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Molecules and Cells

Silibinin Inhibits Osteoclast Differentiation Mediated by TNF Family Members

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Silibinin is a polyphenolic flavonoid compound isolated from milk thistle (*Silybum marianum*), with known hepatoprotective, anticarcinogenic, and antioxidant effects. Herein, we show that silibinin inhibits receptor activator of NF-κB ligand (RANKL)-induced osteoclastogenesis from RAW264.7 cells as well as from bone marrow-derived monocyte/macrophage cells in a dose-dependent manner. Silibinin has no effect on the expression of RANKL or the soluble RANKL decoy receptor osteoprotegerin (OPG) in osteoblasts. However, we demonstrate that silibinin can block the activation of NF-κB, c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein (MAP) kinase, and extracellular signal-regulated kinase (ERK) in osteoclast precursors in response to RANKL. Furthermore, silibinin attenuates the induction of nuclear factor of activated T cells (NFAT) c1 and osteoclast-associated receptor (OSCAR) expression during RANKL-induced osteoclastogenesis. We demonstrate that silibinin can inhibit TNF-α-induced osteoclastogenesis as well as the expression of NFATc1 and OSCAR. Taken together, our results indicate that silibinin has the potential to inhibit osteoclast formation by attenuating the downstream signaling cascades associated with RANKL and TNF-α.

INTRODUCTION

Bone is continuously remodeled by osteoclasts and osteoblasts, and their balanced activity is important for maintaining bone density. Osteoclasts serve a crucial function in bone, possessing the unique ability to resorb bone matrix. Osteoblasts support osteoclast formation from monocyte precursors of hematopoietic origin in response to osteotropic factors such as parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (Suda et al., 1999). These osteotropic factors induce osteoclast differentiation by up-regulating the expression of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL), while concomitantly reducing the levels of the soluble RANKL decoy receptor osteoprotegerin (OPG) in osteoblasts (Rho et al., 2004; Suda et al., 1999; Walsh et al., 2006).

RANKL, a TNF family member, supports osteoclast differentiation, survival, and activation. Binding of RANKL to its receptor, receptor activator of nuclear factor κB (RANK), initiates signals mediated by tumor necrosis factor receptor-associated factor (TRAF) adaptors and activates NF-κB, JNK, p38 MAP kinase, ERK, and Akt (Boyle et al., 2003; Kwak et al., 2008; Lee and Kim, 2003). RANKL induces activation of transcription factors including Mitf, c-Fos, and NFATc1. In particular, NFATc1 is thought to be a key regulator of RANKL-induced osteoclastogenesis, because ectopic expression of NFATc1 causes precursor cells to efficiently differentiate into osteoclasts in the absence of RANKL, whereas NFATc1-deficient embryonic stem cells fail to differentiate into osteoclasts in response to RANKL. Costimulatory signals mediated by immunoreceptor tyrosine-based activation motifs (ITAMs) are necessary for RANKL-induced osteoclastogenesis. Costimulatory receptors, such as triggering receptor expressed in myeloid cells 2 (TREM2), signal-regulatory protein β1 (SIRPβ1), paired Ig-like receptor A (PIR-A), and osteoclast-associated receptor (OSCAR), interact with ITAMs such as DAP12 or FcRγ. The activation of ITAMs through costimulatory receptors enhances calcium signaling and subsequent RANKL-induced osteoclastogenesis.

Silibinin is a major bioactive flavonone present in milk thistle (*Silybum marianum*) plant, which has been used as a traditional medicine for treating hepatitis and cirrhosis and for protecting liver from toxic substances. Besides its hepatoprotective role, silibinin is a potent chemopreventive agent against skin cancer in mouse models (Singh and Agarwal, 2002) and human prostate cancer xonograft growth in a nude mouse model (Singh et al., 2002). Furthermore, silibinin can induce growth arrest in many human epithelial cancer cell types including breast, colon, and skin (Singh and Agarwal, 2005). Although a number of studies have established the various role of silibinin in both *in vitro* and *in vivo* models, the effect of silibinin on osteoclast differentiation and function has yet to be revealed.

We demonstrate that silibinin attenuates RANKL-induced osteoclast differentiation, but that silibinin has no effect on osteoclast function such as bone resorption and osteoclast survival. Silibinin blocks RANKL-induced early signaling pathways, including JNK, p38, ERK and NF-κB, as well as induction of NFATc1 and OSCAR. In addition, silibinin inhibits TNF-α-induced osteoclastogenesis by attenuating its downstream signaling cascades. Thus, this study shows that silibinin has the

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potential therapeutic application for the treatment of bone diseases such as osteoporosis and rheumatoid arthritis.

