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# Suppression of RANKL-Dependent Heme Oxygenase-1 is Required for High Mobility Group Box 1 Release and Osteoclastogenesis

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## ABSTRACT

The differentiation of osteoclasts is regulated by several essential cytokines, such as receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony-stimulating factor. Recently, high mobility group box 1 (HMGB1), a chromatin protein, also has been identified as one of these osteoclast differentiation cytokines. However, the molecular mechanisms that control HMGB1 release from osteoclast precursor cells are not known. Here, we report that RANKL-induced suppression of heme oxygenase-1 (HO-1), a heme-degrading enzyme, promotes HMGB1 release during osteoclastogenesis. In contrast, induction of HO-1 with hemin or curcumin in bone marrow-derived macrophages or RAW-D murine osteoclast precursor cells inhibited osteoclastogenesis and suppressed HMGB1 release. Since an inhibitor for p38 mitogen-activated protein kinase (MAPK) prevented the RANKL-mediated HO-1 suppression and extracellular release of HMGB1, these effects were p38 MAPK-dependent. Moreover, suppression of HO-1 in RAW-D cells by RNA interference promoted the activation of caspase-3 and HMGB1 release, whereas overexpression of HO-1 inhibited caspase-3 activation as well as HMGB1 release. Furthermore, these effects were regulated by redox conditions since antioxidant *N*-acetylcysteine abolished the HO-1/HMGB1/caspase-3 axis. These results suggest that RANKL-dependent HO-1 suppression leads to caspase-3 activation and HMGB1 release during osteoclastogenesis. J. Cell. Biochem. 113: 486–498, 2012. 

**KEY WORDS:** HEME OXYGENASE 1; HIGH MOBILITY GROUP BOX 1; CASPASE-3; RANKL; OSTEOCLASTOGENESIS

Osteoclasts, which are multinucleated cells that resorb bone, are formed by the fusion of monocyte-macrophage precursor cells. Osteoclast differentiation is regulated by several cytokines, such as macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor κB ligand (RANKL), and osteoprotegerin (OPG). M-CSF is a secreted cytokine that promotes the differentiation of hemopoietic stem cells into macrophages and osteoclasts (Elford et al., 1987). RANKL, a member of the tumor necrosis factor (TNF) superfamily, is a key cytokine that regulates osteoclastogenesis and bone resorption (Lacey et al., 1998; Yasuda et al., 1998). The interaction of RANKL and its receptor activates several signaling pathways, such as NF-κB, extracellular signal-regulated kinase (Erk), p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), and phosphoinositide 3-kinase (PI3K) (Boyle et al., 2003). In contrast, OPG, which is a soluble decoy receptor that competes with RANK for RANKL (Simonet et al., 1997), inhibits these pathways. In addition to these cytokines, some proinflammatory cytokines, such as TNF-α and interleukin 1 (IL-1), are involved in osteoclast differentiation (Wei et al., 2005). Since these cytokines play an important role in osteoclast differentiation or activation of

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inflammatory osteolysis, they are known as "osteoclastogenic cytokines."

Recently, high mobility group box 1 (HMGB1) has been identified as one of these osteoclastogenic cytokines (Zhou et al., 2008). HMGB1 has diverse functions. In the cell, it binds chromatin and participates in transcriptional regulation (Agresti and Bianchi, 2003). HMGB1 also is released from injured or dead cells (Bianchi, 2007) and secreted by activated macrophages (Lotze and Tracey, 2005), natural killer cells (DeMarco et al., 2005), and mature dendritic cells (Dumitriu et al., 2005). Extracellular HMGB1, which acts as a proinflammatory cytokine, can bind to cell-surface receptors, such as the receptor for advanced glycation end products (RAGE) and Toll-like receptors 2 and 4 (TLR2 and TLR4), to mediate cellular responses to infection, injury, and inflammation (Dumitriu et al., 2005). Moreover, extracellular HMGB1 stimulates the differentiation of osteoclast precursors in the presence of RANKL in vitro and in vivo (Zhou et al., 2008). However, the regulatory mechanism underlying the HMGB1 release from osteoclasts is not fully understood.

The osteoclast differentiation is also regulated by other factors as well as several cytokines. Recent studies have shown that iron homeostasis (Ishii et al., 2009; Tsay et al., 2010), and oxidative stress (Ha et al., 2004; Lee et al., 2005) play important roles in osteoclastogenesis. Heme oxygenase 1 (HO-1), which is an inducible enzyme that is involved in both of these processes, degrades cellular heme into carbon monoxide (CO), free iron, and biliverdin. Subsequently, biliverdin reductase converts biliverdin to bilirubin, which is a cytoprotective antioxidant. In addition, HO-1 has anti-inflammatory functions, such as induction of IL-10, an anti-inflammatory cytokine, and suppression of TNF-α, a proinflammatory cytokine, and nitric oxide synthase-2 in activated macrophages (Lee and Chau, 2002; Drechsler et al., 2006). Although the previous study (Zwerina et al., 2005) reported that hemin-induced HO-1 inhibits osteoclast differentiation with spleen cells in vitro and in TNF-α transgenic mice in vivo, the molecular mechanisms underlying HO-1 mediated regulation of osteoclast differentiation is not clear.

As a result, we aimed to determine the effect of HO-1 on HMGB1-mediated osteoclast differentiation. We showed that RANKL downregulates HO-1 expression by activating p38 MAPK and stimulates HMGB1 release. Furthermore, inhibition of HO-1 with pharmacological inhibitors or RNA interference promoted extracellular HMGB1 release and caspase-3 activation during osteoclastogenesis. These results suggest that HO-1 downregulation is a key initiator of RANKL-induced osteoclastogenesis.

## MATERIALS AND METHODS

### REAGENTS AND ANTIBODIES

Synthetic Stealth™ small interfering RNA (siRNA) for HO-1 mRNA and Stealth™ RNAi negative control oligo were purchased from Invitrogen (Carlsbad, CA). M-CSF was purchased from Kyowa Hakko Kogyo (Tokyo, Japan), and OPG was obtained from R&D Systems (Minneapolis, MN). NF-κB essential modulator (NEMO)-binding domain-binding peptide, LY294002, PD98059, SB203580, SP600125, and U0126 were purchased from Calbiochem (San Diego, CA). Staurosporine was purchased from Fermentek Ltd (Jerusalem, Israel). Cobalt protoporphyrin IX (CoPPIX) was purchased from Alexis Biochemicals (San Diego, CA). All other reagents, including curcumin, carbon monoxide releasing molecule 2 (CORM2), hemin, PMSF, and protease inhibitor cocktail were purchased from Sigma–Aldrich (St. Louis, MO). The Osteo Assay Plate was purchased from Corning (Corning, NY).

Antibodies (Abs) against Bcl-2 interacting mediator of cell death (Bim), B-cell lymphoma extra-large (Bcl-xL), Bcl-2-associated X protein (Bax), cleaved caspase-3, phospho-p38 (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185), phospho-Akt (Ser473), phospho-IκBα (Ser32), and phospho-Erk (Thr202/Tyr204) were purchased from Cell Signaling Technology (Danvers, MA). We used Abs against HO-1 and HO-2 (Stressgen, Ann Arbor, MI), HMGB1 (Abcam, Cambridge, UK), Src (Upstate, Lake Placid, NY), and β-actin (Sigma–Aldrich). Anti-cathepsin K Ab was prepared as described previously (Kamiya et al., 1998).

### CELL CULTURE

We obtained 5-week-old male BALB/c mice from Clea Japan, Inc (Tokyo, Japan) and handled them in our facilities according to the approved protocols of the Nagasaki University Animal Care Committee. Recombinant RANKL was prepared as described previously (Hu et al., 2008). To isolate bone marrow-derived macrophages (BMMs), marrow cells from the femurs and tibias of the mice were cultured overnight in α-minimal essential medium (α-MEM) (Wako Pure Chemicals, Osaka, Japan) containing 10% FBS with 100 U/ml of penicillin and 100 μg/ml of streptomycin in the presence of M-CSF (50 ng/ml) at 37 °C in 5% $CO_2$. Nonadherent cells were harvested to culture stroma-free bone marrow cells with 50 ng/ml of M-CSF. After 3 days, the adherent cells were harvested as BMMs. These cells were replated and then further cultured with fresh medium containing M-CSF (30 ng/ml) and RANKL (50 ng/ml) for various times.

