Published in final edited form as:

Ann N Y Acad Sci. 2011 December ; 1240(1): 18–25. doi:10.1111/j.1749-6632.2011.06286.x.

HDAC inhibitor, trichostatin A suppresses osteoclastogenesis by up-regulating the expression of C/EBP- β and MKP-1

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Abstract

Histone deacetylases (HDACs) remove the acetyl groups from the lysine residues of histone tails, leading to the formation of a condensed and transcriptionally silenced chromatin. HDAC inhibitors (HDACi) block this action and can result in hyperacetylation of histones, leading to a less compact and more transcriptionally active chromatin and thereby, gene expression. Previously, we have shown that HDACi inhibit osteoclast differentiation. However, which genes are transcriptionally activated following hyperacetylation of histones, and lead to the suppression of osteoclastogenesis, has yet to be elucidated. In this study, we show that a HDACi, trichostatin A (TSA), inhibits receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL) stimulated TNF- α production, NF- κ B activation and bone resorbing pit formation and down-regulates cFos and NFATc1 in RAW264.7 cells. Interestingly, anti-osteoclastogenic factors CCAAT enhancer binding protein (C/EBP)- β and mitogen activated protein kinase phosphatase (MKP)-1 expression were significantly up-regulated in TSA-treated RANKL-stimulated RAW264.7 cells. These findings suggest that TSA up-regulates the expression of C/EBP- β and MKP-1 which may down-regulate pro-osteoclastogenic factors and signaling molecules, ultimately suppressing osteoclastogenesis.

Introduction

Bone remodeling depends on a delicate balance between bone formation and bone resorption. Tipping this balance in favor of bone resorption (osteoclasts) leads to pathologic bone diseases like osteoporosis and affects 10 million Americans over the age of 50 and results in 1.5 million fractures annually [1]. Osteoclasts are multinucleated cells that differentiate from myeloid precursors in response to macrophage colony stimulating factor (M-CSF) and RANKL. NFATc1 is a target of RANKL signaling. During osteoclastogenesis, NFATc1 expression is induced and this transcription factor can be identified at the promoters of osteoclast specific genes [2]. It has been shown that NFATc1 is the key to RANKL-regulated osteoclast differentiation [3]. RANKL also induces c-Fos by an as yet unknown mechanism. Indeed, the essential role of c-Fos pathway in osteoclastogenesis, as determined by gene targeting studies, is well documented [4]. c-Fos has also been found to be induced by RANKL-RANK signals [4].

Histone acetylation, in concert with other histone modifications, has been described as a major epigenetic regulator for controlling cell fate. Histone acetyltransferases transfer acetyl groups to core histones, resulting in local expansion of chromatin and increased accessibility of DNA binding proteins, leading to transcriptional activation [5]. Histone deacetylases (HDACs) are known to counteract the activity of histone acetyltransferases, thus functioning

