

*Full Paper***Fisetin Inhibits Osteoclastogenesis Through Prevention of RANKL-Induced ROS Production by Nrf2-Mediated Up-regulation of Phase II Antioxidant Enzymes**

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Abstract. Osteoclasts (OCLs) are multinucleated bone-resorbing cells that are differentiated by stimulation with receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor. We recently demonstrated that regulation of heme-oxygenase 1 (HO-1), a stress-induced cytoprotective enzyme, also functions in OCL differentiation. In this study, we investigated effects of fisetin, a natural bioactive flavonoid that has been reported to induce HO-1 expression, on the differentiation of macrophages into OCLs. Fisetin inhibited the formation of OCLs in a dose-dependent manner and suppressed the bone-resorbing activity of OCLs. Moreover, fisetin-treated OCLs showed markedly decreased phosphorylation of extracellular signal-regulated kinase, Akt, and Jun N-terminal kinase, but fisetin did not inhibit p38 phosphorylation. Fisetin up-regulated mRNA expression of phase II antioxidant enzymes including HO-1 and interfered with RANKL-mediated reactive oxygen species (ROS) production. Studies with RNA interference showed that suppression of NF-E2-related factor 2 (Nrf2), a key transcription factor for phase II antioxidant enzymes, rescued fisetin-mediated inhibition of OCL differentiation. Furthermore, fisetin significantly decreased RANKL-induced nuclear translocation of cFos and nuclear factor of activated T cells cytoplasmic-1 (NFATc1), which is a transcription factor critical for osteoclastogenic gene regulation. Therefore, fisetin inhibits OCL differentiation through blocking RANKL-mediated ROS production by Nrf2-mediated up-regulation of phase II antioxidant enzymes.

Keywords: osteoclast, fisetin, reactive oxygen species (ROS), NF-E2-related factor 2 (Nrf2), nuclear factor of activated T cells cytoplasmic-1 (NFATc1)

Introduction

Osteoclasts (OCLs) are bone-resorbing multinucleated giant cells formed by fusion of mononuclear progenitors of the monocyte/macrophage lineage (1). Macrophages differentiate into OCLs in the presence of 2 major cytokines: receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). Binding of RANKL to its receptor RANK promotes OCL differentiation via several signaling

pathways such as nuclear factor of activated T cells cytoplasmic-1 (NFATc1), nuclear factor kappa B (NF- κ B), phosphatidylinositol 3-kinase (PI3K)/Akt, Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (Erk), and p38 mitogen-activated protein kinase (MAPK) (2 – 4). In addition to these signaling pathways, recent studies have also shown that generation of reactive oxygen species (ROS) is required for MAPK activation and OCL differentiation (5, 6). More recently, we have reported that the RANKL-induced suppression of heme-oxygenase 1 (HO-1), a phase II antioxidant cytoprotective enzyme against oxidative stress, is essential for OCL differentiation (7). Overexpression of HO-1 by transient gene vector suppresses OCL differentiation,

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while down-regulation of HO-1 by RNA interference enhances osteoclastogenesis (7). In addition, pharmacological induction of HO-1 by curcumin or kahweol, a coffee-specific diterpene, in macrophage progenitors inhibits osteoclastogenesis (7, 8). Therefore, regulation of HO-1 expression during osteoclastogenesis is an important factor for OCL differentiation, suggesting that HO-1 inducer could be used as new therapeutic agent against bone-lytic diseases.

Fisetin (3,3',4',7-tetrahydroxyflavone) is a natural bioactive flavonoid extracted from various plants, fruits, and vegetables (9). Fisetin has been shown to exhibit various pharmacological activities, including anti-oxidative (10, 11), anti-inflammatory (12–14), and anti-carcinogenic activities (15, 16). These cellular protective effects are due to up-regulation of HO-1, which plays a critical role in cellular protection against oxidative stress. This idea is based on the report that fisetin induces HO-1 up-regulation through PKC δ - and p38 MAPK-dependent pathways in human umbilical vein endothelial cells (HUVECs) (17). These findings suggest that fisetin may inhibit osteoclastogenesis via an HO-1-dependent pathway. However, the effects of fisetin on osteoclastogenesis have not yet been investigated. We report herein the inhibitory mechanisms of fisetin on OCL differentiation using *in vitro* OCL culture systems.

Materials and Methods

Reagents

Fisetin was purchased from Wako Pure Chemicals (Osaka). Human M-CSF was purchased from Kyowa Hakko Kogyo (Tokyo). Recombinant human soluble RANKL was prepared as described previously (18). Antibody (Ab) against β -actin (Cat. No. A5060, rabbit polyclonal Ab, 1:20000) was from Sigma-Aldrich, St. Louis, MO, USA and Ab against Src (Cat. No. 05-184, mouse monoclonal Ab, 1:1000) was from Upstate Biotechnology, Lake Placid, NY, USA. Anti-c-fms (Cat. No. sc-692, rabbit polyclonal Ab, 1:1000), anti-RANK (Cat. No. sc-9072, rabbit polyclonal Ab, 1:1000), anti-NF-E2-related factor 2 (Nrf2; Cat. No. sc-13032, rabbit polyclonal Ab, 1:1000), anti-cFos (Cat. No. sc-52, rabbit polyclonal Ab, 1:5000), anti-Lamin A/C (Cat. No. sc-6215, goat polyclonal Ab, 1:5000), and anti-NFATc1 (Cat. No. sc-7294, mouse monoclonal Ab, 1:1000) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Abs specific for Erk (Cat. No. 9102, rabbit polyclonal Ab, 1:2000), phospho-Erk1/2 (Cat. No. 9101S, Thr202/Tyr204, rabbit polyclonal Ab, 1:1000), Akt (Cat. No. 9272, Rabbit polyclonal Ab, 1:2000), phospho-Akt (Cat. No. 9271S, Ser473, rabbit polyclonal Ab, 1:1000), JNK (Cat. No. 9252S, rabbit polyclonal Ab, 1:2000),

