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cAMP 길항제와 PKA 억제제 및 Adenylate Cyclase 촉진제의 백서 파골세포에서 Cathepsin K 생성에 대한 효과

심 연 수
연세대학교 치과대학 치과생체재료공학교실

Inhibitory Effect of Camp Antagonist and Pka Inhibitors, and Stimulatory Effect of Adenylate Cyclase Agonist on Cathepsin K Processing in Cultured Mouse Osteoclasts

Youn-Soo Shim
Department and institute of Dental Biomaterials & Bioengineering, College of Dentistry, Yonsei University

ABSTRACT Cathepsin K (cat K) is the major cysteine protease expressed in osteoclasts and was thought to play a key role in matrix degradation during bone resorption. It was shown that the intracellular maturation of cat K was prevented by the cAMP antagonist, Rp-cAMP, and the protein kinase A (PKA) inhibitors of KT5720 and H89. In contrast, forskolin, a adenylate cyclase agonist, rather induced Cat K processing and maturation in osteoclasts. Furthermore, to determine whether cat K processing and maturation signaling involves protein kinase C (PKC), mouse total bone cells were treated with calphostin C, a specific inhibitor of PKC, however, no effect was observed, indicating that calphostin C did not affect to osteoclast-mediated cat K processing and maturation. Thus, it is indicated that the cAMP-PKA signaling pathway regulates cat K maturation in osteoclasts. Since secreted proenzymes have the potential to reenter the cell via M6P receptor, to prevent this possibility, it was tested cAMP antagonist Rp-cAMP and the PKA inhibitors KT5720 and H89 in the absence or presence of M6P. Inhibition of cat K processing by Rp-cAMP, KT5720, or H89 was observed in a dose-dependent manner. Furthermore, the addition of M6P resulted in enhanced potency of Rp-cAMP, KT5720 and H89. These dose-dependently inhibited *in vitro* bone resorption with a potency similar to that observed for inhibition of cat K processing.

Key words Cathepsin, Protease, Biosynthesis, Rp-cAMP, KT5720

INTRODUCTION

Bone resorption requires the directional secretion of proteolytic enzymes into the resorption lacunae for degradation of the organic matrix proteins. Cathepsin L, B and K are involved in this process of bone resorption[1]. Of them, cathepsin K is the key enzyme involved in the resorptive process[2,3].

Cathepsin K (cat K) (EC 3.4.22.38) is belongs to the papain superfamily of lysosomal cysteine proteases which include cathepsin B, L, H, and S.

These enzymes are thought to participate in a variety of physiological processes including cartilage proteoglycan breakdown[4], matrix modification[5], bone remodeling[6], and embryonic development[7]. In addition, cysteine proteases have been implicated in various pathological settings, such as rheumatoid arthritis[8], tumor invasion[9] and metastasis[10], inflammation[11], and osteoporosis[12].

Cathesin K is a lysosomal cysteine protease that efficiently cleaves peptide bonds in various proteins including collagen, elastin, and gelatin[13].

Cathepsin K cDNA has been cloned from rabbit osteoclasts[14], human osteoclastoma[15], mouse[16-18], and encodes prepropeptides of 329 to 334 residues. The mouse cathepsin K gene contains a 990-bp coding for 329 amino acid prepropolypeptide. The structure of the protein included a 15-amino acid presignal, a 99-aa preprotein, and a 215-aa mature enzyme[16] with two potential N-glycosylation sites[16]. Mouse cathepsin K mRNA is selectively expressed in osteoclast[18]. Mature enzyme cathepsin K appeared to be a monomeric protein with an apparent molecular mass of about 29 kDa with three putative N-glycosylation sites, which presumably facilitates its lysosomal targeting via the mannose-6-phosphate receptor-mediated pathway. Biochemical characterization of recombinant human cathepsin K has revealed that the enzyme is capable of degrading triple helical type I collagen and osteonectin, and also has strong

†Corresponding author
Tel: 031-350-2419
Fax: 031-350-2418
E-mail: shim-21@hanmail.net

elastase and gelatinase activities[13]. Cathepsin K activity had a pH optimum of 6.1.

Cat K was first identified as a cysteine protease in osteoclasts[19-21]. Osteoclasts are specialized bone cells whose primary function is to resorb bone and provide sites for new bone formation. The resorptive phase of bone remodeling is initiated by the attachment of osteoclasts to the bone surface. Furthermore, it has been shown that mature cat K has proteolytic activity against several extracellular matrix substrates[22-23], including type I collagen, and the most abundant matrix protein in bone.

Glycoproteins and core proteins of proteoglycans are susceptible to several proteases. The degradation of native fibrillar collagens is dependent on specific collagenolytic enzymes, collagenases and other matrix metalloproteinases (MMPs), and lysosomal cysteine proteinases, such as cathepsins[24]. Resorption of extracellular matrix occurs both extracellularly and intracellularly[25].

