

# Rutin inhibits osteoclast formation by decreasing reactive oxygen species and TNF- $\alpha$ by inhibiting activation of NF- $\kappa$ B

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Abbreviations: BMM, bone marrow-derived macrophages; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DPI, diphenyleneiodonium; RANKL, receptor activator of NF- $\kappa$ B ligand; ROS, reactive oxygen species

## Abstract

**Rutin, a glycoside of flavonol, inhibits osteoclast formation induced by receptor activator of NF- $\kappa$ B ligand (RANKL) in bone marrow-derived macrophages. It reduces reactive oxygen species produced by RANKL and its inhibitory effect results from reduced levels of TNF- $\alpha$ . Rutin also lowers NF- $\kappa$ B activation in response to RANKL.**

**Keywords:** macrophages; NF- $\kappa$ B; osteoclasts; RANK ligand; reactive oxygen species; rutin; tumor necrosis factor  $\alpha$

## Introduction

Osteoclasts, the cells responsible for bone resorption, are formed from hematopoietic stem cells. Osteoclast precursors have features in common with the precursors of the monocyte and macrophage cell lineage. The mononuclear precursors of osteoclasts fuse to each other to form multinucleated osteoclasts, and become active osteoclasts as the result of a differentiation process (Suda *et al.*, 1999). Although differentiation is regulated by various systemic and local factors including hormones, growth factors, and cytokines, osteoclastogenesis is governed mainly by RANKL/RANK signaling (Suda *et al.*, 1999). Receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) binding to RANK in osteoclast precursors activates

intracellular signaling pathways including the NF- $\kappa$ B pathway (Wong *et al.*, 1998).

At high concentrations, reactive oxygen species (ROS) cause oxidative stress and damage major cellular constituents, but at low concentrations ROS play a critical role in regulating signaling processes (Droge, 2002). They have been implicated in bone metabolism; NADPH oxidase, a ROS generating enzyme, has been detected in osteoclasts (Steinbeck *et al.*, 1994), and RANKL was found to generate ROS in osteoclast cells via TRAF6 and NADPH oxidase (Lee *et al.*, 2005). Moreover bone resorption is inhibited by removing ROS with an antioxidant, and increased intracellular ROS causes bone loss together with augmented TNF- $\alpha$  expression (Jagger *et al.*, 2005; Lean *et al.*, 2005).

The possible involvement of ROS in osteoclast differentiation and bone resorption has led to the use of antioxidants to protect from bone loss. Oxidative stress levels are reported to be negatively associated with bone mineral density and a reduced level of antioxidants has been noted in osteoporosis patients (Basu *et al.*, 2001; Maggio *et al.*, 2003). In addition, N-acetyl cysteine and vitamin C reduced ovariectomy-induced bone loss in mice (Lean *et al.*, 2005).

The potential role of vegetable consumption, rich in flavonols, in protection of osteoporosis suggests that flavonols may have a positive effect on bone remodeling (Muhlbauer and Li, 1999; New *et al.*, 2000). Rutin has been reported to inhibit ovariectomy-induced osteopenia in rats (Horcajada-Molteni *et al.*, 2000), although the potential effect of rutin on bone cells has not been clearly elucidated. Rutin, a non-toxic flavonoid glycoside, has been reported to suppress free radical-mediated processes (Kozlov *et al.*, 1994; Afanas'ev *et al.*, 1995).

In this study, we focused on the effect of rutin, a ROS scavenger, on osteoclast formation in bone marrow-derived macrophages (BMM). We found that rutin inhibited osteoclast formation at an early stage by reducing levels of ROS and TNF- $\alpha$ , which are elevated via RANKL. Rutin also reduced RANKL-induced NF- $\kappa$ B activation in a dose-dependent manner.

## Materials and Methods

### Reagents

Recombinant mouse macrophage-colony stimulat-

ing factor (M-CSF) and RANKL were obtained from R & D Systems, Inc. (Minneapolis, MN). Antibodies against CD11b, F4/80, CD3, and CD45R were from eBioSciences (San Diego, CA), and BD Pharmingen (San Diego, CA). The acid phosphatase kit, Ficoll-Hypaque, diphenyleneiodonium (DPI), and Meyer's hematoxylin were from Sigma Chemical Co. (St. Louis, MO).