phospho-JNK (Cat. No. 9751S, Thr183/Tyr185, rabbit polyclonal Ab, 1:1000), p38 MAPK (Cat. No. 9212, rabbit polyclonal Ab, 1:2000), phospho-p38 (Cat. No. 9211S, Thr180/Tyr182, rabbit polyclonal Ab, 1:1000), inhibitor of nuclear factor kappa B alpha (I κ B α) (Cat. No. 9242, rabbit polyclonal Ab, 1:2000), and phospho-I κ B α (Cat. No. 2859S, Ser32, rabbit polyclonal Ab, 1:1000) were purchased from Cell Signaling Technology, Danvers, MA, USA. Cathepsin K Ab was prepared as described previously (19). The Osteo Assay Plate was purchased from Corning (Corning, NY, USA). All other reagents, including phenylmethylsulfonyl fluoride and the protease inhibitor cocktail were obtained from Sigma-Aldrich.

Cell culture

Five-week-old male BALB/c mice were obtained from CLEA Japan, Inc. (Tokyo) and handled in our facilities under protocols approved by the Nagasaki University Animal Care Committee. Bone marrow-derived macrophages (BMMs) were isolated as described previously (7). The BMMs were replated in culture plates and incubated in α -minimal essential medium (α -MEM) [Code: 135-15175, bicarbonate buffered with L-glutamine; Wako Pure Chemicals containing 10% FBS with 100 U/mL of penicillin and 100 μ g/mL of streptomycin in the presence of M-CSF (50 ng/mL) and RANKL (50 ng/mL)] for 60 or 72 h until the cells differentiated into multinucleated mature OCLs.

The cells were fixed with 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) activity using a previously described method (20). TRAP-positive red-colored cells with 3 or more nuclei were considered mature OCLs. Murine monocytic RAW-D cells were kindly provided by Prof. Toshio Kukita (Kyushu University, Fukuoka) and cultured in α -MEM containing 10% FBS with RANKL (50 ng/mL) (21). For bone resorption pit formation, BMMs were seeded onto Osteo Assay Plates coated with thin calcium phosphate films (Corning) and incubated with M-CSF and RANKL for 5 days until the mature OCLs resorbed the calcium phosphate film. Cells were dissolved in 5% sodium hypochlorite. Images of the resorption pit were taken with a reverse-phase microscope (Olympus, Tokyo). The ratios of the resorbed areas to the total areas were calculated using Image J image-analysis software (<http://rsbweb.nih.gov/ij/>) as described previously (22).

Cell viability assay

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

Western blot analysis

BMMs were stimulated with or without 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]. Nuclear extracts were prepared from RAW-D cells using the nuclear extraction kit from Active Motif (Carlsbad, CA, USA) following the manufacturer's protocol.

The protein concentration of each sample was measured with BCA Protein Assay Reagent (Thermo Pierce, Rockford, IL, USA). An equal amount of protein (5 μ g) was applied to each lane. After SDS-PAGE, proteins were electroblotted onto a polyvinylidene difluoride membrane. The blots were blocked with 5% BSA/TBST for 1 h at room temperature, probed with various Abs overnight at 4°C, washed, incubated with horseradish peroxidase-conjugated secondary Abs (anti-rabbit IgG, Cat. No. 7074, 1:2000 and anti-mouse IgG, Cat. No. 7076, 1:2000, from Cell Signaling Technology; and anti-goat IgG, Cat. No. P0160, 1:2000, from Dako, Glostrup, Denmark) and finally detected with ECL-Plus (GE Healthcare Life Sciences, Tokyo). The immunoreactive bands were analyzed by LAS1000 (Fuji Photo Film, Tokyo).

Reverse transcription and real-time quantitative PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using oligo(dT)₁₅ primer (Promega, Madison, WI, USA) and Revertra Ace (Toyobo, Osaka). Quantitative real-time PCR was performed using a MX3005P QPCR system (Agilent Technology, La Jolla, CA, USA). cDNA was amplified using Brilliant III Ultra-Fast SYBR QPCR Master Mix (Agilent Technology) according to the manufacturer's instructions. The following primer sets were used: *β -actin*: 5'-ACCCAG ATCATGTTTGAGAC-3' forward and 5'-GTCAGG ATCTTCATGAGGTAGT-3' reverse, *Hemoxygenase-1 (HO-1)*: 5'-CACGCATATACCCGCTACCT-3' forward and 5'-CCAGAGTGTTCATTCGAGCA-3' reverse, *NAD(P)H:quinone oxidoreductase-1 (NQO-1)*: 5'-AAG AGCTTTAGGGTCGTCTTGGA-3' forward and 5'-AGCCTCCTTCATGGCGTAGTTGAA-3' reverse, *Glutamate-cysteine ligase catalytic subunit (GCLC)*: 5'-TTGATGTGGACACCCGATGCAGTA-3' forward and 5'-TCTCATCCACCTGGCAACAGTCAT-3' reverse, *Glutathione S-transferase (GST)*: 5'-AACTGCACC GAGGAAGTGGAGAAT-3' forward and 5'-AGCTCC AGGCTGATGATGTCTTT-3' reverse.

Assessment of intracellular ROS

The intracellular formation of ROS was assessed using the cell-permeable fluorescent probe 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCF-DA, Invitrogen) with FACS analysis. Cells were treated with fisetin or vehicle (DMSO) for 24 h and then incubated with 5 μ M CM-H₂DCFDA at 37°C for 30 min. After washing to remove the nonincorporated probe, cells were stimulated with RANKL (50 ng/ml) for 5 min to enhance intracellular ROS in RAW-D cells. The cells were then collected and resuspended in cold PBS before flow microfluorometry. All samples were analyzed on a FACSCanto™ II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with 488-nm laser excitation. Fluorescence emission was observed through a 530/30-nm filter, and 10,000 events were analyzed for peak shift using FACSDiva software (Becton Dickinson).