PI3-kinase is known to be involved in growth factor signal transduction and vesicular membrane trafficking in a variety of systems. Wortmannin(WT), an PI3-kinase inhibitor, has been previously shown to inhibit cat K processing in osteoclast and bone resorption both in vitro[26]. WT inhibited in a dose dependent manner cat K processing in mouse osteoclasts, and eventually resulted in the secretion of unprocessed pro cat K into the culture media.

It was also known that prostaglandins interact with specific cell surface receptors, which result in activation of adenylate cyclase, a rapid increase in intracellular cAMP, and cAMP-dependent protein kinase (PKA). Also, prostaglandins mediate hydrolysis of phosphatidylinoshitol 4,5-bisphosphate, with subsequent production of diacylglycerol and inositol 1,4,5-triphosphate. These second messengers activate protein kinase-C (PKC), and calcium levels are increased from intracellular organelles[27]. In rat osteosarcoma cell line, it was suggested that PGE_2 stimulates the PKA signal transduction pathway and activates the intracellular calcium-dependent pathway, and also stimulates MMP-1 gene expression[28].

The study was focused on Cat K, since Cathesin K is a lysosomal cysteine protease and was first identified as a cysteine protease in osteoclasts.

The formation of matured Cat K is an important even during bone resorption since it has proteolytic activity against several extracellular type I collagen in bone. The present study was henceforth aimed to investigate the inhibition mechanism of the Cat K processing by the cAMP antagonist (Rp-cAMP), the PKA inhibitors (KT5720 and H89), PI-3K inhibitor (WT), adenylate cyclase agonist (forskolin), and PKC inhibitor (Calphostin C).

MATERIALS AND METHODS

1. Reagents

Forskolin and Bovine Serum Albumin(BSA) were purchased from Sigma chemical Co. (St. Louis, MO, USA). Rp-cAMP was obtained from RBI (Natick, MA, USA), and KT5720, H89, and calphostin C from Biomol (Plymouth, PA, USA). Wortmannin(WT), mannose-6-phosphate(M6P), and the cysteine protease inhibitor, E64, were purchased from Sigma chemical Co. (St. Louis, MO, USA). Purified recombinant mouse Escherichia coli-expressed cat K were generated in-house by RT-PCR.

2. PCR cloning of a mouse cathepsin K gene-encoding cDNA fragment and expression in E. coli

Sense and antisense oligonucleotide primers based on the previously cloned sequence[17] were synthesized on an Applied Biosystems Instrument (ABI) Model 394 DNA Synthesizer by CoreBio System Co. (Seoul, Korea). cDNA carrying the mouse full cathepsin k gene was amplified from mouse fetal cDNA library which was prepared in our house laboratory, as described in previous paper[29]. These primers were used to PCR-amplify a fragment from first-strand cDNA which was generated from fetal mouse brain total RNA by reverse transcription.

Sequences for the RT-PCR primers were derived from the previously cloned mouse cathepsin K cDNA sequences (GenBank /EMBL accession number X94444)[17]. Primers were designed for the mature enzyme size of 215 amino acid residues. The cloned gene was expressed in E. coli carrying a recombinant plasmid pETCatK-1, whch was constructed in expression vector, pET3a. The expressed cathepsin K was about 32 kDa with proenzyme (data not shown).

3. Mouse osteoblast culture

Explants of mouse calvarial bone were cultured and the cells obtained have been routinely characterized and shown to express an osteoblast-like phenotype in culture. The population released during the last three digestions was highly enriched in cells expressing two markers of the osteoblast lineage, alkaline phosphatase and osteocalcin[30]. Cells released by collagenase digestions were washed and grown to confluent in 75 cm^2 culture flasks(Falcon) in Dulbecco's modified Eagle's medium(DMEM) supplemented with antibiotics (penicillin and streptomycin) and 10% fetal calf serum (FCS; Gibco, BRL, Bethesda, MD, USA). Incubations were carried out at 37°C in a humidified atmosphere of 5% CO_2/95% air; the medium was changed every 2-3 days. Cells were grown to confluence at 37°C and cultured in duplicate or triplicate wells for an additional 24h in serum-free medium supplemented with Polymixin B sulfate to prevent endotoxin effects prior to treatment.

4. Preparation of rabbit Cat K antibody (CHK-2)

Polyclonal antibody CHK2 was generated against a peptide corresponding to the unique sequence in mouse cat K (data not shown). This antibody demonstrated similar reactivity to recombinant pro and mature cat K by immunoblot (data not shown).

