RESEARCH ARTICLE

# Caffeic Acid Inhibited Receptor Activator of Nuclear Factor κB Ligand (RANKL)-Tumor Necrosis Factor (TNF) α-TNF Receptor Associated Factor (TRAF) 6 induced Osteoclastogenesis Pathway

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## Abstract

**BACKGROUND:** Caffeic acid was reported in our previous study to have potential in inhibiting osteoclastogenesis through inhibition of nuclear factor κB (NFκB). Here in our current study, we would like to investigate further the caffeic acid-affected signaling pathway leading to NFκB inhibition. Since tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) plays important role in osteoclastogenesis, we applied TRAF6-trasfected RAW264 cells D-Clone (RAW-D) cells as model in this study.

**METHODS:** Caffeic acid in various concentrations was added to in vitro osteoclastogenesis of receptor activator nuclear factor κB ligand (RANKL)- TNFα-induced TRAF6-transfected RAW-D cells. Cells were collected, lysed and immunoblotted to detect TRAF6 expression. To detect tartrate resistant acid phosphatase $(TRAP)^+$ polynucleated cells (PNCs), TRAP staining was performed. Meanwhile, to measure NFκB Activity, cells were transfected with pNFκB-TA-Luc and subjected to Dual Luciferase Reporter Assay System.

**RESULTS:** Caffeic acid did not influence TRAF6 expression of RANKL-TNFα-induced TRAF6-transfected RAW-D cells. Caffeic acid diminished NFκB activity of RANKL-TNFα-induced TRAF6-transfected RAW-D cells in a concentration dependent manner. Significant NFκB activity inhibitions were seen under treatment of 1 and 10 μg/ml caffeic acid. By adding 10 μg/ml caffeic acid in

## Abstrak

**LATAR BELAKANG:** Potensi *caffeic acid* dalam menghambat osteoklastogenesis dengan inhibisi *nuclear factor κB* (NFκB) telah dilaporkan pada penelitian kami sebelumnya. Pada penelitian kali ini, kami meneliti pengaruh *caffeic acid* pada jalur persinyalan yang mengarah pada inhibisi NFκB. Oleh karena *tumor necrosis factor* (TNF) *receptor-associated factor 6* (TRAF6) berperan penting dalam osteoklastogenesis, kami menggunakan sel *RAW264 D-Clone* (RAW-D) yang ditransfeksi dengan TRAF6 sebagai model penelitian.

**METODE:** *Caffeic acid* dalam berbagai konsentrasi ditambahkan pada osteklastogenesis *in vitro* dengan menggunakan induksi *receptor activator nuclear factor κB ligand* (RANKL)-TNFα pada sel RAW-D. Sel dikoleksi dan dilisiskan, serta dilakukan imunoblot untuk mendeteksi ekspresi TRAF6. Untuk mendeteksi sel polinuklear (SPN) *tartrate resistant acid phosphatase* $(TRAP)^+$, dilakukan pengecatan TRAP. Sedangkan untuk memeriksa aktivitas NFκB, sel ditransfeksi dengan *pNFκB-TA-Luc* dan diperiksa menggunakan *Dual Luciferase Reporter Assay System*.

**HASIL:** *Caffeic acid* tidak mempengaruhi ekspresi TRAF6 pada sel RAW-D yang diinduksi dengan RANKL-TNFα dan ditransfeksi dengan TRAF6. Aktivitas NFκB pada sel RAW-D yang diinduksi dengan RANKL-TNFα dan ditransfeksi dengan TRAF6, dihambat oleh *caffeic acid* dalam ketergantungan dengan konsentrasi. Inhibisi aktivitas NFκB yang bermakna terlihat pada pemberian *caffeic acid*

RANKL-TNFα-induced TRAF6-transfected RAW-D cells, $TRAP^+$ PNCs number was significantly suppressed.

**CONCLUSION:** Caffeic acid inhibited RANKL-TNFα-TRAF6-induced osteoclastogenesis pathway. Since caffeic acid did not influence TRAF6 expression, TRAF6-RANK interactions and/or TRAF6 downstream signaling pathway should be further pursued to disclose inhibition mechanism of caffeic acid.

**KEYWORDS:** caffeic acid, osteoclastogenesis, TRAF6, RANKL, TNFα, NFκB, RAW-D

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dengan konsentrasi 1 dan 10 µg/ml. Dengan menambahkan *caffeic acid* 10 µg/ml pada sel RAW-D yang diinduksi dengan RANKL-TNFα dan ditransfeksi dengan TRAF6, jumlah SPN $TRAP^+$ berkurang secara bermakna.