Small interfering RNA (siRNA)

The target sequence of murine Nrf2 siRNA was TTGGGATTCACGCATAGGAGCACTG (Nrf2 siRNA). Briefly, RAW-D cells plated at 5×10^4 cells on 60-mm plates were cultured with or without 10 μ M fisetin 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 10 pmol of siRNA for 24 h. For TRAP staining, we incubated the cells for an additional 5 days.

Statistical analysis

All values are expressed as the means \pm standard deviations (S.D.) for 3 independent experiments. The Tukey-Kramer method was used to identify differences between concentrations when ANOVA indicated a significant difference (* P < 0.05 or ** P < 0.01).

Results

Fisetin suppresses osteoclastogenesis in mouse BMMs and RAW-D cells

The structure of fisetin is shown in Fig. 1. To determine whether fisetin prevents osteoclastogenesis, we first investigated its effects on OCL differentiation of native

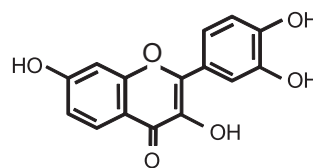


Fig. 1. Structure of fisetin.

BMMs treated with M-CSF (30 ng/mL) and RANKL (50 ng/mL). TRAP staining analysis, which is the most popular method to confirm OCL formation, showed that fisetin suppressed the formation of mononuclear and multinuclear OCLs in a dose-dependent manner (Fig. 2A). The number of TRAP-positive multinucleated OCLs was

decreased after fisetin treatment even at concentrations as low as 1 μ M (Fig. 2B). However, the viability of OCLs treated with 1 – 5 μ M fisetin was comparable with the untreated cells, although the viability of cells treated with fisetin at 10 or 20 μ M was significantly decreased (Fig. 2C).

