Cobalt protoporphyrin represses osteoclastogenesis through blocking multiple signaling pathways

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Abstract.

Cobalt protoporphyrin (CoPP) is a metallo-protoporphyrin that works as a powerful inducer of heme oxigenase-1 (HO-1) in various tissues and cells. Our recent studies have demonstrated that induction of HO-1 by several reagents inhibited differentiation and activation of osteoclasts (OCLs), which are multinucleated bone resorbing cells. However, the effects of CoPP on osteoclastogenesis remain to be elucidated. In this study, we report that CoPP inhibits receptor activator of nuclear factor κB ligand (RANKL)-induced OCL formation in a dose dependent manner. Importantly, CoPP had little cytotoxicity, but rather enhanced cell proliferation of OCLs. CoPP suppressed the protein levels of nuclear factor of activated T cells cytoplasmic-1 (NFATc1) as well as those of OCLs markers such as Src and cathepsin K, which are transcriptionally regulated by NFATc1 in mature OCLs. Western blot analyses also showed that CoPP abolished RANKL-stimulated phosphorylation of several major signaling pathways such as IκB, Akt, ERK, JNK and p38 MAPKs in OCL precursor cells. Thus, our results show that CoPP represses osteoclastogenesis through blocking multiple signaling pathways.

Keywords: osteoclasts, cobalt protoporphyrin, hemeoxigenase-1, RANKL

Introduction

Osteoclasts (OCLs) are bone resorbing multinucleated cells that are matured from monocyte/macrophage precursor cells (Teitelbaum, 2000). The differentiation of OCLs is mainly modulated by the two important factors; receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). The RANKL receptor termed as receptor activator of nuclear factor kappa-B (RANK) activates essential signaling pathways for OCL differentiation. The pathways include nuclear factor of activated T cells cytoplasmic-1 (NFATc1), nuclear factor kappa B (NF-κB), phosphatidylinositol 3-kinase (PI3K)/Akt, Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK) (Darnay et al., 1999; Matsumoto et al., 2000; Zhang et al., 2001). Previously, reactive oxygen species (ROS) was identified as an additional essential factor for the OCL differentiation (Ha et al., 2004; Lee et al., 2005). The RANKL stimulation leads to robust increased ROS through a signaling pathway mediated by TRAF 6, Rac 1, and NADPH oxidase 1 (Nox 1). Concerning the redox conditions, we recently demonstrated that the RANKL-mediated suppression of anti-oxidant enzyme heme-oxigenase 1 (HO-1) promoted OCL differentiation (Sakai et al., 2012). Induction of HO-1 by ectopic gene expression or pharmacological treatment impedes OCL differentiation. Indeed, treatment with bioactive polyphenols, such as curcumin (Sakai et al., 2012) , kahweol (Fumimoto et al., 2012), fisetin (Sakai et al., 2013) prevents osteoclastogenesis. Moreover, our recent study has shown that treatment with hemin, which is known as ferric protoporphyrin, inhibits osteoclastogenesis, suggesting that a HO-1 inducer may be useful for regulating agents for OCL differentiation (Sakai et al., 2012). However, the effects of metalloprotoporphyrins other than hemin on osteoclastogenesis remain to be clarified.

Cobalt protoporphyrin (CoPP) is known as one of potent HO-1 inducers in various cells and tissues (Shan et al., 2000). Induction of HO-1 treated with CoPP has been shown to protect apoptosis in the liver from ischemia/reperfusion (Amersi et al., 1999) ;(Kim et al., 2012) or in human cardiac stem cells (Cai et al., 2012). The CoPP-mediated HO-1 induction has inhibitory effects on inflammation or oxidative damages in diabetic rats (Elmarakby et al., 2012; Nicolai et al., 2009), or cartilage cells from osteoarthritic patients (Guillen et al., 2008); (Clerigues et al., 2012). Moreover, CoPP serves as an immune-modulator that regulates differentiation of macrophages. CoPP exerts inhibitory effects on differentiation of macrophages into dendritic cells (Chauveau et al., 2005; Mashreghi et al., 2008). In contrast to the inhibitory effects, CoPP promotes the differentiation from human monocyte THP-1 into macrophage-like cells that express several differentiation maker proteins (Song et al., 2011). Given that modulation of CoPP in macrophage differentiation, it remains unknown whether CoPP inhibits OCL

differentiation or not. In this study, we evaluated effects of CoPP on OCL differentiation using *in vitro* culture systems, and demonstrated that CoPP inhibits OCL differentiation.

