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Regulation of Collagenolytic Protease Secretion through c-Src in Osteoclasts

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The role of pp60c-src activity in the synthesis and secretion of the collagenolytic cysteine proteases (CCPs), cathepsin K (CAK), cathepsin L (CAL), and cathepsin B (CAB), by osteoclasts was investigated. Synthesis and secretion of CAL were up-regulated by 1α,25-$(OH)_2D_3$, but neither those of CAK, dominant relative to CAL, nor CAB, barely detectable, levels changed in the experiments. Though PP1, a pp60$^{c\text{-}src}$ inhibitor, had no effect on CCPs synthesis, suppressed the CAK and CAL secretion. Wortmannin, a phosphatidylinositol 3-kinase (PI3-kinase) inhibitor that works as a second messenger for pp60$^{c\text{-}src}$, and cytochalasin B, an inhibitor of actin polymerization, suppressed the secretion of both CAK and CAL without suppressing synthesis. Hydroxyproline release, an indicator of degradation of type-I collagen, and F-actin ring formation, a structure linked to osteoclastic bone resorption, were suppressed by PP1, cytochalasin B or wortmannin. These results suggested inhibition of pp60$^{c\text{-}src}$ activity affected the osteoclastic cytoskeleton, which in turn reflected the suppression of bone resorption.

Key Words: **bone resorption; pp60$^{c\text{-}src}$; cathepsin K; cathepsin L; cathepsin B; F-actin.**

Bone resorption by osteoclasts has been reported to depend on the kinase activity of pp60$^{c\text{-}src}$, which must be expressed without suppression of osteoclast differentiation. It was reported that there existed multinucleous osteoclasts lacked ability of bone resorption and this lead to osteopetrosis in c-*src*-deficient mice, but did not effect other kinds of cells (1). As pp60$^{c\text{-}src}$ might act upstream of signaling pathways in osteoclasts, the activity of pp60$^{c\text{-}src}$ might regulate bone resorption by suppressing the functions of osteoclasts such as secretion of demineralizing acid or CCPs, suggested to play a role in the last step of bone resorption. Although the function of CCP in osteoclasts is not well understood, CAK (2, 3), CAL (4) or CAB (7–10) are likely to be involved in the degradation of type-I collagen in bone matrix (5, 6). As the mechanisms of synthesis and secretion of these proteases remain unknown, we focused on the kinase activity of pp60$^{c\text{-}src}$ in synthesis or secretion of CCPs especially CAK, CAL and CAB. We also attempted to clarify whether the suppression of pp60$^{c\text{-}src}$ activity induced normalization of abnormally stimulated bone resorption through suppression of CCP release.

Phosphorylated p60$^{c\text{-}src}$ affects the reorganization of cytoskeleton (11), such as actin polymerization (12, 46). In osteoclasts, the function (i.e. bone resorption) and cell shape (i.e. ruffled border) is known to be closely correlated (13, 14) and formation of F-actin ring might also be linked to ruffled border formation (15, 16).

As these results suggested that synthesis and/or secretion of collagenolytic cysteine proteases are correlated with the activity of pp60$^{c\text{-}src}$, we investigated this possibility in the present study.

MATERIALS AND METHODS

Animals. Time-mated ICR mice from Charles River Japan (Tokyo) and ddY mice from Japan SLC (Shizuoka, Japan) were used. They were housed in a room maintained at 26°C. The animals were given a commercial diet (CE-2, Clea Japan, Inc. Tokyo) and water ad libitum in a temperature-controlled (23 ± 2°C) and humidity-controlled (55 ± 5%) room.

Chemicals. Sources for the materials used in this study were: PP1, Calbiochem (La Jolla, CA); cytochalasin B and wortmannin, Sigma Chemical Co. (St. Louis, MO); α-MEM (phenol red free) and fetal calf serum (FCS), GIBCO (Grand Island, NY); molecular biology reagents, Bethesda Research Laboratories (Gaithersburg, MD); tissue culture plates, Costar (Cambridge, MA); antibodies against mouse CAK, CAL, and CAB, Transduction Laboratories (Lexington, KY). Other chemicals were purchased from Wako Pure Chemicals, Ltd. (Osaka, Japan).

Osteoclast differentiation by coculture of bone marrow stromal cells and osteoblastic cells. Mouse osteoclast-like multinucleated cells were prepared according to the coculture method described by Akatsu *et al.* (17) with modifications. Bone marrow cells were collected from the femora and tibiae of 6-week-old ddY mice, washed

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twice with α-MEM, and resuspended in α-MEM supplemented with 10% FCS at a final density of 4×10^6 cells/ml. Primary osteoblastic cells were obtained from 1-day-old ddY mice calvariae by collagenase digestion and suspended in α-MEM supplemented with 10% FCS. Culture dishes (10 cm in diameter) were precoated with 4 ml of 0.2% collagen gel matrix (Nitta Gelatin Co., Osaka, Japan), and the bone marrow cells and osteoblastic cells were cocultured in the dishes in the presence of 10^{-8} M 1α,25-$(OH)_2D_3$ at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were fed every 2 days with 0.3 ml of fresh α-MEM supplemented with 10% FCS and 10^{-8} M 1α,25-$(OH)_2D_3$. After 6 days, the cells were washed with phosphate-buffered saline (PBS) and separated from the dishes by treatment with 4 ml of 0.2% collagenase. Osteoclast-like multinucleated cells were concentrated by 35% percoll (Pharmacia Biotech, Tokyo, Japan) density gradient centrifugation. The presence of osteoclast-like multinucleated cells was confirmed by positive tartrate-resistant acid phosphatase (TRAP) staining.

