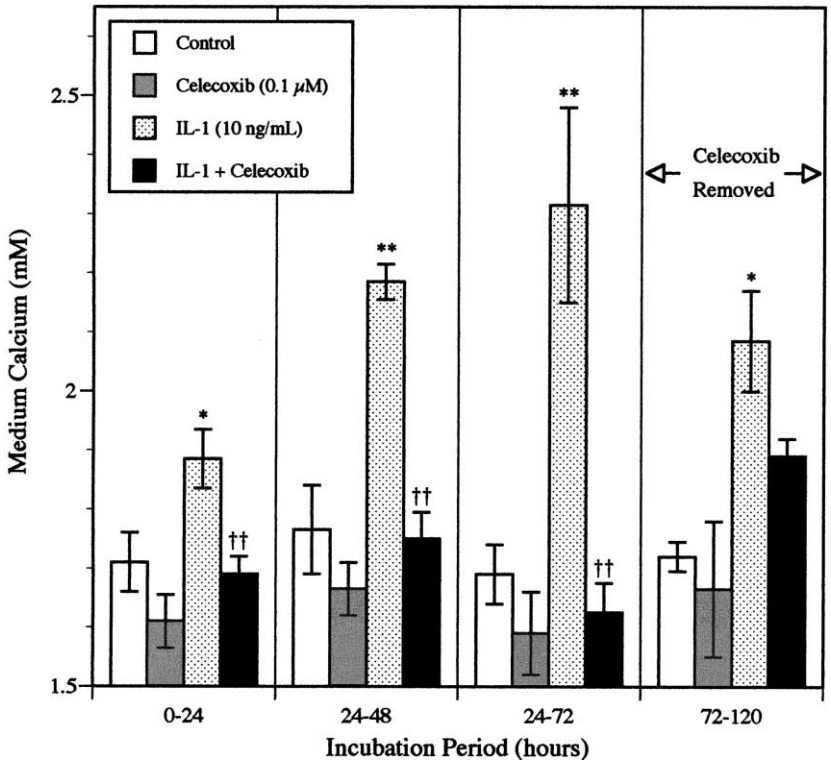

Fig. 3. Time course and reversibility of the effect of celecoxib on IL-1β-stimulated bone resorption in neonatal mouse calvaria. Each column represents the mean ± SEM of responses from three calvaria. (*) $P < 0.05$ and (**) $P < 0.01$: significant increase compared with the control; (††) $P < 0.01$: significant inhibition compared with treatment without celecoxib.