Cells were fixed with 4% paraformaldehyde and were stained for tartrate-resistant acid phosphatase (TRAP) activity as described previously (Hotokezaka et al., 2002). TRAP-positive cells with 3 or more nuclei were considered mature osteoclasts. The RAW-D murine monocytic cell line was cultured in α-MEM with 10% FBS and RANKL (50 ng/ml) (Watanabe et al., 2004). The bone-resorbing activity of osteoclasts was assayed using the Osteo-Assay Plate for 5 days of culture. The resorption area was determined by Image J software.

### CELL PROLIFERATION ASSAY

Cells seeded in 96-well cell culture plates were incubated with the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) for 1 h, and then the absorbance at 450 nm was measured with a microplate reader (Bio-Rad iMark™, Hercules, CA).

### WESTERN BLOT ANALYSIS

BMMs were stimulated with or without RANKL in the presence of M-CSF for various times. Afterward, the cells were rinsed twice with ice-cold PBS and lysed in a cell lysis buffer (50 mM Tris–HCl, pH 8.0; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% SDS; 150 mM NaCl; 1 mM PMSF; and protease inhibitor cocktail). We measured

the protein concentration of each sample by using bicinchoninic acid (BCA) protein assay reagent (Thermo Pierce, Rockford, IL). We loaded 5 μg of lysate into each lane.

For HMGB1, the culture supernatant was collected and the same amount of protein was loaded in each lane. After SDS–PAGE, proteins were electroblotted onto a PVDF membrane. The blots were blocked with 5% BSA/TBST for 1 h at room temperature, probed with specific Abs overnight at 4°C, washed, incubated with HRP-conjugated anti-mouse or anti-rabbit IgG (Cell Signaling), and probed with ECL-plus (GE Healthcare, Uppsala, Sweden). We detected the immunoreactive bands by using an image analyzer (LAS1000; Fuji Photo Film, Tokyo, Japan).

### SMALL INTERFERING RNA

The target sequences of murine HO-1 siRNA were: GGCAGTGG-GAATTTATGCCATGTAA (HO-1 siRNA1) and CAGCTCTATCGTGC-TCGAATGAACA (HO-1 siRNA2). Briefly, RAW-D cells plated $5 \times 10^4$ cells on 60-mm plates were cultured with or without curcumin in the presence of RANKL in antibiotic-free media. The siRNA was transfected into RAW-D cells using Lipofectamine RNAiMAX™ transfection reagent (Invitrogen). BLOCK-iT™ Alexa Fluor Red Fluorescent Oligo was used to optimize the delivery of siRNA. The cells were incubated with either 6 or 10 pmol of siRNA for 24 h, followed by isolation of total RNA for RT-PCR. For TRAP staining, we incubated the cells for another 5 days.

### PLASMID CONSTRUCTION AND TRANSFECTION INTO RAW-D CELLS

To generate a pcDNA expression plasmid with a GFP-tagged wild-type HO-1 (pcDNA/DEST47-HO-1), we PCR-amplified full-length mouse HO-1 cDNA from DNAFORM (RIKEN, Kanagawa, Japan) as a Functional Annotation of Mouse (FANTOM) clone (Carninci et al., 2005) and then cloned the products into the pENTR/D-TOPO entry vector (Invitrogen). Subsequently, the cDNA region was recombined into pcDNA-DEST47 by using Gateway LR clonase. Plasmids (1 μg) were transiently transfected into RAW-D cells using GenePORTER3000 transfection reagent (Genlantis, San Diego, CA). The transfected cells were incubated for 4 h in serum-free medium, and then the medium was changed to fresh medium with 10% FBS. After incubation for 48 h, the cells were incubated with fresh media containing RANKL for another 3 or 4 days.

### STATISTICAL ANALYSIS

Statistically significant differences were determined by using Student's *t*-test and *P*-values $<0.05$ were considered to be statistically significant.

## RESULTS

### HEMIN INHIBITS OSTEOCLASTOGENESIS, UPREGULATES HO-1, AND SUPPRESSES THE RELEASE OF HMGB1

Previously, Zwerina et al. (2005) demonstrated that hemin inhibits osteoclast formation. However, their experiment used osteoclasts that were derived from spleen cells in vitro. To determine whether similar effects could be observed in BMMs, we first investigated the effects of hemin on BMMs that were stimulated with M-CSF and RANKL, and then performed TRAP staining. Consistent with the results of Zwerina et al. (2005), hemin significantly inhibited osteoclastogenesis in a dose-dependent manner (Fig. 1A, B). Although RANKL decreased cell viability slightly, the addition of hemin overcame this suppression (Fig. 1C). In addition, hemin significantly inhibited bone resorbing activity (Fig. 1D). Furthermore, hemin increased expression of HO-1 in a dose-dependent manner, whereas HO-2, an internal control, was not increased by hemin (Fig. 1E). It has reported the relationship between HO-1 and HMGB1 in an experimental sepsis model (Takamiya et al., 2009). To examine the role of HMGB1 in the differentiation of BMMs into osteoclasts, we analyzed effects of hemin on levels of extracellular HMGB1 after stimulation with M-CSF and RANKL. RANKL increased the extracellular concentration of HMGB1; however, the addition of hemin reversed this effect in a dose-dependent manner. These results suggest that upregulation of HO-1 can reduce the release of HMGB1 and osteoclastogenesis in RANKL-stimulated BMMs.

### EFFECT OF HEME METABOLITES ON THE RANKL-INDUCED RELEASE OF HMGB1 AND OSTEOCLASTOGENESIS

Since HO-1 produces bilirubin and CO, we also elucidated their effects on the RANKL-induced release of HMGB1 and osteoclastogenesis. Bilirubin markedly inhibited RANKL-induced osteoclast formation (Fig. 2A) without reducing cell growth (Fig. 2B) and bone resorbing activity (Fig. 2C). In addition, bilirubin inhibited the RANKL-induced release of HMGB1 in a dose-dependent manner (Fig. 2D). In contrast, CORM2 did not have as large of an effect on osteoclast formation as bilirubin and decreased cell growth in a dose-dependent manner (Fig. 2E,F), and bone-resorbing activity was inhibited by treatment with 25 μM CORM2 (Fig. 2G). In addition, CORM2 did not decrease the release of HMGB1 in RANKL-stimulated BMMs (Fig. 2H).

### CURCUMIN REDUCES RANKL INDUCED-RELEASE OF HMGB1 AND OSTEOCLASTOGENESIS BY UPREGULATING HO-1

Curcumin is a natural extract of turmeric which is known to induce HO-1 (Balogun et al., 2003; Hsu et al., 2008). Next, we examined whether curcumin inhibits the RANKL-induced release of HMGB1 and osteoclastogenesis in RAW-D cells. Similar to hemin, curcumin inhibited osteoclastogenesis in a dose-dependent manner (Fig. 3A). In addition, 10 μM curcumin significantly enhanced the RANKL-induced decrease in the proliferation of RAW-D cells (Fig. 3B). RANKL also downregulated the expression of HO-1; however, the addition of curcumin reversed this effect (Fig. 3C). Similarly, the addition of curcumin reversed the RANKL-induced increase in HMGB1 release (Fig. 3C). To determine whether these inhibitory effects are mediated by the induction of HO-1, we knocked down HO-1, with RNA interference. As shown in Figure 3D,E, suppression of HO-1 promoted osteoclastogenesis and the release of HMGB1. When the cells were cultured with CoPPIX, a potent HO-1 inducer, HO-1 expression was also increased. Similarly, suppression of HO-1 by RNA interference promoted HMGB1 release (Fig. 3F). These results suggest that downregulation of HO-1 is sufficient for the RANKL-induced release of HMGB1 and osteoclastogenesis.

