RESEARCH ARTICLE

Caffeic Acid Inhibits RANKL and TNF-α-induced Phosphorylation of p38 Mitogen-activated Protein Kinase in RAW-D Cells

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Abstract

BACKGROUND: Caffeic acid inhibits osteoclastogenesis by downregulating expression of Cathepsin K and Nuclear Factor of Activated T cells (NFATc)1, as well as inhibiting activity of Nuclear Factor κB (NFκB). Meanwhile TNF Receptor-associated Factor (TRAF)6 was not influenced by caffeic acid. In order to investigate further caffeic acid's mechanism in inhibiting osteoclastogenesis, regulation of caffeic acid on p38 Mitogen-activated Protein Kinase (MAPK) was investigated.

METHODS: RAW-D cells were pretreated with/without caffeic acid and treated with/without 20 ng/mL RANKL and 1 ng/mL TNFα for 0.2, 1, 6, and 12 hour. Tartrate Resistant Acid Phosphatase (TRAP) staining was performed. Then, western blot analysis was performed to detect p38 MAPK and phosphorylated-p38 MAPK. Resulted protein bands were quantified and statistically analyzed.

RESULTS: Under induction of 20 ng/mL RANKL and 1 ng/mL TNF-α, RAW-D cells were successfully differentiated into $TRAP^+$ osteoclast-like polynuclear cells. Under treatment of 20 ng/mL of RANKL and 1 ng/mL of TNF-α for 0.2 or 1 hour, significant (p=0,000, T test) increment of phosphorylated p38 MAPK was observed as compared with control. Pretreatment of 10 μg/mL caffeic acid significantly (p=0.000, T test) suppressed the 20 ng/mL of RANKL and 1 ng/mL of TNF-α-induced phosphorylation of p38 MAPK.

CONCLUSION: RANKL and TNF-α are potent osteoclastogenesis inductors in RAW-D cells, meanwhile caffeic acid could inhibit the RANKL and TNFα-induced osteoclastogenesis through p38 MAPK.

KEYWORDS: caffeic acid, osteoclastogenesis, RANKL, TNF-α, p38, MAPK, RAW-D cells

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Introduction

Osteoclastogenesis, the development of osteoclast from precursor polynuclear cells (PNCs), could be induced by several factors, including Tumor Necrosis Factor (TNF) and Receptor Activator of Nuclear Factor κB (RANK) Ligand (RANKL).(1,2) The osteoclastogenesis related factors could be produced in the physiological development process (3), inflammatory process (3,4) and intraosseous tumor development process (5). After the binding of osteoclastogenesis ligands to their receptors, osteoclastogenesis signaling pathways were activated. The bindings of RANKL to RANK and TNF to TNF receptor (TNFR) can further lead to the activation of the Mitogen-activated Protein Kinase (MAPK) family.(6,7) Among the MAPK family, p38 MAPK has been reported to play an important role in osteoclastogenesis.(8,9)

Caffeic acid attracts much special attention because of its ability to protect the human from several diseases,

including cancer (10,11) and bone resorption (12-14). Caffeic acid induced osteosarcoma cells into apoptosis by activation of Caspases, including Caspase-8, -9 and -3.(10,11) Meanwhile in order to protect from bone resorption, caffeic acid targets osteoclastogenesis.(12-14) Caffeic acid did not induce apoptosis in PNCs (12), but it inhibited osteoclastogenesis by inhibiting expression of Cathepsin K and Nuclear Factor of Activated T cells (NFATc)1 (13), as well as inhibiting activity of Nuclear Factor κB (NFκB) (12). However, caffeic acid did not target the expression of TNF Receptor-associated Factor (TRAF)6.(14) In order to investigate further caffeic acid's mechanism in inhibiting osteoclastogenesis, regulation of caffeic acid on p38 MAPK was investigated.

Methods

Cell Culture

RAW-D cells 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% CO_2.

in vitro Osteoclastogenesis

Six thousand RAW-D cells were cultured in each well of a 96-well plate with the complete culture medium. Pretreatment of 10 µg/mL caffeic acid (Wako, Osaka, Japan) was conducted for 2 hours prior to the treatment of 20 ng/mL RANKL (PeproTech, London, UK) and 1 ng/mL TNF-α (Roche Molecular Biochemicals, Mannheim, Germany). Three days later, Tartrate Resistant Acid Phosphatase (TRAP) staining using Leukocyte Acid Phosphatase Kit (Sigma-Aldrich, St. Louis, MO) was performed. $TRAP^+$ PNCs were documented under an inverted microscope.

