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# IL-1α Stimulates Cathepsin K Expression in Osteoclasts *via* the Tyrosine Kinase-NF-κB Pathway

## ABSTRACT

Interleukin-1α (IL-1α) is a powerful activator of osteoclast cells. However, the underlying mechanism for this activation is unknown. In this study, we reveal that IL-1α up-regulates the expression of cathepsin K protein, a key protease in bone resorption, by five-fold. Northern blot analysis and promoter analysis show that this induction occurs at the transcriptional level, in a dose-responsive and time-dependent manner. No increase in expression occurs in the presence of either pyrrolidine dithiocarbamate (PDTC), a selective inhibitor of NF-κB, or Genistein, a protein tyrosine kinase inhibitor, suggesting that IL-1α up-regulation may be *via* the tyrosine kinase-NF-κB pathway to regulate cathepsin K expression. Antisense oligonucleotides to p65, but not the p50 subunit of NF-κB, suppress the IL-1α-induced expression of cathepsin K. We therefore conclude that IL-1α up-regulates cathepsin K gene expression at the transcription level, and this regulation may be *via* the tyrosine-kinase-NF-κB pathway.

**KEY WORDS:** interleukin-1alpha, cathepsin K, osteoclast, NF-κB, tyrosine kinase.

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## INTRODUCTION

The bone-resorptive activity of osteoclasts is an essential part of bone remodeling and occurs in response to the bone micro-environment. In normal physiological conditions, stromal and osteoblast cells secrete local factors—such as macrophage-colony-stimulating factor (M-CSF), receptor activator of NF-κB ligand (RANKL) (Lacey *et al.*, 1998; Yasuda *et al.*, 1998; Kudo *et al.*, 2002), and TNFα (Kudo *et al.*, 2002)—which have been shown to regulate osteoclast differentiation and activity. However, the bone micro-environment is also associated with pathological conditions of bone. For example, under local inflammatory conditions, T-cells secrete interleukin-1-alpha (IL-1α), which stimulates bone resorption and results in many diseases related to elevated bone resorption (Romas *et al.*, 2002). Similarly, Wang and Stashenko (1993) reported a role for IL-1α in the pathogenesis of periapical bone destruction. Moreover, since IL-1α increases the expression of RANKL (Romas *et al.*, 2002), these local factors may act in concert to promote osteoclast recruitment, activation, and osteolysis, since they stimulate the same NF-κB pathway (Jimi *et al.*, 1998, 1999). However, it is unclear which of the genes downstream of the IL-1α-induced activation of the NF-κB pathway activate osteoclasts. Cathepsin K has been shown to have a very important role in bone resorption (Gelb *et al.*, 1996). In this study, we sought to characterize the mechanism by which IL-1α induces osteoclast bone resorption through cathepsin K gene expression regulation.

## MATERIALS & METHODS

### Cell Culture and Assay for Dentin Resorption

The osteoclast precursor cell lines, mouse osteoclast precursor cells (MOCP-5), and mouse bone marrow were co-cultured with mouse stromal cell line (MS-12) cells as previously described (Chen and Li, 1998; Kamolmatyakul *et al.*, 2001). For the pure osteoclast population, MOCP-5 were prepared at a density of 4 x $10^3$ cells/mL in α-MEM containing 10% (v/v) FBS. RANKL (50 ng/ mL) was added for 5 days, so that mature osteoclasts could be obtained. On day 6, different doses of recombinant mouse IL-1α (Genzyme Corporation, Cambridge, MA, USA) were added. Dentin resorption assays were performed as previously described (Chen and Li, 1998).

### Northern Blot Analysis

The total RNA of the osteoclast population was isolated as described previously (Chomczynski and Sacchi, 1987). Northern blot analysis was performed as described previously (Li *et al.*, 1995).