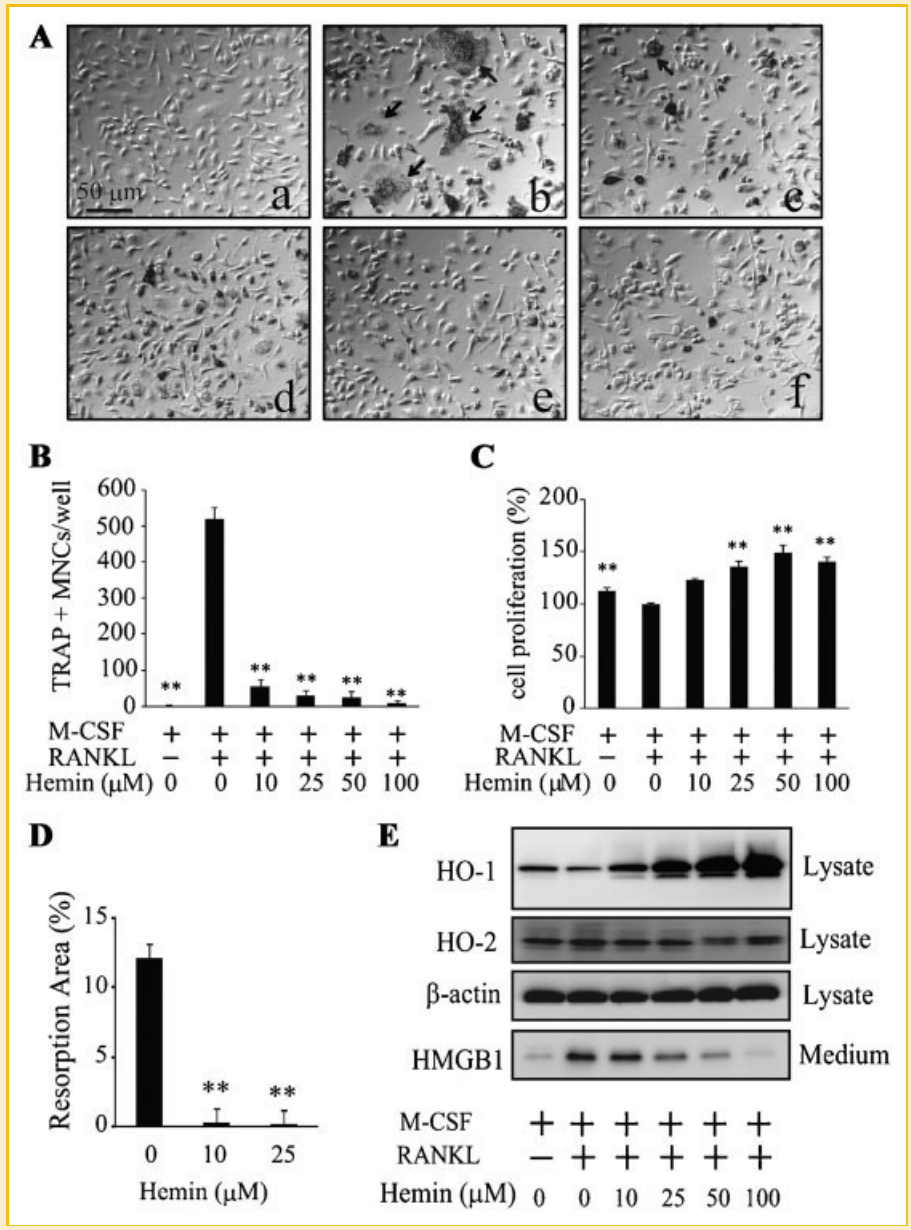



Fig. 1. Hemin inhibits osteoclastogenesis, upregulates heme oxygenase 1 (HO-1), and suppresses the release of high mobility group box 1 (HMGB1). Bone marrow-derived macrophages (BMMs) were cultured with M-CSF (30 ng/ml), RANKL (50 ng/ml), and various concentrations of hemin for up to 72 h, and then stained for tatrate-resistant acid phosphatase (TRAP). A: Cells were cultured for 60 h with (a) M-CSF; (b) M-CSF and RANKL; (c–f) M-CSF, RANKL, and 10 μM (c); 25 μM (d); 50 μM (e); 100 μM (f) of hemin. Arrows indicate multinucleated osteoclasts (MNCs). B,C: Effect of RANKL and increasing concentrations of hemin on the number of TRAP-positive MNCs (B) and cell proliferation (C) after culturing cells for 60 h in the presence of M-CSF. Results are shown as mean (SD) ($n = 4$). D: Effect of hemin on bone resorption. Cells were cultured for 5 days on the Osteo Assay Plate with indicated concentrations of hemin in the presence of M-CSF and RANKL. E: Western blot analysis of the effect of hemin on HO-1 and HO-2 expression in the cell lysate and extracellular HMGB1 in the medium after 60 h of culture. β-actin was used as a loading control. $^{**}P < 0.01$ versus treatment with M-CSF and RANKL. The results are representative of three independent experiments.

### RANKL DOWNREGULATES HO-1 AND INDUCES THE RELEASE OF HMGB1 VIA A P38 MAPK-DEPENDENT PATHWAY

To determine whether alteration of the expression level of HO-1 is characteristic of osteoclastogenesis and whether RANKL regulates HO-1, we analyzed the expression of HO-1. The expression of HO-1 in cells after treatment with RANKL for 24, 48, or 72 h was markedly lower than that in untreated cells (Fig. 4A). Next, we determined whether the RANK ligand-receptor interaction mediated this downregulation. Our results demonstrated that RANKL upregulated osteoclast differentiation markers, such as c-Src and cathepsin K; however, OPG completely prevented this upregulation. OPG also prevented the RANKL-mediated downregulation of HO-1 (Fig. 4B). As reported in previous papers, RANKL stimulates several signaling pathways, such as a p38 MAPK, a Jun N-terminal kinase (JNK), a PI3K/Akt, a NF-κB, and an extracellular signal-regulated kinase (Erk) (Fig. 4C) (Boyle et al., 2003). Moreover, inhibitors for these