Materials and Methods

Reagents

Cobalt protoporphyrin (CoPP) IX was purchased from Wako Pure Chemicals (Osaka, Japan). M-CSF was purchased from Kyowa Hakko Kogyo (Tokyo, Japan). Recombinant RANKL was prepared as previously described (Hu et al., 2008). Antibodies (Abs) were purchased as follows: β-actin (Sigma-Aldrich, St. Louis, MO, USA), Src (Upstate, Lake Placid, NY, USA), c-fms, RANK, and NFATc1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), HO-1 (Stressgen, Ann Arbor, MI, USA), phospho-ERK1/2 (Thr202/Tyr204), phospho-JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-IκBα (Ser32), and phospho-Akt (Ser473) (Cell Signaling Technology, Danvers, MA, USA). Cathepsin K Ab was prepared as previously described (Kamiya et al., 1998). The Osteo Assay Stripwell Plate was purchased from Corning (Corning, NY, USA). All other reagents including PMSF and protease inhibitor cocktail, were obtained from Sigma-Aldrich.

Cell culture

Five-week-old male C57BL/6 mice were obtained from CLEA Japan, Inc. (Tokyo, Japan), and were maintained in an air-conditioned room with a 12 h light/dark cycle, and given a basal diet, MF (Oriental Yeast, Co., Tokyo, Japan) and water *ad libitum*. All animals were handled in our facilities under the approved protocols of the Nagasaki University Animal Care Committee. To isolate BMMs, we cultured marrow cells from femurs and tibias of the mice 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. By harvesting the non-adherent cells, stroma-free bone marrow cells were cultured in the presence of 50 ng/mL of M-CSF. After 3 days, the non-adherent cells were washed out, and the adherent cells were used as BMMs. The BMMs were replated, and then further cultured with new medium containing M-CSF (30 ng/mL) and RANKL (50 ng/mL) for the times indicated. The cells were fixed with 4% paraformaldehyde and stained for TRAP activity using a previously described method (Hotokezaka et al., 2002) TRAP-positive cells with 3 or more nuclei were regarded as mature osteoclasts. The bone-resorbing activity of OCLs was assayed using the Osteo Assay Stripwell Plate for 7 days of culture. The resorption area was determined using Image J

software (http://rsbweb.nih.gov/ij/).

Cell viability 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^{TM}, Hercules, CA, USA).

Western blot analysis

BMMs were stimulated with RANKL in the presence of M-CSF for the indicated amount of time. 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 proteinase inhibitor cocktail). The protein concentration of each sample was measured with BCA Protein Assay Reagent (Thermo Pierce, Rockford, IL, USA). Five micrograms of lysate protein was applied to each lane. After SDS-PAGE, proteins were electroblotted onto a polyvinylidene difluoride membrane. The blots were blocked with 3 % skim milk for 1 h at room temperature, probed with various antibodies overnight at 4°C, washed, incubated with HRP-conjugated secondary antibodies, and finally detected with ECL-Plus (GE Healthcare Life Sciences). The immunoreactive bands were analyzed by LAS4000mini (Fuji Photo Film, Tokyo, Japan).

Statistical analysis

All values were expressed as means ± standard deviations. Tukey-Kramer method was used to identify differences between concentrations when ANOVA indicated that a significant difference (*$P < 0.05$ or **$P < 0.01$) existed.

Results

CoPP is a HO-1 inducer in OCLs

CoPP is known to be a powerful HO-1 inducer in various cell types (Shan et al., 2000). To test whether CoPP induces expression of HO-1 in OCLs or not, we first examined its effects on OCL differentiation of native BMMs treated with M-CSF (30ng/mL) and RANKL (50ng/mL). As shown in Fig.

2, CoPP induced the expression of HO-1 in RANKL-stimulated OCLs in a dose-dependent manner.

CoPP inhibits osteoclastogenesis *in vitro*

Next, we investigated whether CoPP inhibits OCL differentiation from native BMMs stimulated with M-CSF and RANKL. TRAP activity staining showed that CoPP inhibited the mononuclear and multinuclear formation of OCLs in a dose-dependent manner (Fig. 3A). When counted the number of TRAP-positive and multinucleated OCLs, they were significantly decreased after CoPP treatment at the lower concentration of 1.0 μM (Fig. 3B). However, the cell viability of OCLs measured by CCK-assay showed that CoPP increased viability of OCLs (Fig. 3C). Similar results with a different method by MTT assay were observed (Data not shown). These results indicate that CoPP inhibits osteoclastogenesis with increased viability of the cells.

Effects of CoPP on the bone resorbing activity of OCLs

To further test whether CoPP decreases the bone resorbing activity of OCLs, we performed a pit formation assay with BMM-derived OCLs after the stimulation with M-CSF and RANKL under 2 different conditions. One was that CoPP was added at the beginning of stimulation with RANKL (50ng/mL) and M-CSF (30ng/mL) (Fig. 4A). The other was that CoPP was added after 3days activation when BMMs were differentiated into active OCLs (Fig. 4B). Under these different conditions, CoPP significantly suppressed bone resorbing activities of OCLs (Fig. 4A and B). The calculated resorption area of CoPP-treated OCLs was decreased in a dose-dependent manner compared with that of untreated OCLs (Fig. 4A and B). These results indicate that CoPP inhibits the physiological bone resorbing activity of OCLs.