Western Blot

Treated cells were harvested and incubated with lysis buffer containing 10 mM Tris buffer (pH 7.4), 150 mM NaCl, 1% Triton-X100 and 100 µL protease inhibitor cocktail (Sigma-Aldrich, St.Louis, MO). The proteins in the samples were separated by sodium dodecyl sulfate (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 membrane were probed with 1:1000 diluted rabbit polyclonal anti-phospho-p38 MAPK (Thr180/Tyr182) antibody (Cell Signaling Technology, Danvers, MA). The secondary antibody was 1:2000 diluted horseradish peroxidase-conjugated donkey anti-rabbit antibody (Cell Signaling Technology). The bound antibodies were visualized using Immun Star HRP Chemiluminescent Kit (Bio-Rad Laboratories, Hercules, CA). Membrane was then stripped with Seppro stripping buffer (Sigma-Aldrich), blocked with 5 % skim milk in PBS, probed with rabbit polyclonal anti-p38 MAPK (Cell Signaling Technology), bound with same secondary antibody and visualized with the chemiluminescent kit. All visualized bands were captured using Alliance 4.7 (UVItech, Cambridge, UK) and quantified using UVIband software (UVItech).

Statistical Analysis

Analyses were performed using IBM SPSS for Windows version 20.0 (IBM Corp., Armonk, NY). 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 inhibited RANKL and TNFα-induced osteoclastogenesis in RAW-D Cells

Under induction of 20 ng/mL RANKL and 1 ng/mL TNFα, RAW-D cells were successfully differentiated into $TRAP^+$ osteoclast-like PNCs (Figure 1B). However, by adding 10 µg/mL caffeic acid prior to induction of 20 ng/mL RANKL

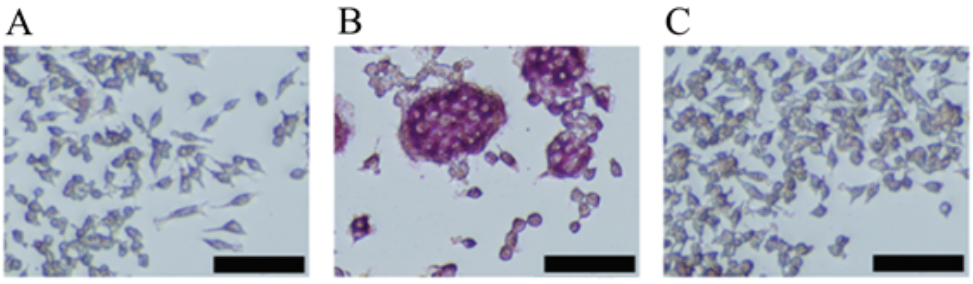

Figure 1. Caffeic acid inhibited RANKL and TNF-α-induced osteoclastogenesis in RAW-D cells. A: Untreated RAW-D cells. B: RAW-D cells were treated with 20 ng/mL of RANKL and 1 ng/mL of TNF-α. C: RAW-D cells were pretreated with 10 µg/mL caffeic acid and treated with 20 ng/mL of RANKL and 1 ng/mL of TNF-α. Bar: 100 µm.

and 1 ng/mL TNFα, TRAP$^+$ osteoclast-like PNC was not found (Figure 1C).

Caffeic Acid inhibited RANKL and TNFα-induced Phosphorylation of p38 MAPK in RAW-D Cells

Under treatment of 20 ng/mL of RANKL and 1 ng/mL of TNF-α for 0.2 or 1 hour, significant (p=0,000, T test) increment of phosphorylated p38 MAPK was observed as compared with control (Figure 2). Treatment of 20 ng/mL of RANKL and 1 ng/mL of TNF-α for 6 and 12 hours did not induce phosphorylation of p38 MAPK. Pretreatment of 10 μg/mL caffeic acid significantly (p=0,000, T test) suppressed the 20 ng/mL of RANKL and 1 ng/mL of TNF-α-induced phosphorylation of p38 MAPK.