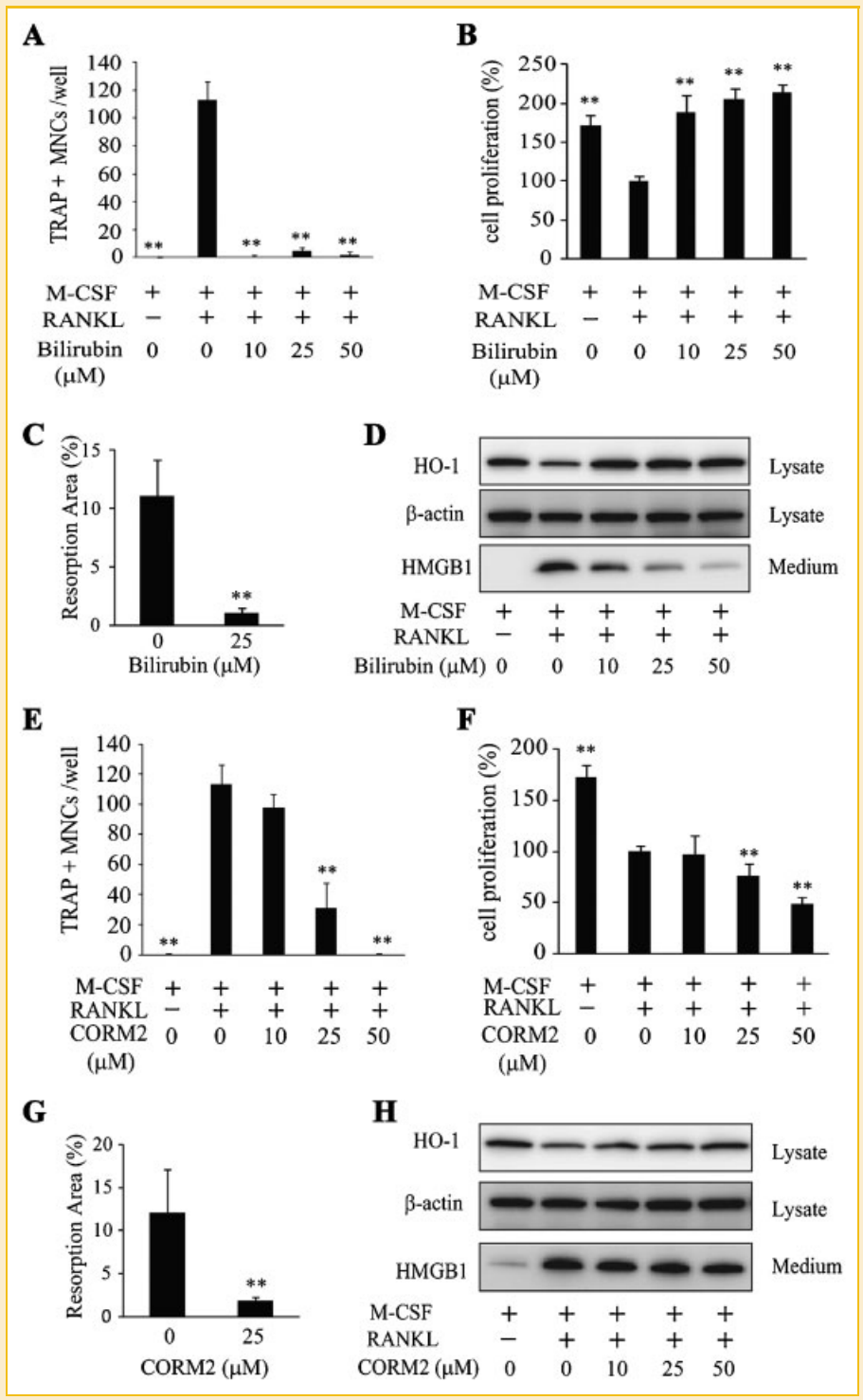



Fig. 2. Effects of bilirubin and carbon monoxide releasing molecule 2 (CORM2) on osteoclastogenesis and HMGB1 release. BMMs were cultured with M-CSF (30 ng/ml) in the absence or presence of RANKL (50 ng/ml) and various concentrations of bilirubin or CORM2 for up to 5 days. Cells were stained for TRAP after 60 h of culture. A,B: Effect of bilirubin on the number of TRAP-positive multinucleated osteoclasts (MNCs) (A) and cell proliferation (B). Results are shown as mean (SD) ($n = 3$–4). $^{**}P < 0.01$ versus treatment with M-CSF and RANKL. C: Effect of bilirubin on the bone resorption. Cells were cultured for 5 days on the Osteo Assay Plate with or without 25 μM of bilirubin in the presence of M-CSF and RANKL. D: Western blot analysis of HO-1 (48 h) and HMGB1 (96 h) in the cell lysate and medium, respectively, of the cells that were cultured in (A) and (B). β-actin was used as a loading control. E,F: Effect of CORM2 on the number of TRAP-positive MNCs (E) and cell proliferation after 60 h of culture (F). Results are shown as mean (SD) ($n = 3$–4). $^{**}P < 0.01$ versus treatment with M-CSF and RANKL. G: Effect of CORM2 on the bone resorption. Cells were cultured for 5 days on the Osteo Assay Plate with or without 25 μM of CORM2 in the presence of M-CSF and RANKL. H: Western blot analysis of HO-1 (48 h) and HMGB1 (96 h) in the cell lysate and medium, respectively, of the cells that were cultured in (E) and (F). β-actin was used as a loading control. The results are representative of three independent experiments.

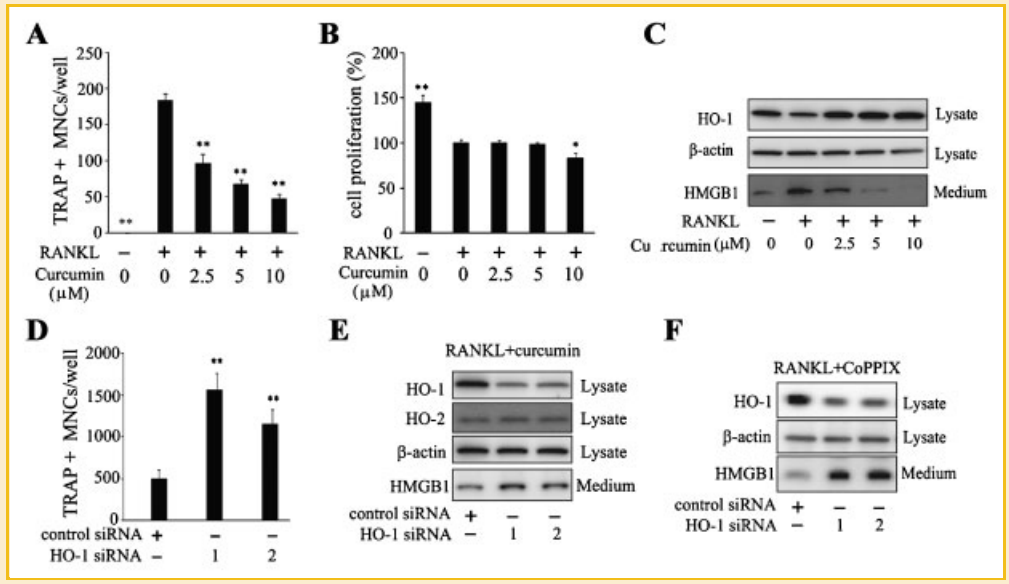
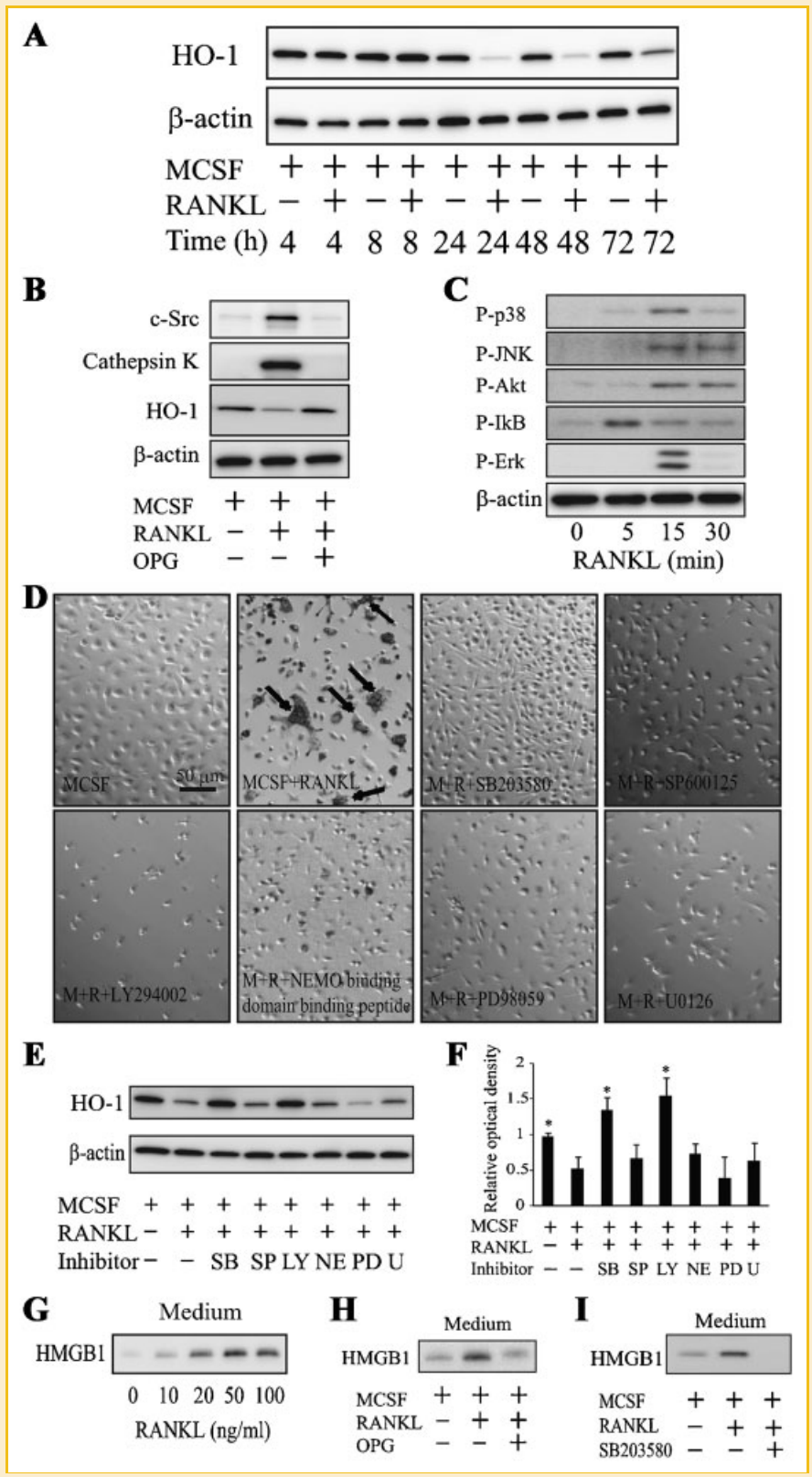