Effects of CoPP on intracellular signaling and expression levels of marker proteins of OCLs

To further evaluate the effects of CoPP on NFATc1, which is a master regulator for OCL differentiation through Ca^{2+}/calmodulin-dependent calcineurin (Takayanagi et al., 2002), we analyzed the expression levels of NFATc1 of OCLs by western blotting. As shown in Fig. 5, the protein levels of NFATc1 were decreased, and disappeared at concentrations of more than 0.5μM CoPP.

We also analyzed the expression levels of other marker proteins than NFATc1 of OCLs by western blotting (Fig. 5). RANK is a cell surface receptor for RANKL, whereas c-fms is an M-CSF receptor.

c-Src is a tyrosine kinase that regulates the formation of actin-rich podosomes in the OCLs (Kim et al., 2010). Cathepsin K is a lysosomal cysteine proteinase specifically expressed in OCLs (Delaisse et al., 2003). c-fos is the proto-oncogene essential for OCL differentiation. The protein levels of RANK, c-fms, and c-fos were gradually decreased, but detected at 10 μM of CoPP. The levels of c-Src and cathepsin K were also decreased at 0.5-1.0 μM CoPP, and disappeared at 5 μM CoPP (Fig. 5).

To examine the effects of CoPP on RANKL-induced intracellular signaling during the OCL differentiation of BMMs, we performed western blotting to evaluate the phosphorylation of p38 MAPK, JNK, IκBα, ERK, and Akt (Fig. 6). When BMMs were pre-incubated with 2.5 μM CoPP for 12 h, the cells were subsequently stimulated with RANKL for the indicated times. CoPP treatment totally inhibited phosphorylation of signal pathways such as Akt, ERK, p38 MAPK, JNK, and IκBα (Fig. 6). These findings suggest that CoPP blocks all of 6 major signaling pathways including Akt, Erk, p38 MAPK, JNK, IκBα and NFATc1 in RANKL-stimulated OCLs.

Discussion

In this study, we showed that CoPP inhibited OCL differentiation from BMMs into mature OCLs, and markedly inhibited the bone resorbing activity of OCLs. CoPP had little cytotoxicity, but rather enhanced cell proliferation of OCLs. CoPP-treated OCLs exhibited markedly abolished multiple signaling pathways such as Akt, ERK, p38 MAPK, JNK, and IκBα. Moreover, upon CoPP treatment, NFATc1 and its target proteins, such as Src and cathepsin K, were down-regulated in OCLs.

Previous studies have shown that induction of HO-1 by ectopic gene expression or pharmacological treatment with HO-1 inducible reagents prevents OCL differentiation (Zwerina et al., 2005). Moreover, our recent study has shown that treatment with hemin, which is known as ferric protoporphyrin, inhibits osteoclastogenesis, suggesting that a HO-1 inducer is useful for regulating agents for OCL differentiation (Sakai et al., 2012). Consistent with these findings, the HO-1 inducer CoPP inhibited OCL differentiation from BMMs into mature OCLs, and the bone resorbing activity of OCLs. In addition to osteoclastogenesis, it is likely that CoPP has also inhibitory effects of differentiation of macrophages into dendritic cells. The HO-1 induction by CoPP inhibits lipopolysaccharide–induced maturation in human and rat dendritic cells (Chauveau et al., 2005). However, the inhibition of macrophage into dendritic cells by CoPP may be HO-1 independent mechanisms. A recent study has indicated that the inhibition of dendritic maturation with CoPP depends on STAT3 activation, but not HO-1 induction with HO-1-deficient dendritic cells (Mashreghi et al., 2008). In spite of involvement of HO-1, CoPP treatment represses dendritic maturation. It is of interest to determine whether HO-1 independent mechanisms also

occur in OCLs or not.

Decreased phosphorylation of 5 major signaling pathways, such as Akt, ERK, p38 MAPK JNK and IκBα by CoPP is characteristic in RANKL-stimulated OCLs. For example, in lipopolysaccharide-activated murine macrophage RAW264.7 cells, CoPP suppresses phosphorylation of JNK, but not ERK, whereas CoPP alone has no effect on both phosphorylation of ERK and JNK in unstimulated cells (Lin et al., 2009). In rat heart or human colon cancer CaCo2 cells (Busserolles et al., 2006), CoPP induces phosphorylation of Akt, whereas in human breast cancer cells it reduces phosphorylation of Akt (Lee et al., 2014). Thus, CoPP has unique inhibitory effects on RANKL-stimulation OCL differentiation through blocking multiple signaling pathways in OCLs.