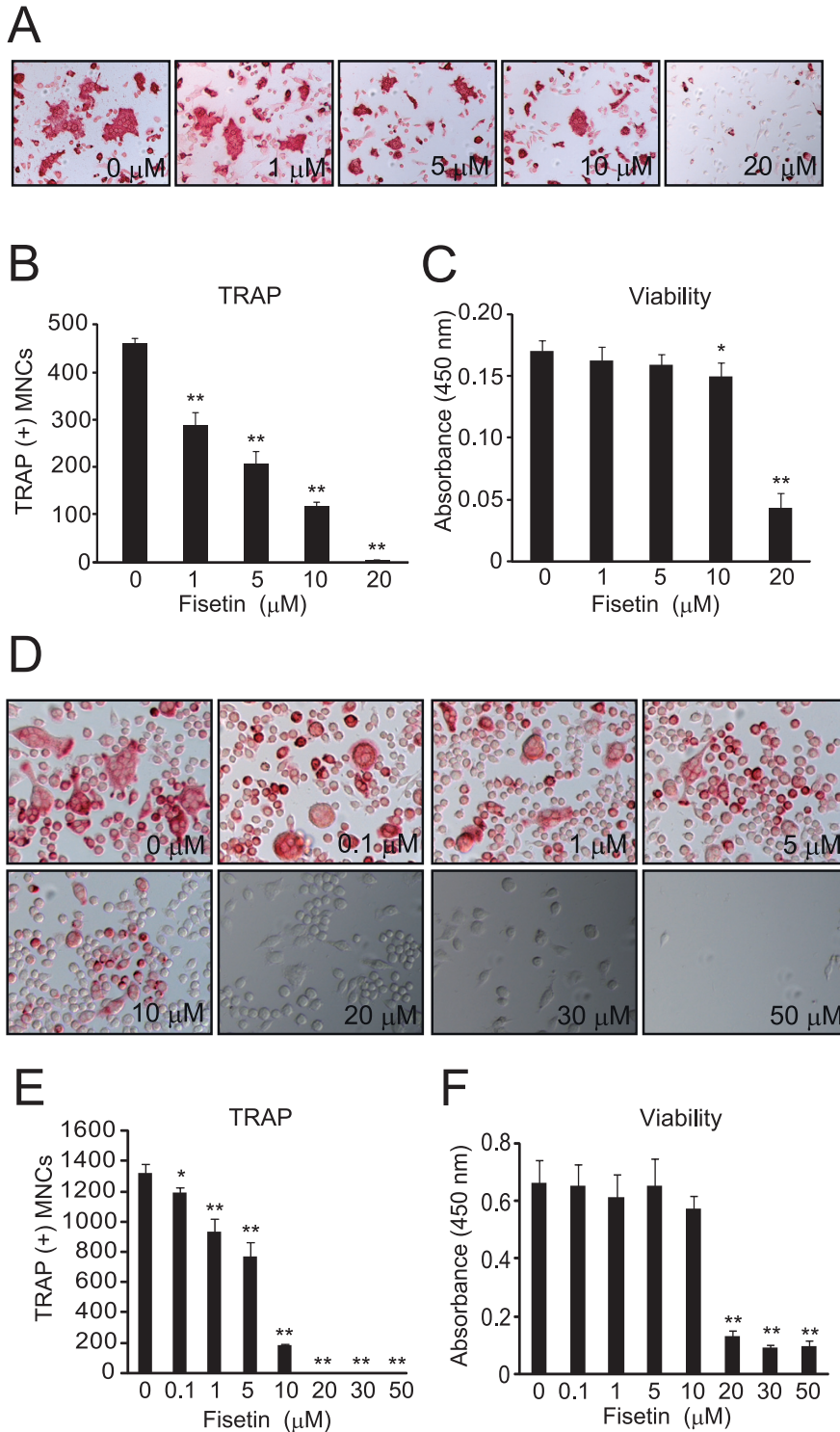


Fig. 2. Effects of fisetin on OCLs differentiated from BMMs or RAW-D cells. A) BMMs were cultured for 72 h with the indicated concentrations of fisetin in the presence of M-CSF (30 ng/mL) and RANKL (50 ng/mL). TRAP staining was performed after fixation. The data are representative of 3 independent experiments. B) The number of TRAP-positive multinucleated OCLs after 72 h of culture was counted. C) The cell viability of BMM-derived OCLs after 72 h of culture was analyzed using the Cell Counting Kit. The data shown in panels B and C are the mean S.D. (standard deviations) of 3 independent experiments. The asterisks indicate statistical significance compared to the control cells without fisetin, * P < 0.05, ** P < 0.01. D) RAW-D cells were cultured for 72 h with the indicated concentrations of fisetin in the presence of RANKL (50 ng/mL). TRAP staining was performed after fixation. The data are representative of 3 independent experiments. E) The number of TRAP-positive multinucleated OCLs after 72 h of culture was counted. F) The cell viability of the RAW-D-cell-derived OCLs after 72 h of culture was analyzed using the Cell Counting Kit. The data shown in panels E and F are the mean S.D. of 3 independent experiments. The asterisks indicate statistical significance compared with the control cells without fisetin, * P < 0.05, ** P < 0.01.

To further investigate whether fisetin has similar effects on RANKL-induced osteoclastogenesis of RAW-D cells, a monocyte macrophage cell line, we treated these cells with fisetin. Treatment of RANKL-induced RAW-D cells with fisetin inhibited OCL formation (Fig. 2D). The number of TRAP-positive RAW-D-derived OCLs was decreased significantly after treatment with more than 0.1 μM fisetin (Fig. 2E). The viability of OCLs treated with 0.1 – 10 μM of fisetin was indistinguishable from untreated cells, although that of OCLs treated with 20 – 50 μM of fisetin was decreased (Fig. 2F). Taken together, fisetin inhibits osteoclastogenesis at non-toxic concentrations in a RANKL-induced in vitro culture system.

Effects of fisetin on the bone-resorption activity of OCLs

To determine whether fisetin decreases the bone-resorption activity of OCLs, we performed a pit formation assay with BMM-derived OCLs after stimulation of M-CSF and RANKL. When the untreated OCLs yielded

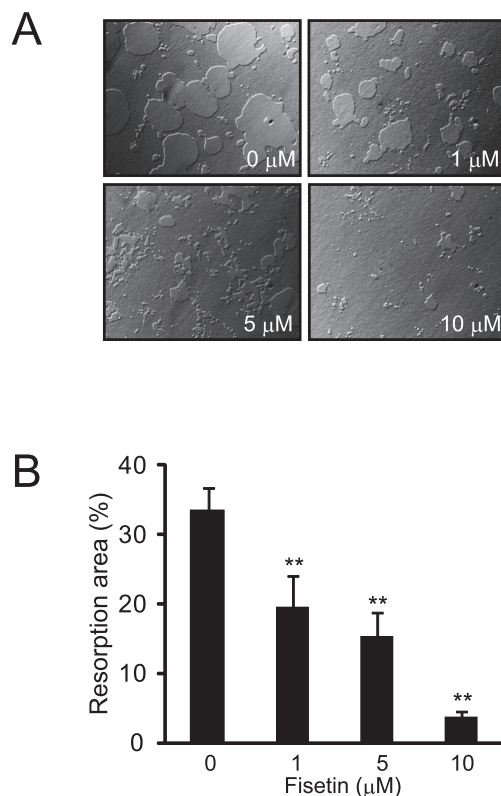


Fig. 3. Effects of fisetin on the bone-resorption activity of OCLs. BMMs were cultured for 5 days with the indicated concentrations of fisetin in the presence of M-CSF (30 ng/mL) and RANKL (50 ng/mL). A) A photograph of the bone-resorption activity of OCLs. B) The resorption area was determined using Image J software. The data are shown as the mean S.D. of 3 independent experiments. The asterisks indicate statistical significance compared to the control cells without fisetin, ** $P < 0.01$.

a moderate resorption pit area, OCLs treated with 1, 5, or 10 μM fisetin exhibited markedly decreased resorption activity (Fig. 3A). The calculated resorption area of 1, 5, or 10 μM fisetin-treated OCLs was markedly lower than that of the untreated OCLs (Fig. 3B). Thus, fisetin inhibits the physiological bone-resorption activity of OCLs.

Effects of fisetin on RANKL-induced intracellular signaling

We next investigated the effects of fisetin on RANKL-induced intracellular signaling during OCL differentiation of BMMs. Various studies have shown that RANKL-induced OCL differentiation is regulated by 6 important signaling pathways: NFATc1, NF- κB , PI3K/Akt, JNK, Erk, and p38 MAPK (23). We studied the effects of fisetin on signaling pathways other than NFATc1, namely phosphorylation of I κ B α , Akt, JNK,