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as transcriptional repressors. However, genome-wide mapping of HATs and HDACs reveals that both HATs and HDACs are associated in active genes with acetylated histones [6]. HDACs and HATs have histones and many non-histone proteins as targets that have a role in regulating gene expression [7–9]. There are two major roles for HDACs. One is their function in active genes, where high levels of HDACs act to remove the acetyl group added by high levels of HATs during the process of transcriptional initiation and elongation and reset the chromatin structure required for the next round of transcription. The other is their function in primed genes, where transient binding of HDACs removes the acetyl group resulted from transient binding of HATs, maintains a low level of acetylation, and prevents Pol II binding, thereby maintaining promoters in an inactive state. In humans and mice, the 18 HDAC enzymes are grouped into four classes. Classical HDACs (class I, II and IV) share sequence similarity and are dependent on Zn^{2+} for enzymatic activity, whereas the class III sirtuins act through a distinct NAD^+ -dependent mechanism [10]. Class I HDACs (HDAC1, 2, 3 and 8) are generally localized to the nucleus and, with the exception of HDAC3, the absence of a nuclear export signal [10]. Class I HDACs have been most widely studied in their classical role as histone modifiers and transcriptional repressors. The class II enzymes have been subdivided into class IIa (HDAC4, 5, 7 and 9) and IIb (HDAC6 and 10) based on domain organization [10]. Class IIa HDACs possess N-terminal domains that interact with transcription factors. They also possess C-terminal nuclear export signals, which enable shuttling between the nucleus and cytoplasm. Nuclear export prevents class IIa HDACs from acting as transcriptional repressors, thus resulting in inducible gene expression. Class IIa HDACs primarily control gene expression by recruiting other proteins (corepressors or coactivators) [6, 10]. Class IIb HDACs (HDAC6 and HDAC10) are distinguished from the class IIa sub-family in possessing tandem deacetylase domains, although the second domain of HDAC10 is reported to be nonfunctional [6]. HDAC6 is unique amongst the classical HDAC family in that it is predominantly cytoplasmic, whereas HDAC10 is found in both the nucleus and cytoplasm [6]. HDAC11 is the sole class IV HDAC. HDACs which target histones as well as non-histone proteins as substrates have the potential to regulate gene expression. HDACi are known to modulate the expression of genes by increasing histone acetylation and thereby, regulating chromatin structure and transcription [11]. Current evidence indicates that HDACi act not only to block the catalytic activity of the enzyme but may also affect the protein-protein interaction of specific HDACs with various critical protein partners [8]. These target proteins are involved in many cell pathways including gene expression, cell proliferation, differentiation, cell migration and cell death and have a role in angiogenesis and immune response. HDACi are emerging as a new class of potential therapeutic agents for the treatment of solid and hematologic malignancies [12]. HDACi have also recently been identified as potential anti-arthritic agents. In animal models of rheumatoid arthritis (RA), HDACi inhibited joint swelling, synovial inflammation and subsequent bone and cartilage destruction [13]. HDACi include several structurally diverse natural products. Currently, there are several classes of HDACi, including butyrate, hydroxamic acid, benzamide, and cyclic peptides. A hydroxamic acid, TSA, is a classical HDAC inhibitor that blocks the activity of all isoforms with similar affinities, except class IIa HDACs, and was originally isolated as a fungistatic antibiotic from the *Streptomyces platensis* strain [14]. Anti-inflammatory properties of HDACi including TSA are well characterized [12]. Various HDACi are reported to enhance osteogenic differentiation in multiple cell types, including osteoblasts, bone marrow mesenchymal cells, and adipose derived stromal cells [15, 16]. Previously, we have shown that both sodium butyrate (NaB) and trichostatin A (TSA) are able to inhibit osteoclast differentiation in the monocyte macrophage cell line RAW264.7 and mouse and rat primary bone marrow cell cultures [17]. HDACi may inhibit osteoclastogenesis by altering the structure of many proteins targets of HDACs, i.e. histones and non-histone proteins. However, due to hyperacetylation of histone and non-histone proteins, transcriptional up-regulation of those genes responsible for the suppression of osteoclast differentiation has yet to be elucidated. In this study, we

determined that anti-osteoclastogenic factors, CCAAT enhancer binding protein (C/EBP)- β and mitogen activated protein kinase phosphatase (MKP)-1 are up-regulated by TSA in RANKL stimulated RAW264.7 cells.

Materials and Methods

Materials

All media components were purchased from GIBCO (Invitrogen Corp., Carlsbad, CA). TSA was purchased from Sigma (St. Louis, MO). Murine sRANKL was obtained from Pepro Tech, Inc. (Rocky Hill, NJ). The bicinchoninic acid kit for protein determination was from Pierce Chemical Co. (Rockford, IL). Rabbit polyclonal antibodies against NFATc1, p65 NF- κ B; MKP-1, C/EBP- β and goat polyclonal antibody against actin, Lamin B and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Chicken polyclonal antibody against c-Fos was purchased from Abcam, Inc. (Cambridge, MA).