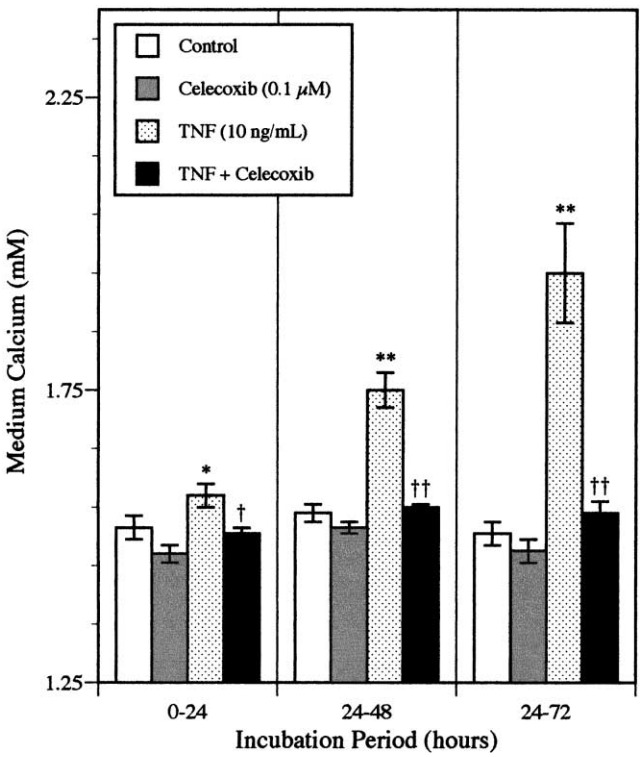

Fig. 4. Effect of celecoxib on TNF-α-stimulated bone resorption in neonatal mouse calvaria. Each column represents the mean ± SEM of responses from five calvaria. (*) $P < 0.05$ and (**) $P < 0.01$: significant increase compared with the control; (†) $P < 0.05$ and (††) $P < 0.01$: significant inhibition compared with treatment without celecoxib.

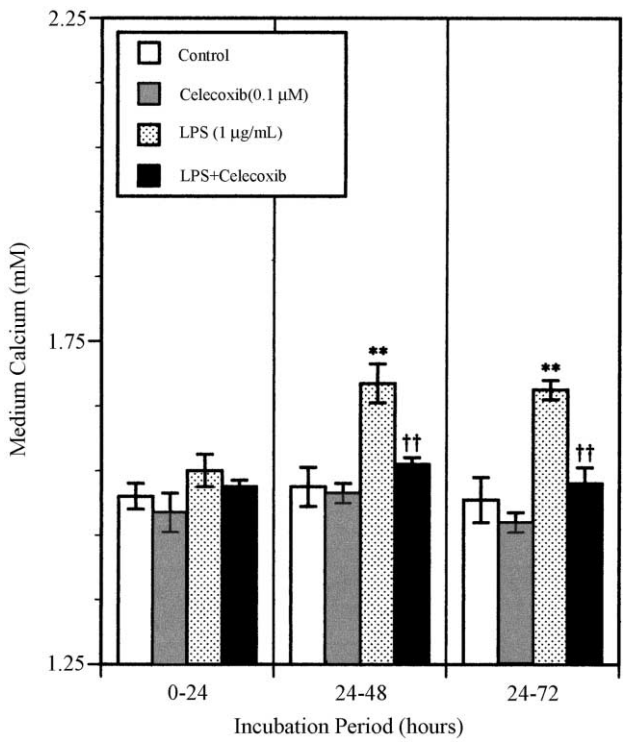

Fig. 5. Effect of celecoxib on LPS-stimulated bone resorption in neonatal mouse calvaria. Each column represents the mean ± SEM of responses from three calvaria. (**) $P < 0.01$: significant increase compared with the control; (††) $P < 0.01$: significant inhibition compared with treatment without celecoxib.

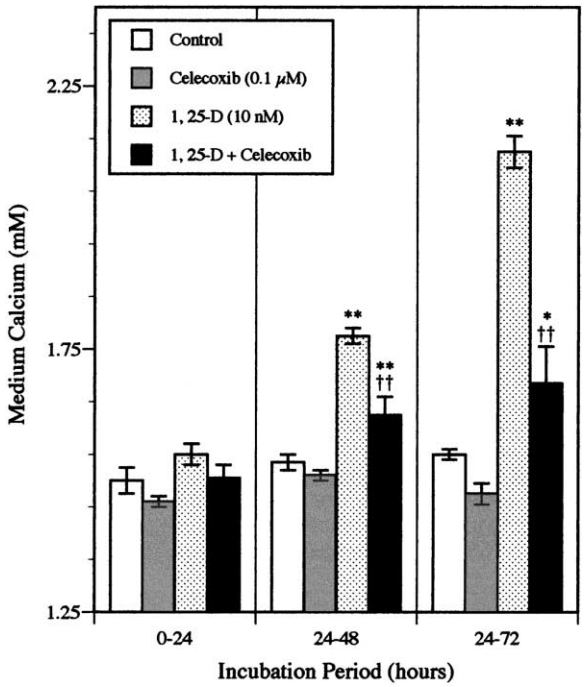

Fig. 6. Effect of celecoxib on 1,25-$(OH)_2D_3$ (1,25-D)-stimulated bone resorption in neonatal mouse calvaria. Each column represents the mean ± SEM of responses from four calvaria. (*) $P < 0.05$ and (**) $P < 0.01$: significant increase compared with the control; (††) $P < 0.01$: significant inhibition compared with treatment without celecoxib.