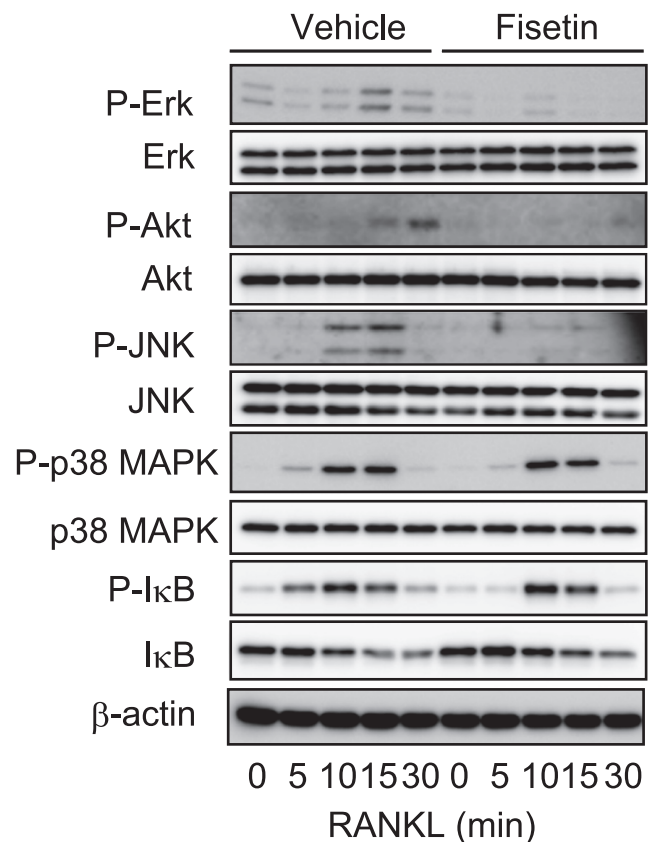
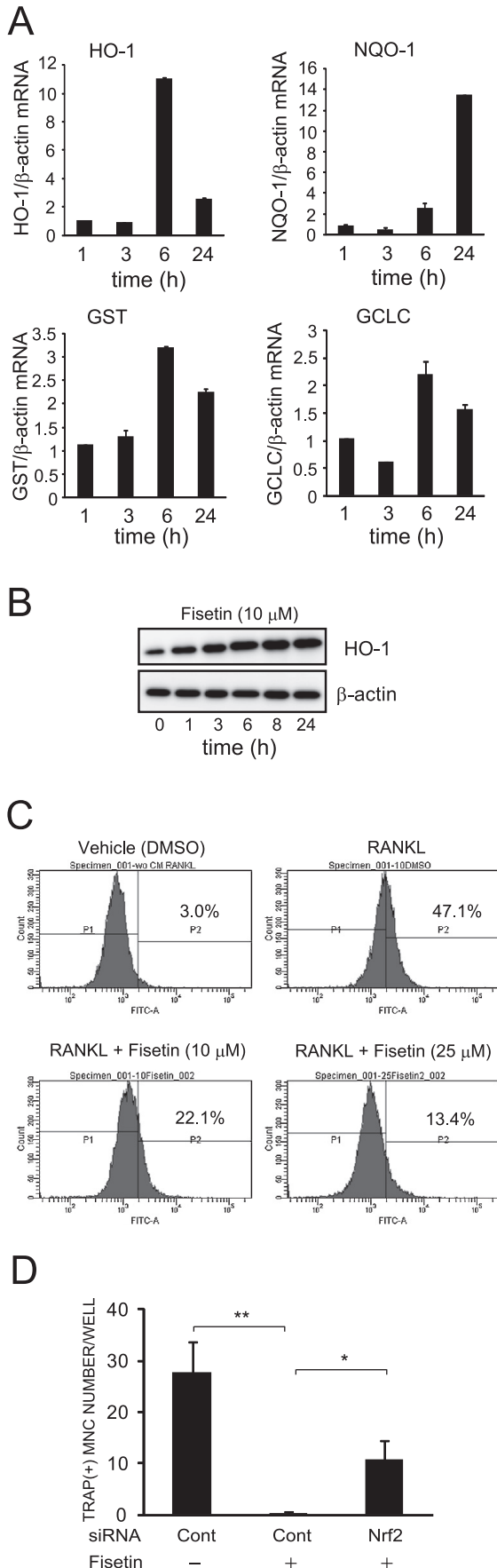


Fig. 4. Effects of fisetin on the essential signaling pathways of OCL differentiation. BMMs were cultured with M-CSF (30 ng/mL) for 12 h in the presence or absence of 10 μM fisetin. After 2 h of cell culture in serum-free media, the cells were subsequently stimulated with RANKL (50 ng/mL) for the indicated times (0, 5, 10, 15, and 30 min). Cell lysates with equal amounts of protein were subjected to SDS-PAGE, followed by western blotting with Abs to p-Erk, Erk, p-Akt, Akt, p-JNK, JNK, p-p38 MAPK, p38 MAPK, p-I κ B α , and I κ B α . β -Actin was used as a loading control. The data are representative of 3 independent experiments.



Erk, and p38 MAPK by western blotting. The effects of fisetin on the expression of NFATc1 are described in Figs. 6 and 7. The phosphorylation levels of BMMs pre-incubated with 10 μ M fisetin for 12 h and stimulated with RANKL were compared to those of vehicle (DMSO)-treated cells. As shown in Fig. 4, phosphorylation of Erk was markedly inhibited by fisetin treatment. Similarly, fisetin treatment suppressed the phosphorylation of Akt and JNK. However, the phosphorylation peak of I κ B α at 10 min was not suppressed by fisetin treatment, although the marginal phosphorylation of I κ B α at 5 min was inhibited. Interestingly, the phosphorylation levels of I κ B α and p38 MAPK were slightly increased. These results indicate that fisetin affects various RANKL-stimulated signaling cascades; in particular, fisetin decreases the phosphorylation of Erk, Akt, and JNK.