### Osteoclast formation

Bone marrow cells were isolated from 4-5-week-old C57BL/6J mice as described (Lee *et al.*, 2007). All mice were housed in the specific pathogen-free animal facility of the Immunomodulation Research Center. Animal experimentation protocols were approved by the Institutional Animal Care and Use Committee of the University of Ulsan Immunomodulation Research Center. Femora and tibiae were aseptically removed and dissected free of adherent soft tissues. The bone ends were cut, and the marrow cavity was flushed out from one end of the bone with  $\alpha$ -modified minimum essential medium ( $\alpha$ -MEM) using a sterile 21-gauge needle. The bone marrow suspension was carefully agitated with a plastic Pasteur pipette to obtain single cells. These were washed twice and resuspended in  $\alpha$ -MEM containing 10% FBS, and the suspension was added to plates along with M-CSF (20 ng/ml) for 16 h. Then non-adherent cells were harvested, layered on a Ficoll-Hypaque gradient, and the cells at the interface were collected, washed, and re-suspended in  $\alpha$ -MEM containing 10% FBS. The isolated cells were seeded at a density of  $10^5$  cells/well in 48-well plates. To each of the wells was added additional medium containing M-CSF (20 ng/ml) for 2 d. A small number of non-adherent cells were removed by washing the dishes with PBS, and the remaining adherent BMM were harvested by vigorous pipetting. The cells were confirmed by FACS as positive for CD11b and F4/80, and negative for CD3 and CD45R (data not shown), and the absence of contaminating stromal cells was confirmed by lack of growth when M-CSF was omitted (data not shown). Additional medium containing M-CSF (20 ng/ml) and RANKL (40 ng/ml) in the presence or absence of rutin was added to each of the wells. After 3 d, the cells were fixed in 10% formalin for 10 min, and stained for tartrate-resistant acid phosphatase (TRAP) as described (Kobayashi *et al.*, 2000). Numbers of TRAP-positive multinucleated cells (MNC) (three or more nuclei) were scored.

### RNA isolation and RT-PCR

Expression of TNF- $\alpha$  and GAPDH mRNAs was

assessed by RT-PCR analysis. RNA was isolated from BMM using TRI reagent (Sigma Chemical Co.). Total RNA was used for cDNA synthesis using the reverse transcriptase supplied with the cDNA synthesis kit (Invitrogen, San Diego, CA). cDNA was amplified by PCR for 30 cycles (TNF- $\alpha$ ), and 25 cycles (GAPDH), with the following specific PCR primers: TNF- $\alpha$ , 5'-TTCTGTCTACTGAACTT-CGGGGTGATCGGTCC-3' (forward) and 5'-GTA-TGAGATAGCAAATCGGCTGACGGTGTGGG-3' (reverse); mouse GAPDH, 5'-ACCACAGTCCAT-GCCATCAC-3' (forward) and 5'-TCCACCACCCT-GTTGCTGTA-3' (reverse). Each cycle consisted of 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 30 s of extension at 72°C. GAPDH was used as internal control. The sizes of the PCR products for mouse TNF- $\alpha$  and GAPDH were 354 and 452 bp, respectively.

### Measurement of intracellular ROS

Intracellular ROS were assayed as described (Lee *et al.*, 2005). BMM cells were washed with  $\alpha$ -MEM lacking phenol red, and incubated in the dark for 10 min in Krebs-Ringer solution containing 10  $\mu$ M 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Leiden, Netherlands). Then the cells were stimulated with RANKL (40 ng/ml) or RANKL (20 ng/ml) and TNF- $\alpha$  (20 ng/ml) for 10 min. Where required, antioxidants were added 30 min before the addition of DCFH-DA. The relative fluorescence intensity of DCFH-DA was measured with an Olympus FV500 confocal laser scanning microscope 6 min after each stimulus, with excitation at 480 nm and emission at 530 nm.