### Cathepsin K Protein Determination

The co-culture systems were incubated with IL-1α (250 U/mL or 1.4 nM) for 10 and 15 hrs. We determined cathepsin K protein production by multinucleated osteoclast-like cells (OCLs) by assaying the media using ELISA, as previously described (Kamolmatyakul *et al.*, 2001). In brief, the purified rabbit IgG

**Table.** Phosphorothiooligodeoxynucleotides Used in This Study as Described (Jimi *et al.*, 1998)

| Gene | Sequences* | Nucleotide |
|---|---|---|
| p50 sense | 5′-CTGCGCATCTTCACCATGGCA-3′ | 277-297 |
| p50 antisense | 5′-TGCCATGGTGAAGATGCGCAG-3′ | |
| p65 sense | 5′-GGGACCCTGACCATGGACGAT-3′ | 49-69 |
| p65 antisense | 5′-ATCGTCCATGGTCAGGGTCCC-3′ | |

* The oligodeoxynucleotide sequences correspond to the 5′ ends of respective mRNAs and include 3 or 4 nucleotides present upstream of the initiation codon.

containing anti-cathepsin K was coated onto 96-well plates (ICN Biomedicals, Aurora, OH, USA). The test supernatants (100-10000x dilution) were applied, followed by HRP-conjugated anti-human cathepsin K. The concentration of cathepsin K in the test solution was interpolated from the standard curve generated from the standard antigen solution.

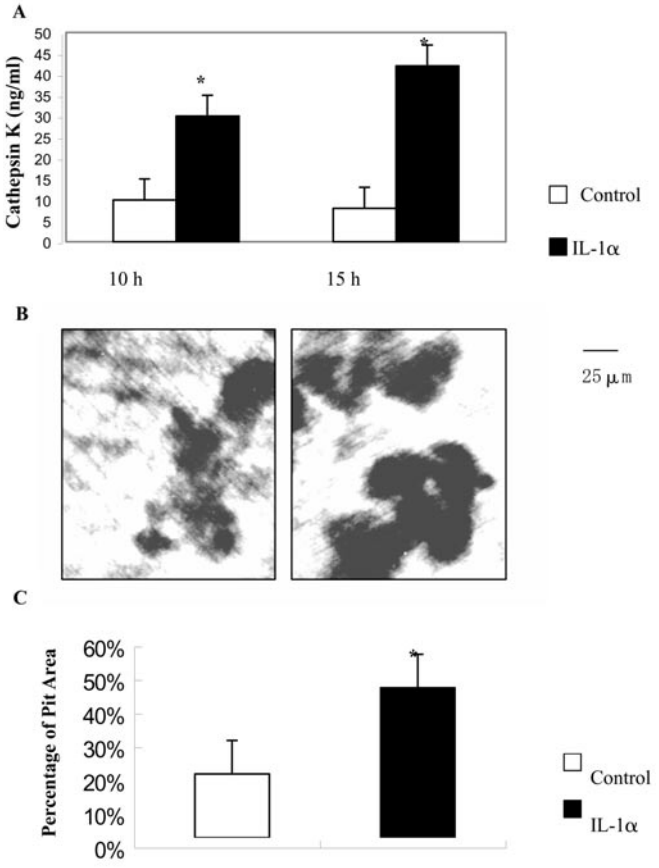


**Figure 1.** Cathepsin K protein expression and bone resorption activity of osteoclasts are up-regulated by IL-1α *in vitro*. **(A)** Application of IL-1α (1.4 nM) to OCL cells resulted in a three- and four-fold increase in cathepsin K protein expression after 10 and 15 hours, respectively, as determined by ELISA assay. Experiments were performed in triplicate (N = 3), and 3 independent experiments yielded similar results ($p < 0.05$). **(B)** Demonstration of resorption pits formed by OCLs. Pre-formed OCLs were applied to dentin slices and cultured for 3 days in the absence (a) or presence (b) of IL-1α. Cells were then removed and resorption pits stained with an anticollagen type I polyclonal antibody. **(C)** Application of IL-1α (1.4 nM) to mouse OCLs resulted in a 2.3-fold increase in Pit Area (N = 3).

## Cycloheximide and Actinomycin D Study

Mature osteoclasts (approximately 5 x $10^4$) were treated with 10 μg/mL cycloheximide (CHX; Sigma, St. Louis, MO, USA) or 5 μg/mL actinomycin D (Act D; Sigma, St. Louis, MO, USA) and then exposed to IL-1α (1.4 nM; Genzyme Corporation, Cambridge, MA, USA) an hour later. In parallel, control cells were treated with PBS for 1 hr before being exposed to IL-1α.

## Cathepsin K Promoter Constructs

A cathepsin K promoter-CAT fusion gene (pMCCAT) containing a 1978-bp XbaI/EcoRI fragment (-1400 to +357, numbered relative to the cap site) of the mouse cathepsin K 5′-flanking region was used as to generate the cathepsin K promoter CAT fusion gene construct. The XbaI/EcoRI fragment was subcloned into pCAT-3 Basic reporter vector (Promega, Madison, WI, USA) to produce the cathepsin K promoter constructs and named pMCCAT-1400.