MATERIALS AND METHODS

Reagents

All cell culture media and supplements were obtained from HyClone (USA). Soluble recombinant mouse RANKL and human M-CSF were purchased from PetroTech (USA). $1,25(OH)_2D_3$, PGE_2, and silibinin were purchased from SigmaAldrich (USA). TGF-β and TNF-α were obtained from R&D Systems (USA). Antibodies specific for phospho-p38, p38, phospho-ERK, ERK, phospho-Akt, Akt, IκB, and JNK were purchased from Cell Signaling Technology (USA); phospho-JNK and NFATc1 were purchased from BD Biosciences (USA); actin was purchased from Sigma-Aldrich. Polyclonal antibodies specific for OSCAR were prepared as previously described (Kim et al., 2002).

Osteoclast formation

Murine osteoclasts were prepared from bone marrow cells as previously described (Kim et al., 2007; Lee et al., 2006). In brief, bone marrow cells were obtained by flushing femurs and tibiae from 6-8-wk-old ICR mice. Bone marrow cells were cultured in α-MEM containing 10% FBS with M-CSF (5 ng/ml) for 16 h. Nonadherent cells were harvested and cultured with M-CSF (30 ng/ml). After 3 days in culture, floating cells were removed and adherent cells (bone marrow-derived macrophages [BMMs]) were used as osteoclast precursors. To generate osteoclasts, BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 3 d in the presence of various concentrations of silibinin. To generate osteoclasts by means of TNF-α-stimulation, bone marrow cells were cultured for 3 d in the presence of M-CSF (30 ng/ml) and TGF-β (1 ng/ml). BMMs were further cultured with M-CSF (30 ng/ml) and TNF-α (10 ng/ml) for 3 d in the presence of various concentrations of silibinin. To generate osteoclasts from coculture with osteoblasts and bone marrow cells, primary osteoblasts were prepared from calvariae of newborn mice as previously described (Suda et al., 1997). Bone marrow cells and primary osteoblasts were cocultured for 6 d in the presence of $1,25(OH)_2D_3$ (2×10^{-8} M). To generate osteoclasts from the murine myeloid RAW264.7 cell line, cells were cultured with RANKL (100 ng/ml) for 4 d. Cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) as previously described (Suda et al., 1997).

Survival assay

Bone marrow cells and osteoblasts were co-cultured in α-MEM containing 10% FBS with $1,25(OH)_2D_3$ (1×10^{-8} M) and PGE_2 (1×10^{-6} M) in 100-mm-diameter dishes precoated with type I collagen gel (cell matrix type-IA; Nitta Gelatin Inc., Japan) (Suda et al., 1997). Osteoclasts were formed within 6 days in co-culture, and all cells were removed from the dishes by treatment with 0.1% collagenase (Wako Pure Chemical Industries Ltd., Japan). The crude osteoclast preparation was replated in 48-well culture plates. After 6-8 h in culture, osteoblasts were removed by treatment with 0.05% trypsin and EDTA for 5 min (Suda et al., 1997). The purified osteoclasts were cultured for an additional 24 h in the presence of RANKL (100 ng/ml) or vehicle (control) with varying concentrations of silibinin. Cultured cells were fixed and stained for TRAP and $TRAP^+$ MNCs containing more than three nuclei were counted as viable osteoclasts.

Pit formation assay

Osteoclasts were prepared from co-culture as described above. Crude osteoclasts were placed on dentine slices in 48-well culture plates and cultured for 2 d in the presence of RANKL (300 ng/ml) and varying concentrations of silibinin. After wiping the cells off dentine slices with cotton, the slices were immersed in Mayer's hematoxylin (Sigma-Aldrich) to stain for the resorption pits formed by osteoclasts.

Fusion assay

Fusion assays were performed as previously described (Kim et al., 2008). In brief, BMMs were cultured for 48 h with M-CSF (30 ng/ml) and RANKL (100 ng/ml) to generate pre-osteoclasts. Pre-osteoclasts were cultured for an additional 24 h with M-CSF (30 ng/ml) and varying concentrations of silibinin in the absence (Vehicle) or presence of RANKL (150 ng/ml). Cultured cells were subsequently fixed and stained for TRAP.

Western blot analysis

For immunoblotting analysis, cells were harvested after washing with ice-cold PBS and then lysed in extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.01% protease inhibitor cocktail). Cell lysates were subjected to SDS-PAGE and Western blotting. Signals were detected and analyzed by LAS3000 luminescent image analyzer (Fuji photo film Co., Japan).