on inflammation and pain, recent studies suggest that COX-2 inhibitors might be useful in the treatment or prevention of other types of diseases such as colon cancer [13]. The present study demonstrated that celecoxib is also an efficacious inhibitor of bone resorption. Celecoxib completely and reversibly inhibited IL-1β-stimulated bone resorption at a concentration of 0.1 μM. The inhibitory effect was comparable to that of a powerful non-selective COX inhibitor, indomethacin (data not shown). Again there was complete inhibition by celecoxib of the bone resorption induced by TNF-α and LPS. Celecoxib also inhibited 1,25-$(OH)_2D_3$-stimulated bone resorption, although the inhibition was not complete. These results indicate that in neonatal mouse calvarial cultures, COX-2-dependent prostaglandin synthesis is essential for the bone resorption induced by IL-1β, TNF-α, and LPS, and is involved in the resorption elicited by 1,25-$(OH)_2D_3$. This is consistent with previous studies which have shown that these cytokines, endotoxin, and hormone exert their effects on bone resorption to some extent by a prostaglandin-dependent mechanism [7,14–18] and that the stimulation of prostaglandin production is largely through the induction of COX-2 expression [19–22]. On the other hand, the present results contrast with previous reports in which the resorptive effect of IL-1β was not inhibited by indomethacin [20,23]. This might be due to a difference in the experimental conditions such as the length of the preincubation period [24]. In the calvarial cultures, prostaglandin synthesis can be readily elicited. Medium PGE_2 concentrations at 72 hr ranged from 0.05 to 0.13 ng/mL in control cultures, and increased in IL-1β (1.75 ng/mL)-stimulated cultures, ranging from 4.16 to 6.00 ng/mL (unpublished data, measured by an enzyme immunoassay). Therefore, the inhibitory effect of celecoxib might be less apparent in another system such as fetal rat limb bone cultures in which