## DNA Transfection and CAT (Chloramphenicol acetyltransferase) Assay

Transient transfection of plasmid DNA into MOCP-5 was performed with the lipofectAMINE reagent (Invitrogen, Gaithersburg, MD, USA) as described (Chen and Li, 1998). In all experiments, the effect of IL-1α (250 U/mL or 1.4 nM) on pMCCAT-1400 promoter activity was tested in triplicate, either with or without RANKL (20 ng/mL). CAT assays were performed on lysates as described (Neumann *et al.*, 1987). CAT activity in transfected cultures was standardized by normalization to β-galactosidase activity of cotransfected pSV-β-galactosidase constructs (Promega, Madison, WI, USA) (Li and Stashenko, 1993). CAT assay results were standardized to the protein concentration of cell layer extracts by means of a protein assay kit (Bio-Rad, Richmond, CA, USA).

## Signaling Pathway Inhibitor Studies

When OCLs were fully developed, the following signaling pathway inhibitors were added: 100 μM PDTC (pyrrolidine dithiocarbamate, from Sigma, St. Louis, MO, USA) dissolved in water, 100 μM Genistein (Sigma, St. Louis, MO, USA), 20 μM SB 203580 (Calbiochem, La Jolla, CA, USA), and 20 μM PD 98059 (Biomol, Plymouth Meeting, PA, USA) dissolved in DMSO (dimethylsulfoxide, Sigma, St. Louis, MO, USA). After 2 hrs of culturing with these inhibitors, IL-1α (125 U/mL or 0.7 nM) was added. The medium was discarded 6 hrs later. The OCL population was washed in PBS, and total RNA was then isolated.

## Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extraction was performed as described (Li *et al.*, 1995). The sequence of the NF-κB binding oligonucleotide used as a radioactive DNA probe was 5′-AGCTTGGGGACTTTCCGAC-3′.

## Antisense Oligodeoxynucleotide Studies

We performed antisense blocking experiments to characterize and confirm that IL-1α acts through NF-κB to regulate cathepsin K expression as described (Jimi *et al.*, 1998). In brief, when OCLs were fully developed on day 5, the sense and antisense oligonucleotides were added (see Table for oligo sequences): p50 antisense (5 μM) and p65 antisense (10 μM). Prior to that addition, we mixed 100 nM Tfx™-50 (Promega, Madison, WI, USA), polycationic liposome, with these antisense oligonucleotides as the S-ODN (synthetic phosphorothioate oligodeoxynucleotides), as previously described (Inui *et al.*, 1997). After nine-hour incubation with these antisense oligonucleotides, IL-1α (0.7 nM) was added. Control cells received an equal

concentration of corresponding sense oligonucleotides or just fresh medium without oligonucleotides. After 6 hrs of culture, the medium was discarded. The OCL population was washed in PBS, total RNA was isolated, and Northern blot analysis was carried out as described above. The expression of p50 or p65 mRNA was detected by RT-PCR with the use of gene-specific PCR primers as described (Jimi *et al.*, 1998).

### Statistical Analysis

Statistical significance was evaluated by the Student's *t* test. The results were considered significant for $p < 0.05$.

## RESULTS

### IL-1α Up-regulates Cathepsin K Protein

We examined whether IL-1α up-regulates cathepsin K protein expression in osteoclasts by using osteoclast-like cells (OCLs) which derived from MOCP-5 (Chen and Li, 1998), or mouse bone marrow cells. Using the ELISA technique, we found that incubating OCLs with IL-1α (250 U/mL or 1.4 nM) for 10 and 15 hrs increased cathepsin K protein production by about three- and four-fold, respectively, compared with controls (Fig. 1A). We then examined the effects of IL-1α on inducing bone-resorbing activity of osteoclasts by a dentin resorption assay. Fig. 1B shows the resorption pits formed by OCLs in the absence (a) or presence (b) of IL-1α. It has been demonstrated that the resorbed surface area is a suitable parameter in studies of osteoclasts to quantitate pit formation (Chen and Li, 1998). As shown in Fig. 1C, incubating OCLs with IL-1α increased the resorbed surface area by about 2.5-fold, compared with controls, indicating that IL-1α significantly increased the pit-forming activity of OCLs.