5. Preparation of fetal mouse long bone organ tissue culture system containing both of osteoclast and osteoblasts

The fetal mouse long bone organ tissue culture system

was based on that described by Raisz[31]. Mouse long bone preparation was also described in previous paper[32]. The method for disaggregating osteoclasts from fresh long bone organ tissue and their subsequent use for in vitro assays has also been described in detail[31,33,34]. Briefly, cell suspensions derived from collagenase digestion were washed and osteoclasts enriched by negative selection using magnetic beads coated with a murine antimouse HLA-DR MAb. The isolated cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and resuspended in RPMI media containing 10% FBS (Jeil-Biotech Service, Co. Ltd. Taegu, Korea). The cells were then plated on either bone slices or on plastic tissue culture dishes and incubated at 37°C prior to treatment.

6. Bone resorption assay

The fetal mouse long bone organ tissue culture system was based on that described by Raisz[31]. Fetal bones were labeled with ^{45}Ca by injecting the mother with 200 μCi ^{45}Ca (NEN, Boston, MA, USA) on the eighteenth day of gestation. Radii and ulnae bone shafts were obtained from 19 day fetuses by microdissection. The shafts were cut just beyond the calcified zone and therefore contained short lengths of cartilage at the ends. Bones were cultured on sunk Millipore filter dots in 24-well Limbro plates. The shafts were first cultured in 0.5 ml BGJ_b medium(Gibco Laboratories, Grans Island, NY, USA) containing 1.0 mL/mL bovine serum albumin, 100 units/ml penicillin G, and 1 μg/mL polymyxin B for 1 day to reduce exchangeable ^{45}Ca. One bone from a pair (right and left radii or right and left ulnae from a single fetus) was then transferred into medium containing agonist(s) (treatment) and the contralateral bone was placed into identical medium without agonist(s) (control). A typical test group consisted of 5 pairs of bones. Bones were cultured for 5 days in a 95% air / 5% CO_2 incubator at 37°C and 95% humidity with one change of media after 2 days. The percentage of ^{45}Ca released from a bone into the medium during the 5-day culture was determined by measuring the radioactivity in medium 1, medium 2, and the trichloroacetic acid solubilized bone using a liquid scintillation counter. Stimulated resorption was expressed as the paired difference between treatment and control bone percent ^{45}Ca released from during the 5-day culture. Dead bone ^{45}Ca release in this system was apprximately 10%. BGJ_b control ^{45}Ca release was 16-20% and maximum IL-1 β ^{45}Ca release was 60-80%. Since "stimulated" release is expressed as the mean difference between paired BGJ_b control bones (C%) and treated bones (T%), the T%-C% for an inactive treatment is zero, and a maximum IL-1 β response is approximately 40-60%. Each bone was labeled with approximately 20,000 CPM ^{45}Ca.

7. Biosynthetic radiolabeling of targent proteins by ^{35}S-isotope

Osteoclast containing cultures were washed with DPBS containing 5 mg/mL bovine serum albumin (BSA) and cultured in cysteine/methionine-free α-minimal essential medium (α-MEM) containing HEPES (pH 7.2) for 60min at 37°C. The medium was removed and replaced with cysteine/methionine-free α-MEM containing 250 mCi/mL Trans [^{35}S]-Label (−1000 Ci/mmol; ICN Biomedicals, Costa Mesa, CA, USA) for 30 min at 37°C. Cultures were washed three times with ice-cold DPBS and resuspended in RPMI media containing 5% fetal bovine serum (FBS) and 20 μmol/L HEPES (pH 7.4) in the presence or absence of test agents and incubated for the indicated times.

8. Sample collection and preparation

Cell cultures were lysed with 500 μL of 50 μM Tris-HCl (pH 7.4), containing 150 μM NaCl, 1% Triton X-100, 1 μM EDTA, 1 μM phenylmethysulfonylflouride (PMSF), 50 μM leupeptin, 50 μM E-64, and 1 μM pepstatin for 10 min at 4°C. Cultures were scraped with a rubber policeman, lysates were centrifuged for 5min at 20,000 g at 4°C and supernatants were transferred to new tubes. Culture supernatants were collected and centrifuged to remove cell debris and transferred to tubes containing protease inhibitors.

9. Mmunoprecipitation, SDS-polyacrylamide gel electrophoresis and immunoblot analysis of the proteins

Cell lysate and media samples (450 μL) were incubated with 5 μL of polyclonal antibody CHK-2 (0.5 mg/mL stock), which recognizes both the pro and mature enzyme, followed by addition of 20 μL of protein A agarose and placed on a rotator overnight at 4°C. Precipitates were centrifuged and washed twice with lysis buffer and once with lysis buffer without Triton X-100. Precipitated proteins were resuspended in 30 μL of 4 × sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer without 2-mercaptoethanol (nonreducing) and boiled for 5 min, as described by Laemmli[35]. Samples were applied to 12.5% SDS-PAGE gels and ran at 30 mA/gel. Protein determination of the samples was carried out as described by Lowry *et al.*[36].