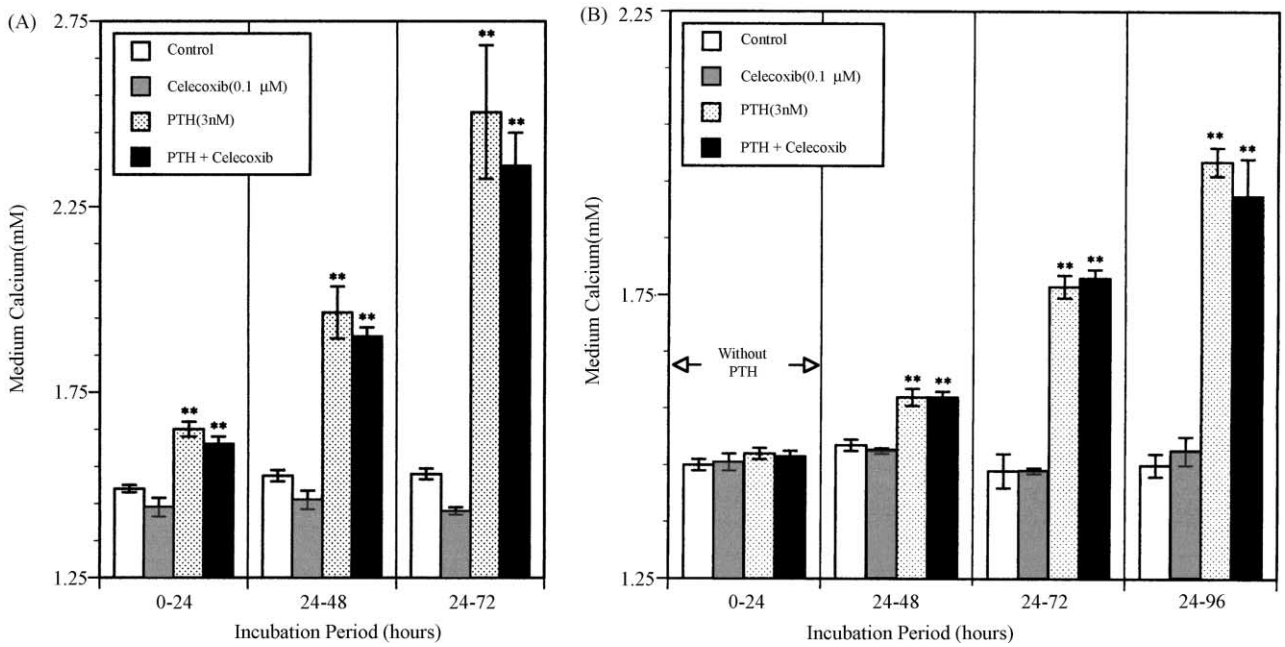

Fig. 7. Lack of effect of celecoxib on PTH-stimulated bone resorption in neonatal mouse calvaria. (A) Celecoxib was present together with PTH during the whole experimental period. Each column represents the mean ± SEM of responses from four calvaria. (**) $P < 0.01$: significant increase compared with the control. (B) Celecoxib was added 24 hr prior to adding PTH. Each column represents the mean ± SEM of responses from three calvaria. (**) $P < 0.01$: significant increase compared with the control.