In spite of the multiple inhibition of signaling pathways, CoPP had little cytotoxicity, but rather enhanced cell proliferation of OCLs. Similar results are observed in the ferric protoporphyrin hemin-treated OCLs. Our previous study has shown that the hemin-treated OCLs exhibit a 1.5-fold increase in cell viability, although the intracellular signaling mechanisms of hemin-treated OCLs were uninvestigated (Sakai et al., 2012). The detailed mechanisms of cell viability increased by CoPP or hemin remain unknown, since CoPP treatment decreased intracellular signaling pathways, such as Akt, ERK, p38 MAPK and JNK. However, it is possible that HO-1 induced by CoPP may work as cytoprotective protein during osteoclastogenesis.

In conclusion, CoPP inhibited OCL differentiation through blocking multiple pathways and down-regulation of NFATc1. Taken together, it is likely that CoPP inhibits OCL differentiation via multiple pathways, including Akt, ERK, p38 MAPK, JNK, IκBα, and NFATc1-dependent pathways.

Acknowledgments

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure Legends

Fig. 1. Structure of CoPP.

Fig.2. Effects of CoPP on the protein expression of HO-1.
BMMs were cultured with M-CSF (30 ng/mL) and RANKL (50 ng/mL) for 72 h in the presence of CoPP at the indicated concentration (0, 0.2, 0.5, 1, 5, and 10 μM). The cell lysates were subjected to SDS-PAGE followed by western blotting with antibodies to HO-1 and ß-actin. Cont: a cell culture without vehicle.

Fig. 3. Effects of CoPP on OCL differentiation from BMMs cultured for 72 h.
A, BMMs were cultured for 72 h with M-CSF (30 ng/mL), RANKL (50 ng/mL), and CoPP at the indicated concentrations. TRAP staining was performed. B, The number of TRAP-positive OCLs was counted. The data are shown as the mean + standard deviations (significance compared with 0 μM CoPP. $^{**}p < 0.01$). C, Cell proliferation of the BMM-derived-OCLs was analyzed using the Cell Counting Kit. The data are shown as the mean + standard deviations (significance compared with 0 μM CoPP. $^{**}p < 0.01$). Results are representative of 3 independent experiments.

Fig. 4. Effects of CoPP on the bone resorbing activity of OCLs.
BMMs were cultured with M-CSF (30 ng/mL) and RANKL (50 ng/mL). A. CoPP was added at the beginning of stimulation with RANKL and M-CSF at the indicated concentrations for 5 days. Photograph of the bone resorbing activity of OCLs. B. CoPP was added after 3days activation when BMMs were differentiated into active OCLs Photograph of the bone resorbing activity of OCLs. C. The calculated resorption area of data from A was determined using Image J software. (significance compared with 0 μM CoPP. ** $p < 0.01$). D. The calculated resorption area of data from B was determined using Image J software. (significance compared with 0 μM CoPP. ** $p < 0.01$).

Fig.5. Effects of CoPP on protein expression of OCL marker proteins.

BMMs were cultured with M-CSF (30 ng/mL) and RANKL (50 ng/mL) for 72 h in the presence of CoPP at the indicated concentrations (0, 0.2, 0.5, 1, 2.5, 5, and 10 μM). The cell lysates were subjected to SDS-PAGE, followed by western blotting with antibodies specific to NFATc1, RANK, c-fms, c-fos, c-Src, cathepsin K, and ß-actin. C: a cell culture without vehicle.

Fig.6. Effects of CoPP on the essential signaling of OCL differentiation. BMMs were cultured with M-CSF (30 ng/mL) for 12 h in the presence of vehicle or 2.5 μM CoPP. The cells were subsequently stimulated with RANKL (300 ng/mL) for the indicated times (0, 5, 15, and 30 min). The cell lysates were subjected to SDS-PAGE, followed by western blotting with antibodies to p-Akt, p-ERK, p-p38 MAPK, p-JNK, p-IκBα, and ß-actin. The results are representative of 3 independent experiments.