TRAP staining of osteoclasts. Cells were fixed with 10% formalin in PBS for 10 min, permeabilized with 1:1 (vol/vol) acetone/ethanol for 1 min, and stained for TRAP for 15 min at room temperature. The TRAP staining solution contained 50 mM sodium acetate, 30 mM tartrate, 0.3 mg of fast red-violet LB per ml, and 0.1 mg of naphthol AS-MX (pH 5.0) per ml.

Staining of the F-actin ring of osteoclasts. F-actin was stained according to the method of Barak *et al.* (18) with modifications. The cells were fixed with 3% paraformaldehyde for 30 min, washed with PBS, and permeated with 0.1% Triton X-100 in PBS for 5 min. The cells were then washed and incubated with rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR) at a final concentration of 20 units/ml at 37°C for 30 min. The labeled cells were gently rinsed with PBS 5 times, and examined using a fluorescent microscope.

Calvarial organ culture. Calvariae were cultured according to the method described previously (19). In brief, calvariae were dissected from 1-day-old fetal mice and cultured in a 48-well plastic tissue culture plate in 250 μl α-MEM medium containing 1 mg/ml BSA, 100 μg/ml ascorbic acid, and 1 mM proline. The culture dishes were placed on a rocking platform in a 37°C incubator equilibrated with 5% CO_2 in air.

Measurement of CCP activity and hydroxyproline content. Enzymatic activity of CCP was assayed using 20 μM Z(benzyloxycarbonyl)-Phe-Arg-MCA(4-methyl-7-coumarylamide) (Sigma Chemicals, Co., MO) as the substrate, as described by Barrett and Kirschke (20). The fluorescence of the free aminomethylcoumarin was determined by excitation at 365 nm and emission at 450 nm using a Fluorescence Concentration Analyzer (Baxter Healthcare Co., IL). The culture medium was hydrolyzed with 6 N HCl at 130°C for 3 h. After neutralization, hydroxyproline in the hydrolyzate was measured by the method of Woessner (21).

Western blotting. Cells were washed extensively with PBS and lysed in PBS (1%) Triton X-100 (0.5%) deoxycholate. After initial clarification by centrifugation, pellets were resuspended in reduced sample buffer and electrophoresed in 10–20% SDS–PAGE gradient gels. The electrophoresed proteins were then transferred to nitrocellulose by means of a Transblot apparatus (Bio-Rad Laboratories, Richmond, CA). After treating the membrane with blocking solution (25 mM Tris–HCl, pH 7.4, containing 154 mM NaCl and 5% BSA), primary antibody staining was performed with rabbit anti-mouse CAK, CAL, or CAB overnight at room temperature. Secondary antibody staining was performed under similar conditions using alkaline phosphatase-coupled goat anti-rabbit IgG for an hour and detected using BCIP-NBT (Promega, Madison, WI).

Reverse transcription polymerase chain reaction (RT–PCR). Total RNA was extracted from calvariae using the guanidine isothiocyanate method. Calvariae were homogenized in 2 ml of 4 M guanidinium isothiocyanate, 25 nM sodium citrate, pH 7, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Sodium acetate, pH 4 (0.2 ml), 2 ml water-saturated phenol, and 0.4 ml of a mixture of chloroform and isoamylalcohol (24:1) were added to the homogenate. The aqueous phase was precipitated with 1 vol isopropanol, reextracted with the guanidinium isothiocyanate solution described above, and precipitated with 1 vol isopropanol. Total RNA was dissolved in distilled water, heated at 65°C, and quantitated by absorbance at 260 nm. Ten μg of total RNA was reverse-transcribed in a 20 μl reaction volume using an oligo primer (28 cycles in a linear range). Specific primers were as follows: for cathepsin K, forward 5′-TGGGGGCTCAAGGTTCTGCT-3′, reverse 5′-CATCTTGGGGAAGCTGGCCA-3′; for cathepsin L, forward 5′-AATCTTTTACTCCTTTTGGC-3′, reverse 5′-ATTCACGACAGGATAGCTGG-3′; for cathepsin B, forward 5′-TGGTGGTCCTTGATCCTTCT-3′, reverse 5′-GAATCTTCCCCAGTACTGGT-3′. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as an internal control, were purchased from Clontech (Palo Alto, CA).

Statistical analysis. All data are expressed as the mean ± standard error of the mean (SEM). Statistical differences between the groups were evaluated with the Dunnett type test or Student's *t*-test. *P* values of less than 0.05 were considered to be significant.

RESULTS

Effects of pp60$^{c\text{-}src}$ Inhibitors on Synthesis of Cathepsins

As it was difficult to obtain adequate number of pure osteoclasts for applying to non-PCR methods, expression of mRNA was investigated semiquantitative PCR (RT-PCR) normalized by GAPDH expression. Messenger RNA of CAK, CAL, and CAB were extracted from osteoclasts, differentiated by the coculture of bone marrow stromal and osteoblastic cells of mice, in the presence of 1α,25-$(OH)_2D_3$ (10^{-9}–10^{-8} M) for 24 h (Fig. 1). The expression of mRNA for CAL increased on addition of 1α,25-$(OH)_2D_3$ in a dose-dependent manner. This increase of CAL mRNA changed little on suppression of pp60$^{c\text{-}src}$ activity by PP1 (10^{-5} M). As for CAK, expression of the mRNA was little affected by the addition of 1α,25-$(OH)_2D_3$ or PP1 (10^{-5} M). Expression of CAB mRNA, barely detected, also exhibited no changes on addition of 1α,25-$(OH)_2D_3$ and/or PP1. Same results were gained using quantitative PCR (Perkin-Elmer ABI Prism 7700 Sequence Detection System, ABI-Perkin-Elmer, Foster City, CA) (data not shown).