**KESIMPULAN:** *Caffeic acid* menghambat jalur osteoklastogenesis yang diinduksi oleh RANKL-TNFα-TRAF6. Oleh karena *caffeic acid* tidak mempengaruhi ekspresi TRAF6, sebaiknya dilakukan penelitian lebih lanjut pada interaksi TRAF6-RANK dan/atau jalur persinyalan di bawah TRAF6, sehingga mekanisme hambatan *caffeic acid* dapat diketahui.

**KATA KUNCI:** *caffeic acid*, osteoklastogenesis, TRAF6, RANKL, TNFα, NFκB, RAW-D

## Introduction

Osteoclast is a unique bone-resorbing cell derived from the pluripotent hematopoietic stem cell, which gives rise to a myeloid stem cell that can further differentiate into megakaryocytes, granulocytes, monocytes/macrophages and osteoclasts.(1) Loss of osteoclast function or differentiation will inhibit bone marrow cavity formation, an osteopetrotic phenotype.(2) However, osteoclasts is dispensable for HSC mobilization and may function as negative regulators in the hematopoietic system.(2)

Osteoclastogenesis is a complicated procedure that includes many stages, such as commitment, differentiation, multinucleation, and activation of immature osteoclasts.(1) Receptor activator of nuclear factor κB ligand (RANKL), a member of the tumor necrosis factor (TNF) superfamily, is a type 2 transmembrane residing protein, which activates receptor activator of nuclear factor κB (RANK) on osteoclast precursors in a trimeric symmetric complex.(1,3) RANKL induces osteoclast differentiation(3), interacting with an adaptor molecule TNF receptor-associated factor 6 (TRAF6)(1,4). Meanwhile TNFα, which is a member of the TNF ligand superfamily and a multifunctional cytokine, induces osteoclast differentiation and bone resorption by activating osteoclast progenitors.(5) TNFα did not effectively induce osteoclast differentiation from osteoclast progenitor cells derived from TRAF6-deficient mice into mature multinucleated osteoclasts, although c-jun N-terminal kinase (JNK) and TNFα activation was observed in osteoclast progenitor cells.(5) Thus, TRAF6 is involved in TNFα-induced osteoclastogenesis.(5)

TRAF6 plays essential roles in both the differentiation and maturation of osteoclasts by activating various kinases via its multiple domains.(6) TRAF6-deficient mice show severe osteopetrosis due to impaired osteoclastogenesis. (1) Upon recruitment of TRAF6, nuclear factor κB (NFκB) and c-Jun N-terminal kinase (JNK) pathways will be further activated.(4) Mapping of the structural requirements for TRAF/RANK interaction revealed multiple TRAF binding sites clustered in two distinct domains in the RANK cytoplasmic tail, which are functionally important for the RANK-dependent induction of NFκB and JNK activities. (7)

In our attempt to search for a natural agent having potent activity in inhibiting osteoclastogenesis, we found that sweet potato leaf extract could inhibit osteoclastogenesis. (8) The extract was then further investigated and we found some potential contents including caffeic acid, a major representative of hydroxycinnamic acids.(8) Since caffeic acid potentially inhibited osteclastogenesis, we perfomed a study using a synthetic high purified caffeic acid.(9) Then we found the synthetic one could inhibit osteoclastogenesis as well. The caffeic acid osteoclastogenesis inhibition studies were performed in RANKL-TNFα-macrophage colony stimulating factor (M-CSF)-induced bone marrow-derived monocyte/macrophage precursor cells (BMMs) as well as RANKL-TNFα-induced RAW264 cells D-Clone (RAW-D) cells.(9) Later we found that caffeic acid inhibited osteoclastogenesis of RAW-D cells by suppressing NFκB activity.(9) Since TRAF6 plays important role in osteoclastogenesis, we further investigated the inhibition effect of caffeic acid on osteoclastogenesis pathway of TRAF6-transfected RAW-D cells.

## Methods

### Cell Cultures

RAW-D cells, a high RANKL responsive osteoclast precursor cell line clone (10), were cultured in α-MEM (GIBCO-BRL, Grand Island, NY) with 10% FBS (Biosource, Camarillo, CA) at 37 °C in a humidified incubator with 5% CO2.