For western blot analysis, proteins were transferred onto nitrocellulose membrane, blocked with 5% skim milk in PBST, and probed with a polyclonal anti-cat K antibody (1 : 1000) in PBST containing 0.1% BSA for 2 h. The membrane was washed three times for 15 min with PBST and developed by ECL (Amersham Pharmacia Biotech., Upssala, Sweden). For quantitative analysis of radiolabeld cat K, gels were fixed, dried, and exposed to a phosphor screen, and then scanned using a phosphorimager analyzer (Fuji Co. Ltd. Tokyo, Japan).

10. Mouse osteoclast resorption assay

Osteoclast number was adjusted to 2×10^3 cells/mL and preincubated with test agent or with vehicle control for 30 min at 37°C and then seeded onto bovine cortical bone slices (0.5 ml/well) in a 48 well plate. Following incubation for 48 h at 37°C, culture supernatants were harvested and resorption was measured using a competitive ELISA, according

to the manufacturer's protocol (Osteometer A/S, Rodovre, Denmark). The assay measures carboxyterminal telopeptides of the α1 chain of human type I collagen that are released during the resorption process[22]. The results were expressed as percent inhibition of resorption compared to supernatants derived from untreated osteoclast cultures.

RESULTS

1. Cat K-recognizing polyclonal (CHK-2) antibodies and expression of cat K enzyme in mouse osteoclasts

A mouse polyclonal antibody (CHK-2) that can recognize the pro or mature forms of mouse cat K was generated against two peptide mixtures corresponding to the unique sequences in mouse cat K pro and mature protein. This antibody demonstrated similar reactivity to recombinant pro and mature cat K by immunoblot. An activity staining technique using zymography was performed on total bone cells including osteoclasts and osteoblsts using a synthetic cathepsin K/L substrate (Z-Leu-Arg-4MNA). This activity was completely inhibited by the addition of E64, a nonselective cysteine protease inhibitor. However, no inhibition was observed using the aspartate protease inhibitor, pepstatin A, and the serine protease inhibitor phenylmethylsulfonylfluoride (PMSF), indicate that the enzyme activity is a cysteine protease.

To confirm the specific expression of cat K in osteoclasts, immunoblot analysis was performed using cell extracts of total bone cells (osteoclast + osteoblast + stromal cells), as well as osteoclast-enriched (vitronectin receptor, $\alpha_v \beta_3$-positive osteoclasts) and osteoclast-depleted $\alpha_v \beta_3$-negative cell mixtures (osteoblasts + stromal cells). As shown in (Fig. 1), cat K expression is observed in both the total cell mixtures(lanes 1 and 2) and osteoclast-enriched cell populations (lanes 3 and 4), but not in the osteoclast-depleted cells (lane 5). This observation is entirely consistent with the osteoclast-selective expression of cat k observed by immunolocalization and in situ hybridization as described by Drake *et al*[19]. Furthermore, the majority of cat K detected by immunob lot analsys in osteoclast lysates was mature enzyme, which was processed preoteolyically.

Processing and activation of cysteine proteases often occurs in lysosomal compartments under highly acidic environments. Osteoclasts were treated with chloroquine, which increases lysosomal pH, to address that cat K is processed in lysosomes. Also, osteoclasts were treated with monensin, which disrupts Golgi apparatus structure and lysosomes. Treatment with 20 uM chloroquine and 20 uM monensin almost prevented the proteolytic processing of cat K (Fig. 2), suggesting that cat K processing takes place in lysosomes. Many proenzymes are glycosylated and contain mannose residues that facilitate their delivery to the lysosome for proteolytic processing, modification and maturation. For example, Mcqueney *et al*.[37] and Rieman *et al*.[26] have reported that pro cat K expressed in baculovirus is glycosylated.

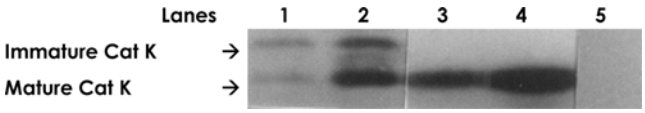

Fig. 1. Differential expression of pro and mature cat K in cultured mouse osteoblasts and non-osteoclastic cells as confirmed by western blot analsysis.
Osteoclasts were immunologically isolated from calvarial and fetal long bone cells using magnetic beads coated with an antivitronectin receptor MAb as described in Methods Section. Lysates of total bone cells (lane 1, 5 ug ; lane 2, 10 ug), osteoclast-enriched (lane 3, 5 ug ; lane 4, 10 ug) or osteoclast- depleted cells (lane 5) were electrophoresed on a 12.5% SDS-PAGE gel. The gel was subjected the immunoblot analysis using an anti-cat K polyclonal antibody. Pro cat K (Pro) and mature cat K (Mat) are distinguished by their mobilities as indicated.