Effects of pp60$^{c\text{-}src}$ Inhibitors on Secretion of Cathepsins

Activity of CCP, including CAK, CAL and CAB activity, was increased about 50–90% within 48 h by 1α,25-$(OH)_2D_3$ (10^{-9}–10^{-8} M) in the culture medium of calvaria (Fig. 2A). This increase of protease activity was suppressed by the addition of PP1 (10^{-5} M), but not lost completely. The CCP activity in the calvaria increased significantly on addition of PP1 relative to the activity without PP1. CAL, secreted into the culture medium, was increased by 1α,25$(OH)_2D_3$ (10^{-8} M), and this increase was suppressed by cotreatment of PP1 (10^{-5} M) (Fig. 2B). On the other hand, CAL in

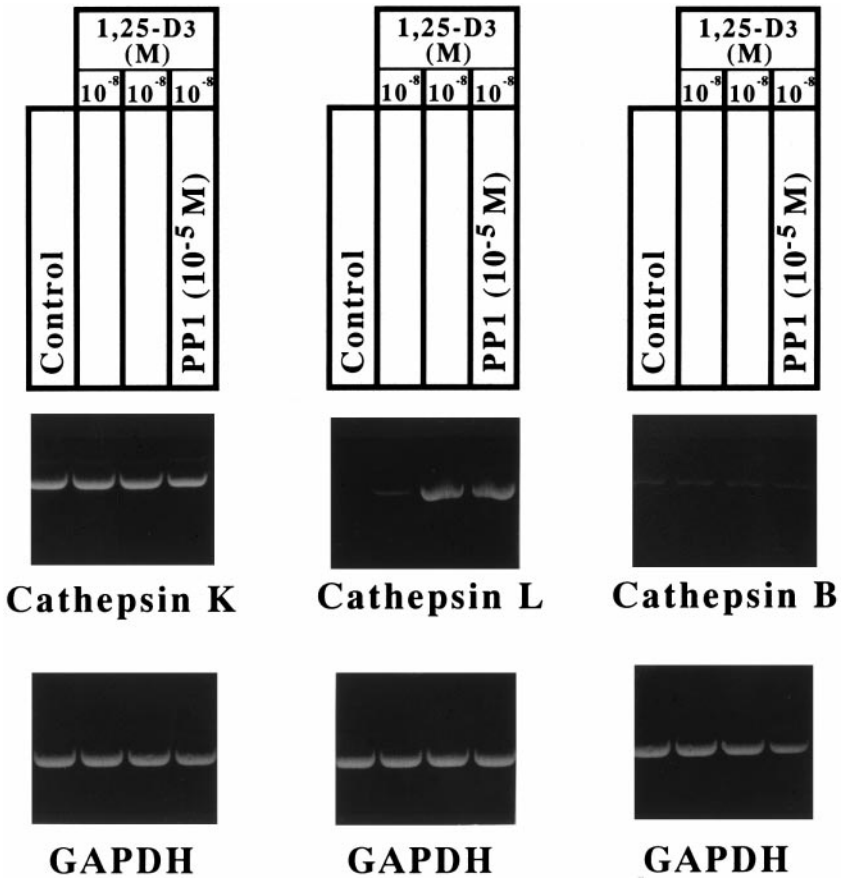

FIG. 1. Effect of pp60$^{c\text{-}src}$ inhibitor, PP1, on synthesis of cathepsins in osteoclasts. Osteoclasts, differentiated by coculture of bone marrow stromal cells and osteoblastic cells, were treated with the indicated concentration of 1α,25-$(OH)_2D_3$ in the presence or absence of PP1 (10^{-5} M) for 24 h. Total RNA (10 μg) was isolated from the cells and examined by RT-PCR analysis for cathepsin K, cathepsin L and cathepsin B.

calvarial osteoclasts, not secreted into culture medium, was increased by cotreatment of PP1 as revealed by Western blot analysis. CAK, secreted into the culture medium 10 times the rate of CAL, was not affected in the presence of 1α,25$(OH)_2D_3$, but was suppressed by PP1 (10^{-5} M). CAK in calvarial osteoclasts, not secreted into culture medium, was increased by cotreatment of 1α,25$(OH)_2D_3$ and PP1 as revealed by Western blot analysis. CAB was barely detectable in the experiments.

Effects of Actin Polymerization Inhibitor on Synthesis and Secretion of Cathepsins

The increase of CAL mRNA, extracted from osteoclasts differentiated by the coculture of bone marrow stromal and osteoblastic cells of mice in the presence of 1α,25-$(OH)_2D_3$ (10^{-9}–10^{-8} M) for 24 h, was not changed by cytochalasin B (10^{-6} M), an inhibitor of polymerization of actin (data not shown). The mRNA levels of CAK and CAB also did not change with or without cytochalasin B. On the other hand, the collagenolytic CCP activity in the medium of calvarial organ culture was suppressed, but not lost completely (Fig. 3A). The CCP activity in osteoclasts increased on suppression of actin polymerization by cytochalasin B (10^{-6} M). Levels of secreted CAL, into the culture medium, stimulated by 1α,25$(OH)_2D_3$, was found to decrease by cotreatment of cytochalasin B (10^{-6} M) by Western blot analysis (Fig. 3B). On the other hand, the level of CAL in calvarial osteoclasts, not secreted into culture medium, increased by cotreatment of 1α,25$(OH)_2D_3$ and cytochalasin B. CAK, secreted into the culture medium, not affected in the presence of 1α,25$(OH)_2D_3$, was suppressed by cytochalasin B (10^{-6} M). CAK in calvarial osteoclasts, not secreted into culture medium, was increased on cotreatment of 1α,25$(OH)_2D_3$ and cytochalasin B as revealed by Western blot analysis. Trace amount of CAB was found to have no effects. These results were matched those obtained using PP1.