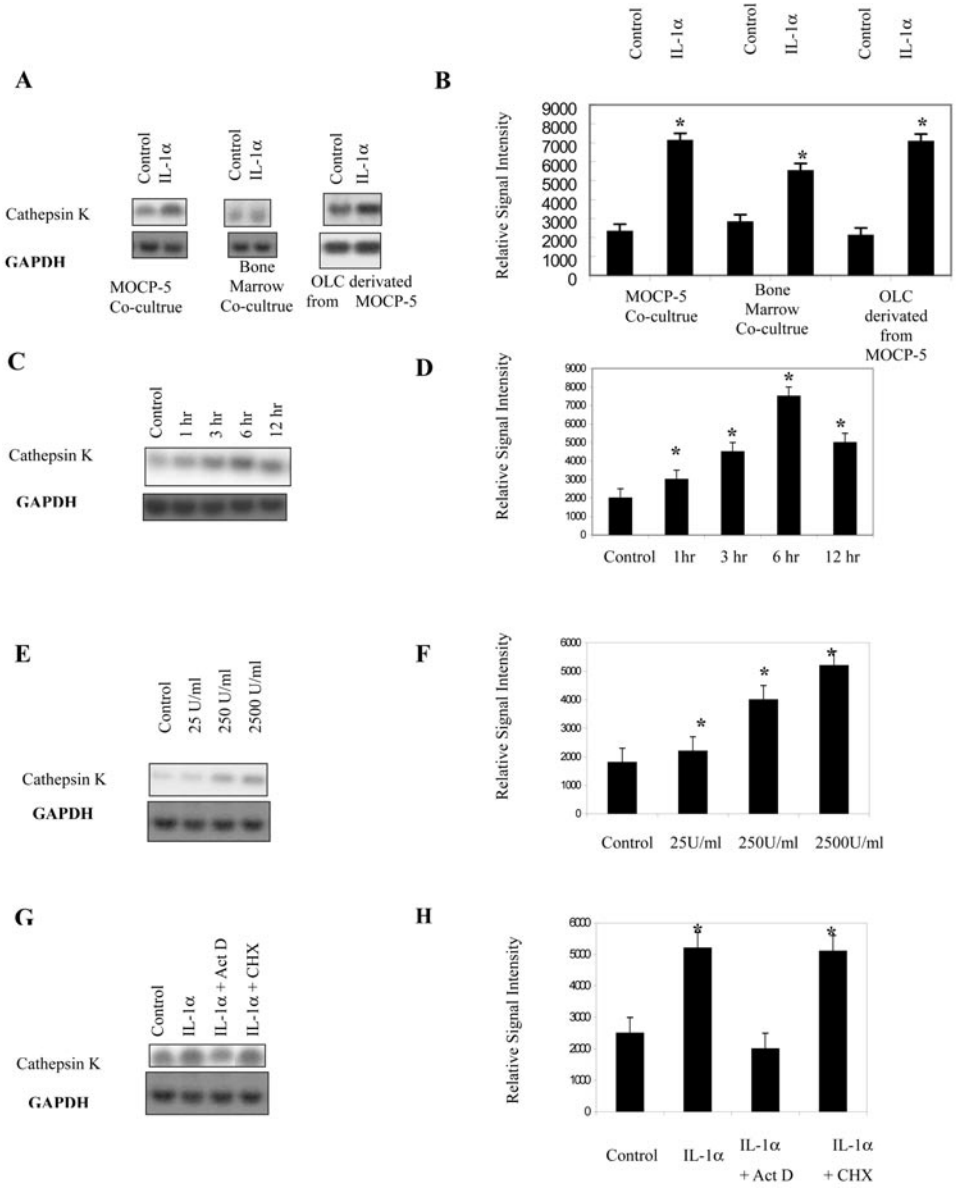


**Figure 2.** Northern analysis of cathepsin K gene regulation. **(A)** Up-regulation of cathepsin K expression as determined by Northern blot analysis. IL-1α (1.4 nM) treated (6 hrs) or untreated total RNA (15 μg) from mature osteoclasts was used as the sample. The lower panels in A, C, E, and G represent hybridization of blots to GAPDH to standardize loading. **(B)** Relative abundance of cathepsin K mRNA on Northern blotting, as evaluated by densitometric scanning. $*p < 0.05$, compared with control. **(C,D)** Cathepsin K mRNA expression in response to increasing exposure to IL-1α (1.4 nM), as indicated. Controls cells were cultured for 6 hrs. **(E,F)** Cathepsin K mRNA expression in MOCP-5 cells after 6 hrs of exposure to increasing amounts of IL-1α. **(G,H)** Northern blot analysis of cathepsin K mRNA expression in mature osteoclast cells (control, lane 1), and following IL-1α treatment for 6 hrs, preceded by 1 hr of pre-incubation with PBS (lane 2), Act D (5 μg/mL; lane 3), or CHX (10 μg/mL; lane 4). These figures represent at least 4 independent experiments (N = 4) that yielded similar results.