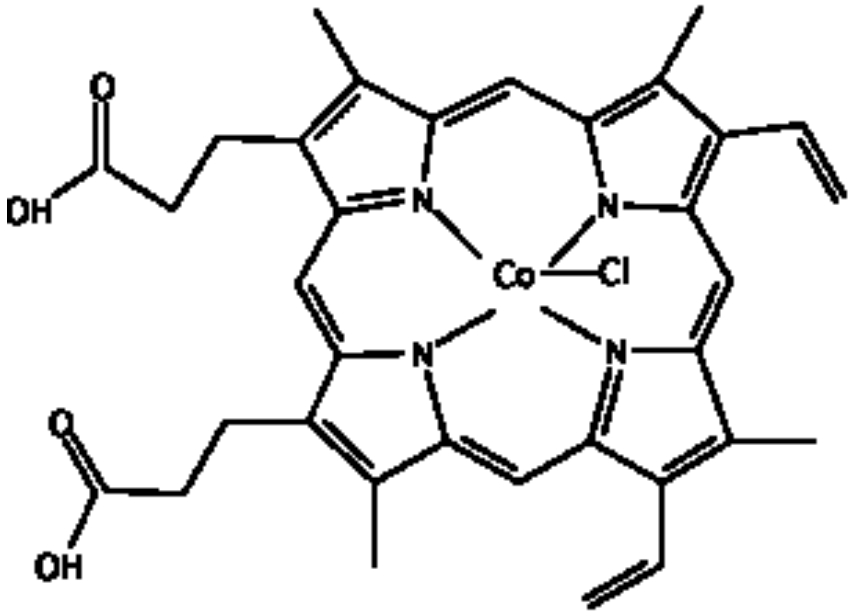


Figure 1

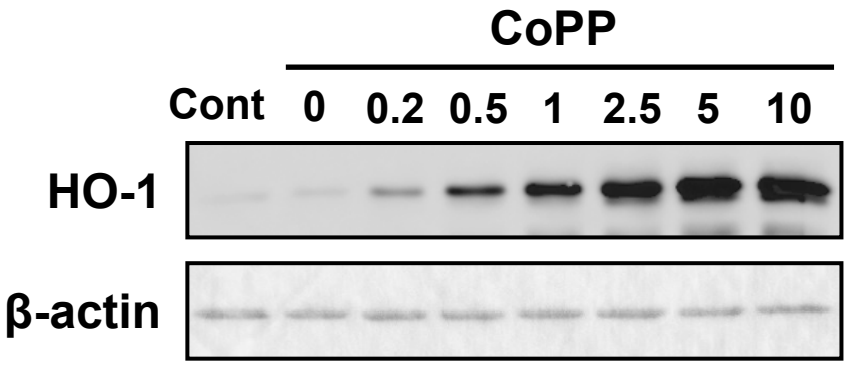
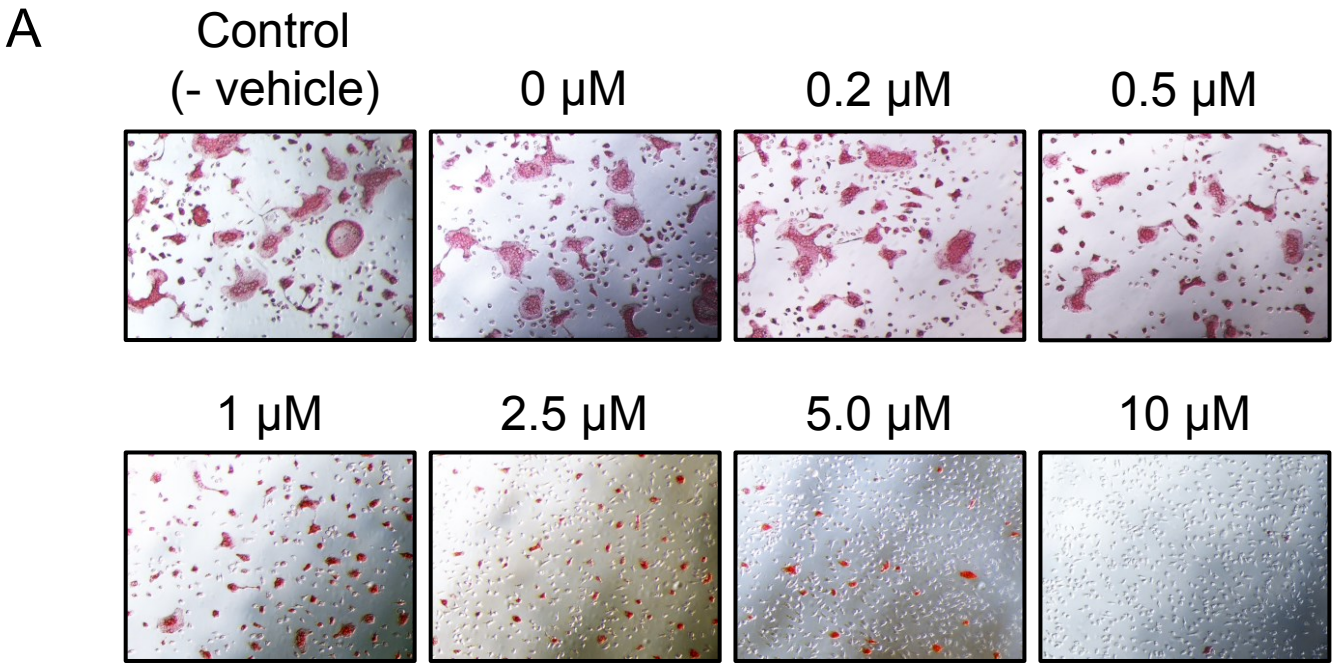
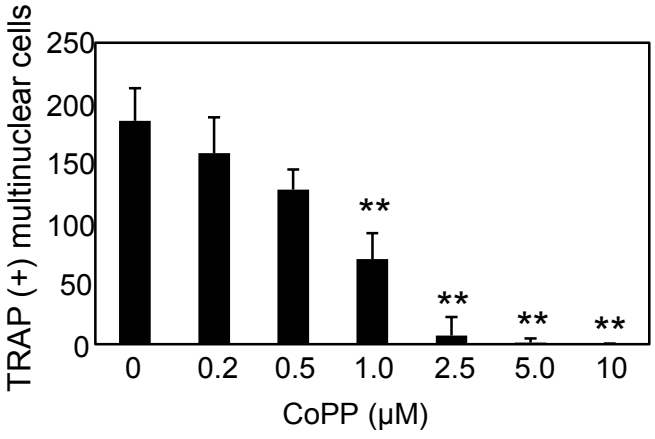