Effects of PI3-kinase Inhibitor on Synthesis and Secretion of Cathepsins

The increase of CAL mRNA, extracted from osteoclasts differentiated by the coculture of bone marrow

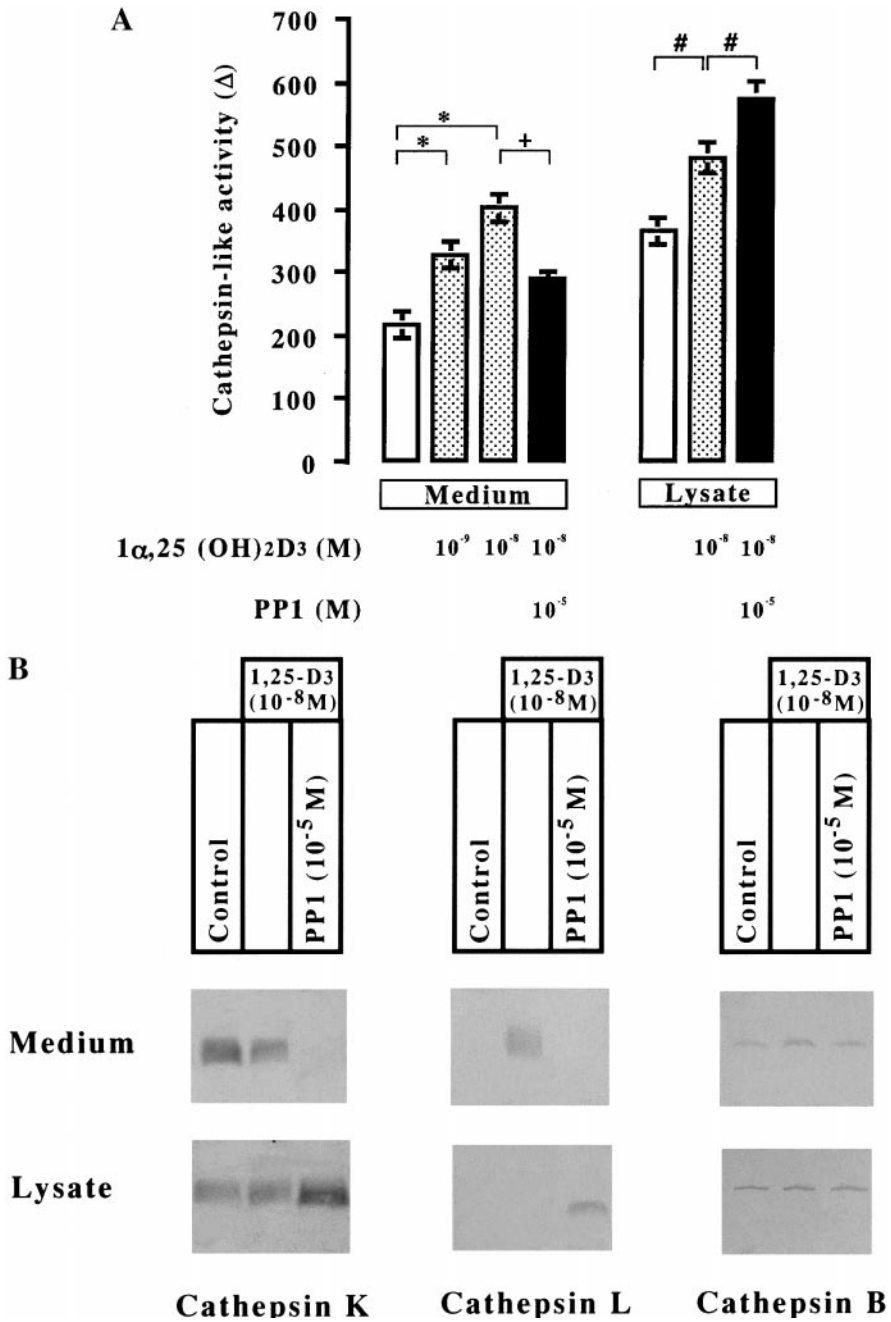

FIG. 2. Effect of pp60$^{c\text{-}src}$ inhibitor, PP1, on secretion of cathepsins in mouse calvarial organ culture. Calvaria were cultured with the indicated concentration of 1α,25-$(OH)_2D_3$ in the presence or absence of PP1 (10^{-5} M) for 48 h. Cathepsin-like activities in the culture medium, and in the calvaia were measured (A). Cathepsin K, cathepsin L, and cathepsin B in mouse calvarial organ culture media, and in the calvaria were detected by western blot analysis (B). *$P < 0.05$ 1α,25-$(OH)_2D_3$ vs control. Dunnett type test, $n = 6$. +$P < 0.05$ 1α,25-$(OH)_2D_3$ (10^{-8} M) vs 1α,25-$(OH)_2D_3$ (10^{-8} M) + PP1. Student's *t*-test, $n = 6$. #$P < 0.05$ 1α,25-$(OH)_2D_3$ (10^{-8} M) vs 1α,25-$(OH)_2D_3$ (10^{-8} M) + PP1 or control. Student's *t*-test, $n = 6$.