Fig. 3. Curcumin inhibits the extracellular release of HMGB1 and osteoclastogenesis in RAW-D murine monocytic cells by upregulating HO-1. RAW-D cells were cultured for 5 d in the absence or presence of 50 ng/ml of RANKL and various concentrations of curcumin and were then stained for TRAP. A,B: Effect of RANKL and curcumin on the number of TRAP-positive multinucleated osteoclasts (MNCs) (A) and cell proliferation (B). Results are shown as mean (SD) ($n = 3$–4). $^{*}P < 0.05$, $^{**}P < 0.01$ versus treatment with only RANKL. C: Western blot analysis of HO-1 and HMGB1 in cell lysate and medium, respectively, from cultured cells. β-actin was used as a loading control. D,E: The effect of HO-1 siRNA1 and 2 (see Materials and Methods section for sequences) or negative control siRNA on the number of TRAP-positive MNCs after 120 h (D) or the expression of HO-1 after 48 h and HMGB1 after 120 h (E). Cells were cultured with 5 μM curcumin. The results in (D) are shown as mean (SD) ($n = 3$). $^{**}P < 0.01$ versus control siRNA. HO-2 and β-actin were used as a loading control. F: RAW-D cells were cultured for 48 h in the presence of 50 ng/ml of RANKL and 25 μM of cobalt protoporphyrin IX (CoPPIX). The effect of HO-1 siRNA1 and siRNA2 or negative control siRNA on the expression of HO-1 and HMGB1. The results are representative of three independent experiments.

molecules markedly reduced osteoclastogenesis (Fig. 4D). To elucidate the pathway that is involved in the RANKL-mediated suppression of HO-1, we also tested the effect of these inhibitors on the expression of HO-1. SB203580, a p38 MAPK inhibitor, and LY294002, a PI3K inhibitor, increased HO-1 expression (Fig. 4E, F). However, SP600125, a JNK inhibitor, NEMO binding-domain binding peptide, an NF-κB inhibitor, and PD98059 and U0126, which are mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitors, did not affect the RANKL-mediated suppression of HO-1. RANKL also increased the extracellular release of HMGB1 (Fig. 4G), and this release was prevented by OPG (Fig. 4H). Similarly, SB203580 effectively inhibited the RANKL-induced release of HMGB1 (Fig. 4I). Although LY294002 and SB203580 enhanced HO-1 expression to the same extent (Fig. 4E, F), LY294002 strongly suppressed cell growth (Fig. 4D). As a result, LY294002 did not inhibit HMGB1 release (data not shown). Collectively, these results suggest that the p38 MAPK signaling pathway mediates the RANKL-mediated suppression of HO-1 and promotion of HMGB1 release.

### SUPPRESSION OF HO-1 IS ASSOCIATED WITH CASPASE-3 ACTIVATION AND RELEASE OF HMGB1

Since HMGB1 is released from dying cells, we investigated whether RANKL induces cell death. As shown in Figure 5A, RANKL significantly inhibited the proliferation of BMMs after 48 h of culture. This inhibitory effect of RANKL was dose-dependent up to the half maximal inhibitory concentration ($IC_{50}$) of 50 ng/ml, but higher concentrations did not have any further effect. In addition, RANKL did not strongly inhibit cell proliferation after 72 h of culture (Fig. 5B). Since previous study reported that caspase-3 activation is required for osteoclast differentiation (Szymczyk et al., 2006), we tested whether RANKL activates caspase-3. As shown in Figure 5C,D, RANKL activated caspase-3 in cultured BMMs. In addition, RANKL slightly downregulated Bcl-xL, an anti-apoptotic protein, and upregulated Bim, a pro-apoptotic protein; however, OPG reversed these effects (Fig. 5D). RANKL did not change the expression of Bax significantly. As shown in Figure 5E,F, bilirubin inhibited RANKL-induced caspase-3 activation, Bim expression, and HMGB1 release. In contrast, CORM2 did not have as large of an effect on caspase-3 activation, Bim expression, and HMGB1 release as bilirubin. These findings motivated us to determine whether caspase-3 is involved in the RANKL-induced release of HMGB1. To determine whether caspase-3 participated in HMGB1 release, we examined whether the activation of caspase-3 by staurosporine stimulates HMGB1 release without RANKL. Staurosporine, which induces apoptosis, not only activated caspase-3 activation but also promoted HMGB1 release (Fig. 5G). Although HMGB1 release was promoted by staurosporine, BMMs did not proliferate and differentiate into osteoclasts because almost all cells shrank and died. We also determined the effect of HO-1 on caspase-3 activation and HMGB1 release. In RAW-D cells, knockdown of HO-1 promoted caspase-3 activation and HMGB1 release compared with negative control siRNA (Fig. 5H). These results suggest that HO-1 downregulation is sufficient for the caspase-3 activation and HMGB1 release.


A
HO-1
β-actin
MCSF + + + + + + + + + +
RANKL − + − + − + − + − +
Time (h) 4 4 8 8 24 24 48 48 72 72
B
c-Src
Cathepsin K
HO-1
β-actin
MCSF + + +
RANKL − + +
OPG − − +
C
P-p38
P-JNK
P-Akt
P-IkB
P-Erk
β-actin
0 5 15 30
RANKL (min)
D
MCSF
50 μm
MCSF+RANKL
M+R+SB203580
M+R+SP600125
M+R+LY294002
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M+R+PD98059
M+R+U0126
E
HO-1
β-actin
MCSF + + + + + + + +
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Relative optical density
2
1.5
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0
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G
Medium
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RANKL (ng/ml)
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Medium
HMGB1
MCSF + + +
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Fig. 4.