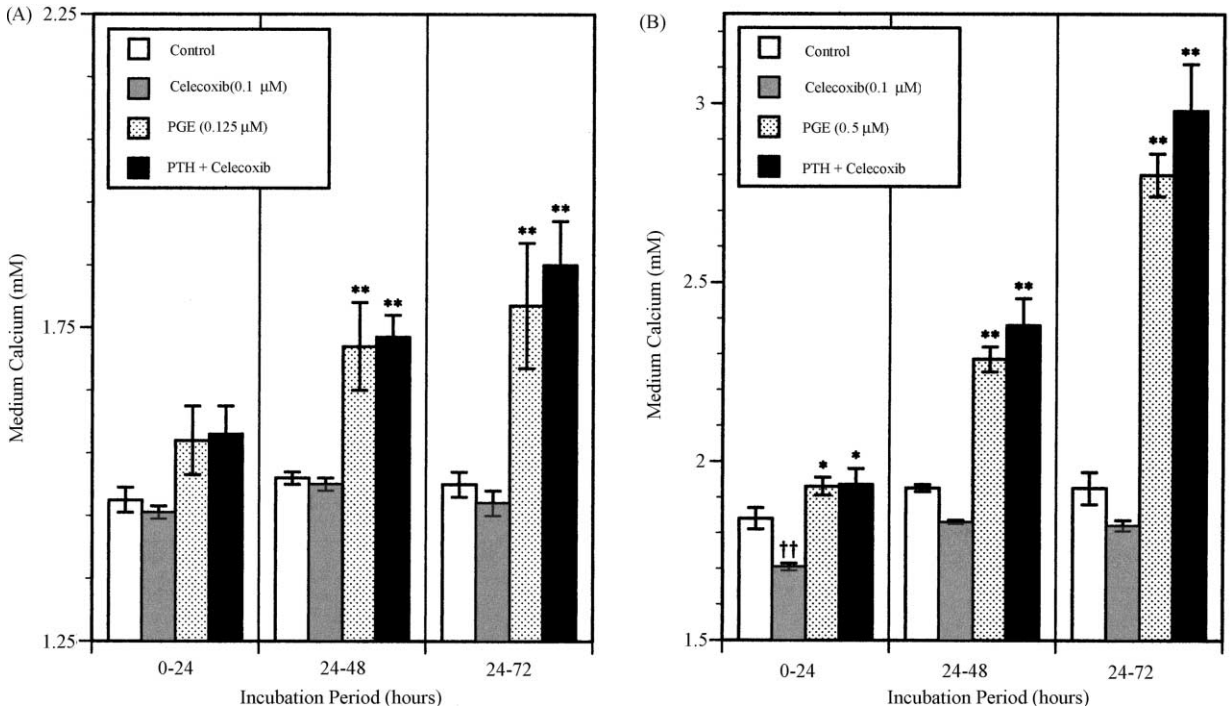

Fig. 8. Lack of effect of celecoxib on PGE_2-stimulated bone resorption in neonatal mouse calvaria. (A) 0.125 μM PGE_2. Each column represents the mean ± SEM of responses from five calvaria. (**) $P < 0.01$: significant increase compared with the control. (B) 0.5 μM PGE_2. Each column represents the mean ± SEM of responses from three calvaria. (*) $P < 0.05$ and (**) $P < 0.01$: significant increase compared with the control; (††) $P < 0.01$: significant inhibition compared with treatment without celecoxib.

bone resorption is largely prostaglandin-independent [8]. Although PTH is the most potent stimulator of prostaglandin production in bone [25] and the stimulation is mainly through induction of COX-2 expression [19], celecoxib failed to inhibit the bone resorption elicited by PTH, indicating that its effect on bone resorption is independent of COX-2 in the present experimental model. This lack of dependence on prostaglandin has also been found by others

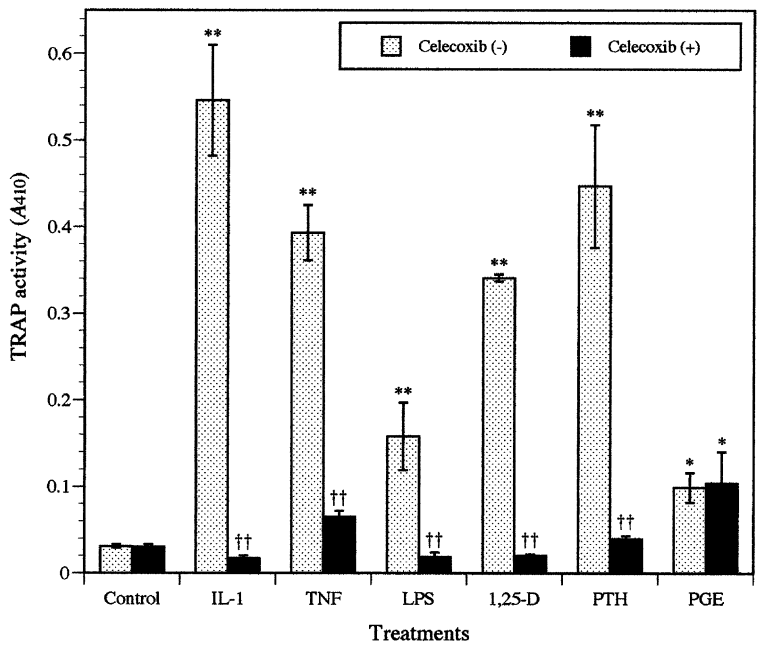

Fig. 9. Effect of celecoxib (0.1 μM) on osteoclastogenesis induced by various stimulators of bone resorption in 6-day cocultures of bone marrow cells and osteoblastic cells prepared as described in Section 2. Each column represents the mean ± SEM of responses from three or four wells. IL-1: IL-1β (10 ng/mL); TNF: TNF-α (30 ng/mL); LPS: LPS (1 μg/mL); 1,25-D: 1,25-$(OH)_2D_3$ (10 nM); PTH: PTH1-34 (10 nM); PGE: PGE_2 (1 μM). (*) $P < 0.05$ and (**) $P < 0.01$: significant increase compared with the control; (††) $P < 0.01$: significant inhibition compared with treatment without celecoxib.