### IL-1α Up-regulates the mRNA Levels of Cathepsin K in a Dose-responsive and Time-dependent Manner at the Transcription Level

To determine whether IL-1α up-regulates cathepsin K expression at the transcriptional level, we examined cathepsin K mRNA levels following IL-1α application. IL-1α (1.4 nM) was found to increase cathepsin K mRNA levels by two-fold in the MOCP-5 and stromal cells co-culture system (Chen and Li, 1998), two-fold in the bone marrow cells co-culture system, and 2.5-fold in osteoclast-like cells derived from MOCP-5 cells induced by RANKL, respectively, within 6 hrs, as determined by Northern analysis (Figs. 2A, 2B). We used the MOCP-5 co-culture system in all other experiments. A time-course examining cathepsin K expression following IL-1α (1.4 nM)

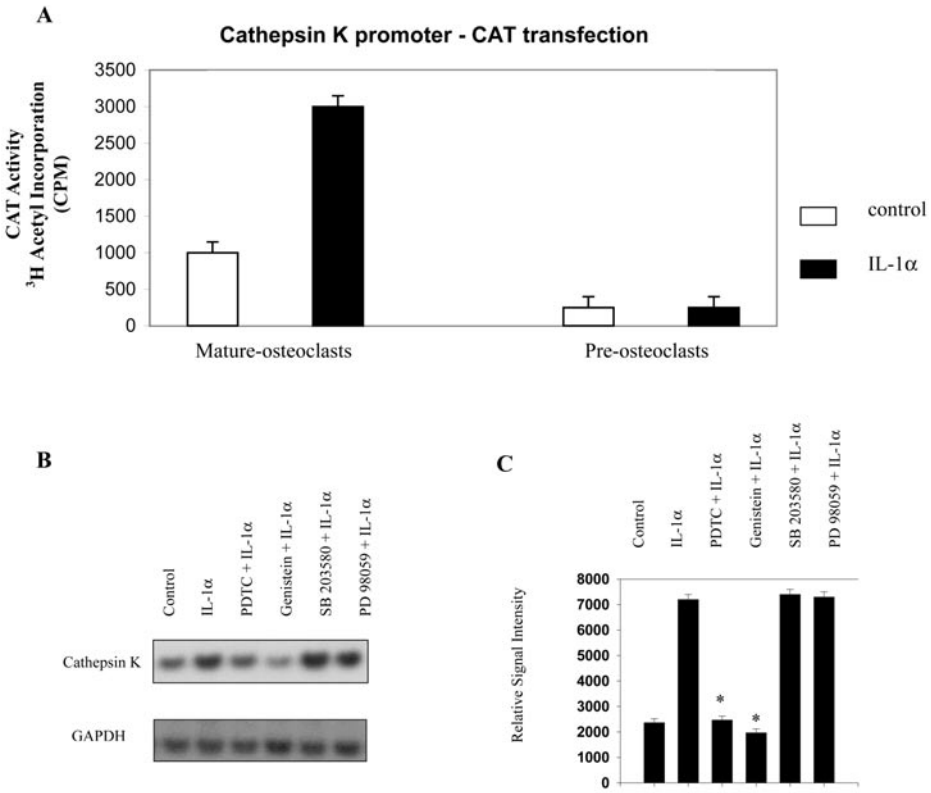


**Figure 3.** IL-1α increases cathepsin K promoter-CAT fusion gene activity, and NF-κB activation inhibitor and tyrosine kinase inhibitor inhibit IL-1α induction cathepsin K expression. **(A)** MOCP-5 cells were transiently transfected with pMCCAT by means of the lipofectAMINE reagent, and reporter CAT activity was determined in premature and mature osteoclasts. A three-fold increase in CAT activity was detected after 7 hrs of IL-1α treatment. **(B)** Northern blot analysis of cathepsin K mRNA expression in mature osteoclast cells (control, lane 1), and following IL-1α (0.7 nM) treatment for 6 hrs preceded by a two-hour incubation with PBS (lane 2), PDTC (100 μM; lane 3), Genistein (100 μM; lane 4), SB 203580 (20 μM; lane 5), or PD 98059 (20 μM; lane 6). We hybridized blots to GAPDH to standardize loading (lower panel). Experiments were performed in triplicate (N = 3). **(C)** Relative abundance of cathepsin K mRNA on Northern blots, as evaluated by densitometric scanning. $^*p < 0.05$ compared with control pre-treated samples stimulated with IL-1α.