	Control	Chloroquine	Monensin
Labeled Mature Cat K	6.5	1.3	0.6
	8.4	2.3	1.5
	7.8	2.6	1.3

Fig. 2. Effect of lysosome-targeting agents of chlroroquine and monensin on cat K processing and maturation. Osteoclast cultures were treated with each 20uM chloroquine or monensin for 60 min prior to and during pulse-chase labelling. After 4h chase, cells were harvested and cat K was analyzed as described in text. Radiolabeled cat K is expressed as total mature cat K processed.

2. Cat K processing and maturation stimulated by osteoclasts is induced by adenylate cyclase agonist, forskolin, indicating that the maturation is mediated by a PKA pathway in mouse total long bone cells

It was previously reported that PI3-kinase plays a key role in cat K processing as elucidated by using the PI3-kinase inhibitor, WT[26]. Osteoclasts were pulse-labeled and pulse-chased in the presence or absence of WT. After a 4h chase with WT, intracellular processing of immature cat K were significantly inhibited, indicating that WT disrupt the intracellular trafficking of pro cat K[26].

Thus, osteoclasts-containing total bone cells were grown to confluence and treated for 4h with adenylate cyclase agonist, forskolin, to examine whether cAMP is involved in the induction of cat K maturation. The role of adenylate cyclase in cat K processing was investigated using the adenylate cyclase agonist, forskolin. Osteoclasts were pulse-labeled and pulse-chased in the presence or absence of forskolin(1 μM) for 4 h and radiolabeled cat K was analyzed by immunoprecipitation and SDS-PAGE. As shown in (Fig. 3), following a 30 min pulse (T = 0, lane 1), the pro form of cat K was detected in the osteoclast cell lysate. After a 4 h chase in the presence of forskolin, significant intracellular processing of immature cat K was observed (lane 4), suggesting that forskolin induces the intracellular trafficking of pro cat K. These showed that forskolin induced cat K processing and maturation in osteoclasts. The results were contrast to those made by WT[26].

3. Cat K processing and maturation stimulated by osteoclasts is inhibited by cAMP antagonist Rp-cAMP or PKA inhibitors KT5720 and H89, confirming that the maturation is also mediated by a PKA pathway in mouse total long bone cells

On the other hand, the cells were treated with either cAMP antagonists or PKA inhibitors to examine the role of cAMP and cAMP-dependent kinases in cat K processing and maturation signaling. As shown (Fig. 4), cat K processing and maturation were inhibited by the PKA antagonist, Rp-cAMP, and the PKA inhibitors, KT 5720 and H89 (20μM), suggesting that these inhibitors targeted specifically the cat K processing and maturation pathway stimulated by osteoclasts.

4. No effect of calphostin C, a specific inhibitor of PKC, on cat K processing and maturation stimuated by osteoclasts

Furthermore, to determine whether cat K processing and maturation signaling involves PKC, mouse total bone cells were treated with calphostin C, a specific inhibitor of PKC[38], however, was effected not observed (Fig. 5), indicating that calphostin C did not affect to osteoclast-mediated cat K processing and maturation signaling. These results also strongly suggested that cat K processing and maturation in osteoclasts are mediated by cAMP-dependent PKA pathway in mouse osteoclast cells.

5. Rp-cAMP, KT 5720 and H89 inhibit cat K proessing, while forskolin induces the cat K processing, and no effect of calphostin C, a specific inhibitor of PKC

It is well known that secreted proenzymes have the potential to reenter the cell via M6P receptors, and can be delivered to the lysosome for proteolytic processing and modification. To prevent this reentrance and delivery of proenzyme into lysosome by M6P receptor is important for inhibition and blocking of pro cat K processing, and finally to prevent bone resorption raised by processed cat K mature enzyme.

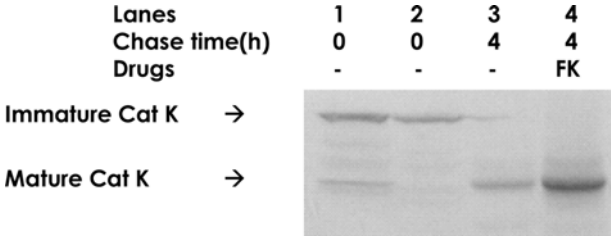

Fig. 3. Stimulating activity of forskolin, an adenylate cyclase agonist, on cat K processing and maturation from immature proenzyme to mature form in mouse total bone cells including both osteoblasts and osteoclasts.

Osteoclasts were pulse radiolabeled and chased for the indicated times in the presence or absence of forskolin (Fk,1 μ M). Cells and media were harvested at 0 and 4h after chase and cat K was immunoprecipitated with polyclonal antibody CHK-2 and analyzed by SDS-PAGE. Lanes 1 and 3(10ug); Lanes 2 and 4 (5ug).