### Immunoblotting

Cells were harvested and incubated with lysis buffer containing 20 mM Tris buffer (pH 7.4), 5 mM EDTA, 1% Triton-X, 50 mM sodium fluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1mM p-amidinophenyl methanesulfonyl fluoride hydrochloride and protease inhibitor cocktail (Sigma-Aldrich, St.Louis, MO). Samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Biorad, Richmond, CA). After blocking with 5% skim milk in phosphate buffer saline (PBS) (pH 7.4), the sheets were probed with the following 1:1000 diluted antibodies: mouse monoclonal anti-TRAF6 (D-10, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal anti-Actin (H-300, Santa Cruz Biotechnology). The secondary antibody was horseradish peroxidase-conjugated 1:2000 diluted sheep anti-mouse (Amersham, Buckinghamshire, UK) or 1:1000 diluted donkey anti-rabbit (Amersham) IgG antibody. The bound antibodies were visualized using the ECL system (Amersham).

### Tartrate Resistant Acid Phosphatase (TRAP) Staining

TRAP staining using Leukocyte Acid Phosphatase Kit (Sigma-Aldrich) was performed according to kit protocol. $TRAP^+$ polynucleated cells (PNCs) (>3 nuclei) were counted.

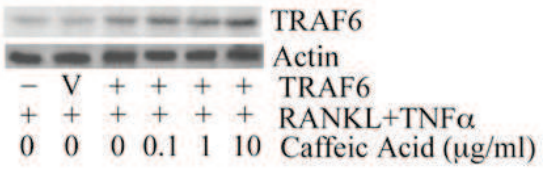



**Figure 1. TRAF6 expression of RANKL-TNFα-caffeic acid-treated TRAF6-transfected RAW-D cells.** One hundred thousand RAW-D cells were transfected with/without TRAF6 or pcDNA3 vector only. After 30 hours, transfected cells were treated with 20 ng/ml RANKL, 1 ng/ml TNFα and 0-10 μg/ml caffeic acid as indicated in the panel for 12 hours. Cells were lysed and subjected to immunoblotting assay using anti-TRAF6 and anti-Actin antibodies. Data represent a typical result from 3 independent experiments. V: pcDNA3 (empty vector of TRAF6).

### Cell Transfection and NFκB Activity Luciferase Assay

RAW-D cells were transfected with pNFκB-TA-Luc (Clontech, Palo Alto, CA) and/or pcDNA3 or pcDNA vector only in a serum free α-MEM using FuGene6 (Roche Molecular Biochemicals, Mannheim, Germany). For internal control, pRLTK (an internal control plasmid containing the herpes simplex thymidine kinase promoter linked to a constitutively active Renilla luciferase reporter gene) was also transfected. After transfection, cells were treated with 20 ng/ml RANKL, 1 ng/ml TNFα and 0-10 μg/ml caffeic acid for 12 hours. Luciferase assay was performed using the Dual Luciferase Reporter Assay System (Promega, Madison, WI). Briefly, after rinse with PBS, 1X passive lysis buffer (PLB) was added to cells. Cells were rocked for 15 minutes at room temperature. Then 20 μl of each cell lysate was immediately applied to 96-well plate for measuring NFκB luciferase activity using TR717 Microplate Luminometer (Tropix, Bedford, MA).

### Statistical Analysis

Analyses were performed using IBM SPSS for Windows version 19.0 (IBM Corp., Armonk, NY). Student's t-test was used to determine the statistical differences between the means of experiments. A probability value $<0.05$ was considered to be statistically significant.

## Results

**Caffeic acid did not influence TRAF6 expression of TRAF6-transfected RAW-D cells.**

As shown in Figure 1, successful RAW-D cells transfection was achieved. Elevated TRAF6 expression of TRAF6-transfected RAW-D cells was observed (lane 3). Meanwhile, TRAF6 expression of pcDNA3 vector control-transfected RAW-D cells (lane 2) was similar to endogenous TRAF6 expression of wild type RAW-D cells (lane 1). Under induction of 20 ng/ml RANKL, 1 ng/ml TNFα and 0-10 μg/ml caffeic acid, expressions of TRAF6 did not show distinct differences.

**Caffeic acid inhibited RANKL-TNFα-induced NFκB luciferase activity in TRAF6-transfected RAW-D cells.**

By induction of 20 ng/ml RANKL and 1 ng/ml TNFα for 12 hours, NFκB activity of wild type RAW-D cells was elevated (Figure 2). The NFκB activity elevation was further escalated in TRAF6-transfected RAW-D cells under the same induction of RANKL and TNFα. Caffeic acid, which was shown previously to inhibit osteoclastogenesis(8), was added in this assay. The results showed that caffeic

acid diminished NFκB activity of RANKL-TNFα-induced TRAF6-transfected RAW-D cells in a concentration dependent manner. Significant NFκB activity inhibitions were seen under treatment of 1 and 10 µg/ml caffeic acid with $p<0.05$ and $p<0.01$, respectively.