treatment showed that cathepsin K mRNA levels peaked at 6 hrs (Figs. 2C, 2D). As the concentration of IL-1α treatment was increased, cathepsin K expression steadily rose to an IL-1α concentration of 14 nM, where it reached a plateau (Figs. 2E, 2F). These findings indicate that IL-1α regulates cathepsin K expression in a time-dependent and dose-responsive manner.

To determine whether IL-1α regulated cathepsin K expression through inducing protein expression or regulating DNA transcription, we first pre-treated mature osteoclasts in the MOCP-5 cell co-culture system, that were exposed to IL-1α, with 10 μg/mL cycloheximide (CHX), a protein synthesis inhibitor, or actinomycin D (Act D), a transcription blocker. CHX failed to prevent the IL-1α-induced increase in cathepsin K expression (Figs. 2G, 2H), indicating that no new protein was required for this induction. Moreover, mature osteoclasts treated with Act D blocked the IL-1α-induced increase in cathepsin K mRNA (Figs. 2G, 2H), indicating that IL-1α up-regulates cathepsin K expression at the transcription level.

## IL-1α Up-regulates the Cathepsin K Promoter-CAT Fusion Gene Only in Mature Osteoclast Cells

To determine whether the cathepsin K promoter is the region that IL-1α uses to regulate cathepsin K transcription, we performed transfection studies with a cathepsin K promoter-CAT fusion gene (pMCCAT). Pre-osteoclast (MOCP-5) and mature osteoclast cells were transiently transfected with pMCCAT by means of the lipofectAMINE reagent, and reporter CAT activity was determined following IL-1α treatment. In mature osteoclast cells, CAT activity was up-regulated approximately three-fold in response to 7 hrs of IL-1α treatment, compared with controls (Fig. 3A), and no effect was observed in pre-osteoclast cells, indicating that the IL-1α signal acted on the cathepsin K promoter region, and this action occurred only in mature osteoclast cells.

## NF-κB Activation Inhibitor and Tyrosine Kinase Inhibitor Inhibit the Induction of IL-1α on Cathepsin K Gene Expression

To characterize the signaling pathway that is used by IL-1α to regulate cathepsin K expression, we used signaling pathway inhibitors in our study: an NF-κB activation inhibitor (PDTC), a tyrosine kinase inhibitor (Genistein), and 2 mitogen-activated protein kinase (MAPK) pathway inhibitors (pyridinyl-imidazole, SB 203580, a specific inhibitor of p38 mitogen-activated and 2′-amino-3′-methoxyflavone, PD 98059, for MEK/ERK inhibition). The MAPK pathway inhibitors showed no effect on cathepsin K expression following IL-1α treatment (Figs. 3B, 3C). In contrast, after pre-treatment with either NF-κB activation inhibitor or tyrosine kinase inhibitor for 2 hrs, the up-regulation of cathepsin K mRNA level was completely blocked, as determined 6 hrs after IL-1α treatment (Figs. 3B, 3C). This suggests that IL-1α regulates cathepsin K expression *via* the tyrosine kinase-NF-κB signal pathway.

## IL-1α Activates NF-κB in Osteoclasts

The DNA-binding activation of NF-κB was strongly detected in nuclear extracts derived from OCLs following 30 min of induction by IL-1α (Fig. 4A, lane 3). The binding activity was partially reduced with 10-fold (lane 4) and almost completely diminished with 100-fold excess unlabeled NF-κB binding oligonucleotide (lane 5), signifying specific binding. These findings indicate that NF-κB is activated in response to IL-1α stimulation, and that IL-1α activates NF-κB in osteoclasts.