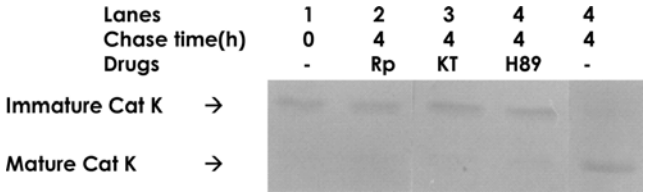

Fig. 4. Inhibitory activity of cAMP antagonist Rp-cAMP or the PKA inhibitors KT5720 and H89, on cat K processing and maturation from immature proenzyme to mature form in the mouse total bone cells including both osteoblasts and osteoclasts.

Osteoclasts were pulse radiolabeled and chased for the indicated times in the presence or absence of Rp-cAMP (5.0 uM) or 5 μM (KT5720) or H89 (20 μM). Cells and media were harvested at 0 and 4h after chase and cat K was immunoprecipitated with polyclonal antibody CHK-2 and analyzed by SDS-PAGE.

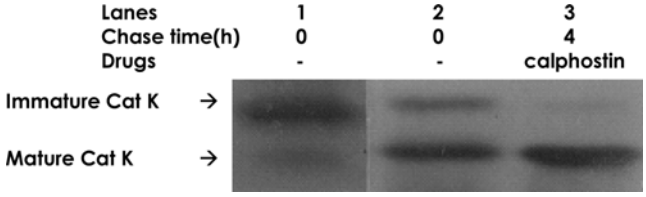

Fig. 5. Effect of calphostin C, a specific inhibitor of PKC, on cat K processing and maturation stimulated by osteoclasts in the mouse total bone cells including both osteoblasts and osteoclasts.

Osteoclasts were pulse radiolabeled and chased for the indicated times in the presence or absence of PKC inhibitor Calphostin C (CP, 0.2 μM). Cells and media were harvested at 0 and 4 h after chase and cat K was immunoprecipitated with polyclonal antibody CHK-2 and analyzed by SDS-PAGE.

Therefore, we tested Rp-cAMP, KT 5720, H89, forskolin, and calphostin C at various doses in the absence or presence of M6P (5 umoL/L). As shown in (Fig. 6, 7 and 8), inhibition of cat K processing by Rp-cAMP, KT 5720 or H89 alone was dose-dependent with an IC_{50} of 5.6 uM, 0.01 uM, and 0.5 uM, respectively.

However, forskolin rather induced the cat K processing while calphostin C did not was affected (Fig. 7). Furthermore, the addition of M6P increased potency of Rp-cAMP, KT 5720 or H89. On the other hand, forskolin induced the bone resorption via cat K processing, while calphostin C, a specific inhibitor of protein kinase C (PKC), did not affect to the bone resorption induced by osteoclasts.

6. Dose-dependent inhibition of in vitro bone resorption by Rp-cAMP, KT5720 and H89.

WT has previously been shown to inhibit bone resorption[26]. Therefore, it was thought to determine the ability of Rp-cAMP, KT5720 and H89 to prevent in vitro bone resorption under similar conditions. Rp-cAMP, KT5720 and H89 dose-dependently inhibited in vitro bone resorption Fig. 9 with a potency similar to that observed by inhibition of cat K processing.

Concentration		
Rp-cAMP(μM, □)	KT5720 (μM, ■)	H89 (μM, △)
0 0.1 0.5 5.0	0 0.001 0.004 0.01	0 0.001 0.06 0.5
↓		
0 3.4 21.4 46.7	0 0.4 14.3 44.6	0 2.5 13.6 50.2
Processing (% inhibition)		

Fig. 6. **Dose-dependent inhibition of cat K processing by Rp-cAMP, KT5720, and H89 in the absence of M6P. Pulse-radiolabeled osteoclasts were chased with increasing doses of Rp-cAMP, KT5720 and H89 in the absence of M6P (5 μmol/L) for 4h. Cell extracts were immunoprecipitated with an anti-cat K antibody as described. Cat K was quantitated using a phosphorimager and the results are expressed as percent inhibition of processing. M6P alone had no effect on cat K processing. Rp-cAMP (□), KT5720 (■), H89 (△)**

Forskolin (μM, □)	Calphostin C (μM, ■)
0 0.1 0.2 1.0	0 0.01 0.2 1.0
↓	↓
0 8.1 11.6 23.7	0 0.2 0.3 0.2
Processing (% induction)	

Fig. 7. **Effect of forskolin and calphostin C on cat K processing in the absence of M6P. Pulse-radiolabeled osteoclasts were chased with increasing doses of forskolin and calphostin C in the absence of M6P (5 μM) for 4 h. Cells extracts were immunoprecipitated with an anti-cat K antibody as described. Cat K was quantitated using a phosphorimager and results are expressed as percent inhibition (or induction for forskolin) of processing. M6P alone had no effect on cat K processing. Forskolin (□), calphostin(■)**