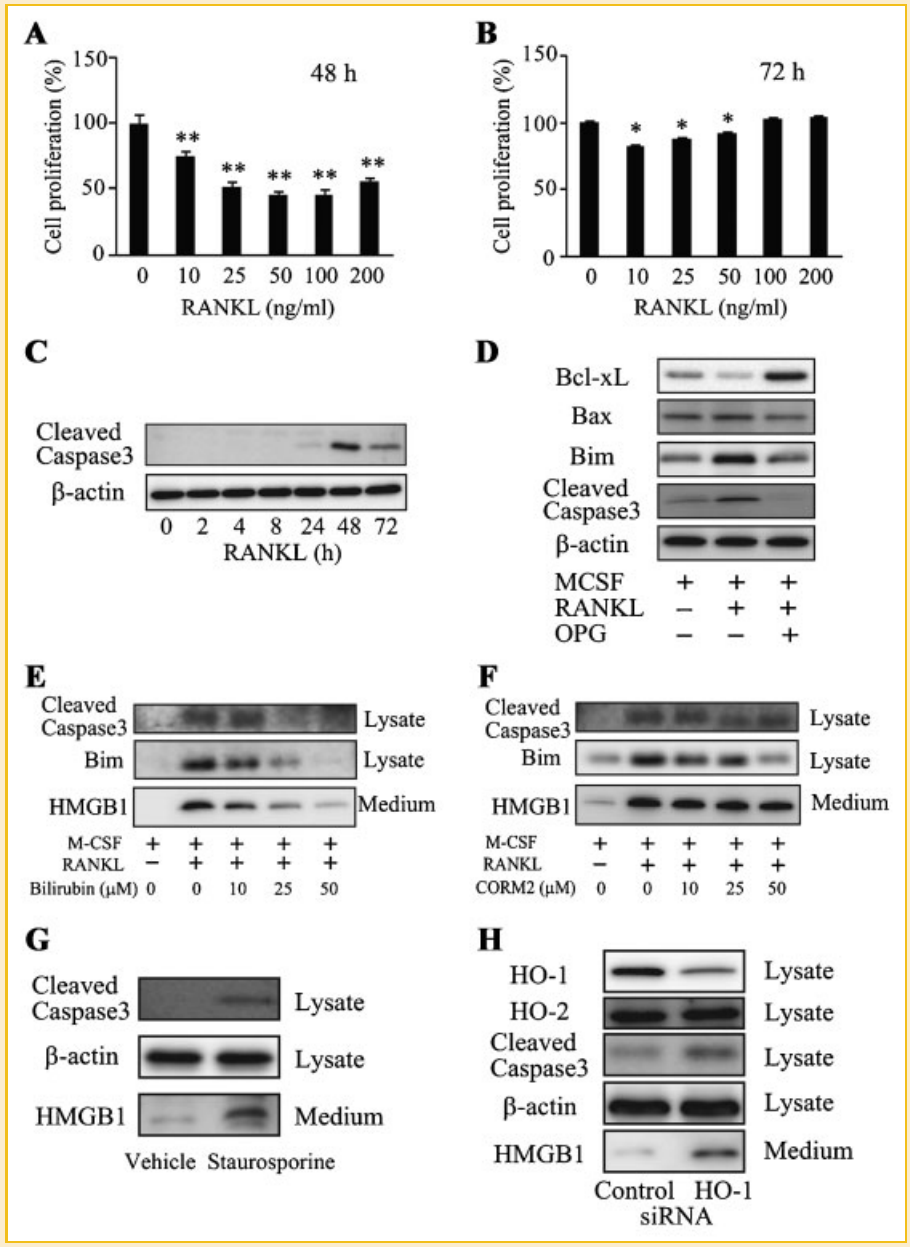


Fig. 5. Downregulation of HO-1 promotes caspase-3 activation and HMGB1 release. Effects of various concentrations of RANKL on the proliferation of BMMs after 48 h (A) or 72 h (B) of culture in the presence of M-CSF (30 ng/ml). Results are shown as mean (SD) ($n = 3$–4). $^{*}P < 0.05$, $^{**}P < 0.01$ versus treatment with M-CSF and RANKL. C: Western blot analysis of the active (cleaved) form of caspase-3 after culturing BMMs with M-CSF (30 ng/ml) and RANKL (50 ng/ml) for up to 72 h. D: Western blot analysis of B-cell lymphoma extra-large (Bcl-xL), Bcl-2 associated X protein (Bax), and Bcl-2 interacting mediator of cell death (Bim) after culturing BMMs with M-CSF (30 ng/ml) in the absence or presence of RANKL (50 ng/ml) and OPG (300 ng/ml) for 72 h. E,F: Effects of bilirubin or CORM2 on RANKL-induced caspase-3 activation, Bim expression, and HMGB1 release in the cell lysate and medium of the cells that were cultured in Figure 2A or 2C. G: Western blot analysis of the cleaved form of caspase-3 and HMGB1 in the cell lysate and medium, respectively, of BMMs that were cultured with staurosporine (1 μM) or vehicle (methanol) in the presence of M-CSF (30 ng/ml) for 24 h. H: Western blot analysis of HO-1, cleaved caspase-3, and HMGB1 in the cell lysate or medium of RAW-D cells that were transfected with HO-1 siRNA1 (see Materials and Methods section for sequence) or a negative control siRNA, and were then cultured without RANKL. HO-2 and β-actin were used as loading control. Results are representative of three independent experiments.

Fig. 4. RANKL downregulates the expression of HO-1 and promotes the release of HMGB1 via a p38 MAPK-dependent pathway. A: Western blot analysis of HO-1 in BMMs that were cultured with or without RANKL (50 ng/ml) in the presence of M-CSF (30 ng/ml) for 72 h. The same amount of protein was loaded in each lane. β-actin was used as a loading control. B: Western blot analysis of various osteoclast differentiation markers in BMMs that were cultured with or without RANKL (50 ng/ml) and osteoprotegerin (OPG; 300 ng/ml) in the presence of M-CSF (30 ng/ml) for 72 h. β-actin was used as a loading control. C: BMMs were incubated with serum-free media for 2 h; subsequently, they were stimulated with RANKL (100 ng/ml) for the indicated times (0, 5, 15, and 30 min). The cell lysates with equal amounts of protein were subjected to SDS-PAGE, followed by Western blotting with antibodies to p-p38 MAPK, p-JNK, p-Akt, p-IκBα, p-Erk, and β-actin. D: TRAP-stained BMMs that were cultured for 72 h in the presence of M-CSF (30 ng/ml), RANKL (50 ng/ml), and 10 μM of one of the indicated inhibitors. Arrows indicate TRAP-positive MNCs. E: Western blot analysis of HO-1 in BMMs that were cultured for 48 h as described in (D). F: The ratio of the relative optical density of HO-1 to that of β-actin was calculated using ImageJ software (National Institute of Health, http://rsbweb.nih.gov/ij). Results are shown as mean (SD) ($n = 3$). $^{*}P < 0.05$ versus treatment with M-CSF and RANKL. G–I: Western blot analysis of HMGB1 in BMMs that were cultured with various concentrations of RANKL for 48 h (G), with or without RANKL (50 ng/ml) and OPG (300 ng/ml) for 72 h (H) or RANKL (50 ng/ml) and SB203580 (10 μM) for 72 h (I). All cells were cultured with 30 ng/ml of M-CSF. The results are representative of three independent experiments.

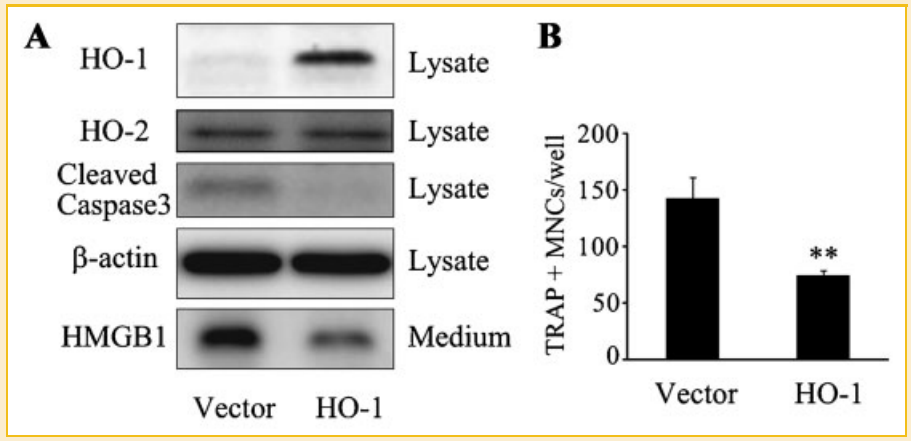


Fig. 6. Overexpression of HO-1 inhibits caspase-3 activation, HMGB1 release, and osteoclastogenesis. A: Western blot analysis of HO-1, cleaved caspase-3, and HMGB1 in the cell lysate or medium of RAW-D cells that were transfected with the HO-1-GFP overexpression vector or a control vector for 24 h and then cultured in the presence of RANKL (50 ng/ml) for 72 h. HO-2 and β-actin were used as loading controls. B: Effect of HO-1 overexpression on the number of TRAP-positive MNCs after culturing for 72 h. Results are shown as mean (SD) ($n = 3$). $^{**}P < 0.01$ versus control vector. The results are representative of three independent experiments.