### Electrophoretic mobility shift assay (EMSA)

BMM cells were pretreated with rutin (10, 50  $\mu$ M) for 30 min and stimulated with RANKL in the presence of M-CSF for 1 h, and nuclear extracts were then prepared. NF- $\kappa$ B-binding studies were performed using double-stranded oligonucleotides (Santa Cruz Biotechnology, Santa Cruz, CA) containing an NF- $\kappa$ B consensus binding site. The oligonucleotides were end-labeled with [ $\gamma$ - $^{32}$ P] ATP using T4 polynucleotide kinase (Promega, Madison, WI). Five  $\mu$ g of nuclear extract was incubated at 30°C for 20 min with 1 ng of  $^{32}$ P-labeled NF- $\kappa$ B probe in 10  $\mu$ l of binding buffer containing 1  $\mu$ g of poly (dI:dC), 15 mM HEPES, pH 7.6, 80 mM NaCl, 1 mM EGTA, 1 mM DTT, and 10% glycerol. DNA-protein complexes were separated from the DNA probe by electrophoresis on a native 5% polyacrylamide gel, and the gel was vacuum-dried and subjected to autoradiography with an intensifying

screen at  $-80^{\circ}\text{C}$ .

### Statistical analysis

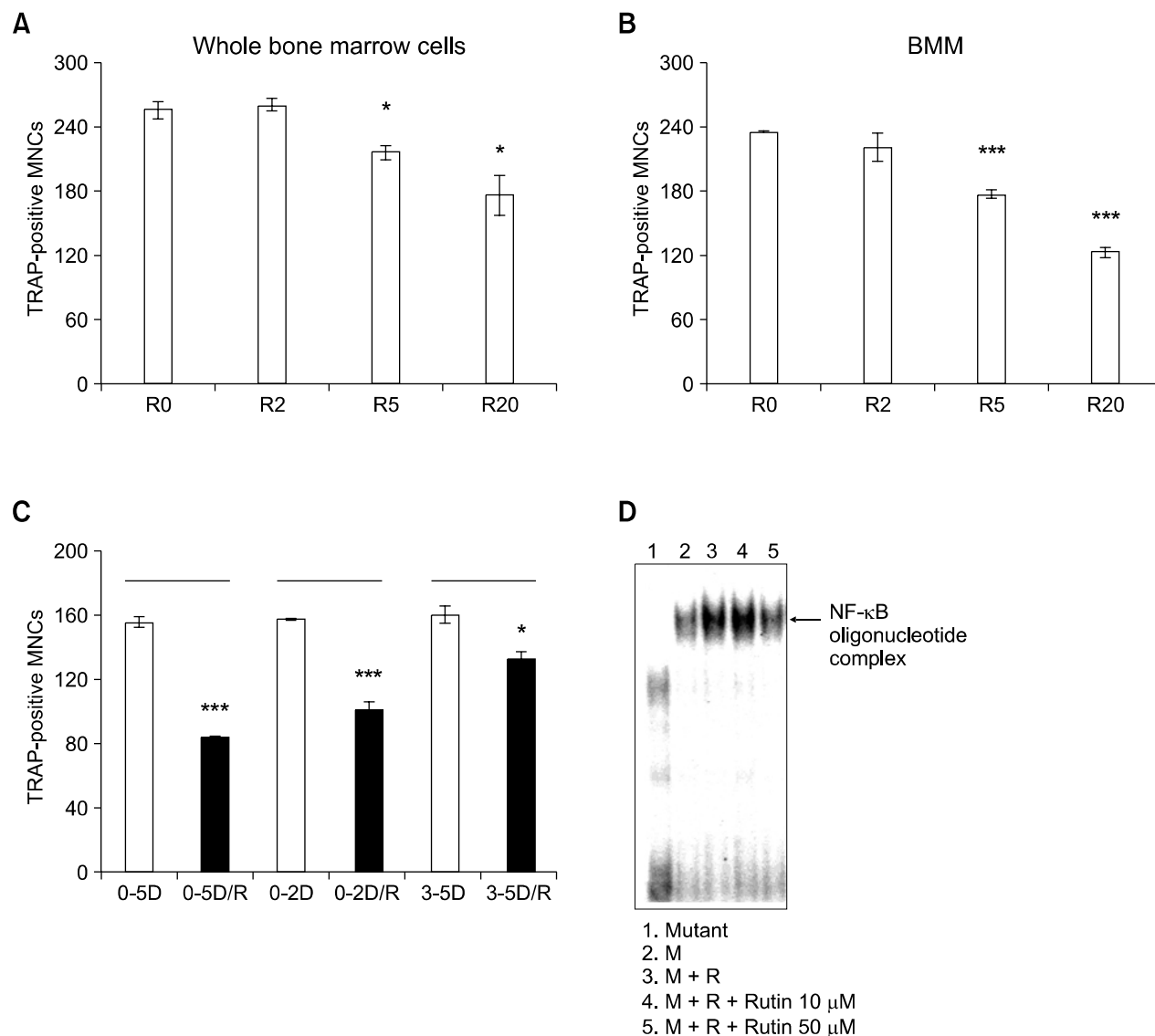
All values are expressed as means  $\pm$  SEM. Student's *t*-test was used to evaluate differences between samples of interest and the respective controls. *P* values of less than 0.05 were considered

statistically significant.