Semiquantitative RT-PCR

RT-PCR analysis was performed as previously described (Kim et al., 2005b; 2008). The following primers were used: RANKL-sense, 5′-CCT GAG ACT CCA TGA AAC GC-3′; RANKL-antisense, 5′-TAA CCC TTA GTT TTC CGT TGC-3′; OPG-sense, 5′-CAG TGA TGA GTG TGT GTA TTG CAG-3′; OPG-antisense, 5′-TTA TAC AGG GTG CTT TCG ATG AAG-3′; RANK-sense, 5′-TAC TAC AGG AAG GGA GGG AAA G-3′; RANK-antisense, 5′-CCT GCT GGA TTA GGA GCA GTG-3′; c-fms-sense, 5′-AGT GTG GGT AAC AGC TCT CAG TAC-3′; c-fms-antisense, 5′-TCC TAG AGT CTT ACC AAA CTG CAG-3′; NFATc1-sense, 5′-CTC GAA AGA CAG CAC TGG AGC AT-3′; NFATc1-antisense, 5′-CGG CTG CCT TCC GTC TCA TAG-3′; OSCAR-sense, 5′-CTG CTG GTA ACG GAT CAG CTC CCC AGA-3′; OSCAR-antisense, 5′-CCA AGG AGC CAG AAC CTT CGA AAC T-3′; TRAP-sense, 5′-CAG TTG GCA GCA GCC AAG GAG GAC-3′; TRAP-antisense, 5′-GTC CCT CAG GAG TCT AGG TAT CAC-3′; HPRT-sense, 5′-GTA ATG ATC AGT CAA CGG GGG AC-3′; HPRT-antisense, 5′-CCA GCA AGC TTG CAA CCT TAA CCA-3′.

RESULTS

Silibinin inhibits osteoclast differentiation, but not osteoclast function

To examine the effects of silibinin on osteoclasts, varying concentrations of silibinin were added to bone marrow-derived macrophage cells (BMMs) cultures in the presence of M-CSF and RANKL. Consistent with previous results (Hsu et al., 1999; Lacey et al., 1998; Suda et al., 1999; Yasuda et al., 1998), RANKL induced formation of TRAP-positive multinuclear osteoclasts ($TRAP^+$ MNCs) (Fig. 1A). Silibinin inhibited RANKL-induced osteoclastogenesis in a dose-dependent manner. Moreover, when increasing concentrations of silibinin were added to osteoblasts co-cultured with bone marrow cells in the presence of $1,25(OH)_2D_3$, addition of silibinin resulted in a dramatic reduction of $TRAP^+$ MNCs. Of note, higher concentration of silibinin was required to achieve similar inhibitory effects in osteoclasts than that in BMM culture (Fig. 1B). It has been shown that a monocyte/macrophage cell line RAW264.7 can