Figure 2


A
Control
(- vehicle)
0 μM
0.2 μM
0.5 μM
1 μM
2.5 μM
5.0 μM
10 μM


B
TRAP (+) multinuclear cells
250
200
150
100
50
0
**
**
**
**
0
0.2
0.5
1.0
2.5
5.0
10
CoPP (μM)

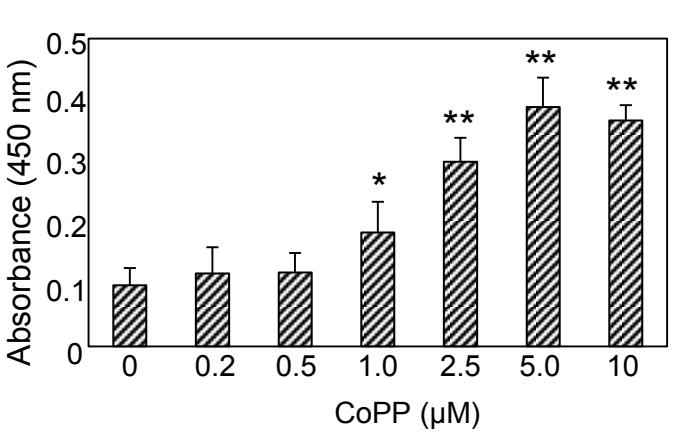
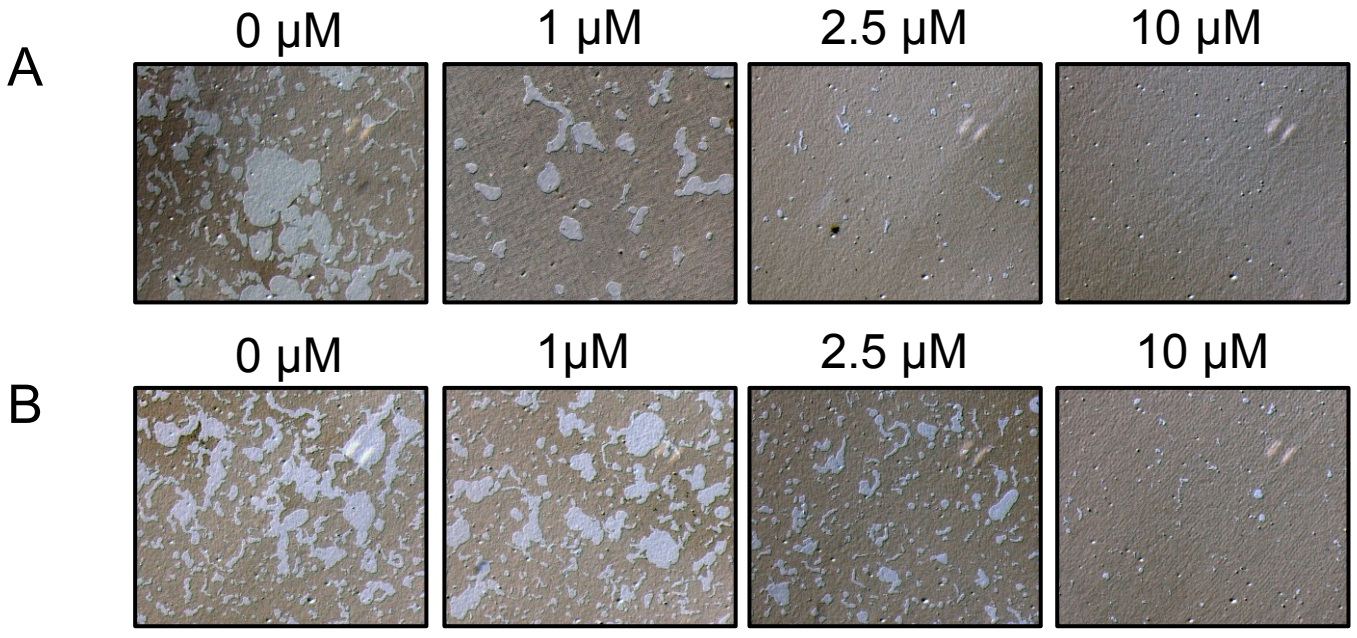
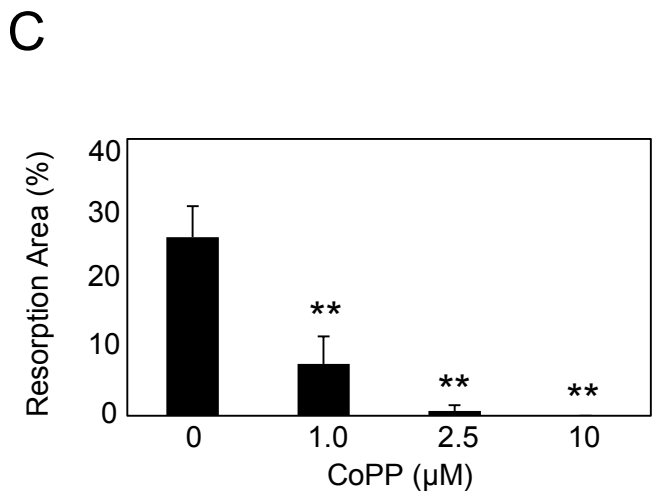