Effects of fisetin on the expression levels of oxidative stress response enzymes and RANKL-induced ROS production by OCLs

Our recent studies have shown that RANKL-induced suppression of HO-1, a phase II antioxidant cytoprotective enzyme, is required for OCL differentiation. Although over-expression of HO-1 by transient gene vector suppresses the OCL differentiation, down-regulation of HO-1 by RNA interference enhances osteoclastogenesis (7). In addition, a recent study has shown that fisetin induces HO-1 in HUVECs (17). Therefore, we investigated whether fisetin up-regulates *HO-1* gene expression in OCLs, thereby inhibiting OCL differentiation. As shown in Fig. 5A, fisetin enhanced expression of *HO-1*

Fig. 5. Effects of fisetin on the mRNA levels of phase II antioxidant enzymes and ROS production via the Nrf2 pathway in OCLs. **A)** RAW-D cells were cultured with fisetin (10 μ M) or vehicle (DMSO) in the presence of RANKL (50 ng/mL) for the indicated times (1, 3, 6, and 24 h). The mRNA expression levels were determined by real time PCR using specific primers for *HO-1*, *NQO1*, *GST*, and *GCLM*. β -Actin was used as a control. The graph shows the fold induction of each genes as compared to vehicle-treated cells at each time point. The results are representative of 3 independent experiments. **B)** BMMs were cultured with M-CSF (30 ng/mL) and RANKL (50 ng/mL) for the indicated times (0, 1, 3, 6, 8, and 24 h). The protein expression levels of HO-1 were determined by western blotting with specific Abs. β -Actin was used as a loading control. The data are representative of 3 independent experiments. **C)** Intracellular levels of ROS upon stimulation with RANKL (50 ng/mL) in the presence or absence of fisetin were determined by flow cytometric analysis after staining with CM-H₂DCFDA. **D)** Nrf2 siRNA rescues fisetin-mediated inhibition of OCL differentiation. RAW-D cells were transfected with Nrf2-specific (siNrf2) or control siRNA. After 24 h of transfection with siRNA, cells were cultured with fisetin (10 μ M) or vehicle (DMSO) for an additional 5 days. The number of TRAP-positive, multinucleated OCLs was counted. The data are shown as the mean S.D. of 3 independent experiments. The asterisks indicate statistical significance compared to the control siRNA and fisetin-treated cells, ** P < 0.01.

mRNA especially after the 6-h incubation period. We then analyzed other genes of oxidative stress response enzymes such as *NQO-1*, *GST*, and *GCLC*. Fisetin induced the mRNA levels of all these oxidative-stress response enzymes, although their expression patterns were different (Fig. 5A). Moreover, fisetin enhanced the expression of HO-1 protein (Fig. 5B). These results suggest that fisetin activates ROS scavenging by inducing oxidative-stress response enzymes.

To monitor intracellular ROS levels in OCLs, flow cytometric analysis with the oxidative stress indicator CM-H₂DCFDA was performed. As shown in Fig. 5C, RANKL treatment induced higher levels of ROS in RAW-D cells (upper right) than did vehicle treatment (upper left). These results were consistent with those of a previous study (5). Under these conditions, fisetin suppressed RANKL-induced ROS production in a dose-dependent manner (Fig. 5C, lower left and right).

Generally, gene expression of phase II antioxidant enzymes such as *HO-1*, *NQO-1*, *GST*, and *GCLC* is regulated by Nrf2, a key transcription factor for oxidative stress. A recent study has reported that fisetin treatment in HUVEC enhances expression of the *Nrf2* gene (17). To test whether Nrf2 is involved in fisetin-mediated suppression of osteoclastogenesis, we performed knock-down experiments with siRNA and examined the effects on OCL differentiation. As shown in Fig. 5D, treatment with fisetin (10 μ M) completely suppressed TRAP-positive multi-nucleated cell (MNC) formation. However,

suppression of Nrf2 by RNA interference rescued the fisetin-mediated inhibition of OCL differentiation, although not completely (Fig. 5D), suggesting that fisetin inhibits osteoclastogenesis, at least in part, via an Nrf2-dependent pathway.

Effects of fisetin on the expression levels of OCL marker proteins

To investigate whether fisetin has other pharmacological effects on osteoclastogenesis besides elimination of ROS via the Nrf2 pathway, we analyzed protein levels of various OCL marker proteins by western blotting. RANK, a RANKL receptor, is a type I membrane protein that is expressed on cell surface of OCLs, and the M-CSF receptor c-fms is also expressed on the cell surface of OCLs, both molecules have been implicated in osteoclastogenesis (23). NFATc1 is a master regulator of OCL differentiation through the Ca²⁺/calmodulin/calcineurin-dependent pathway (24). c-Src is a non-receptor-type tyrosine kinase that regulates the formation of actin-rich podosomes in the OCLs (25). Cathepsin K is an OCL-specific lysosomal cysteine proteinase (26). As shown in Fig. 6, the expression of RANK in BMM-derived OCLs was unaffected at 10 μ M fisetin, while the expression of c-fms slightly up-regulated in a dose-dependent manner. However, the protein levels of NFATc1 were markedly decreased upon treatment with fisetin at concentrations greater than 0.1 μ M (Fig. 6). Consistently, the expression levels of c-Src and cathepsin K, which are transcriptionally regulated by NFATc1, were also decreased after fisetin treatment, although those of c-Src were comparatively detected at higher concentrations of fisetin (0.1 – 1 μ M) (Fig. 6). These results indicate that fisetin down-regulates the expression of NFATc1 and its regulated proteins such as c-Src and cathepsin K.

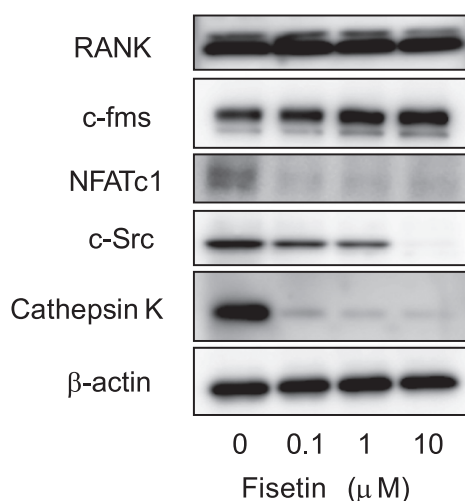


Fig. 6. Effects of fisetin on the expression of OCL marker proteins. BMMs were cultured with M-CSF (30 ng/mL) and RANKL (50 ng/mL) for 60 h in the presence or absence of fisetin at the indicated concentrations (0, 0.1, 1, and 10 μ M). Cell lysates in equal amounts were subjected to SDS-PAGE, followed by western blotting with specific Abs. β -Actin was used as a loading control. The results are representative of 3 independent experiments.