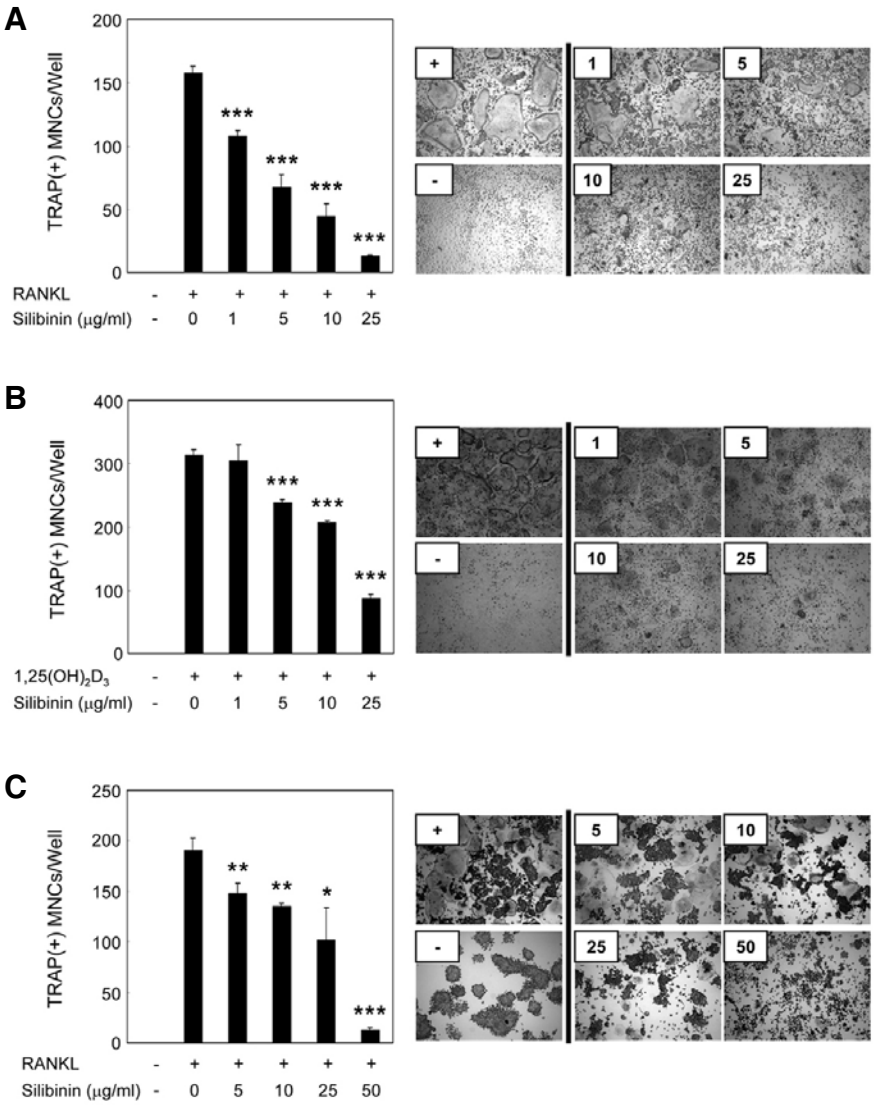

Fig. 1. The effects of silibinin on osteoclast differentiation. (A) BMMs were derived from bone marrow cells cultured for 3 d in the presence of M-CSF. BMMs were cultured for an additional 3 days with M-CSF in the absence (-) or presence (+) of RANKL with increasing concentrations (1-25 μg/ml) of silibinin as indicated. (B) Primary calvarial osteoblasts and bone marrow cells were co-cultured for 6 d in the absence (-) or presence (+) of $1,25(OH)_2D_3$ with increasing concentrations (1-25 μg/ml) of silibinin as indicated. (C) RAW264.7 cells were cultured for 4 d in the absence (-) or presence (+) of RANKL with increasing concentrations (1-50 μg/ml) of silibinin as indicated. (A-C) Cultured cells were fixed and stained for TRAP (right panel). Numbers of TRAP-positive multinucleated cells were counted (left panel). Data represent means ± SDs of triplicate samples. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. positive control. The results are representative of at least three independent sets of similar experiments.

become $TRAP^+$ MNCs upon stimulation with RANKL (Hsu et al., 1999), and we found that addition of silibinin to this culture system inhibited the formation of $TRAP^+$ MNCs in a dose-dependent manner (Fig. 1C). Together, these results indicate that silibinin can directly inhibit osteoclast differentiation.

To investigate whether silibinin can affect osteoclast function, we performed both survival and pit formation assays with purified mature osteoclasts in the presence or absence of silibinin. Consistent with published results (Suda et al., 1999), RANKL supported the survival of mature osteoclasts whereas medium alone could not (Fig. 2A). Silibinin treatment showed no effects on survival in the presence of RANKL and no detectable effect on RANKL-induced pit formation (Fig. 2B). However, when increasing concentrations of silibinin were added at time 0 to BMMs cultured on dentine slices in the presence of RANKL, silibinin inhibited pit formation in a dose-dependent manner (Fig. 2C), which is presumably due to silibinin's inhibitory effect on osteoclastogenesis as opposed to function. To examine whether silibinin plays a role in the fusion of $TRAP^+$ mononuclear pre-osteoclasts, we performed fusion assays using isolated $TRAP^+$ mononuclear preosteoclasts. Consistent with previous results (Kim et al., 2008; Suda et al., 1999), RANKL-induced the fusion of $TRAP^+$ mononuclear pre-osteoclasts to form $TRAP^+$ MNCs (Fig. 2D). The number of $TRAP^+$ MNCs by fusion was not reduced by the addition of silibinin at concentrations less than or equal to 10 μg/ml, however a silibinin-mediated inhibitory effect was observed when higher concentration of silibinin (25 μg/ml) were used. Taken together, these results indicate that silibinin can have a direct effect on osteoclast differentiation, but has no apparent effect on osteoclast function.

Silibinin affects early RANKL signaling pathways

To investigate the potential mechanism of silibinin's inhibitory effect on osteoclast differentiation, we examined whether silibinin affects RANKL signaling. When RAW264.7 cells are treated with RANKL, RANKL induces the degradation of IκB within 10 min, whereas pretreatment with silibinin attenuated IκB degradation in the presence of RANKL (Fig. 3A). Moreover, the RANKL-mediated phosphorylation of JNK, p38, and ERK was also inhibited by treatment with silibinin. However, silibinin did not inhibit RANKL-mediated phosphorylation of Akt which may be the underlying reason why no effect on sibilin on osteoclast survival was observed in other assays (Fig. 3A). Similar to