Cell cultures

RAW264.7 cells were cultured in α -MEM medium (Sigma) with 10% fetal calf serum (FCS) (Sigma). All media were supplemented with 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin (Sigma).

Pit formation assay

RAW264.7 cells were suspended in α -MEM containing 10% FCS and plated at a concentration of 1×10^4 cells/well on an Osteoclast Activity Assay Substrate plate (OCT USA Inc.) in the presence or absence of 50 ng/ml RANKL and incubated for 24 hours. TSA (20 nM) was then added to the cultures. Half of the medium was replaced with a fresh media once every 2 days. After 7 days of culture, the plates were washed in 6% sodium hypochlorite solution in order to remove the cells. The resorbed areas on the plates were captured with a digital camera attached to a microscope and analyzed by the Metaview Image Analysis System [18]. Resorbed pit area was expressed as % resorption area.

TNF- α production in RAW264.7 cell culture

RAW264.7 cells were suspended in α -MEM containing 10% FCS and plated at a concentration of 1×10^5 cells/well into a 24-well culture dish (Corning, USA) for 24 hours. They were then treated with/without TSA (10 nM and 20 nM) for an additional 24 hours before adding RANKL (50 ng/ml) in order to stimulate TNF- α secretion for 16 hours. Culture medium was collected and analyzed for TNF- α using the ELISA kit [18].

Protein preparation and Western blot analysis

In order to determine the nuclear protein level of p65 NF- κ B, RAW264.7 cells were cultured in α -MEM with 10% FCS. After 24 hours of incubation, cells were treated with TSA (100 nM) for an additional 24 hours and then incubated with sRANKL (50 ng/ml) for several time points (0, 5, 10, 20 and 30 minutes). Cells were then collected and nuclear extracts were prepared, as previously described [17]. For determining the levels of all other factors, RAW264.7 cells were cultured in α -MEM with 10% FCS for 24 hours. sRANKL (50 ng/ml) and TSA (20 nM) were added and incubated for different time points as mentioned. Cells were collected and whole cell extracts and nuclear extracts were prepared as described earlier [18]. Protein concentrations of the nuclear extracts, cytosolic extracts, and whole cell extracts were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). 50 μ g of whole cell extract and 15 μ g of nuclear extract were subjected to Western blot analysis.

Statistical Analysis

Results are expressed as mean $\pm$ SEM. Data was statistically analyzed using either student's t test or one-way ANOVA and $P < 0.05$ was considered statistically significant. Unpaired t test and Newman–Keuls multiple comparison tests were used to test the differences between groups for significance using Graphpad prism for Windows (La Jolla, CA, USA).

Results

Effect of TSA on bone resorption by RAW264.7 cells stimulated with RANKL

Earlier, we reported that TSA inhibited osteoclastogenesis [17]. Here we examined whether TSA has any effect on bone resorbing pit formation in RANKL stimulated RAW264.7 cells cultured in calcium phosphate-coated tissue culture plates. Cultures treated with TSA showed significantly reduced numbers and areas of resorption pits, compared with cultures treated with RANKL alone (Figure 1A).

Effects of TSA on RANKL- stimulated TNF- α production in RAW264.7 cells

TNF- α is a strong pro-osteoclastogenic factor [19, 20]. Therefore, we examined whether TSA can modulate RANKL-stimulated TNF- α production in RAW264.7 cell cultures. Cultures treated with TSA exhibited dose dependent reduction of TNF- α production compared with cultures treated with RANKL alone (Figure 1B).

Effects of TSA on RANKL-stimulated NF- κ B activation in RAW264.7 cells

Previously we showed that TSA inhibits TNF- α induced activation of NF- κ B [17]. RANKL is the most pivotal factors mediating osteoclastogenesis. Therefore, we examined the effect of TSA on RANKL stimulated activation of NF- κ B by determining the nuclear levels of p65 NF- κ B subunit. Interestingly, dramatic reduction of nuclear p65 NF- κ B level was found in TSA treated RAW264.7 cells (Figure 2A). These data support our previous findings that TSA inhibits RANKL stimulated NF- κ B-dependent transactivation in RAW264 cells [17].