stromal and osteoblastic cells of mice in the presence of 1α,25-$(OH)_2D_3$ (10^{-9}–10^{-8} M) for 24 h, was not changed by wortmannin (10^{-7} M), an inhibitor of PI3-kinase (data not shown). The mRNA levels of CAK and CAB also did not change with or without wortmannin (10^{-7} M). On the other hand, the CCP activity in the medium of calvarial organ culture was suppressed, but not lost completely (Fig. 4A). The CCP activity in osteoclasts increased on suppression of PI3-kinase activity by wortmannin (10^{-7} M). Levels of secreted CAL, into the culture medium, stimulated by 1α,25$(OH)_2D_3$, was found to decrease by cotreatment of wortmannin (10^{-7} M) by Western blot analysis (Fig. 4B). On the other hand, the level of CAL in calvarial osteoclasts, not

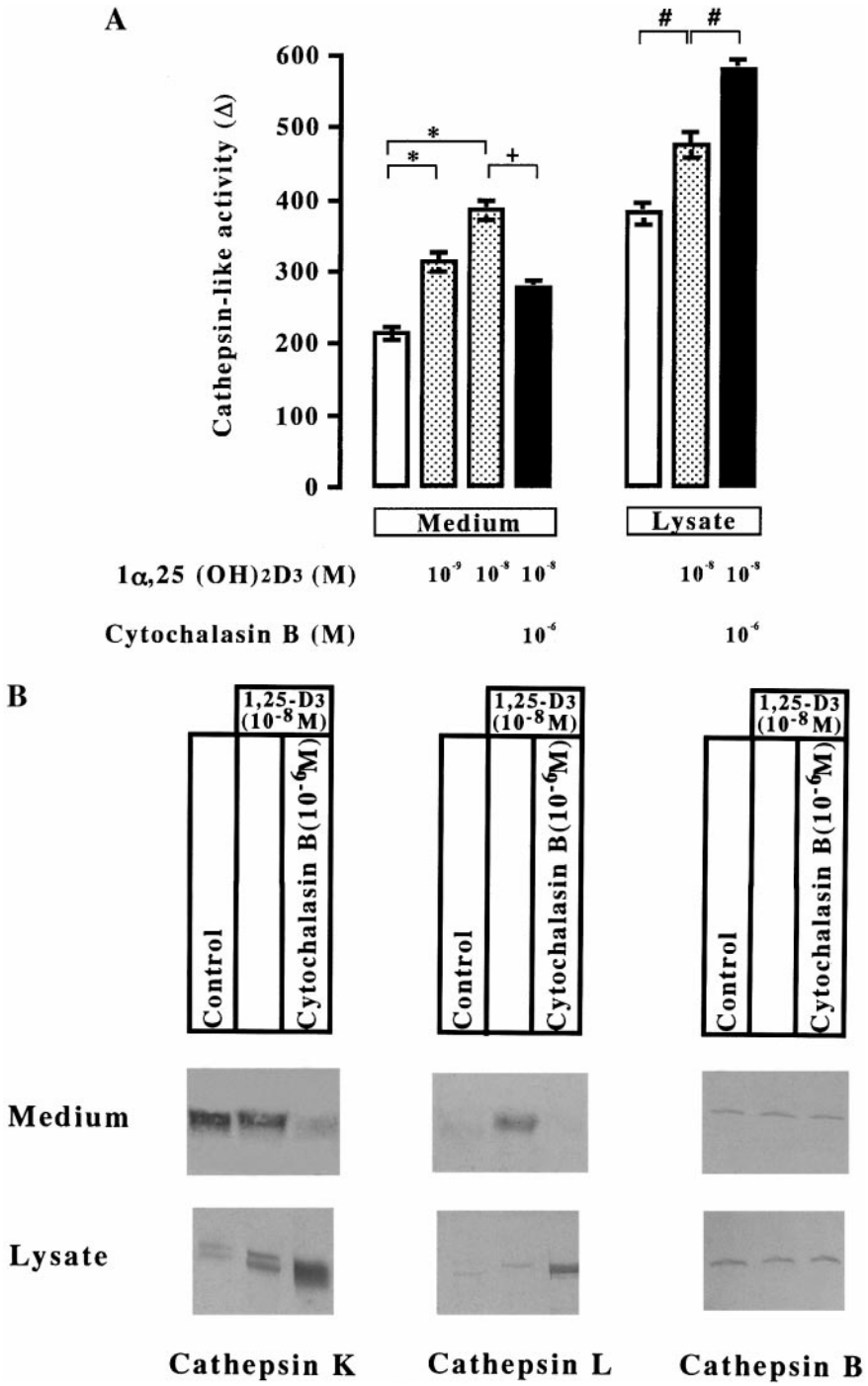

FIG. 3. Effect of actin polymerization inhibitor, cytochalasin B, on secretion of cathepsins in mouse calvarial organ culture. Calvaria were cultured with the indicated concentration of $1\alpha,25\text{-}(OH)_2D_3$ in the presence or absence of cytocharasin B (10^{-6} M) for 48 h. Cathepsin-like activities in the culture medium, and in the calvaia were measured (A). Cathepsin K, cathepsin L and cathepsin B in mouse calvarial organ culture media, and in the calvaria were detected by western blot analysis (B). $*P < 0.05$ $1\alpha,25\text{-}(OH)_2D_3$ vs control. Dunnett type test, $n = 6$. $+P < 0.05$ $1\alpha,25\text{-}(OH)_2D_3$ (10^{-8} M) vs $1\alpha,25\text{-}(OH)_2D_3$ (10^{-8} M) + cytochalasin B. Student's *t*-test, $n = 6$. $\#P < 0.05$ $1\alpha,25\text{-}(OH)_2D_3$ (10^{-8} M) vs $1\alpha,25\text{-}(OH)_2D_3$ (10^{-8} M) + cytochalasin B or control. Student's *t*-test, $n = 6$.