## p65 but not p50 is Responsible for the Transcription Activation of NF-κB.

The activated nuclear form of the NF-κB transcription factor binds to DNA as a heterodimer of 50-kDa (p50) and 65-kDa (p65) polypeptides. We performed antisense blocking

experiments further to characterize and confirm that IL-1α acts through NF-κB to regulate cathepsin K expression as described (Jimi *et al.*, 1998). When MOCP-5 cells were treated with the antisense S-ODNs to p50 or p65, expression of the respective mRNA by MOCP-5 cells was suppressed (Fig. 4B). MOCP-5 cells pre-treated with antisense oligonucleotides to the p65 subunit of NF-κB suppressed the increase in cathepsin K expression by IL-1α approximately 45% compared with controls (Figs. 4C, 4D). In contrast, MOCP-5 cells pre-treated with antisense oligonucleotides to the p50 subunit of NF-κB showed no effect.

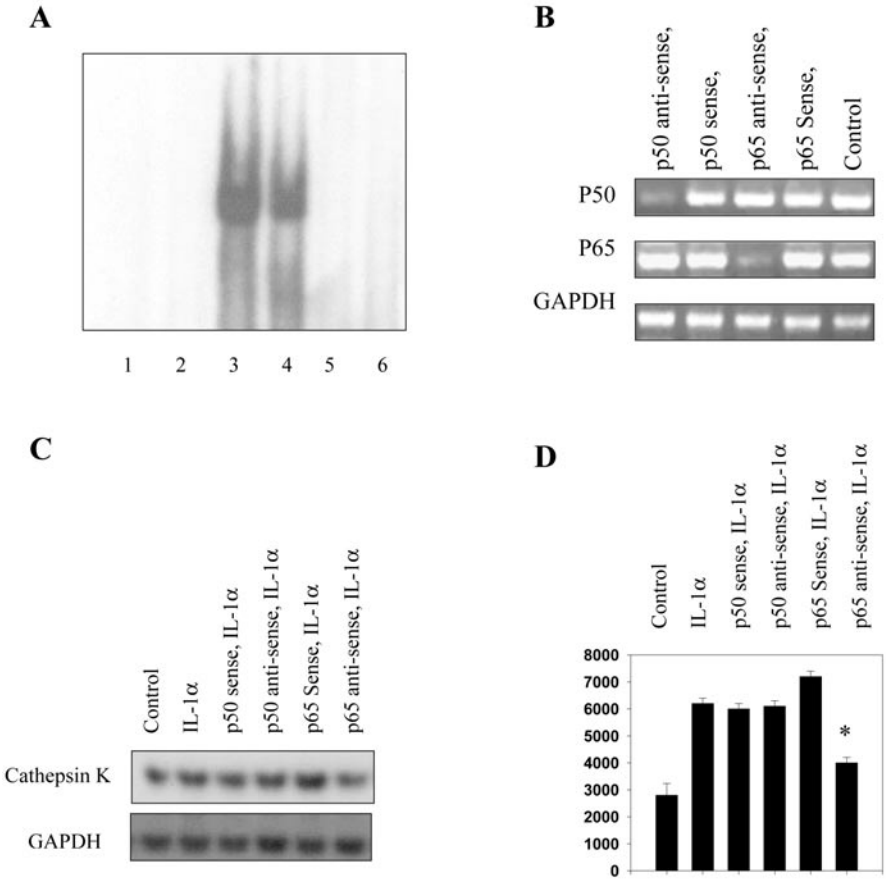


**Figure 4.** IL-1α activates NF-κB in osteoclasts, and the p65 domain of NF-κB mediates IL-1α-induced cathepsin K expression. **(A)** Nuclear extracts from osteoclast cells were analyzed by EMSA for DNA binding to a $^{32}$P-labeled NF-κB consensus oligonucleotide probe. Lane 1, the probe alone; lane 2, nuclear extract and probe; lane 3, nuclear extract from IL-1α-stimulated osteoclast cells and probe; lane 4, nuclear extract from IL-1α-stimulated osteoclast cells and probe, plus 10-fold excess unlabeled NF-κB consensus oligonucleotide; lane 5, nuclear extract from IL-1α-stimulated osteoclast cells and probe, plus 100-fold excess unlabeled NF-κB consensus oligonucleotide; lane 6, probe alone. **(B)** MOCP-5 cells were plated on culture dishes and incubated for 10 hrs in the presence or absence of antisense S-ODNs (*p50AS* and *p65AS*) or sense S-ODNs (*p50S* and *p65S*) to p50 and p65. We then isolated total RNA from some cultures to determine the expression of mRNAs to p50 and p65 by RT-PCR. The expression of GAPDH mRNA was used as the control. **(C)** Northern blot analysis of total RNA (15 μg) from mature osteoclasts pre-treated with oligonucleotides sense to p50 (5 μM; lane 3), antisense to p50 (5 μM; lane 4), sense to p65 (10 μM; lane 5), antisense to p65 (10 μM; lane 6), and then stimulated with 0.7 nM IL-1α for 6 hrs. Cells treated with sense oligonucleotides or the same medium without oligonucleotides were used as controls. The lower panel illustrates loading differences as determined by GAPDH hybridization. Experiments were performed in triplicate (N = 4). **(D)** Relative abundance of cathepsin K mRNA on Northern blots, as evaluated by densitometric scanning. *$p < 0.05$ compared with control pre-treated with sense oligonucleotides.