### OVEREXPRESSION OF HO-1 INHIBITS RANKL-INDUCED CASPASE-3 ACTIVATION, HMGB1 RELEASE, AND OSTEOCLASTOGENESIS

We also investigated whether overexpression of HO-1 affects RANKL induced caspase-3 activation, HMGB1 release, and osteoclastogenesis. Overexpression of HO-1 inhibited RANKL-induced caspase-3 activation and HMGB1 release (Fig. 6A) and decreased the number of TRAP-positive multinucleated osteoclasts (Fig. 6B). These results provided further support that the RANKL-induced downregulation of HO-1 promotes caspase-3 activation, HMGB1 release, and osteoclastogenesis.

### RANKL-INDUCED PRODUCTION OF REACTIVE OXYGEN SPECIES PROMOTES CASPASE-3 ACTIVATION AND HMGB1 RELEASE

Since *N*-acetylcysteine (NAC), a reactive oxygen species (ROS) scavenger, inhibits the RANKL-induced production of ROS and osteoclastogenesis (Lee et al., 2005), we determined the effect of NAC on RANKL-induced caspase-3 activation and HMGB1 release. As shown in Figure 7A, 5 or 10 mM NAC significantly inhibited osteoclast formation. In addition, the RANKL-induced upregulation of Bim, cleaved caspase-3, and extracellular HMGB1 were inhibited by 5 or 10 mM NAC (Fig. 7B). These results suggested that oxidative stress leads to RANKL-mediated cellular apoptosis and HMGB1 release.

## DISCUSSION

In this study, we elucidated a novel HO-1-mediated mechanism of RANKL-induced osteoclastogenesis. Specifically, our results demonstrated that suppression of HO-1 promotes caspase-3 activation and HMGB1 release during osteoclastogenesis. Although previous studies have demonstrated that HO-1 and HMGB1 each have important roles in osteoclastogenesis, this is the first report of a relationship between HO-1 and HMGB1. Moreover, our results indicated that the regulation of HO-1 and HMGB1 was mediated by the activation of caspase-3 and redox conditions during osteoclastogenesis.

Zwerina et al. (2005) revealed that induction of HO-1 is a negative regulator of osteoclastogenesis and suggested that suppression of HO-1 during osteoclastogenesis is necessary for the production of proinflammatory cytokines, such as TNF-α and IL-1β. In addition, they demonstrated, albeit indirectly, that hemin inhibits inflammation-driven osteoclastogenesis in TNF-transgenic mice. In contrast, our results provided direct evidence that HO-1 regulates osteoclastogenesis via HMGB1 release- and caspase-3-dependent pathways. Thus, HO-1 is a regulator of HMGB1 release during osteoclastogenesis.

Zhou et al. (2008) demonstrated that RANKL induces HMGB1 release during an early stage of differentiation from BMMs into osteoclasts. Extracellular HMGB1 acts as an osteoclastogenic cytokine by promoting actin cytoskeleton reorganization and integrin signaling in RANKL-stimulated BMMs. However, the mechanisms by which RANKL mediates HMGB1 release in BMMs are not known. Our results demonstrated that RANKL-mediated HO-1 suppression directly regulates HMGB1 release in BMMs.

Transcriptional regulation of HO-1 is well understood. The 5′-flanking region of the HO-1 gene contains binding sites for the transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2) (Alam et al., 1999). Although we did not clarify how RANKL downregulates the transcription of HO-1 in this study, there is a possibility that the activation of Nrf2 is suppressed by RANKL, especially via p38 MAPK and PI3K pathways (Fig. 4E,F). In addition, we found that *tert*-butylhydroquinone, an Nrf2 activator, upregulated HO-1 and inhibited osteoclastogenesis (unpublished data). Nrf2-mediated transcriptional regulation of HO-1 appears to have a key role in osteoclastogenesis. Further studies will be necessary in the future.

Both bilirubin and CORM2 inhibited RANKL-mediated HO-1 suppression and consequently blocked osteoclastogenesis. However, bilirubin and CORM2 had opposing effects on proliferation. A previous study demonstrated that CO inhibited LPS-induced NF-κB

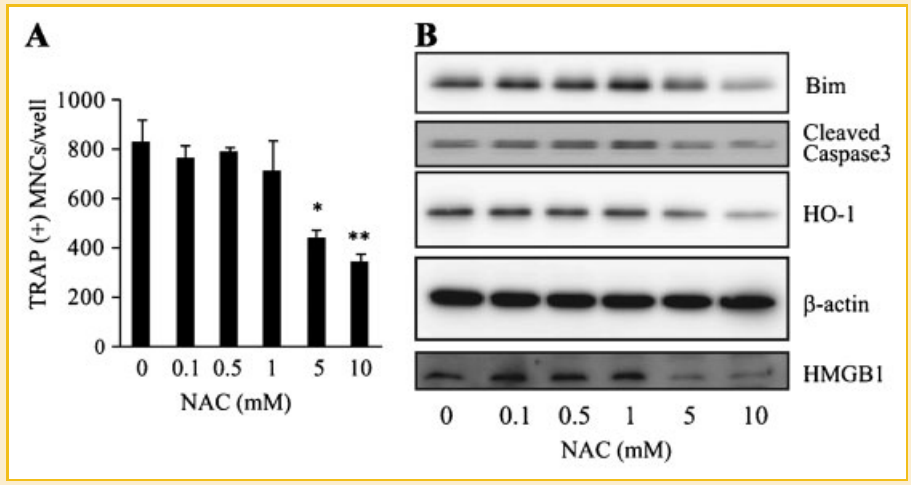


Fig. 7. RANKL-induced production of reactive oxygen species promotes caspase-3 activation, HMGB1 release, and osteoclastogenesis. BMMs were cultured with various concentrations of NAC in the presence of M-CSF (30 ng/ml) and RANKL (50 ng/ml) for 72 h, and then stained for TRAP. A: Effect of NAC on the number of TRAP-positive MNCs. Results are shown as mean (SD) ($n = 3$–4). $^{*}P < 0.05$, $^{**}P < 0.01$ versus treatment with M-CSF and RANKL. B: Western blot analysis of Bim, cleaved caspase-3, HO-1, and HMGB1 in the cell lysate or medium of BMMs that were cultured with NAC in the presence of M-CSF (30 ng/ml) and RANKL (50 ng/ml) for 48 h. β-actin was used as a loading control. The results are representative of three independent experiments.

activation (Lee et al., 2003). In this study, significant cell death was observed among CORM2-treated cells, although HO-1 was upregulated in these cells. It is well known that the activation of NF-κB is necessary for osteoclastogenesis. RANKL-induced NF-κB activation might have been inhibited by CORM2 treatment.

Our observations with SB203580, which pharmacologically blocks p38α MAPK activity, indicated the upregulation of HO-1 in RANKL-stimulated BMMs. The p38 MAPK family is composed of four isozymes: p38α, p38β, p38γ, and p38δ. Among these four isoforms, only p38α is found to be highly expressed in both osteoclast precursors and mature osteoclasts (Böhm et al., 2009). Thus, our results suggest that p38α MAPK activation promotes HO-1 downregulation. Currently, the effect of p38 MAPK on HO-1 gene expression is controversial. Several studies have reported that p38 MAPK activation regulates HO-1 upregulation. For example, genetic suppression of p38α MAPK in embryonic fibroblasts and pharmacological inhibition of p38 MAPK in RAW264.7 macrophage-like cells upregulate HO-1 (Naidu et al., 2009). These findings were consistent with our results. However, our results contradicted other studies reporting that p38 MAPK inhibits HO-1 gene expression (Yu et al., 2000; Keum et al., 2006). These discrepancies might be due to differences in some types of cells, since Yu et al. (2000) and Keum et al. (2006) used HepG2 human hepatoma and Hepa1c1c7 murine hepatoma cells, respectively, whereas we used BMMs. Nevertheless, our results suggested that activation of p38α MAPK promotes HO-1 downregulation, and consequently, HMGB1 release during the differentiation of BMMs into osteoclasts.