Concentration		
Rp-cAMP(μM, □)	KT5720 (μM, ■)	H89 (μM, △)
0 0.1 0.2 0.4	0 0.001 0.004 0.01	0 0.001 0.01 0.3
↓		
0 12.6 26.2 36.7	0 13.6 36.7 49.5	0 14.2 34.5 47.6
Processing (% inhibition)		

Fig. 8. **Dose-dependent inhibition of cat K processing in the presence of M6P by Rp-cAMP, KT5720, and H89 Pulse-radiolabeled osteoclasts were chased with increasing doses of Rp-cAMP, KT5720, and H89 in the presence of M6P (5 μM) for 4h. Cells extracts were immunoprecipitated with an anti-cat K antibody as described. Cat K was quantitated using a phosphorimager and results are expressed as percent inhibition of processing. M6P alone had no effect on cat K processing. Rp-cAMP (□), KT5720 (■), H89 (△).**

DISCUSSION

It had been reported that cat K is a cysteine protease abundantly and selectively expressed in human osteoclasts, and is thought to have an integral role in bone resorption. Cat K, like other members of this protease family, has been shown to exist as a latent precursor that can be processed

Concentration		
Rp-cAMP(μM, □)	KT5720 (μM, ■)	H89 (μM, △)
0 0.1 0.5 5.0	0 0.001 0.004 0.01	0 0.001 0.06 0.5
↓		
0 12.5 25.7 53.4	0 11.3 25.3 53.3	0 8.6 35.2 50.7
Resorption (% inhibition)		

Fig. 9. **Dose-dependent inhibition of in vitro bone resorption by Rp-cAMP, KT5720, and H89. Osteoclasts were cultured on bone particles and treated with various doses of Rp-cAMP (□), KT5720 (■), and H89 (△) for 24 h. Osteoclastic resorption was measured as described in the Materials and Methods. The results are expressed as percent inhibition of resorption compared to supernatants derived from osteoclasts cultured in the absence of inhibitors.**

to the mature catalytically active form in vitro[38]. However, little is known on mechanisms how does the osteoclasts deliver to catalytically active enzyme to bone resorption sites. Thus, the examination for the biosynthesis, and processing of cat K using cultured mouse osteocalsts derived from fetal long bone cells was performed.

The cat K was present within osteoclasts. The pro from of cat K in osteoclasts exhibited a molecular mass slightly larger than the theoretical 35 kDa deduced by gene structure, indicating that the pro enzyme may be posttranslationally modified. The results showed that the pro, but not mature, form of osteoclast cat K was susceptible to N-glycanase F, suggesitng that osteoclast pro cat K, like many mammalian proenzymes, is N-liked glycosylated. Although the nature of the glycosylation is presently unknown, the modification of high-mannose.

oligosaccharides present on many proenzymes facilitates intracellular targeting to the lysosomal trafficvia M6P receptors[40].

Several reports have speculated that, during the bone resorption, osteoclasts directionally secrete cysteine proteases as proenzymes into the bone resorption sites and that activation occurs extracellularly in this acidic milieu[2]. Therefore, this study examined cat K processing in nonadherent osteoclasts, which are devoid of an extracellular acidic compartment. Pro cat K was not detected in the media of these osteoclast cells, indicating that the osteoclasts could proteolytically modify the enzyme and active cat K is processed intracellularly, henceforth, the resulting mature enzyme is released in a catalytically active form.

PI3-kinase is known to be involved in growth factor signal transduction and vesicular membrane trafficking in a variety of systems. WT, an PI3-kinase inhibitor, has been previously shown to inhibit a number of osteoclast functions, including cell attachment and spreading[41], ruffled border formation[37], and bone resorption both in vitro and in vivo[38]. WT inhibited in a dose dependent manner cat K processing in mouse osteoclasts, and eventually resulted in the secretion of unprocessed pro cat K into the culture media[26]. Furthermore, Rp-cAMP, KT5720 and H89 also showed the similar effects as WT on inhibition of pro cat K processing. These observations are consistent with other previous

results[6,10]. These suggest that Rp-cAMP, KT5720, and H 89 can induce the mistargeting of acid hydrolases to lysosomal vesicles. Moreover, the addition of M6P resulted in enhanced potency of Rp-cAMP, KT5720, and H89, suggesting that M6P may prevent the reuptake and delivery of secreted proenzyme to the lysosomes for activation via M6P receptors. The activation of cysteine proteases requires a low pH microenvironment, such as that found in lysosomes. For example, alkalinization of these acidic lysosomal vesicles with either chloroquine or monensin resulted in complete inhibition of cat K processing, suggesting that activation occurs within lysosomes[26]. These results suggest that PI3-kinase plays a role in the delivery of pro cat K to lysosomal vesicles for enzyme activation. The inhibition of bone resorption by Rp-cAMP, KT5720, and H89 occured at concentrations consistent with the inhibition of cat K processing. Therefore, Rp-cAMP, KT5720 and H89 may exert its antiresorptive effects on osteoclasts, in part by reducing lysosomal pools of catalytically active cat K at least.