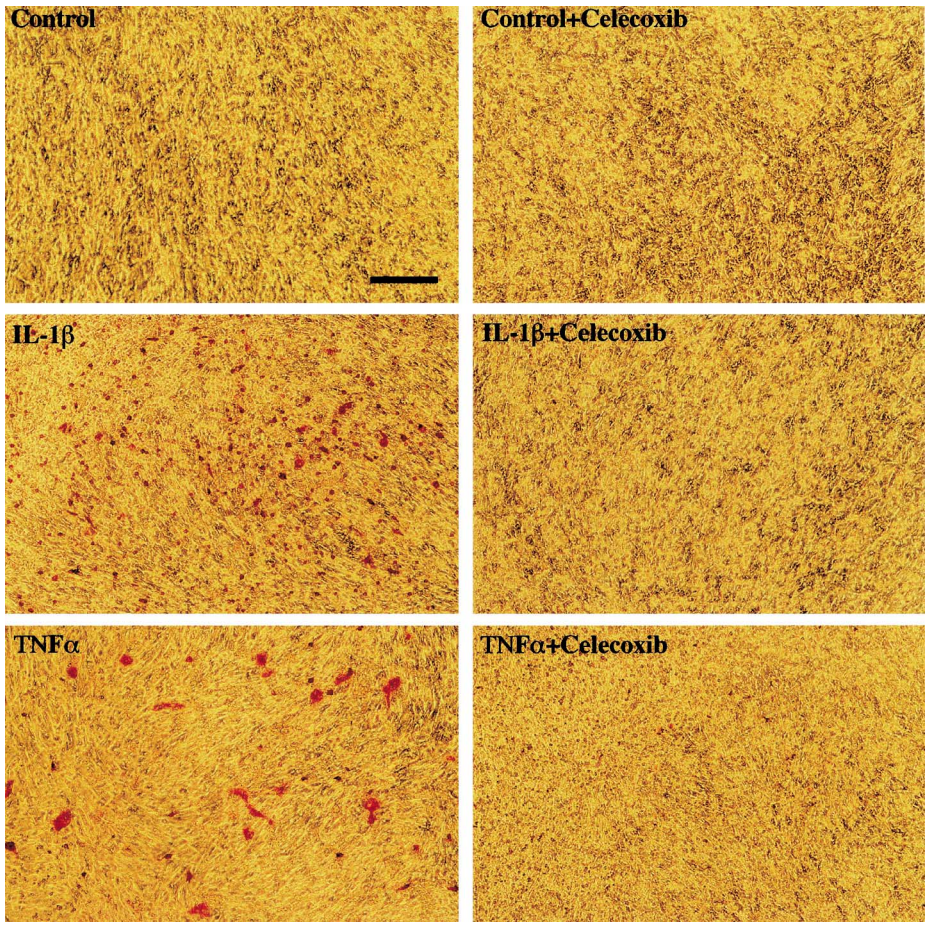

Fig. 10. Photomicrographs of cocultures of bone marrow cells and osteoblastic cells. After culture for 6 days, cells were stained for TRAP. Celecoxib (0.1 μM) inhibited formation of TRAP-positive cells stimulated by IL-1β (10 ng/mL) and TNF-α (30 ng/mL). 1 Bar = 50 μM.