Although the relationship between HMGB1 release and caspase-3 activation is not clear, it is likely that redox conditions in osteoclast precursor cells regulate both processes, since NAC abolished both HMGB1 release and caspase-3 activation (Fig. 7B). Recently, several studies have demonstrated that RANKL-mediated ROS generation is essential for osteoclastogenesis (Ha et al., 2004; Lee et al., 2005). ROS acts as a second messenger that regulates several signaling cascades including JNK, p38 MAPK, and Erk cascades. In a previous study, NAC blocked JNK, p38 MAPK, and Erk signaling (Lee et al., 2005), although Nox1 siRNA blocked the activation of JNK and p38 MAPK, but not Erk. The results indicated that the activation of these 3 kinases is ROS-dependent, the activation of JNK and p38 MAPK is Nox1-dependent, and the activation of Erk is Nox1-independent. It is known that ROS stimulate HO-1 expression, however, our results suggest RANKL-dependent ROS promote the suppression of HO-1. Besides the RANKL-dependent ROS generation, it is known that ROS is continuously produced in aerobic organisms (Finkel and Holbrook, 2000; Valko et al., 2007) under physiologic conditions (Fig. 8A). Our results indicated that 5 or 10 mM NAC further decreased RANKL-mediated HO-1 suppression (Fig. 7B), suggesting that ROS produced from different intracellular sources such as mitochondria and endoplasmic reticulum are involved in the expression of HO-1 in osteoclasts. HO-1 is an important antioxidant molecule and it promotes cytoprotection. The level of ROS formation is in balance with antioxidant capacity and an increased level of ROS promotes apoptotic cell death (Circu and Aw, 2010). In addition, previous reports indicated the accumulation of ROS in HO-1-deficient cells (Poss and Tonegawa, 1997). Our results suggest that the inhibition of HO-1 by RANKL may allow the generation of ROS, and the elevated ROS level may facilitate the upregulation of Bim and the caspase-3 activation. Our observations in this study clearly demonstrated that RANKL can downregulate HO-1 expression via p38 MAPK pathway; and mediate caspase-3 activation; and promote HMGB1 release; and subsequently, osteoclastogenesis (Fig. 8B).

It should be noted that RANKL-induced caspase-3 activation failed to induce cell death in all osteoclasts (Figs. 5 and 6). Previous reports revealed a 38% increase in annexin V-positive cells after 48 h of RANKL treatment (Bharti et al., 2004), and caspase-3

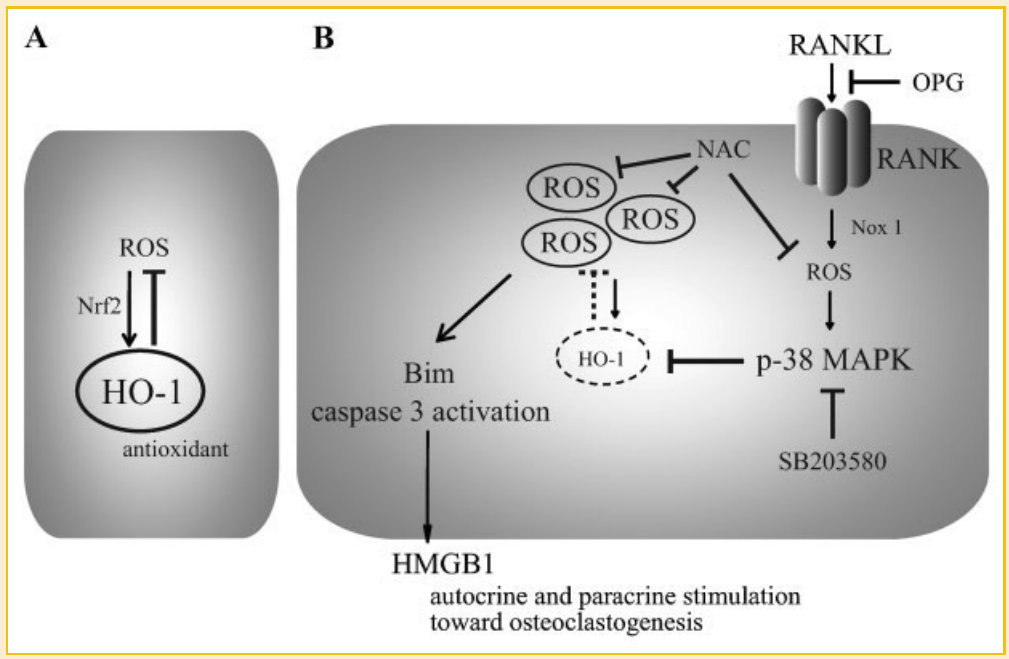


Fig. 8. A hypothetical model of the regulation of osteoclastogenesis by an HO-1-dependent caspase-3 and HMGB1 control mechanism. A: Under physiologic conditions, ROS is continuously produced. HO-1 expression is stimulated by ROS via Nrf2 transcriptional regulation. HO-1 acts as an antioxidant molecule. B: When the RANKL-ROS-p38 MAPK axis is stimulated, the suppression of HO-1 promotes caspase-3 activation, HMGB1 release, and subsequently, osteoclastogenesis.

activation was required for osteoclast differentiation (Szymczyk et al., 2006). As shown in Figure 5A, RANKL inhibited cell proliferation (maximal inhibition at 50 ng/ml) at 48 h of RANKL treatment, although the cell proliferation recovered at 72 h (Fig. 5B). Higher doses of RANKL recovered cell proliferation more than lower doses of RANKL did. RANKL stimulated HMGB1 release in a dose-dependent manner (maximal release at 50 ng/ml; Fig. 4G). There appears to be a correlation between the recovery of cell proliferation and the amount of HMGB1 in the medium. It is believed that the surviving cells were stimulated by HMGB1, which was released from the dead cells and promoted proliferation and differentiation.

We propose that only partial activation of caspase-3 is required for the fusion of monocyte/macrophage precursor cells to form osteoclasts. This hypothesis is supported by several studies. For example, apoptosis is partially induced in myoblasts that fuse to form multinucleated myotubes (Nadal-Ginard, 1978). In addition, caspase-3 was activated during myoblast fusion in a cell culture model (Fernando et al., 2002). Furthermore, a previous report demonstrated that myoblasts express both HMGB1 and RAGE, which decrease proliferation and increase apoptosis and differentiation in myoblasts (Riuzzi et al., 2007). In addition to partial caspase-3 activation, several other cell surface molecules, such as cluster of differentiation 47 (CD47), CD98, cadherin, and dendritic cell-specific transmembrane protein (DC-STAMP), are essential for cell fusion during osteoclastogenesis (Kukita et al., 2004). Therefore, further investigation is needed to elucidate the molecules that are involved in the cell fusion process of osteoclastogenesis.

Previous researchers have investigated the relationship between oxidative stress and bone metabolism by using iron overload (Isomura et al., 2004; Ishii et al., 2009; Tsay et al., 2010). Additionally, our findings that the heme-degrading enzyme HO-1 regulates osteoclastogenesis suggest a novel mechanism involving iron homeostasis, oxidative stress, and bone metabolism.

In conclusion, we demonstrated that RANKL-induced HO-1 suppression causes HMGB1 release during osteoclast differentiation. Furthermore, the regulation of HO-1 and HMGB-1 in osteoclastogenesis is mediated by caspase-3 activation and redox conditions. Thus, HO-1 downregulation is a key initiator of RANKL-induced osteoclastogenesis.

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