## Results

### Rutin inhibits osteoclast formation

We first examined the effect of rutin on RANKL-



**Figure 1.** Effect of rutin on OC formation. (A) Bone marrow cells from 4-5-wk-old C57BL/6 mice were incubated in 48-well plates ( $2 \times 10^5$  cells/well) in the presence of M-CSF (20 ng/ml) and RANKL (40 ng/ml) with various concentrations of rutin (0, 2, 5, and 20  $\mu\text{M}$ ). After 6 d, cells were fixed and stained for TRAP, and the number of TRAP-positive MNC per well was scored. (\**P* < 0.05, *n* = 3). (B) BMM were prepared as described in "Materials and Methods" and incubated in 48-well plates ( $3 \times 10^4$  cells/well) in the presence of M-CSF and RANKL with various concentrations of rutin (0, 2, 5, and 20  $\mu\text{M}$ ). After 3 d, cells were fixed and stained for TRAP, and the number of TRAP-positive MNC per well was scored (\*\*\**P* < 0.001, *n* = 3). (C) BMM ( $3 \times 10^4$  cells/well) were incubated with R (rutin, 20  $\mu\text{M}$ ) over the intervals 0-5D (0-5 day), 0-2D, and 3-5D, in the presence of M-CSF and RANKL. After 5 d, the number of TRAP-positive MNC per well was scored (\**P* < 0.05; \*\*\**P* < 0.001, *n* = 3). (D) Effect of rutin on NF- $\kappa$ B activation. BMM ( $5 \times 10^6$  cells) were incubated in the presence or absence of rutin (0, 10, 50  $\mu\text{M}$ ) for 1 h, and stimulated with medium (lane 2), or R (RANKL, 40 ng/ml) (lane 3-5) in the presence of M-CSF for 1 h. A mutant form of NF- $\kappa$ B oligomer (lane 1) was used as a negative control.

induced osteoclast (OC) formation by whole bone marrow cells. The cells were incubated for 6 d with M-CSF (20 ng/ml) and RANKL (40 ng/ml), with or without rutin. Under these conditions rutin significantly inhibited the RANKL-induced formation of TRAP-positive multinuclear OCs (Figure 1A). The bone marrow cells used in that experiment were heterogeneous. Since cells secrete growth factors and cytokines that might stimulate or inhibit OC formation, we examined the effect of rutin on cultures of OC precursors free of stromal cells and lymphocytes. Maximum numbers of OCs were formed after 3 d in the presence of M-CSF and RANKL, and rutin inhibited OC formation more strongly than was observed with the whole bone marrow cells (Figure 1B). All further experiments were performed with OC precursors free of stromal cells and lymphocytes.

To determine at which stage rutin inhibits osteoclastogenesis, it was added to or removed from separate cultures on day 0 and day 3, and TRAP staining was performed on day 5. Rutin proved to be an efficient inhibitor when added early, but when addition was delayed to day 3, its inhibitory effect was much reduced (Figure 1C). This suggests that it targets early OC precursors.

Since many of the factors that promote osteoclast formation do so by activating the transcription factor, NF- $\kappa$ B, we examined the effect of rutin on NF- $\kappa$ B activation. We found that RANKL activated NF- $\kappa$ B DNA binding activity in the BMM, as deter-