Fisetin inhibits RANKL-mediated nuclear translocation of c-Fos and NFATc1

During RANKL-induced osteoclastogenesis, NFATc1 activates its own transcription by binding to its promoter region, and c-Fos can bind to the promoter region of NFATc1 and regulate NFATc1 expression (27). To determine whether fisetin affects the nuclear translocation of c-Fos, we examined the nuclear fraction of c-Fos in RAW-D cells with or without fisetin treatment. As shown in Fig. 7, western blot analysis showed that addition of fisetin inhibited the nuclear translocation of c-Fos in a dose-dependent manner. Furthermore, fisetin markedly inhibited nuclear translocation of NFATc1, but conversely, fisetin up-regulated the nuclear translocation of Nrf2 in a dose-dependent manner (Fig. 7). Thus, fisetin inhibits the nuclear translocation of c-Fos and NFATc1 in RANKL-stimulated OCLs.

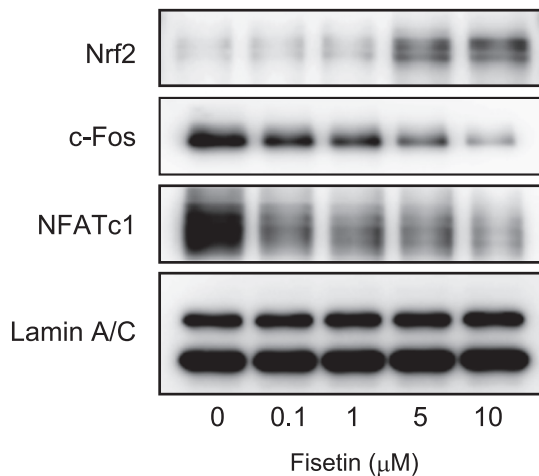


Fig. 7. Fisetin suppresses RANKL-induced nuclear translocation of c-Fos and NFATc1, but up-regulates translocation of Nrf2. RAW-D cells were cultured with the indicated concentrations of fisetin in the presence of RANKL (50 ng/mL) for 6 h. Nuclear extracts were collected and subjected to western blot analysis with the indicated antibody. Lamin A/C was used as a loading control. The results are representative of 3 independent experiments.

Discussion

In this study, we demonstrated that fisetin inhibits the differentiation of BMMs and RAW-D cell-derived OCLs *in vitro*. In addition, fisetin markedly inhibited the bone resorbing activity of OCLs. Fisetin-treated OCLs exhibited markedly reduced phosphorylation of Erk, Akt, and JNK. Fisetin evoked the expression of various oxidative stress-response enzymes such as HO-1, NQO1, GST, and GCLC. Fisetin treatment also dose-dependently suppressed RANKL-induced ROS formation. Fisetin-mediated inhibition of osteoclastogenesis was partially rescued by Nrf2 siRNA. At the protein level, fisetin inhibited the expression of osteoclastogenesis marker proteins such as NFATc1 and its regulated proteins, c-Src and cathepsin K; however, fisetin had no effect on RANK expression. Finally, fisetin inhibited RANKL-induced nuclear translocation of AP-1/c-Fos and NFATc1 in a dose-dependent manner, while fisetin up-regulated the nuclear translocation of Nrf2. Induction of oxidative stress response enzymes is mediated by Nrf2. Therefore, this study demonstrated that fisetin has a powerful inhibitory effect on osteoclastogenesis through Nrf2-mediated up-regulation of oxidative stress-response enzymes and suppression of the nuclear translocation of AP-1/c-Fos and NFATc1 (Fig. 8).

A number of previous studies have reported that fisetin suppresses the maturation and activation of immune-related cells. For example, fisetin blocks lipopoly-

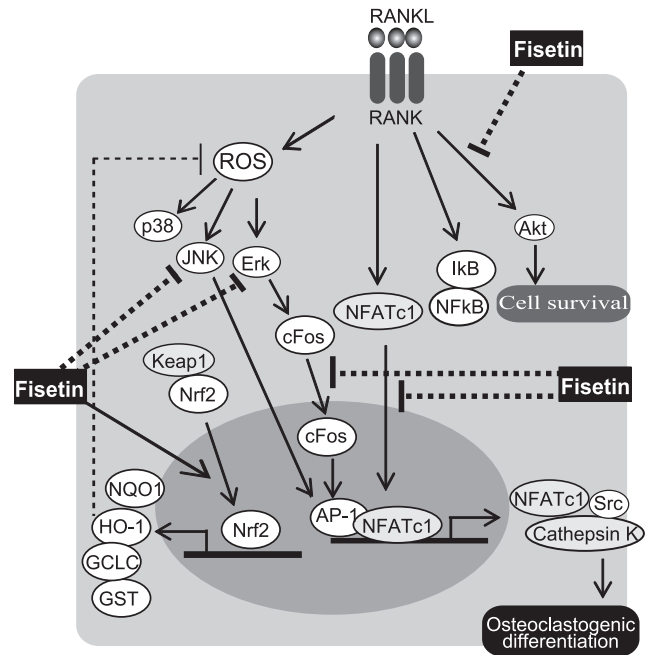


Fig. 8. Possible regulatory mechanism for fisetin-mediated repression of RANKL-induced osteoclastogenesis.

saccharide (LPS)-induced macrophage activation and dendritic cell maturation accompanied by reduced production of pro-inflammatory proteins such as TNF- α , iNOS, and IL-12 and impaired phagocytic activity (28–30). These effects are commonly observed in murine RAW264.7 macrophages and primary immune cells such as peripheral blood mononuclear cells and bone-marrow-derived dendritic cells. Similar suppressive effects for fisetin have also been found in LPS-activated microglia, which are macrophage-like cells in the central nervous system (31). In addition, fisetin impairs the production of Th2-type cytokines, including IL-4 and IL-5, by activated basophils (32) and decreases histamine release and cytokine production by mast cells (33). In this study, we showed that fisetin inhibited OCL differentiation of both BMMs and RAW-D cells and suppressed bone resorbing activity of OCLs. Thus, fisetin has powerful suppressive effects not only on immune-related cells, but also on OCL regulation of bone metabolism.