secreted into culture medium, increased on cotreatment of $1\alpha,25(OH)_2D_3$ and wortmannin. CAK, secreted into the culture medium, not affected in the presence of $1\alpha,25(OH)_2D_3$, was suppressed by wortmannin (10^{-7} M). CAK in calvarial osteoclasts, not secreted into culture medium, was increased on cotreatment of $1\alpha,25(OH)_2D_3$ and wortmannin as revealed by Western blot analysis. Trace amount of CAB was found to have

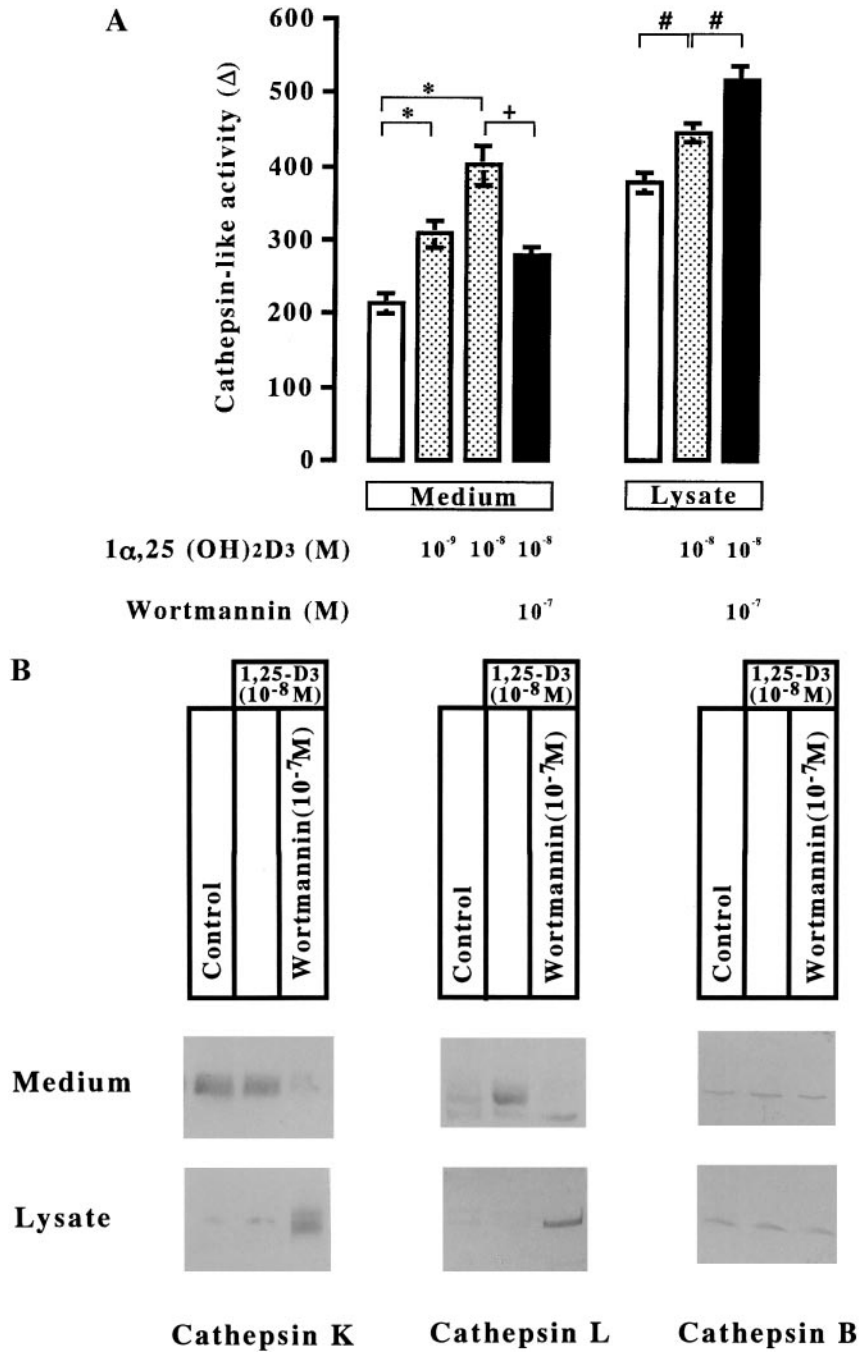

FIG. 4. Effect of PI3-kinase inhibitor, wortmannin, on secretion of cathepsins in mouse calvarial organ culture. Calvaria were cultured with the indicated concentration of 1α,25-$(OH)_2D_3$ in the presence or absence of wortmannin (10^{-7} M) for 48 h. Cathepsin-like activities in the culture medium, and in the calvaia were measured (A). Cathepsin K, cathepsin L, and cathepsin B in mouse calvarial organ culture media, and in the calvaria were detected by western blot analysis (B). $*P < 0.05$ 1α,25-$(OH)_2D_3$ vs control. Dunnett type test, $n = 6$. $+P < 0.05$ 1α,25-$(OH)_2D_3$ (10^{-8} M) vs 1α,25-$(OH)_2D_3$ (10^{-8} M) + wortmannin. Student's t-test, $n = 6$. $\#P < 0.05$ 1α,25-$(OH)_2D_3$ (10^{-8} M) vs 1α,25-$(OH)_2D_3$ (10^{-8} M) + wortmannin or control. Student's t-test, $n = 6$.

no effects. The activity of CCP and amount of CAK or CAL in the culture medium were positively correlated. These results were the same as those obtained using PP1 or cytochalasin B.