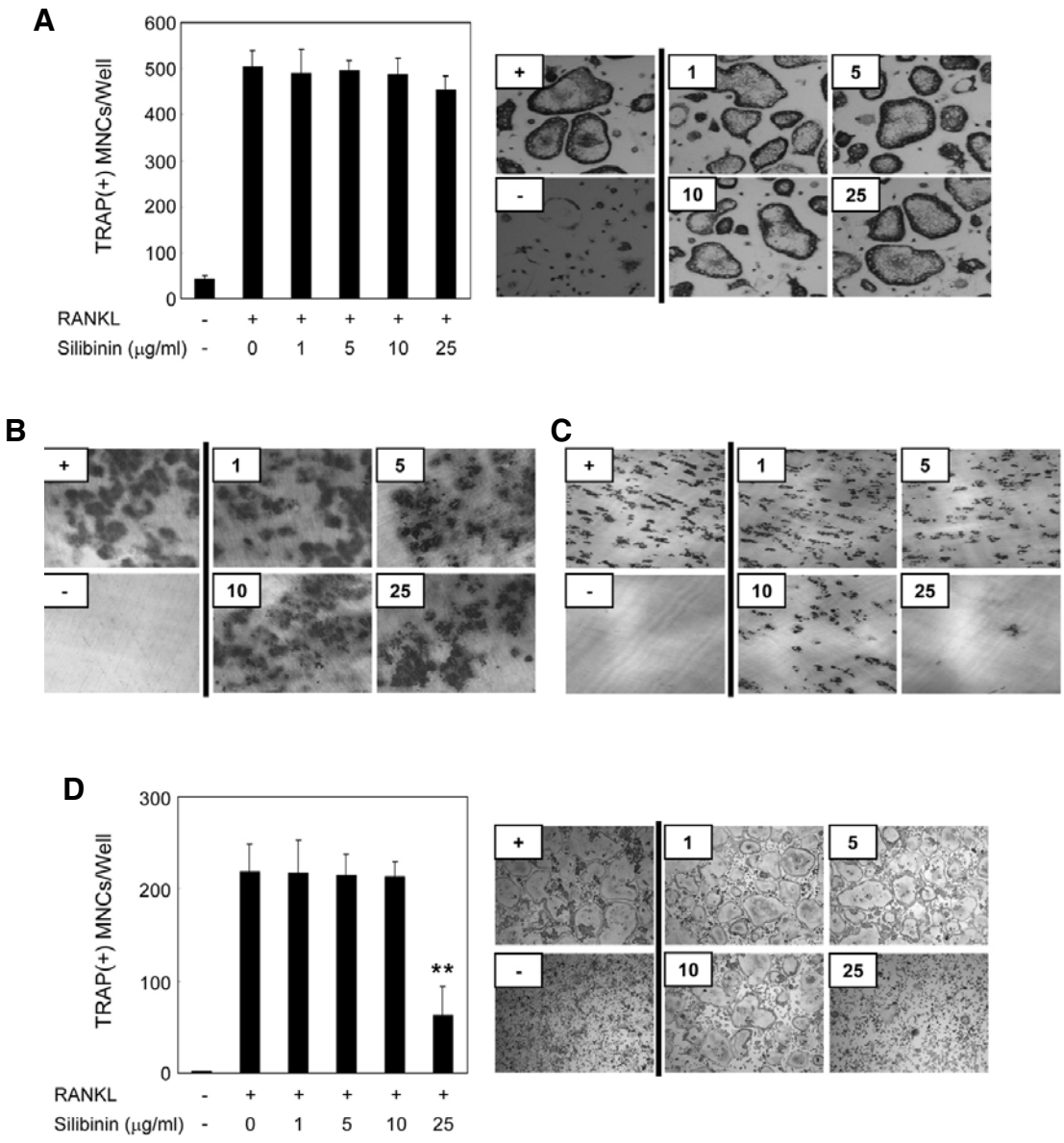

Fig. 2. The effects of silibinin on osteoclast function. (A) Primary calvarial osteoblasts and bone marrow cells were co-cultured for 6 d with $1,25(OH)_2D_3$ and PGE_2. Mature osteoclasts were purified from the cultures as described in "Materials and Methods". The isolated mature osteoclasts were cultured for an additional 24 h in the absence (-) or presence (+) of RANKL with increasing concentrations (1-25 μg/ml) of silibinin as indicated. Cultured cells were fixed and stained for TRAP (right panel). Numbers of TRAP-positive multinucleated cells were counted (left panel). (B) Mature osteoclasts were isolated from co-culture as described above. Mature osteoclasts were placed on dentine slices and cultured for 2 d in the absence (-) or presence (+) of with increasing concentrations (1-25 μg/ml) of silibinin as indicated. The slices were immersed in Mayer's hematoxylin to stain the resorption pits formed by osteoclasts. (C) BMMs were placed on dentine slices and cultured for 5 d with M-CSF in the absence (-) or presence (+) of RANKL with increasing concentrations (1-25 μg/ml) of silibinin as indicated. Pit formation on dentine slices was detected by staining with Mayer's hematoxylin. (D) To generate pre-osteoclasts, BMMs were cultured for 48 h with M-CSF and RANKL. Pre-osteoclasts were incubated for an additional 24 h in the absence (-) or presence (+) of RANKL with increasing concentrations (1-25 μg/ml) of silibinin as indicated. Cultured cells were fixed and stained for TRAP (right panel). Numbers of TRAP-positive multinucleated cells were counted (left panel). Data represent means ± SDs of triplicate samples. $^{**}P < 0.01$ vs. positive control. The results are representative of at least two independent sets of similar experiments.

our results in RAW264.7 cells, we found that silibinin inhibited the RANKL-mediated activation of NF-κB, JNK, p38, and ERK, whereas silibinin only slightly attenuated RANKL-induced Akt phosphorylation in BMMs (Fig. 3B). Together, these results suggest that silibinin inhibits RANKL-induced signaling cascades, which have been to be important for osteoclast differentiation, but silibinin has little effect on Akt activation which is a signaling cascade associated with survival (Lee and Kim, 2003).