Effects of TSA on RANKL- stimulated osteoclast specific transcription factor NFATc1 and c-Fos expression in RAW264.7 cells

NFATc1 and c-Fos are considered to be the most important osteoclast-specific transcription factors and the master regulators of osteoclastogenesis [2, 21]. We examined whether TSA modulates these transcription factors in RANKL-stimulated RAW264.7 cells. RAW264.7 cells treated with TSA significantly decreased the RANKL-stimulated up-regulation of NFATc1 and c-Fos transcription factors (Figure 2B). However, RAW264.7 cell treated with only TSA did not have any effect on the expression of these transcription factors (data not shown).

Effects of TSA on RANKL stimulated C/EBP- β expression in RAW264.7 cells

Very recently, C/EBP- β has been reported to be involved in controlling osteoclastogenesis [22]. Therefore, we determined if TSA up-regulated the expression of C/EBP- β in RAW264.7 cells treated with RANKL. We first determined the levels of C/EBP- β during the course of osteoclast differentiation in RAW264.7 cells in the presence of RANKL. We found that expression of C/EBP- β decreased with time during the course of RANKL-induced osteoclast differentiation (Figure 3A). We determined the levels of C/EBP- β at 0 hour, 6 hour and 5 day time points. At 6 hour, usually there is no osteoclast formation occurred in RAW264.7 cells in the presence of RANKL, whereas at 5 day time point there are a number of osteoclasts formed in RAW264.7 cells treated with RANKL. Interestingly, we did not find any down-regulation of C/EBP- β in 6 hours RANKL treated culture, whereas dramatic inhibition of C/EBP- β expression was noted in 5 day RANKL treated

RAW264.7 cells. However, TSA inhibited this down-regulation of C/EBP- β expression in RAW264.7 cells treated with RANKL (Figure 3B).

Effects of TSA on RANKL stimulated MKP-1 expression in RAW264.7 cells

MKP-1 inactivates MAPKs, which are very important signaling molecules for osteoclastogenesis [23,24]. Therefore, we determined if TSA-induced hyper-acetylation of histone can up-regulate the expression of MKP-1 in RAW264.7 cells. Interestingly, TSA inhibits this down-regulation of MKP-1 expression in RAW264.7 cells treated with RANKL (Figure 4).

Discussion

Here we show that two anti-osteoclastogenic factors, C/EBP- β and MKP-1, were up-regulated in RANKL stimulated RAW264.7 cells in the presence of TSA. Thus HDAC inhibition may inhibit osteoclastogenesis by up-regulating the expression of C/EBP- β and MKP-1. In this study, we showed that TSA inhibited bone resorption in RAW264.7 cell culture. The reduction of bone resorbing pit formation might be the direct result of the reduced number of osteoclast formation. Previously, we showed that TSA inhibits TNF- α induced activation of NF- κ B [17]. Since RANKL is a member of the TNF- α super family and the most important osteoclastogenic factor, here we showed that TSA could also inhibit RANKL-induced activation of NF- κ B. These observations demonstrate that TSA can inhibit both RANKL dependent and independent activation of NF- κ B. Others have reported that TSA decreases the mRNA and protein levels of the pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β , and in contrast, increases the level of the anti-inflammatory cytokine IL-10 in models of autoimmune and inflammatory diseases [25]. Our observations, together with these data, demonstrate that TSA is fundamentally anti-inflammatory in nature.

Previously, we have shown that TSA inhibits osteoclastogenesis by inhibiting the activation of NF- κ B and p38 MAPK signaling. In this study, we found that TSA also reduced RANKL stimulated up-regulation of both c-Fos and NFATc1 expression, the most pivotal osteoclastogenic transcriptional factors, in RAW264.7 cells [21, 26]. In general, HDAC inhibition leads to transcriptional activation. Therefore, the down-regulation of c-Fos and NFATc1 may not be a direct effect of TSA, but rather an indirect effect of TSA, through the up-regulation of factors, that negatively regulate the transcriptional activation of c-Fos and NFATc1.