RANKL-induced OCL differentiation is known to be regulated by 6 important signaling pathways: NFATc1, NF- κ B, PI3K/Akt, JNK, Erk, and p38 MAPK (23). Among these pathways, fisetin markedly inhibited phosphorylation of Erk, Akt, and JNK during OCL differentiation (Fig. 4) and suppressed marginal phosphorylation of I κ B α at 5 min in OCLs. In contrast, fisetin treatment slightly increased the phosphorylation levels of I κ B α and p38 MAPK at 10 min. Similar fisetin-mediated attenua-

tion of the Erk signaling pathway was observed in the human lung cancer cell line A 549 (34). However, signaling pathways other than Erk MAPK have also been implicated in fisetin-induced cellular activation. For example, in LPS-stimulated RAW264.7 macrophages, decreased phosphorylation of JNK and I κ B α , but not Erk and p38 MAPK was found (13, 30). Interestingly, fisetin also attenuates I κ B α and p38 MAPK phosphorylation in LPS-stimulated microglia (31). In HUVECs, the p38 signaling pathway is primarily involved in Nrf2-mediated HO-1 expression induced by fisetin (17). Therefore, fisetin uniquely inhibits OCL differentiation through down-regulation of Erk, Akt, and JNK phosphorylation.

Fisetin has been shown to possess anti-oxidant activity, which is probably due to its free radical-scavenging properties (29) and subsequent regulation of certain cellular signaling pathways. In particular, fisetin induces the transcription factor Nrf2, which results in increased expression of many cellular protective and antioxidant genes in cells other than OCLs (35). A previous study reported that fisetin induces enhanced expression of Nrf2 and HO-1 in primary cultured HUVECs (17). Up-regulation of HO-1 is directly mediated by Nrf2 since Nrf2 siRNA blocked fisetin-induced HO-1 up-regulation. Studies on the detailed mechanisms of this regulation have shown that Nrf2 translocates to the nucleus and binds to the antioxidant response element (ARE) located in the 5'-flanking region of antioxidant genes such as NQO1 and HO-1 (36). Consistent with previous observations, we found that suppression of Nrf2 by RNA interference rescued fisetin-mediated inhibition of OCL differentiation (Fig. 5C). Since fisetin-mediated Nrf2 stabilization is a common event, this further induces antioxidant genes in cultured cells including OCLs. We also found that fisetin activated Nrf2 nuclear translocation (Fig. 7) and up-regulated phase II antioxidant enzymes and further scavenged RANKL-induced ROS production (Fig. 5: A and B). Therefore, it is highly likely that fisetin inhibits osteoclastogenesis through Nrf2-mediated pathways.

Since c-Fos and NFATc1 are crucial transcription factors for osteoclastogenic gene regulation during osteoclastogenesis (24, 37), inhibition of c-Fos and NFATc1 by fisetin is probably sufficient to suppress OCL differentiation directly. In the present study, we showed that fisetin significantly inhibited the nuclear translocation of c-Fos and NFATc1 following RANKL stimulation of RAW-D cells, but the nuclear translocation of Nrf2, a non osteoclastogenic transcription factor, was not inhibited, suggesting that fisetin-mediated inhibition of c-Fos and NFATc1 was critical for inhibition of osteoclastogenesis. Although the precise molecular mechanisms by which fisetin prevents the nuclear

translocation of c-Fos are unknown, our preliminary data show that inhibition of Erk phosphorylation by U0126 prevented the nuclear translocation of c-Fos, suggesting that inhibition of Erk phosphorylation by fisetin may prevent c-Fos activation (data not shown). Taken together, we suggest that by inhibiting RANKL-induced phosphorylation of Erk, fisetin might reduce the nuclear translocation of c-Fos and impair NFATc1 induction by c-Fos and NFATc1 during osteoclastogenesis.

In the present study, we showed that expression of NFATc1 and cathepsin K, markers of osteoclastogenesis, was drastically decreased by fisetin at 0.1 μ M (Fig. 6), while TRAP activity was marginally inhibited by fisetin at same concentration (Fig. 1: D, E). Although we cannot provide a conclusive explanation for the differences at the present time, it may be considered that weak inhibition of NF- κ B is a possible reason.

During preparation of this manuscript, Choi et al. (38) also reported inhibitory effects of fisetin on osteoclastogenesis via down-regulation of p38 signaling. However, our results show that fisetin treatment did not inhibit p38 phosphorylation, but markedly decreased phosphorylation of Erk, Akt, and Jun kinases. This discrepancy may be due to mouse strain differences between ICR in that study and BALB/c in this study. Moreover, those authors did not investigate Akt signaling and did not report detailed molecular mechanisms of Nrf2-mediated control and RANKL-mediated ROS production.

In conclusion, our data demonstrated that fisetin can suppress osteoclastogenesis via the molecular mechanisms by which fisetin inhibits osteoclastogenesis through inhibition of RANKL-mediated ROS production by Nrf2-mediated up-regulation of phase II antioxidant enzymes. In addition, fisetin prevents RANKL-induced nuclear translocation of c-Fos and the expression of NFATc1, c-Src, and cathepsin K. The present study indicates that fisetin may have therapeutic value for bone diseases associated with abnormal stimulation of osteoclastogenesis such as osteoporosis and rheumatoid arthritis.

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