Silibinin attenuates NFATc1 and OSCAR gene expression during osteoclastogenesis

Given our observation that silibinin attenuates osteoclastogenesis

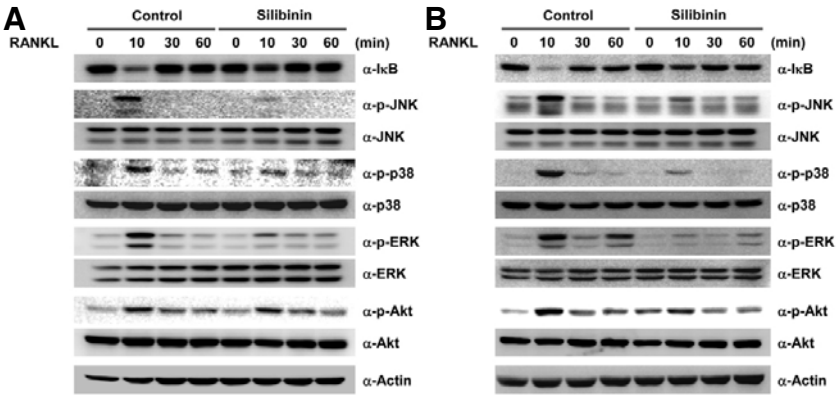

Fig. 3. The effects of silibinin on RANKL-mediated signaling. RAW264.7 cells (A) or BMMs (B) were stimulated with RANKL for the indicated times. Cultures were pre-treated with silibinin (50 mM) or vehicle for 30 min before stimulation with RANKL. Whole cell lysates were subjected to Western blot analysis for detection of the various signaling proteins as indicated. The results are representative of at least two independent sets of similar experiments.

in osteoblasts co-cultured with bone marrow cells in the presence of $1,25(OH)_2D_3$, we examined whether silibinin can affect expression patterns of the osteoclast-associated genes RANKL and OPG which are known to be up-regulated and down-regulated, respectively, by treatment with $1,25(OH)_2D_3$ (Suda et al., 1999). Consistent with previous results (Lacey et al., 1998; Yasuda et al., 1998), when primary osteoblasts derived from calvariae were treated with $1,25(OH)_2D_3$, RANKL expression was readily observed, whereas OPG expression was down-regulated (Fig. 4A). Pre-treatment with silibinin did not affect the expression of RANKL and OPG in the presence of $1,25(OH)_2D_3$ (Fig. 4A), suggesting that silibinin primarily has the capacity to inhibit osteoclast precursors as opposed to mature osteoblasts.

To investigate the effect of silibinin on expression of various osteoclastogenesis-associated genes, we performed RT-PCR using BMMs treated with M-CSF and RANKL in the absence or presence of silibinin (Fig. 4B). Consistent with previous data (Kim et al., 2005a; Takayanagi et al., 2002), RANKL stimulation increased the expression of NFATc1, which is a key modulator of the late-stage RANKL-induced osteoclastogenesis. The induction of NFATc1 gene expression was followed by the expression of TRAP and OSCAR. Compared with the control, silibinin inhibited the expression of NFATc1 as well as OSCAR and TRAP. However, the expression of c-fms and RANK, which are receptors for M-CSF and RANKL, respectively, was not affected by treatment with silibinin. Western blot analyses were consistent with our RT-PCR data, indicating that the induction of NFATc1 and OSCAR by RANKL stimulation was attenuated by the addition of silibinin. These results suggest that silibinin can attenuate the key signaling molecules requisite for osteoclast differentiation.