F-actin Ring Formation

PP1 (10^{-5} M), cytochalasin B (10^{-6} M) and wortmannin (10^{-7} M), treated for 3 h, suppressed the formation

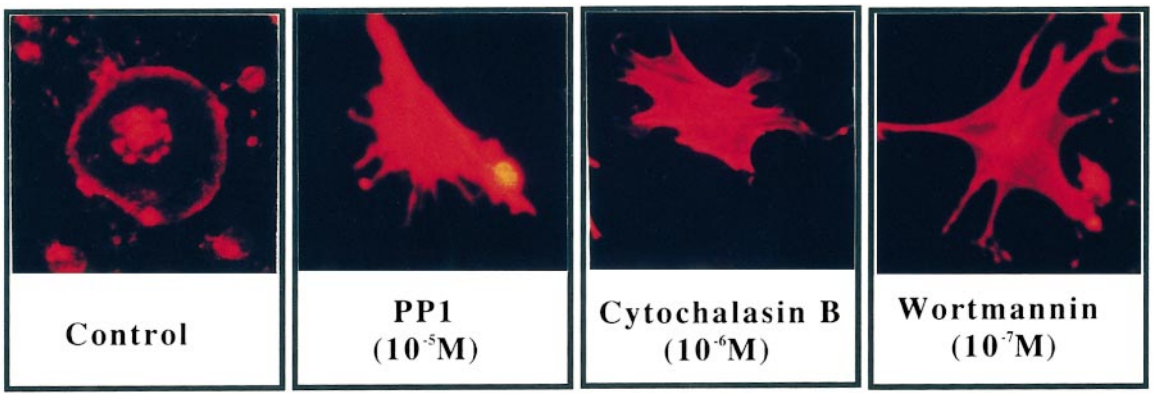

FIG. 5. The effects of PP1 (10^{-5} M), cytochalasin B (10^{-6} M) and wortmannin (10^{-7} M) on F-actin ring formation in osteoclasts. Osteoclasts, fixed on plates, were stained with rhodamine-labeled phalloidin and observed under a fluorescent microscope (×500).

of the F-actin ring of osteoclasts, differentiated by the coculture method (Fig. 5). The degradation of F-actin ring began within minutes after the addition of cytochalasin B. Addition of PP1 at 10^{-5} M, a concentration sufficient to inhibit the activity of pp60$^{c\text{-}src}$, suppressed the formation of F-actin ring.

Hydroxyproline Release

PP1 (5×10^{-5} M), Cytochalasin B (10^{-5} M) and wortmannin (10^{-6} M) suppressed the release of hydroxyproline, which was higher than the concentration necessary for suppression of F-actin ring formation (Fig. 6).

DISCUSSION

CAK, CAL and/or CAB, secreted from osteoclasts, were reported to be involved in degradation of type-I collagen in bone (4, 9, 22), but how the synthesis and secretion of these CCPs is modulated in osteoclasts remains unknown. The consensus sequences of responsive elements of cAMP and pholbolester were found in the promoter region of CAL gene (23), and the synthesis or secretion of the cathepsins might be regulated by estrogen (24) or γ-IFN (25). Past results were mostly obtained using transformed cells or macrophage, other than follicle-stimulating hormone (FSH) and cAMP, found to have regulated the expression of CAL in Sertoli cells (26), and retinoic acid regulated CAK in osteoclasts (27). These cells secrete cathepsins under appropriate conditions just like osteoclasts, though whether the same mechanisms are involved remains to be clarified. As CCPs are known to locate in lysosome and to degrade cytosolic proteins, the mechanisms involved in the secretion of cathepsins might be distinct from those affecting ordinary secreted proteases. So we focused on whether the activity of pp60$^{c\text{-}src}$ participates in the synthesis and/or secretion of cathepsins, and found that inhibitors of pp60$^{c\text{-}src}$ suppressed the secretion of CCP in osteoclasts.

Phosphorylated p60$^{c\text{-}src}$ activity is vital to osteoclasts, facilitating bone resorption without affecting the differentiation of osteoclasts (1). One of the mechanisms involved in the impairment of osteoclast function on disruption of pp60$^{c\text{-}src}$ might depend on the breakdown of ruffled border. It is known that (I) ruffled border is indispensable to and formed before bone resorption (14), (II) both demineralizing acid and collagenolytic proteases are secreted from ruffled border (14, 28, 29), suggesting that bone resorption and ruffled border formation are closely correlated. One of the principal roles of pp60$^{c\text{-}src}$ in bone resorption might be to regulate ruffled border formation through polymerization of actin. Under the condition of ruffled border disruption, caused by the suppression of pp60$^{c\text{-}src}$ activity in osteoclasts, the low pH (around pH4-5) environment, optimal for the degradation of type-I collagen by CAL and CAK (20, 30, 31), might be disappeared via suppression of H^+ secretion into Howship's lacunae. However CAK, the most likely candidate among collagenolytic proteases, is able to degrade type-I collagen under neutralized conditions and to also act as an exoprotease (31, 32). So, the suppression of hydroxyproline release by inhibition of pp60$^{c\text{-}src}$ activity might mostly depend on a reduction of CCPs secretion.