In searching for the anti-osteoclastogenic factors up-regulated in TSA treated RAW264.7 cells, we first examined the expression of C/EBP- β . Recently, the action of C/EBP- β in mesenchymal cell differentiation, including osteoblasts has been addressed in several studies [26–28]. The general function of C/EBP- β in bone homeostasis, including its potential role in osteoclasts, has also been reported. C/EBP- β deficiency enhances bone resorption and exhibits enhanced osteoclast markers [27]. Smink et al. reported that C/EBP- β controls osteoclastogenesis, through mTOR mediated isoforms, liver-enriched activating protein (LAP) which acts as transcriptional activators and liver-enriched inhibitory protein (LIP) which acts as a dominant negative inhibitor of LAP) and MafB, a negative regulator of osteoclastogenesis [22]. C/EBP- β mutant mouse strains exhibit increased bone resorption and attenuates expression of MafB [22]. These observations by others demonstrate that the effect of C/EBP- β on osteoclastogenesis may depend on the LAP and LIP isoform switching. On the contrary, it has been reported that C/EBP- β and NF- κ B cooperatively induce inflammatory gene products [29, 30]. This effect might also be isoform specific. In our study, we observed that the expression of C/EBP- β decreases with time during the course of RANKL-induced osteoclast differentiation, indicating that diminished expression

of C/EBP- β may be a pre-requisite for osteoclastogenesis. Sustained expression of C/EBP- β in RANKL stimulated RAW264.7 cells, in the presence of TSA, may be one of the mechanisms how TSA suppresses osteoclastogenesis. However, further studies are necessary to determine the effect of TSA on C/EBP- β isoform switching.

We then determined the effect of TSA on the expression of another anti-osteoclastogenic factor MKP-1 and found up-regulation of MKP-1 expression by TSA in RANKL treated RAW264.7 cells. MKP-1 dephosphorylates MAPKs [31]. The promoter of genes for MAPK phosphatase is expressed in macrophages and is activated by TSA [32]. Activation of MAPKs such as p38 and JNK are associated with differentiation and activation of osteoclasts [23, 24]. MKP-1 acts as a negative regulator of p38 and JNK. Macrophages from *Mkp-1* knockout mice exhibited prolonged activation of p38 and JNK [31]. *Mkp-1*^{-/-} macrophages produced more pro-inflammatory cytokines, such as TNF- α and IL-6, which are also known to be associated with osteoclastogenesis [31, 33, 34]. Inhibition of pro-inflammatory cytokine biosynthesis by MKP-1 in LPS-stimulated macrophages has also been reported [35, 36]. Furthermore, HDACi reduced inflammation and mortality in WT mice treated with LPS, but failed to protect MKP-1 knockout mice [37]. MKP-1 may limit the inflammatory reaction by inactivating JNK and p38, thus preventing osteoclast differentiation and activation caused by exaggerated inflammatory responses. Further, there is evidence that C/EBP- β plays an important role on transcriptional activation of MKP-1 [38].

In summary, TSA induce the expression of C/EBP- β and MKP-1, which may independently or synergistically suppress the osteoclast differentiation. The data in this study was obtained solely in the RAW cell line system. Further experiments using siRNA and chromatin immunoprecipitation (CHIP) technology using primary bone marrow cells and transgenic and knockout mice are necessary to confirm the activation of C/EBP- β and MKP-1 by HDACi. PCR array should be done to find out other anti-inflammatory genes up-regulated by HDAC inhibitors and possible correlation with osteoclast inhibition. Further studies are also necessary to determine how HDACi modulates the non-histone substrates and the relationship of these non-histone substrate modulations in osteoclastogenesis. HDACi are promising compounds to treat osteoporosis and other inflammatory bone diseases.

Acknowledgments

Supported by NIH-K01-AG034233-02. We acknowledge Dr. Anthony Valente for his kind review of our manuscript for scientific and grammatical corrections.