C
Absorbance (450 nm)
0.5
0.4
0.3
0.2
0.1
0
*
**
**
**
0
0.2
0.5
1.0
2.5
5.0
10
CoPP (μM)

Figure 3


A
0 μM
1 μM
2.5 μM
10 μM
B
0 μM
1μM
2.5 μM
10 μM


C
Resorption Area (%)
40
30
20
10
0
**
**
**
0
1.0
2.5
10
CoPP (μM)

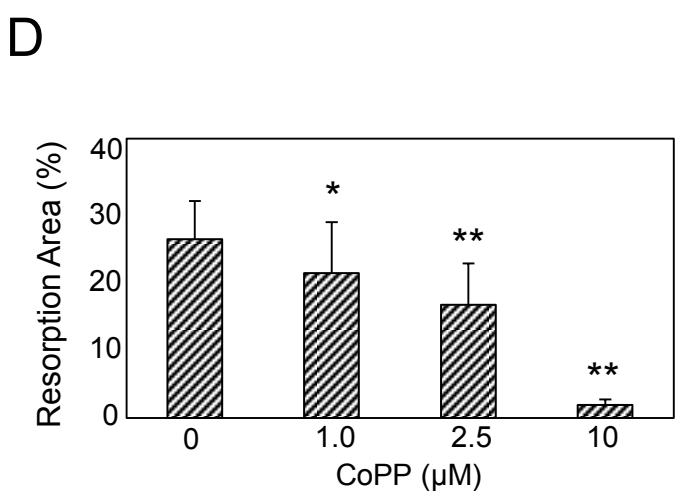

D
Resorption Area (%)
40
30
20
10
0
*
**
**
0
1.0
2.5
10
CoPP (μM)