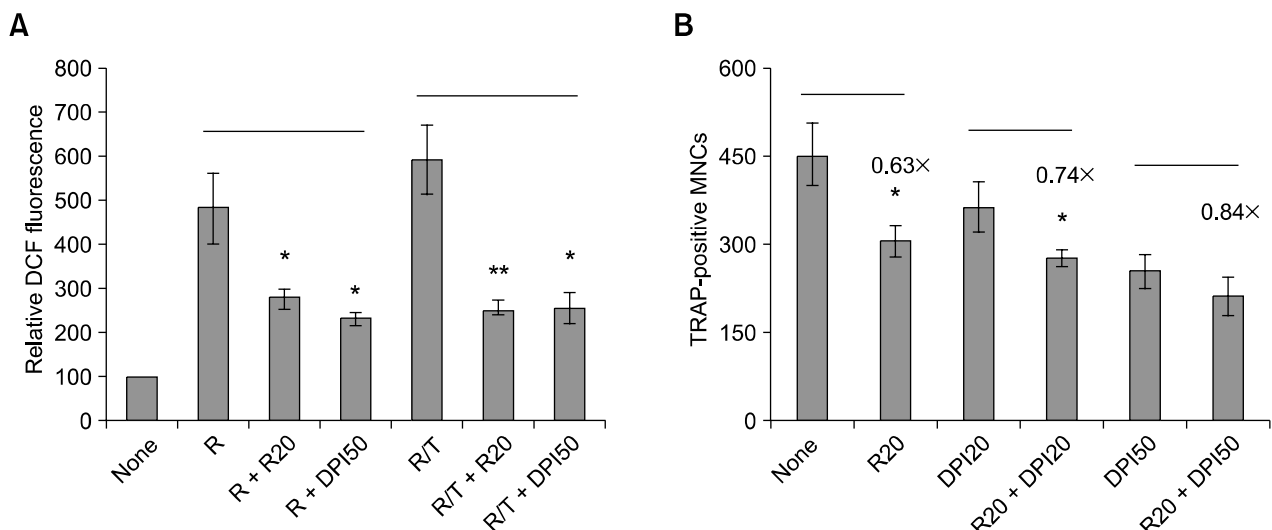
mined by EMSA, and 50  $\mu$ M rutin reduced RANKL-induced NF- $\kappa$ B activation, while not much difference was not observed with 10  $\mu$ M rutin (Figure 1D). The specificity of RANKL-induced NF- $\kappa$ B DNA binding was confirmed by demonstrating that binding was abolished when we used a mutant form of the NF- $\kappa$ B oligomer.

### Rutin inhibits production of reactive oxygen species (ROS)

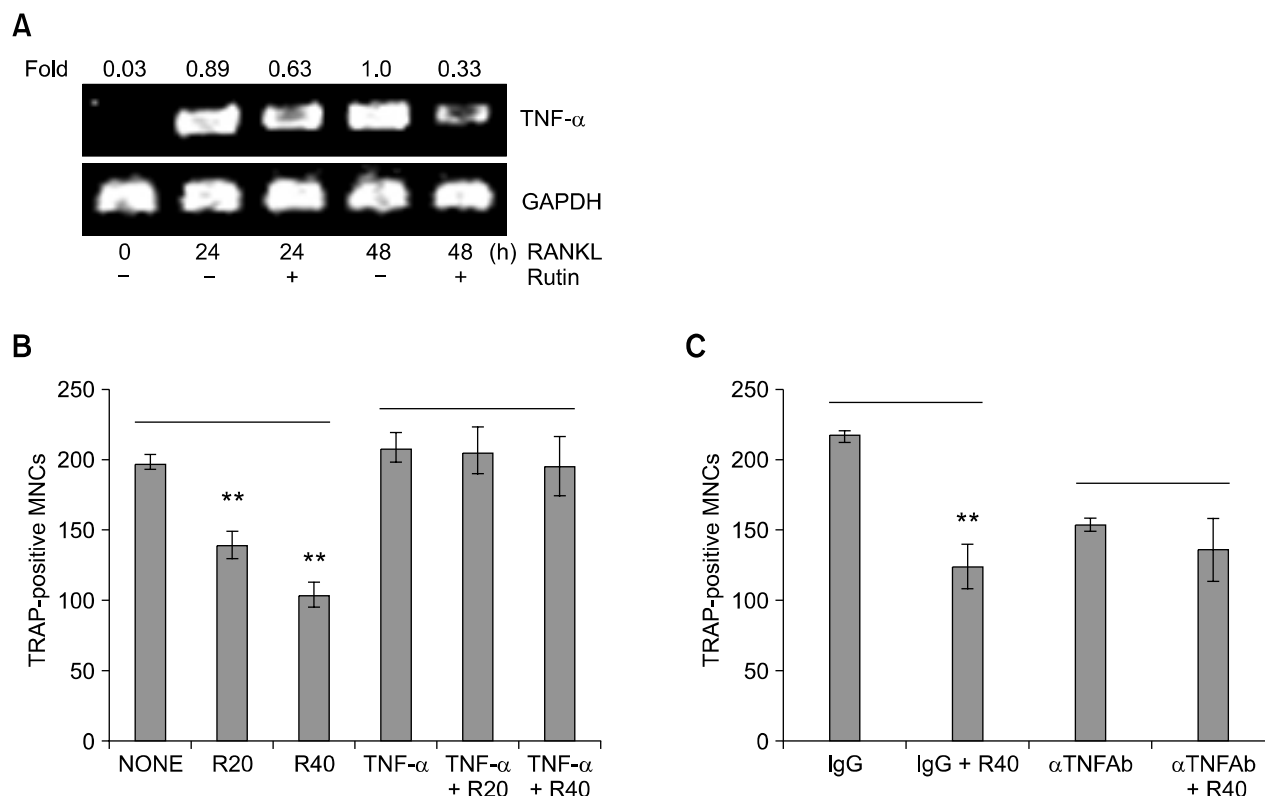
Osteoclasts are known to produce ROS, which are involved in osteoclast formation (Lee *et al.*, 2005). We found that stimulation with RANKL or RANKL and TNF- $\alpha$  resulted in a maximum increase in ROS levels after 6 min, and rutin antagonized this effect (Figure 2A).