References

1. Aliprantis AO, et al. NFATc1 in mice represses osteoprotegerin during osteoclastogenesis and dissociates systemic osteopenia from inflammation in cherubism. *J Clin Invest*. 2008; 118:3775–3789. [PubMed: 18846253]
2. Takayanagi H, et al. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell*. 2002; 3:889–901. [PubMed: 12479813]
3. Ikeda F, et al. Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. *J Clin Invest*. 2004; 114:475–484. [PubMed: 15314684]
4. Grigoriadis AE, et al. c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science*. 1994; 266:443–448. [PubMed: 7939685]
5. Mottet D, Castronovo v. Histone deacetylases: target enzymes for cancer therapy. *Clin Exp Metastasis*. 2008; 25:183–189. [PubMed: 18058245]
6. Wang Z, et al. Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. *Cell*. 2009; 138:1019–1031. [PubMed: 19698979]

7. Shahbazian MD, Grunstein M. Functions of site-specific histone acetylation and deacetylation. *Annu Rev Biochem.* 2007; 76:75–100. [PubMed: 17362198]
8. Marks PA. Histone deacetylase inhibitors: a chemical genetics approach to understanding cellular functions. *Biochim Biophys Acta.* 1799:717–725. [PubMed: 20594930]
9. Spange S, et al. Acetylation of non-histone proteins modulates cellular signalling at multiple levels. *Int J Biochem Cell Biol.* 2009; 41:185–198. [PubMed: 18804549]
10. Shakespear MR, et al. Histone deacetylases as regulators of inflammation and immunity. *Trends Immunol.* 32:335–343. [PubMed: 21570914]
11. Richon VM, O'Brien JP. Histone deacetylase inhibitors: a new class of potential therapeutic agents for cancer treatment. *Clin Cancer Res.* 2002; 8:662–664. [PubMed: 11895892]
12. Dinarello CA. Anti-inflammatory Agents: Present and Future. *Cell.* 140:935–950. [PubMed: 20303881]
13. Chung YL, et al. A therapeutic strategy uses histone deacetylase inhibitors to modulate the expression of genes involved in the pathogenesis of rheumatoid arthritis. *Mol Ther.* 2003; 8:707–717. [PubMed: 14599803]
14. Yoshida M, et al. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. *J Biol Chem.* 1990; 265:17174–17179. [PubMed: 2211619]
15. Cho HH, et al. Induction of osteogenic differentiation of human mesenchymal stem cells by histone deacetylase inhibitors. *J Cell Biochem.* 2005; 96:533–542. [PubMed: 16088945]
16. Xu Y, et al. Inhibition of Histone Deacetylase Activity in Reduced Oxygen Environment Enhances the Osteogenesis of Adipose-derived Stromal Cells. *Tissue Eng Part A.* 2009
17. Rahman MM, et al. Two histone deacetylase inhibitors, trichostatin A and sodium butyrate, suppress differentiation into osteoclasts but not into macrophages. *Blood.* 2003; 101:3451–3459. [PubMed: 12511413]
18. Rahman MM, Bhattacharya A, Fernandes G. Conjugated linoleic acid inhibits osteoclast differentiation of RAW264.7 cells by modulating RANKL signaling. *J Lipid Res.* 2006; 47:1739–1748. [PubMed: 16702601]
19. Kobayashi K, et al. Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. *J Exp Med.* 2000; 191:275–286. [PubMed: 10637272]
20. Lam J, et al. TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *J Clin Invest.* 2000; 106:1481–1488. [PubMed: 11120755]
21. Wang ZQ, et al. Bone and haematopoietic defects in mice lacking c-fos. *Nature.* 1992; 360:741–745. [PubMed: 1465144]
22. Smink JJ, et al. Transcription factor C/EBPbeta isoform ratio regulates osteoclastogenesis through MafB. *EMBO J.* 2009; 28:1769–1781. [PubMed: 19440205]
23. Srivastava S, et al. Estrogen decreases osteoclast formation by down-regulating receptor activator of NF-kappa B ligand (RANKL)-induced JNK activation. *J Biol Chem.* 2001; 276:8836–8840. [PubMed: 11121427]
24. Matsumoto M, et al. Involvement of p38 mitogen-activated protein kinase signaling pathway in osteoclastogenesis mediated by receptor activator of NF-kappa B ligand (RANKL). *J Biol Chem.* 2000; 275:31155–31161. [PubMed: 10859303]
25. Han SB, Lee JK. Anti-inflammatory effect of Trichostatin-A on murine bone marrow-derived macrophages. *Arch Pharm Res.* 2009; 32:613–624. [PubMed: 19407980]
26. Zanotti S, et al. Misexpression of CCAAT/enhancer binding protein beta causes osteopenia. *J Endocrinol.* 2009; 201:263–274. [PubMed: 19218285]
27. Motyl KJ, et al. CCAAT/enhancer binding protein {beta}-deficiency enhances type 1 diabetic bone phenotype by increasing marrow adiposity and bone resorption. *Am J Physiol Regul Integr Comp Physiol.* 300:R1250–R1260. [PubMed: 21346244]
28. Tominaga H, et al. CCAAT/enhancer-binding protein beta promotes osteoblast differentiation by enhancing Runx2 activity with ATF4. *Mol Biol Cell.* 2008; 19:5373–5386. [PubMed: 18843047]