Figure 4

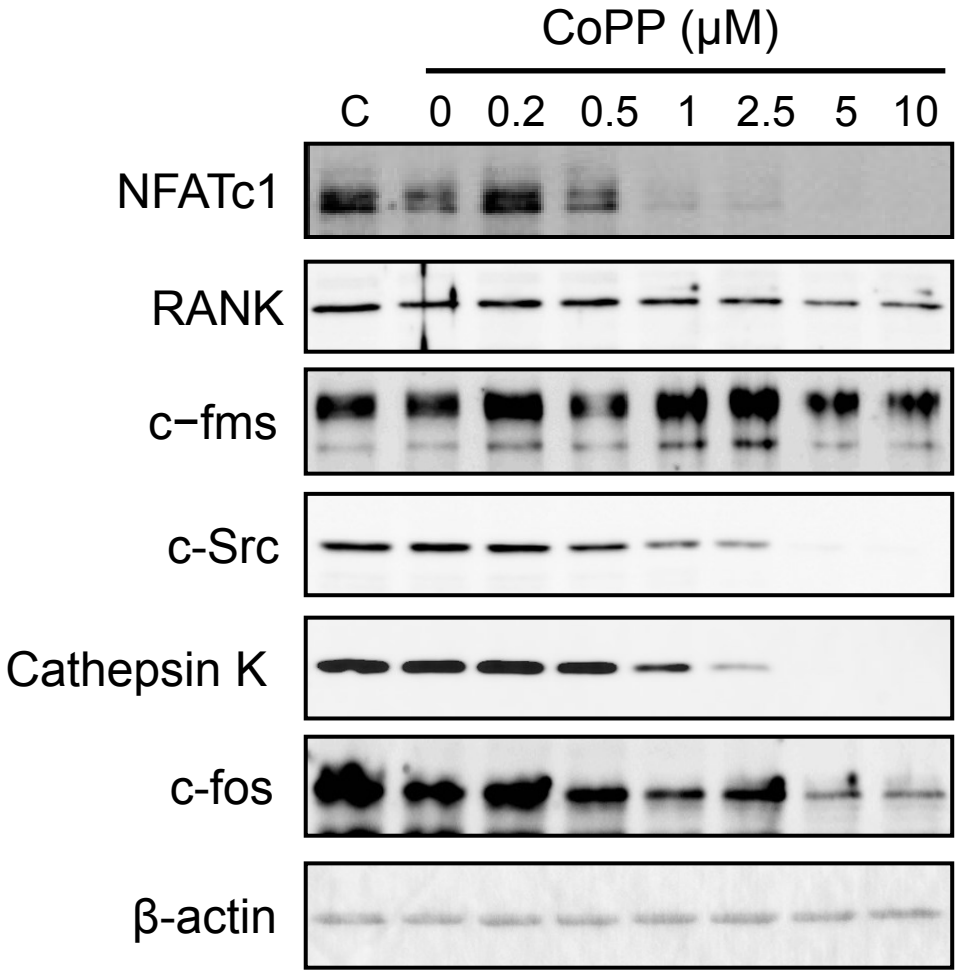

CoPP (μM)
C
0
0.2
0.5
1
2.5
5
10
NFATc1
RANK
c−fms
c-Src
Cathepsin K
c-fos
β-actin

Figure 5

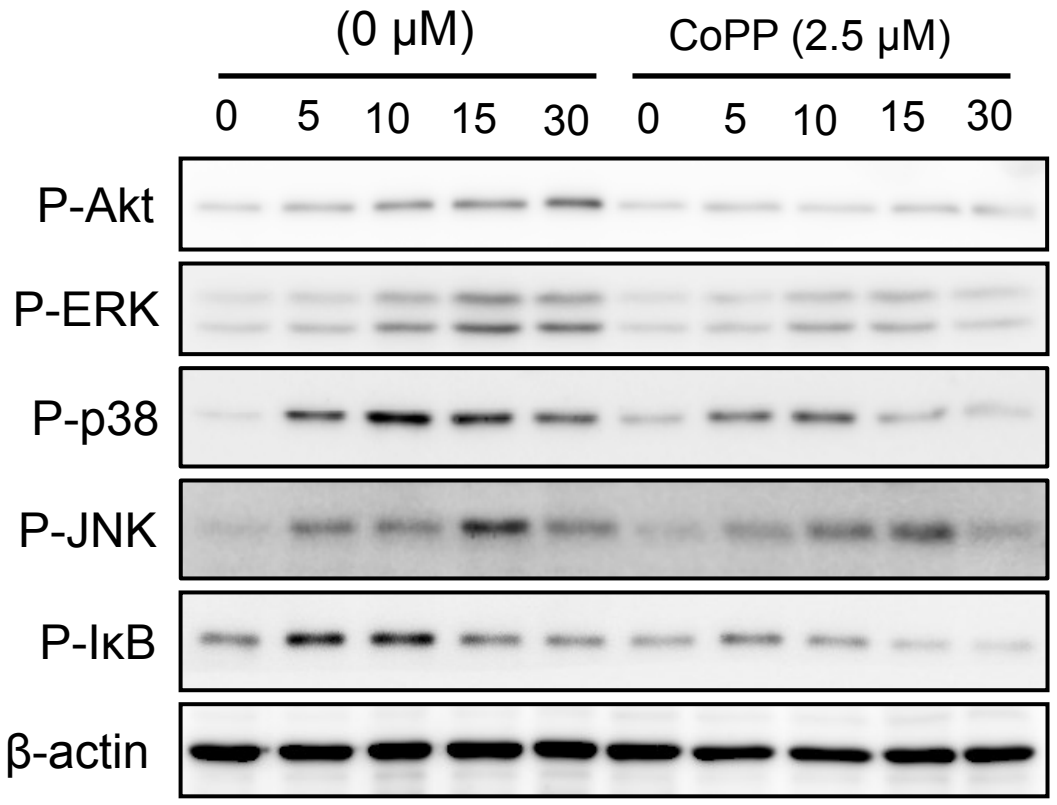

(0 μM)
CoPP (2.5 μM)
0
5
10
15
30
0
5
10
15
30
P-Akt
P-ERK
P-p38
P-JNK
P-IκB
β-actin

Figure 6