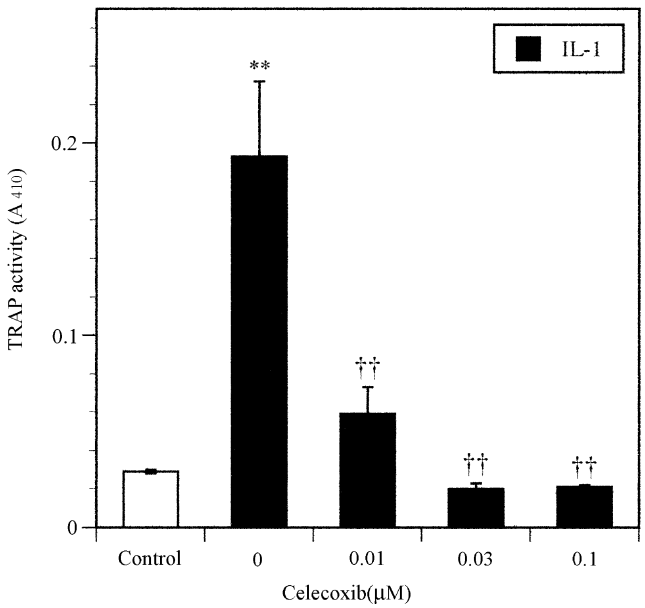

Fig. 11. Concentration-dependent effect of celecoxib on osteoclastogenesis elicited by IL-1β (10 ng/mL) in 6-day cocultures of bone marrow cells and osteoblastic cells. Each column represents the mean ± SEM of responses from three wells. (**) $P < 0.01$: significant increase compared with the control; (††) $P < 0.01$: significant inhibition compared with IL-1β alone.

who have shown that indomethacin did not affect the resorptive effect of PTH in organ culture [20]. Celecoxib did not affect the resorptive effect of PGE_2, a major product of COX enzymes in bone tissues. Previous studies have shown that prostaglandins can increase their own production by inducing COX-2 expression [26], thereby enhancing their resorptive effects [27]. However, our results indicate that the resorption process downstream of prostaglandin production was unaffected by COX-2 in this model system, and confirm that the inhibitory effects elicited by celecoxib in our studies were due to inhibition of COX-2-dependent responses, rather than a more general effect on bone resorption.

Prostaglandins stimulate osteoclast differentiation through both direct and indirect effects on hemopoietic precursors [1,11]. The indirect effect is mediated by sRANKL, which has been shown to serve as a common final mediator through which many resorptive stimuli cause osteoblasts/stromal cells to activate osteoclastogenesis [28]. The present results confirmed that PGE_2 induces osteoclastogenesis. However, the response to PGE_2 was relatively small as compared with that of the other stimulators. Since it has been found that PGE_2 also has direct inhibitory effects on osteoclasts [29], PGE_2 at the relatively