## DISCUSSION

IL-1α activates several signaling cascades, including Akt and ERK (Lee *et al.*, 2002), NF-κB (Jimi *et al.*, 1996; Miyazaki *et al.*, 2000), and JNK (Jimi *et al.*, 1999) pathways. It has been suggested that ERK is responsible for osteoclast survival, whereas NF-κB regulates osteoclast activation for bone resorption (Miyazaki *et al.*, 2000). However, it is not well-known what downstream genes are induced after IL-1α stimulation to promote osteoclastic bone resorption. In the present study, we found that IL-1α up-regulated cathepsin K expression *via* the NF-κB pathway.

In the CAT assay, no increase of CAT activity response to IL-1α was observed in pre-osteoclast cells. This result is consist with our previous finding that cathepsin K mRNA was not detected in pre-osteoclast cells (Li and Chen, 1999). Osteopetrosis in NF-κB1 and NF-κB2 double-knockout mice has identified an important role for NF-κB in osteoclast differentiation (Iotsova *et al.*, 1997). In this study, NF-κB was activated in response to IL-1α stimulation, a result consistent with that of Jimi *et al.* (1996). Furthermore, NF-κB activation inhibitor or tyrosine kinase inhibitor completely blocked the increase of cathepsin K mRNA levels induced by IL-1α treatment. We had previously reported that there is an NF-κB binding site, GGGACTGCCC, located in -8886 to -8877 of the cathepsin K promoter (Li and Chen, 1999). Taken together, these results indicate that IL-1α up-regulates cathepsin K expression *via* the NF-κB pathway.

The cycloheximide experiment showed that no new protein was needed to induce cathepsin K expression. However, cathepsin K expression could not be induced when p65 subunits of NF-κB protein synthesis were blocked in an antisense study. Similar results, in which antisense Hsp60 oligonucleotides reduced Hsp60 expression, while cycloheximide was used to inhibit protein synthesis, did not alter the effect of Hsp60 on IGF-1R signaling, and IGF-1R mRNA levels were not up-regulated by Hsp10 or Hsp60 (Shan *et al.*, 2003). It is possible that CHX inhibited synthesis of all cellular proteins, including proteinases, so p65 is relatively stable to perform IL-1α response stimulation. While cells lack both original p65, due to protein degradation, and new p65 synthesis, due to antisense treatment, blocking failed to respond to IL-1α.

Our results also demonstrate that pre-treatment with antisense oligonucleotides to p65, but not p50, suppresses the up-regulation effect of IL-1α on cathepsin K expression in

osteoclasts. This indicates that the p65, but not the p50, subunit of NF-κB is responsible for the transcriptional regulation of cathepsin K expression induced by IL-1α. This finding is consistent with p65, the DNA-binding component of NF-κB, having the more critical role in initiating transcription, whereas p50 functions to assist in DNA binding (Schmitz and Baeuerle, 1991) and has a primary structure completely divergent from that of p65 (Nolan *et al.*, 1991). This finding is also consistent with the results of Xing *et al.* (2003), that p50 and p52 subunits, which are highly homologous, can have overlapping functions in cell activation, as seen in the impaired osteoclast development only in double-knockout mice. Expression of either NF-κB p50 or p52 in osteoclast precursors is required for IL-1α-mediated formation of mature osteoclasts (Xing *et al.*, 2003).

The results of this study suggest the mechanism by which IL-1α activates bone resorption through its up-regulation on the expression of osteoclast genes. Our findings presented here will add to our understanding of the interplay between the local bone environment and the regulation of expression of osteoclast genes during the normal and pathological bone resorption process, and will help us identify additional targets for therapeutic intervention in bone disorders such as osteoporosis, osteoarthritis, rheumatoid arthritis, periodontal disease, and other bone disorders.

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