In many cases, lysosomal cysteine proteases become trapped in the Golgi apparatus after synthesis at endoplasmic reticulum through binding with mannose 6-phosphate receptor (M6PR), known also as IGF-II receptor (33), without being secreted into the extracellular environment (34, 35). In osteoclastic bone resorption, overproduced CCPs might be secreted from osteoclasts to Howship's lacunae and trapped by M6PR, expressed on ruffled borders in osteoclasts (36), to maintain high concentration of CCPs following abnormal stimulation of bone resorption. Under such conditions, cytochalasin B, which did not inhibit the activity

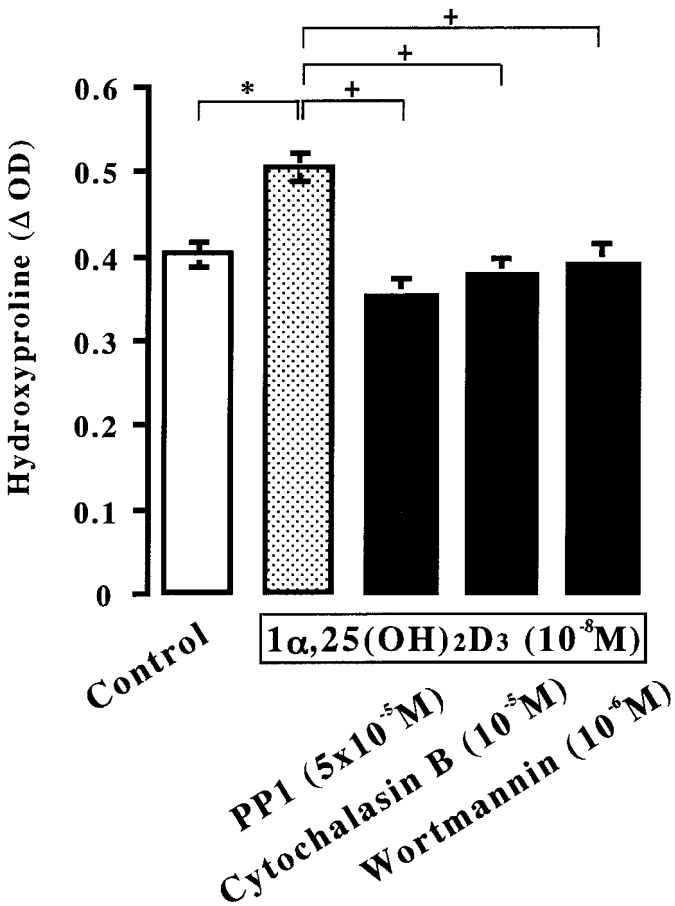

FIG. 6. The effects of PP1 (5×10^{-5} M), cytochalasin B (10^{-5} M) and wortmannin (10^{-6} M) on collagenolysis in mouse calvarial organ culture. Calvaria were cultured with $1\alpha,25\text{-}(OH)_2D_3$ (10^{-8} M) in the presence or absence of each inhibitor for 48 h. Hydroxyproline in the culture medium was measured. $*P < 0.05$ $1\alpha,25\text{-}(OH)_2D_3$ vs control. Student's *t*-test, $n = 6$. $+P < 0.05$ $1\alpha,25\text{-}(OH)_2D_3$ (10^{-8} M) vs $1\alpha,25\text{-}(OH)_2D_3$ (10^{-8} M) + PP1, cytochalasin B or wortmannin. Dunnett type test, $n = 6$.

of pp60$^{c\text{-}src}$, might suppress the formation of ruffled border via breakdown of F-actin ring resulting in the reduction of secretion of cathepsins. So we speculate that failure to form ruffled borders is one of the major mechanisms of disturbance of bone resorption caused after disruption of pp60$^{c\text{-}src}$ activity.

Though the precise mechanism remains unknown, PI3-kinase might play a role in governing ruffled border generation caused in part by c-Src in osteoclasts. Wortmannin, an inhibitor of PI3-kinase (37, 38) which is assumed to be one of the second messengers of pp60$^{c\text{-}src}$ (39–41), suppressed the secretion of cathepsins, as might result from the disruption of ruffled border formation through cytoskeletal rearrangement. As PI3-kinase was reported to act as a regulator of cytoskeletal organization (42) and actin polymerization (43), wortmannin might affect the structure of ruffled border, which then disrupted the secretion of proteases without disturbing their synthesis. Recently, Tanaka *et al.* found that c-Cbl acted on the downstream of pp60$^{c\text{-}src}$ in signaling pathways necessary for bone resorption (44, 45). It is currently under investigation whether PP1 also affects the signaling pathways of c-Cbl. Besides the c-Src and PI3-kinase, Rabs (Rab3 and Rab7), a group of small GTP-binding protein constituting part of the ras superfamily, have reported to be involved in the ruffled border formation in bone-resorbing osteoclasts (47, 48). The results of subcellular colocalization of Rab3B/C, isoforms of Rab3, with c-Src in osteoclasts (48), suggested the possibility that Rab3 might take part in c-Src signaling pathways involved in ruffled border formation.

Inhibitor of pp60$^{c\text{-}src}$ was required at higher concentration to suppress collagenolysis than to suppress the F-actin ring formation and secretion of CCP. The results suggested that the major mechanism by which PP1 suppresses the secretion of cathepsins and bone resorption is the disturbance of ruffled border formation through inhibition of pp60$^{c\text{-}src}$-PI3-kinase signaling pathways.

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