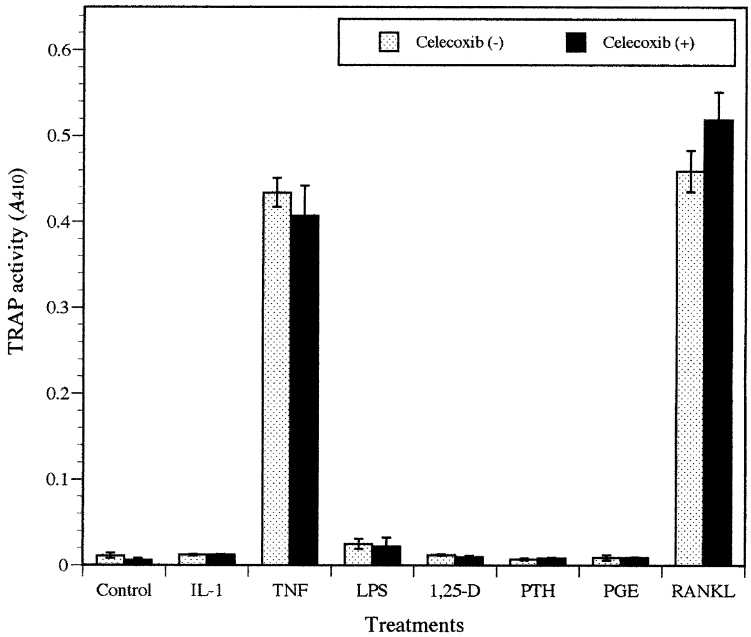

Fig. 12. Lack of effect of celecoxib (0.1 μM) on osteoclastogenesis in M-CSF-dependent bone marrow cell cultures prepared as described in Section 2. Each column represents the mean ± SEM of responses from three wells. IL-1: IL-1β (10 ng/mL); TNF: TNF-α (30 ng/mL); LPS: LPS (1 μg/mL); 1,25-D: 1, 25-$(OH)_2D_3$ (10 nM); PTH: PTH1-34 (10 nM); PGE: PGE_2 (1 μM); RANKL: sRANKL (50 ng/mL). sRANKL-treated cells were incubated in substrate solution for only 5 min due to high TRAP activities.

high concentration (1 μM) used in this study may have inhibited mature osteoclasts, in addition to stimulating osteoclastogenesis. It has been demonstrated that the stimulatory effects of several cytokines and calciotropic hormones on osteoclast formation in cocultures or bone marrow cell cultures are mediated to a greater or lesser extent by endogenous prostaglandin synthesis [15,22,30]. Consistent with these findings, in cocultures of bone marrow cells and osteoblastic cells celecoxib markedly inhibited osteoclastogenesis induced by all of the stimulators of bone resorption tested in the organ cultures except for PGE_2. In contrast, celecoxib failed to inhibit osteoclastogenesis induced by TNF-α and sRANKL in the M-CSF-dependent bone marrow cell cultures. It is worth noting that there was still a small persistent stimulatory effect of TNF-α in the coculture system, even in the presence of celecoxib. These results suggest that in the present osteoclastogenesis model, COX-2-dependent prostaglandin synthesis by osteoblastic cells or stromal cells is essential for the osteoclast differentiation induced by IL-1β, LPS, 1,25-$(OH)_2D_3$, and PTH, and that TNF-α can exert its effects on osteoclastogenesis by both prostaglandin-dependent and -independent mechanisms. The direct stimulatory effect of TNF-α on osteoclast differentiation has been shown previously by others [31].

Since most findings were comparable between the calvarial experiments and coculture experiments, the inhibitory effect of celecoxib on bone resorption could be attributable, at least in part, to the inhibition of osteoclastogenesis. For example, the concentration-response of the inhibitory effect of celecoxib on IL-1β-stimulated bone resorption was similar to that on the osteoclastogenesis induced by IL-1β. On the other hand, there was a dissociation of results obtained in the two systems using PTH as a stimulator. Celecoxib (0.1 μM) completely inhibited osteoclast differentiation induced by PTH (10 nM), whereas it failed to inhibit the bone resorption elicited by PTH (3 nM). The reason for the dissociation is not known. One possible explanation is that in the calvarial cultures PTH may have acted predominantly on preexisting osteoclasts as either an activator or a survival factor by a prostaglandin-independent mechanism rather than promoting formation of new osteoclasts.

In conclusion, the present study suggests that, under certain conditions, COX-2-dependent prostaglandin synthesis is critical for the bone resorption induced by IL-1β, TNF-α, and LPS, and for the osteoclastogenesis induced by these pro-inflammatory molecules and calciotropic hormones. Since it has been suggested that IL-1 and TNF-α have important roles in the pathogenesis of bone loss associated with rheumatoid arthritis [32], the prevention of prostaglandin synthesis by these cytokines in bone cells might contribute to the efficacy of celecoxib in the treatment of rheumatoid arthritis. Our results also suggest that COX-2 inhibitors could be potential therapeutic adjuncts for the treatment of increased bone resorption associated with postmenopausal osteoporosis and periodontal disease, in which inflammatory cytokines and/or endotoxin are involved [33,34]. Very recently, celecoxib has been shown to reduce implant wear debris-induced inflammation and the associated osteolysis in mouse calvaria *in vivo* [35]. Further studies using *in vivo* models of bone resorption

could address the efficacy of celecoxib in these other pathophysiological states.

Acknowledgment

This study was supported by a research grant from Searle/Monsanto.

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