Since NADPH oxidase has been implicated in ROS production in osteoclasts, the effect of the NADPH oxidase inhibitor, DPI, was also determined. DPI also reduced ROS elevation in response to RANKL and RANKL/TNF- $\alpha$  (Figure 2A), suggesting that most of the ROS are generated by NADPH oxidase in this system. Next, we determined whether DPI reduced the inhibitory effect of rutin on osteoclast formation (Figure 2B). DPI reduced the yield of TRAP-positive MNCs, but pretreatment with DPI lessened the inhibitory effect of rutin, suggesting that this is caused by lowering ROS formation in response to RANKL.

Since RANKL stimulates production of TNF- $\alpha$  in



**Figure 2.** Rutin reduces elevation of ROS levels by RANKL and TNF- $\alpha$ . (A) BMMs were stimulated for 10 min with R (RANKL, 40 ng/ml) or R/T (RANKL, 20 ng/ml + TNF- $\alpha$ , 20 ng/ml) in the presence or R20 (rutin, 20  $\mu$ M) or DPI50 (diphenyleneiodonium, 50 nM). The relative fluorescent intensity of the fluorophore DCFH was measured 6 min later (\* $P$  < 0.05; \*\* $P$  < 0.001,  $n$  = 3). (B) BMMs were incubated in 48-well plates ( $3 \times 10^4$  cells/well) in the presence of M-CSF and RANKL with diphenyleneiodonium [20 (DPI20), and 50 nM (DPI50)] plus rutin [0, and 20  $\mu$ M (R20)]. After 3 d, cells were fixed and stained for TRAP, and the number of TRAP-positive MNC per well was scored (\* $P$  < 0.05,  $n$  = 3). The numbers above the histograms are ratios of the number of MNC formed in the presence of rutin to the number formed in its absence.



**Figure 3.** TNF- $\alpha$  opposes the inhibition of osteoclast formation by rutin. (A) BMM were incubated in 24-well plates ( $5 \times 10^5$  cells/well) in the presence of M-CSF (20 ng/ml) and RANKL (40 ng/ml) with rutin (20  $\mu$ M) for the indicated periods. Total RNA was isolated and subjected to RT-PCR analysis for TNF- $\alpha$ . (B) BMMs were incubated in 48-well plates ( $3 \times 10^4$  cells/well) in the presence of M-CSF and RANKL with TNF- $\alpha$  (0, 10 ng/ml) plus rutin (0, 20, and 40  $\mu$ M). (C) BMMs were incubated in 48-well plates ( $3 \times 10^4$  cells/well) in the presence of M-CSF and RANKL with  $\alpha$ TNF- $\alpha$  Ab ( $\alpha$ TNFAb, 0.2  $\mu$ g/ml) or control IgG (IgG 0.2  $\mu$ g/ml) plus rutin (R40, 40  $\mu$ M). After 3 d, cells were fixed and stained for TRAP, and the number of TRAP-positive MNC per well was scored. The differences between groups without TNF- $\alpha$  or with IgG were statistically significant (\*\* $P < 0.01$ ,  $n = 3$ ), whereas there was no significant differences between groups with TNF- $\alpha$  or  $\alpha$ TNF- $\alpha$ Ab.