In contrast, forskolin, a adenylate cyclase agonist, rather induced cat K processing and maturation in osteoclasts and calphostin C, a specific inhibitor of PKC, did not affect. Although forskolin rather induced cat K processing and maturation stimulated by osteoclasts in the mouse total bone cells including both osteoblasts and osteoclasts, PKA inhibitors prevented cat K processing and maturation, indicating that osteoclasts utilize cAMP-PKA-dependent signaling pathway to upregulate cat K processing and maturation in mouse total long bone cells. With respect to the proinflammatory cytokine IL-1β, osteoblasts also utilizes cAMP-PKA-dependent signaling pathway to upregulate IL-1β gene expression. This pathway was strengthened by recent observations reported by McCarthy *et al.*[42] and Millet *et al.*[43] in rat osteoblasts, indicating that the anabolic effects and induction of IL-6 activity by PGE_2 are mediated by a cAMP-PKA signaling pathway. The role of PGE_2 in bone resorption associated with inflammation such as rheumatoid arthritis and bone resorption requires the osteoblast cells by involving the cAMP-dependent PKA signaling pathway[44]. Thus, the results indicated that the cat K processing and maturation and bone resorbing activity of osteoclasts may be mediated in part by the signal pathway similar to IL-1β-mediated bone resorption by osteoblasts. It is hypothesized that PGE_2 induces c-fos and c-jun expression via the PKA-activated transcription factor cAMP response element-binding protein. This factor has been known to mediate activation of c-fos transcription and has also been proposed as an activator of c-jun transcription[44]. Thus, the signaling pathway of cat K processing is needed for further investigation.

In summary, the data of the study suggest that mature, catalytically active cat K is produced and Rp-cAMP, KT5720 and H89 prevented the processing of the enzyme and bone sorption. An understanding of the inhibitory factors that influence the activation and trafficking of cat K in osteoclasts should provide insight into treatment of bone resorption.

요 약

기계적 자극에 의한 골조직의 개조에서 압박측은 일차적으로 파골세포에 의하여 골기질의 흡수를 위한 유전자 발현에 의하여 기시된다. 그러나 기질을 이루는 유기 단백질의 흡수에 관여하는 단백용해효소의 세포 내 작용 기전은 여전히 완전히 이해되지 않고 있다. 이 연구는 파골세포에서 용해용소체효소인 cathepsin k에 주목하여, cAMP 길항제와 PKA 억제제 및 adenylate cyclase 촉진제에 의한 cathepsin k 생성의 촉진 또는 억제 효과의 기전을 해명하는데 그 목적을 두었다.

cAMP 길항제인 Rp-cAMP와 PKA 억제제인 KT5720과 H89는 cathepsin K의 세포 내성숙을 차단하였으며, 대조적으로 adenylate cyclase 촉진제 forskolin은 파골세포에서의 cathepsin K의 생성과 성숙을 유인하는 것으로 나타났다. 특히 cathepsin K의 생성과 성숙에 관여하는 신호전달이 protein kinase C(PKC)와 관련성을 검정하기 위하여 백서의 골세포를 PKC의 선택적 억제제인 calphostin C로 처리하였을 때 아무런 영향이 없는 것으로 나타남으로써 calphostin C는 파골 세포에 의해 매개된 cathepsin K의 생성과 성숙과는 무관한 것으로 밝혀졌다. 이는 파골세포에서의 cathepsin K의 성숙은 cAmp-PKA 신호전달 경로에 의해 조절됨을 의미한다.

분비된 전구효소는 M6P 수용체를 통하여 세포 내로 다시 진입할 수 있는 잠재성을 가지고 있기 때문에 이러한 가능성을 차단하기 위하여 M6P가 존재 또는 결여된 상태에서 cAMP 길항제인 Rp-cAMP와 PKA 억제제인 KT5720 및 H89를 시험하였다. 그 결과 Rp-cAMP, KT5720 또는 H89에 의한 cathepsin K의 M6P용량 비례적 생성 억제가 관찰 되었다. 또한 M6P를 주었을 때 Rp-cAMP, KT5720와 H89의 작용이 증가됨을 보였다.

이상에서와 같이 Rp-cAMP, KT5720와 H89의 cathepsin K 생성 방해를 통한 골흡수 억제는 골다공증 또는 관절염의 치료와 같은 골흡수의 억제를 필요로 하는 분야에서의 임상적 응용 가능성을 시사한다.

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