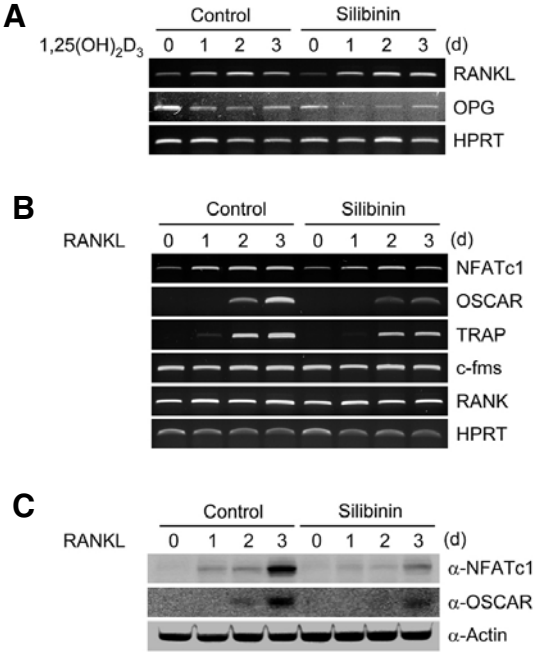

Fig. 4. The effects of silibinin on gene expression. (A) Primary calvarial osteoblasts were cultured with $1,25(OH)_2D_3$ in the absence or presence of silibinin (25 mM) for the indicated times. Total RNA was collected from each time point and analyzed by RT-PCR to assess the expression of RANKL, OPG, and HPRT (control). (B, C) BMMs were cultured with M-CSF and RANKL in the absence or presence of silibinin (25 mM) for the indicated times. RT-PCR (B) and Western blot analyses (C) were performed to assess the expression of the indicated genes. The results are representative of at least two independent sets of similar experiments.

Silibinin inhibits TNF-α-mediated osteoclastogenesis and suppresses NFATc1 and OSCAR expression

Independent of the RANKL-RANK-TRAF6 axis, TNF-α has been shown to induce osteoclast formation in the presence of co-factors such as TGF-β (Kim et al., 2005b; Kobayashi et al., 2000). Since silibinin affects osteoclast differentiation mediated by RANKL, we investigated the effect of silibinin on TNF-α-induced osteoclastogenesis. Consistent with previous results (Kim et al., 2005b), when osteoclasts precursors were isolated from bone marrow cells and initially treated with M-CSF and TGF-β and further cultured in the presence of M-CSF and TNF-α, the formation of $TRAP^+$ MNCs was readily observed (Fig. 5A). Similar to our results obtained in RANKL-induced osteoclastogenesis, silibinin attenuated TNF-α-mediated osteoclast differentiation in a dose-dependent manner. To investigate whether silibinin affects TNF-α-induced signaling cascades, we performed Western blot analyses using BMMs. Consistent with published results (Lee et al., 2001), TNF-α induced degradation of IκB and phosphorylation of JNK, p38, ERK, and Akt within 10 min after stimulation, but in the presence of silibinin this pattern of activation was not observed (Fig. 5B). Furthermore, silibinin attenuated the induction of NFATc1 and OSCAR expression during osteoclastogenesis mediated by TNF-α (Fig. 5C). These data suggest that silibinin can attenuate TNF-α-induced osteoclastogenesis by means of inhibiting the activation of various signaling cascades and ultimately suppressing NFATc1 and OSCAR expression.