BMM (Nakao *et al.*, 2007), we tested whether rutin also inhibited TNF- $\alpha$  production induced by RANKL. As shown in Figure 3A, rutin decreased expression of TNF- $\alpha$  mRNA in a time-dependent manner. Next, we examined whether the addition of TNF- $\alpha$  opposed the inhibitory effect of rutin on osteoclast formation (Figure 3B). Whereas exogenously added TNF- $\alpha$  did not increase osteoclast formation dramatically, the inhibitory effect of rutin was almost completely abolished. To confirm whether TNF- $\alpha$  mediates inhibitory effect of rutin on osteoclast formation, we added neutralizing Ab against TNF- $\alpha$  (or control IgG) to cultures of BMM. As shown in Figure 3C, no significant inhibition by rutin was observed on blockade of TNF- $\alpha$ , comparing with plates treated with control IgG. These results point to role of TNF- $\alpha$  in inhibitory effect of rutin on osteoclastogenesis.

## Discussion

Our findings demonstrate that rutin inhibits RANKL-

induced osteoclast formation *in vitro* at millimolar range by decreasing ROS production. Rutin did not show cytotoxicity to either bone marrow cells or BMM under these conditions (Data not shown). Rassi *et al.* (2005) showed that nanomolar concentration of rutin reduced 1,25-dihydroxyvitamin D<sub>3</sub>-induced bone resorption in porcine bone marrow cells. Rutin could be more sensitive in inhibiting bone resorption activity than in inhibiting osteoclast formation, although we did not measure the effect of rutin on functional activity of osteoclast. RANKL stimulates osteoclast differentiation and is reported to induce production of ROS via the RANKL-TRAF6 pathway (Lee *et al.*, 2005). This indicated that ROS may be involved in osteoclast differentiation. We showed that rutin attenuated ROS production by RANKL or RANKL/TNF and reduced NF- $\kappa$ B activation in osteoclast precursor cells. The involvement of ROS in NF- $\kappa$ B activation has also been demonstrated in other cell types (Schreck *et al.*, 1991; Rhee, 1999; Droge, 2002; Song *et al.*, 2007). Since NF- $\kappa$ B activation is critical for RANKL-

induced osteoclastogenesis, it is plausible that the inhibitory effect of rutin on osteoclastogenesis should be mediated by suppression of NF- $\kappa$ B activation. The antioxidant,  $\alpha$ -lipoic acid, has been shown to inhibit osteoclastogenesis by inhibiting DNA binding by NF- $\kappa$ B (Kim *et al.*, 2006).

TNF- $\alpha$  is produced by monocytes and macrophages and has been implicated in bone resorption. Although the association between RANKL and the direct or indirect effect of TNF- $\alpha$  on osteoclastogenesis is controversial (Azuma *et al.*, 2000; Lam *et al.*, 2000), RANKL does stimulate TNF- $\alpha$  expression in osteoclast precursor cells and homogenous populations of Raw264.7 cells (Nakao *et al.*, 2007). ROS are known to induce TNF- $\alpha$  expression. Hence the dependence of ROS-induced bone loss on TNF- $\alpha$  suggests that ROS act by increasing intracellular signals that induce TNF- $\alpha$  expression, rather than by augmenting signals that stimulate osteoclast formation and function (Jagger *et al.*, 2005). We also demonstrated that TNF- $\alpha$  production in response to RANKL was reduced by rutin at the transcription level. We have tried to determine secretion of TNF- $\alpha$  in response to rutin with no success. The release of TNF- $\alpha$  from C57BL/6-derived BMM was low due to lower translation, comparing with that from BALB/c-derived one (Amcheslavsky *et al.*, 2004).

We conclude that the inhibitory effect of rutin on osteoclastogenesis is at least partly due to reduced ROS and TNF- $\alpha$  levels resulting from inhibition of RANKL-induced NF- $\kappa$ B activation. Since rutin, a flavonol, is widespread in the human diet, our finding provides a rationale for the protective effect of high flavonol intake on post-menopausal bone loss (Horcajada-Molteni *et al.*, 2000; New *et al.*, 2000; Maggio *et al.*, 2003). Further studies could lead to the development of a novel, non-pharmacological, therapy for osteoporosis.

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