29. Ray A, Hannink M, Ray BK. Concerted participation of NF-kappa B and C/EBP heteromer in lipopolysaccharide induction of serum amyloid A gene expression in liver. *J Biol Chem.* 1995; 270:7365–7374. [PubMed: 7706280]
30. Liu S, et al. Lingual antimicrobial peptide and IL-8 expression are oppositely regulated by the antagonistic effects of NF-kappaB p65 and C/EBPbeta in mammary epithelial cells. *Mol Immunol.* 48:895–908. [PubMed: 21255844]
31. Wang X, et al. Knockout of Mkp-1 enhances the host inflammatory responses to gram-positive bacteria. *J Immunol.* 2007; 178:5312–5320. [PubMed: 17404316]
32. Musikacharoen T, Yoshikai Y, Matsuguchi T. Histone acetylation and activation of cAMP-response element-binding protein regulate transcriptional activation of MKP-M in lipopolysaccharide-stimulated macrophages. *J Biol Chem.* 2003; 278:9167–9175. [PubMed: 12511574]
33. Rahman M, et al. Conjugated linoleic acid (CLA) prevents age-associated skeletal muscle loss. *Biochem Biophys Res Commun.* 2009; 383:513–518. [PubMed: 19393220]
34. Rahman MM, et al. Endogenous n-3 fatty acids protect ovariectomy induced bone loss by attenuating osteoclastogenesis. *J Cell Mol Med.* 2009
35. Chen P, et al. Restraint of proinflammatory cytokine biosynthesis by mitogen-activated protein kinase phosphatase-1 in lipopolysaccharide-stimulated macrophages. *J Immunol.* 2002; 169:6408–6416. [PubMed: 12444149]
36. Zhao Q, et al. The role of mitogen-activated protein kinase phosphatase-1 in the response of alveolar macrophages to lipopolysaccharide: attenuation of proinflammatory cytokine biosynthesis via feedback control of p38. *J Biol Chem.* 2005; 280:8101–8108. [PubMed: 15590669]
37. Cao W, et al. Acetylation of mitogen-activated protein kinase phosphatase-1 inhibits Toll-like receptor signaling. *J Exp Med.* 2008; 205:1491–1503. [PubMed: 18504304]
38. Johansson-Haque K, Palanichamy E, Okret S. Stimulation of MAPK-phosphatase 1 gene expression by glucocorticoids occurs through a tethering mechanism involving C/EBP. *J Mol Endocrinol.* 2008; 41:239–249. [PubMed: 18682532]

Figure 1.