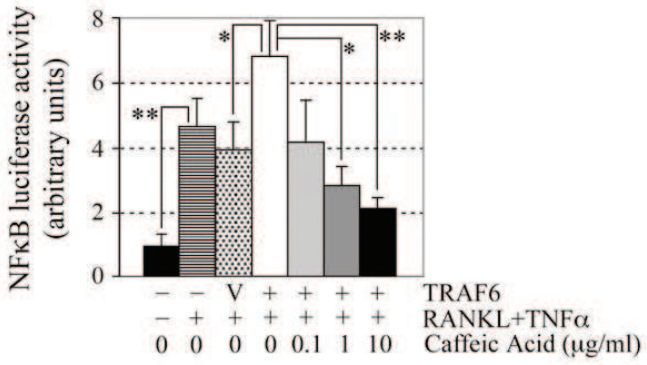
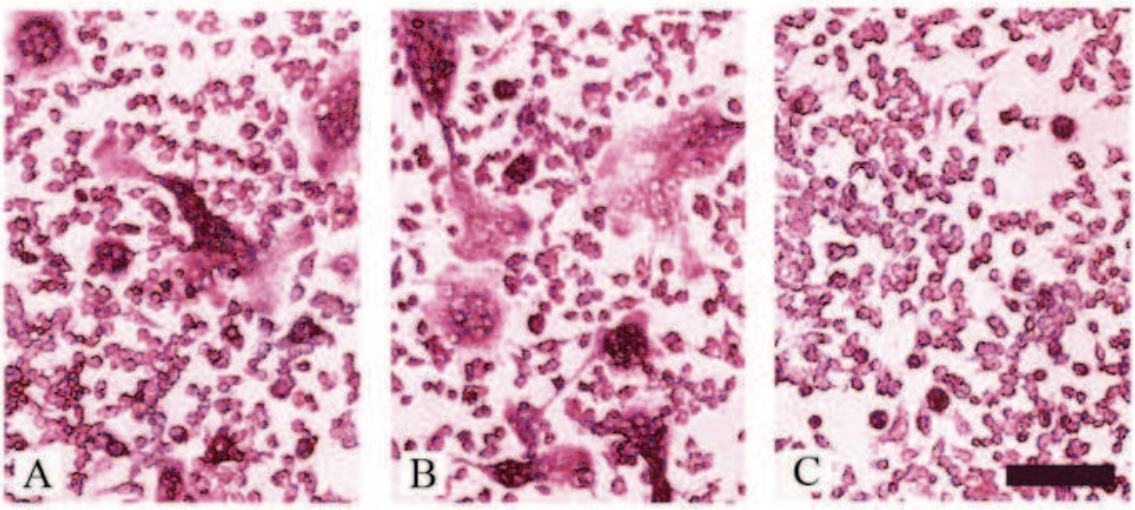



**Figure 2. NFκB luciferase activity of RANKL-TNFα-caffeic acid-treated TRAF6-transfected RAW-D cells.** One hundred thousand RAW-D cells were transfected with pNFκB-TA-Luc and/or pcDNA-TRAF6 or pcDNA3 vector only. After 30 hours, transfected cells were treated with 20 ng/ml RANKL, 1 ng/ml TNFα and 0-10 µg/ml caffeic acid as indicated in the panel for 12 hours. Cells were lysed and subjected to NFκB Luciferase assay. Data were analyzed by Student's t-test, *p<0.05, **p<0.01. Data represent a typical result from 3 independent experiments. V: pcDNA3 (empty vector of TRAF6).

**Caffeic acid inhibited RANKL-TNFα-induced osteoclastogenesis in TRAF6-transfected RAW-D cells.** Under induction of 20 ng/ml RANKL and 1 ng/ml TNFα, wild type RAW-D cells were successfully differentiated into TRAP$^+$ osteoclast-like PNCs (Figure 3A-C). Meanwhile, under the same induction, significantly increased PNCs number was observed in TRAF6-transfected RAW-D cells (Figure 3D). However, by adding 10 µg/ml caffeic acid in RANKL-TNFα-induced TRAF6-transfected RAW-D cells, TRAP$^+$ PNCs number was significantly suppressed.