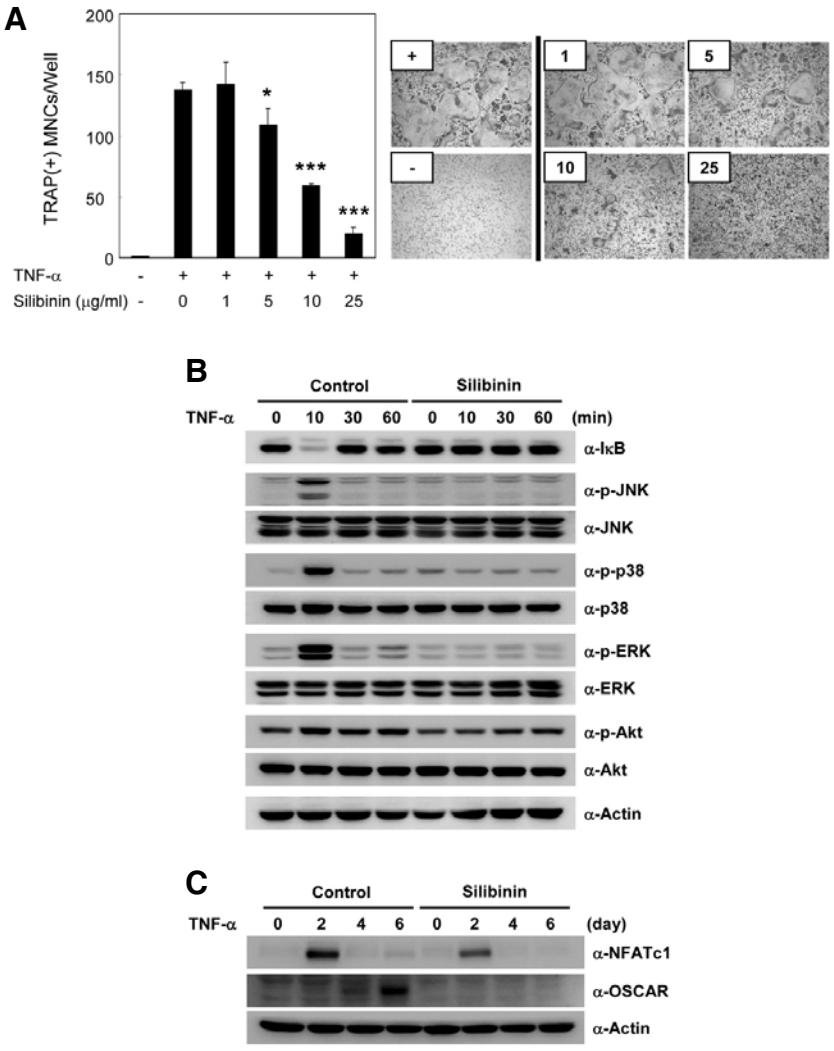

Fig. 5. The effects of silibinin on TNF-α-induced osteoclastogenesis. (A) BMMs were derived from bone marrow cells by culturing them for 3 d with M-CSF and TGF-β. BMMs were cultured for an additional 3 days with M-CSF in the absence (-) or presence (+) of TNF-α with increasing concentrations (1-25 μg/ml) of silibinin as indicated. Cultured cells were fixed and stained for TRAP (right panel). Numbers of TRAP-positive multinucleated cells were counted (left panel). Data represent means ± SDs of triplicate samples. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. positive control. (B) BMMs were stimulated with TNF-α for the indicated times. Cultures were pre-treated with silibinin (50 mM) or vehicle 30 min before stimulation with TNF-α. Whole cell lysates were subjected to Western blot analysis for detection of the various signaling proteins as indicated. (C) BMMs were cultured with M-CSF and TNF-α in the absence or presence of silibinin (25 mM) for the indicated times. Western blot analysis was performed to access the expression of the indicated genes. The results are representative of at least two independent sets of similar experiments.

DISCUSSION

Bone remodeling is tightly regulated by two processes: bone formation by osteoblasts and bone resorption by osteoclasts. The balance between both processes is important for maintaining bone density. When bone resorption exceeds bone formation, an imbalance of skeletal turnover causes bone-resorbing diseases such as osteoporosis, Paget's disease, and periodontal disease. In this study, we report that silibinin, a novel inhibitor in bone, attenuates the formation of $TRAP^{+}$ MNCs from precursor cells mediated by RANKL as well as TNF-α and represents a previously unknown potential regulator of osteoclastogenesis (Figs. 1 and 5).

Pro-osteoclastic factor $1,25(OH)_2D_3$ induces osteoclast formation via up-regulation of RANKL and down-regulation of OPG on the cell-surface of osteoblasts (Suda et al., 1997). We observed that silibinin attenuated osteoclast formation *in vitro* when osteoblasts were co-cultured with osteoclast precursors in the presence of $1,25(OH)_2D_3$ (Fig. 1B). However, the expression level of RANKL and OPG in osteoblasts was not affected by silibinin treatment (Fig. 4A), indicating that silibinin can primarily affect osteoclastogenesis and has little effect on mature osteoclasts. We observed the inhibitory effect of silibinin on osteoclast formation from bone marrow-derived macrophages (BMMs) as well as RAW264.7 murine monocytic cells (Fig. 1). Since RAW264.7 cultures do not contain any osteoblast/bone marrow stromal cells or M-CSF, we provide strong evidence that silibinin directly affects the RANKL signaling pathway. Thus, our data suggest that silibinin can act directly on osteoclast precursors, but has little or no effect on osteoblasts.

Recently, we demonstrated that TNF-α can induce osteoclast formation independent of RANKL-RANKsignaling, and that TNF-α can also activate p38, JNK, ERK, Akt, and NF-κB (Kim et al., 2005b), which are important for osteoclastogenesis (Lee and Kim, 2003). Silibinin attenuated RANKL- and TNF-α-induced activation of these early signaling pathways, including p38, JNK, ERK, and NF-κB (Figs. 3 and 5). Similar to our results, it has been reported that silibinin suppresses activation of NF-κB, JNK, Akt, and MAPK kinase in various types of cancer cells (Li et al., 2006; Manna et al., 1999; Singh et al., 2005).

NFATc1 and OSCAR augment RANKL-induced osteoclastogenesis via a positive feedback regulation (Kim et al., 2005a; Koga et al., 2004; Takayanagi et al., 2002). We found that silibinin could also suppress the expression of NFATc1 and OSCAR during osteoclastogenesis (Figs. 4 and 5). The down-regulation of both genes in the presence of silibinin could be due to silibinin's observed inhibitory effect on early signaling pathways induced by RANKL and TNF-α. Together, our data

indicate that silibinin inhibits osteoclast formation mediated by TNF-α and RANKL by attenuating downstream signaling cascades.

These results are the first time that an effect of silibinin on osteoclastogenesis has been shown. Since silibinin does not show any toxic effects in humans even in the large doses (Hahn et al., 1968), silibinin is being investigated as a therapeutic agent for various cancers and metastases (Ramasamy and Agarwal, 2008). Of note, we confirmed that the viability of cells was not affected by treatment with high concentration (~25 μg/ml) of silibinin (data not shown). In addition to its therapeutic potential in cancer, we report that silibinin has the potential for use in the treatment of bone-resorbing diseases like postmenopausal osteoporosis, Paget's disease, and rheumatoid arthritis.

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