TSA inhibited RANKL (RL)-stimulated bone resorption and TNF- α production of RAW264.7 cells. (A) Demonstration of pit formation on an Osteoclast Activity Assay Substrate plate using RAW264.7 cells stimulated with 50 ng/ml RANKL with or without 20 nM TSA. TSA was added after 24 h of incubation of RAW264.7 cells with RANKL. After 7 days of culture, plates were analyzed for resorption pit formation. Bar diagram shows the percentage resorption area per well measured by image analyzer. Values shown are means $\pm$ SEM of three independent experiments with triplicate cultures. Statistical differences were evaluated between the groups with Students t test. * Significantly different at $p < 0.001$. (B) Dose dependent inhibition of RANKL (RL)-induced TNF- α production of RAW264.7 cells by TSA. RAW264.7 cells were cultured for 24 h. Then they were treated with/without TSA for another 24h before adding RANKL (50 ng/ml) to stimulate TNF- α secretion for 16 h. Medium was collected and analyzed for TNF- α using ELISA kit. Each value represents the mean $\pm$ SEM of three independent quadruplicate cultures. Statistical differences were evaluated among all groups with one-way ANOVA. Value with different superscripts are significantly different at $p < 0.05$ by Newman-Keuls' one way ANOVA with multiple comparison test.

Figure 2.

TSA reduced the expression of p65 NF- κ B and NFATc1 and c-Fos in RAW264.7 cells stimulated with RANKL. (A) RAW264.7 cells were treated without or with TSA (100 nM) for 24 hours, followed by treatment with RANKL for 5, 10, 20 and 30 minutes. Nuclear extracts were prepared and 15 μ g of proteins were analyzed by Western blotting using p65 NF- κ B antibody. (B) RAW264.7 cells were cultured for 24 hours and then RANKL (50 ng/ml) and TSA were added and cultured for another 48 hours. Whole cell lysates were prepared, and 50 μ g of proteins were analyzed by Western blotting using antibodies to nuclear factor of activated T-cells (NFATc1) and c-Fos. Lower panel shows the loading control (actin) for whole cell lysates. The intensity of the bands was determined by densitometry. Values shown are means $\pm$ SEM of two independent experiments. Statistical differences were evaluated among all groups with one-way ANOVA. Value with different superscripts are significantly different at $p < 0.05$ by Newman-Keuls' one way ANOVA with multiple comparison test.

Figure 3.

TSA up-regulated the expression of C/EBP- β in RANKL (RL)-stimulated RAW264.7 cells. **A.** RAW264.7 cells were cultured in the presence of RANKL (50 ng/ml) for 5 days. Nuclear extracts were prepared at indicated time points. **B.** RAW264.7 cells were cultured for 24 hours and then RANKL (50 ng/ml) and TSA were added and cultured for 24 and 48 hours. Nuclear extracts were prepared, and equal amount of proteins (15 μ g) were analyzed by Western blotting using antibodies to C/EBP- β . Lower panel shows the loading control (Lamin B) for nuclear extracts. The intensity of the bands was determined by densitometry. Values shown are means $\pm$ SEM of two independent experiments. Statistical differences were evaluated among all groups with one-way ANOVA. Value with different superscripts

are significantly different at $p < 0.05$ by Newman-Keuls' one way ANOVA with multiple comparison test.

Figure 4.

TSA up-regulated the expression of MKP-1 in RANKL (RL)-stimulated RAW264.7 cells. RAW264.7 cells were cultured for 24 hours and then RANKL (50 ng/ml) and TSA were added and cultured for 24 and 48 hours. Whole cell lysates were prepared, and equal amount of proteins (50 μ g) were analyzed by Western blotting using antibodies to MKP-1. The intensity of the bands was determined by densitometry. Values shown are means $\pm$ SEM of two independent experiments. Statistical differences were evaluated among all groups with one-way ANOVA. Value with different superscripts are significantly different at $p < 0.05$ by Newman-Keuls' one way ANOVA with multiple comparison test.