## Discussion

In this study, we demonstrated that TRAF6 introduction could escalate RANKL-TNFα-induced NFκB activation. As being reported, TRAF6 plays essential roles in both the differentiation and maturation of osteoclast. Osteoclast differentiation from osteoclast progenitor cells into mature



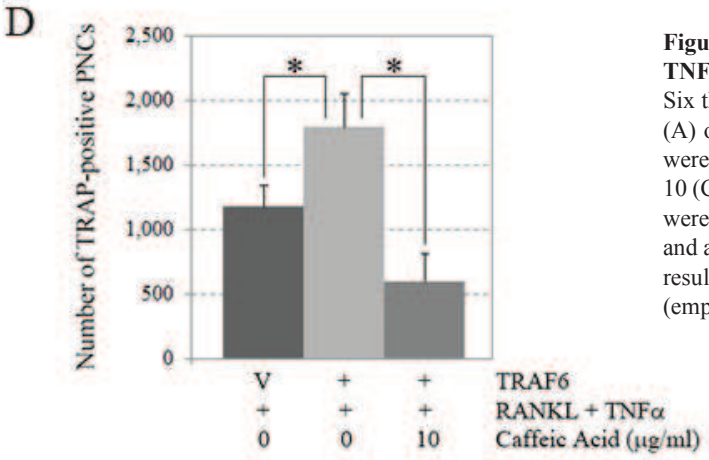




**Figure 3. Expression and number of TRAP$^+$ PNCs of RANKL-TNFα-caffeic acid-treated TRAF6-transfected RAW-D cells.** Six thousand RAW-D cells were transfected with pcDNA3 vector (A) or pcDNA3-TRAF6 (B&C). After 30 hours, transfected cells were treated with 20 ng/ml RANKL, 1 ng/ml TNFα and 0 (B) or 10 (C) µg/ml caffeic acid as indicated in the panel for 3 days. Cells were fixed and stained with TRAP. D: TRAP+ PNCs were counted and analyzed by Student's t-test, *p<0.05. Data represent a typical result from 3 independent experiments. Bar: 100 µm. V: pcDNA3 (empty vector of TRAF6).

multinucleated osteoclasts was disrupted in TRAF6-deficient mice(5) as well as in Si-RNA TRAF6-transfected human macrophages and Si-RNA TRAF6-transfected RAW 264.7 cells(11). By addition of caffeic acid in the model, we observed that NFκB activity was then diminished. This suggests that inhibition of caffeic acid could affect TRAF6-dependent osteoclastogenesis pathway.

Inhibition of TRAF6 could be achieved by ubiquitination, for example carboxyl terminus of heat shock protein (Hsp)70-interacting protein (CHIP) was reported to promote TRAF6 ubiquitination and proteasome degradation.(12) This CHIP-mediated TRAF6 degradation plays an important role in osteoclastogenesis inhibition. However, in our current study we found that TRAF6 expression was not disrupted by caffeic acid. This suggests that caffeic acid could possibly play a role in TRAF6 and RANK/TNF Receptor 1 interactions(13), or in TRAF6 downstream signaling pathway. TRAF6 downstream signaling includes activation of inhibitor of κB (IκB) kinase (IKK), mitogen-activated protein kinase (MAPK) kinase kinase (MKK) and sarcoma viral oncogene homolog c-src-dependent phosphatidylinositol 3-kinase (PI3K).(1,4,11,14-18). Activation of IKK leads to NFκB activation, while MKK activates subsequent stimulation of p38 MAPK, extracellular signal-regulated kinase (Erk), and c-Jun N-terminal kinase (JNK) leading to nuclear translocation of the transcription factors: c-Fos, c-Jun, and nuclear factor of activated T-cells c1 (NFATc1).(1,4,11,14,16) In the PI3K pathway, resulted phosphatidylinositol-3,4,5-triphosphate (PIP3), activates Akt, which then phosphorylates and inactivates the pro-apoptotic protein Bad.(1,15-18)

Under induction of RANKL and TNFα, differentiation of RAW-D cells into TRAP+ osteoclast-like PNCs was well reported.(8,19,20) A significant increase of TRAP+ osteoclast-like PNCs was observed in RANKL-TNFα-induced TRAF6-transfected RAW-D cells. These results are in accordance with previous report showing that increased numbers of TRAF6-binding sites in the cytoplasmic tails leading to a dose-dependent increase and more pronounced osteoclastogenesis (21). When caffeic acid was added in the model of RANKL-TNFα-induced TRAF6-transfected RAW-D cells, significant lower number of osteoclast-like PNCs was observed.

## Conclusion

Taken together, our results showed that caffeic acid inhibited RANKL-TNFα-TRAF6-induced osteoclastogenesis pathway. However, further investigation should be pursued to disclose further the inhibition mechanism of caffeic acid.

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