Discussion

Previous study has shown that caffeic acid did not significantly affect the expression of TRAF6 while significantly inhibited NFκB's activity in RANKL and TNF-α-induced TRAF6 transfected-RAW-D cells.(14) Despite TRAF6, the involvement of MAPK in the osteoclastogenesis signaling process induced by RANK-RANKL has been widely recognized.(6-9) MAPK is an enzyme that converts extracellular stimulus into various cellular responses. p38 MAPK is a member of MAPK family that has an important role in tissue development and homeostasis functions such as cell differentiation, apoptosis, maturation, and cytokine production which initiates osteoclastogenesis.(8,9) After being activated, p38 MAPK will induce important transcription factors such as NFκB and NFATc1.(8,9,12) Inhibition of p38 MAPK was reported to fully diminish RANKL and TNF-α-induced osteoclast formation.(15) In the present study, RANKL and TNF-α induced formation of TRAP$^+$ PNCs, meanwhile pretreatment of caffeic acid could diminish the formation of RANKL and TNF-α-induced TRAP$^+$ PNCs. The upregulated phosphorylation of p38 MAPK was clearly observed in RANKL and TNF-α-induced RAW-D cell, meanwhile pretreatment of caffeic acid could diminish the phosphorylation of p38 MAPK.

RANKL and TNF-α which induce bone loss, can be secreted by activated T and B cells in inflammatory states.(16) Inhibition on RANKL and TNF-α-induced osteoclastogenesis by some agents has been reported, including osteoprotegerin (17), Simon extract (13) and its constituent, caffeic acid (10,12-14). However, mechanism of caffeic acid in inhibiting osteoclastogenesis remained unclear. Current study has shown that p38 MAPK is one of the second messengers that plays an important role in RANKL and TNF-α-induced osteoclastogenesis. A schematic signaling pathway of RANKL-induced osteoclastogenesis in RAW-D cells can be seen in Figure 3. based on current results and previous report, caffeic acid is shown to inhibit activation of NFκB and p38 MAPK. Further investigation for other activated second messengers should be pursued.

Conclusion

Taken together, current results suggested that RANKL and TNF-α are potent osteoclastogenesis inductors in RAW-D cells, meanwhile caffeic acid could inhibit the RANKL and TNF-α-induced osteoclastogenesis through p38 MAPK.

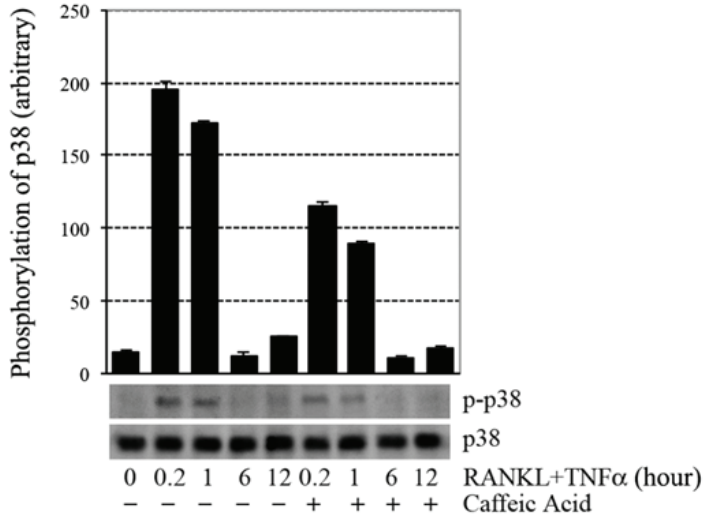

Figure 2. Caffeic acid inhibited RANKL and TNF-α-induced phosphorylation of p38 in RAW-D cells. RAW-D cells were pretreated with/without 10 μg/mL caffeic acid and treated with/without 20 ng/mL of RANKL and 1 ng/ml of TNF-α for 0, 0.2, 1, 6 and 12 hours. Cells were lysed and subjected to immunoblotting assay using anti-p38 and anti-phosphorylated p38 antibodies. Data represent a typical result from 3 independent experiments.

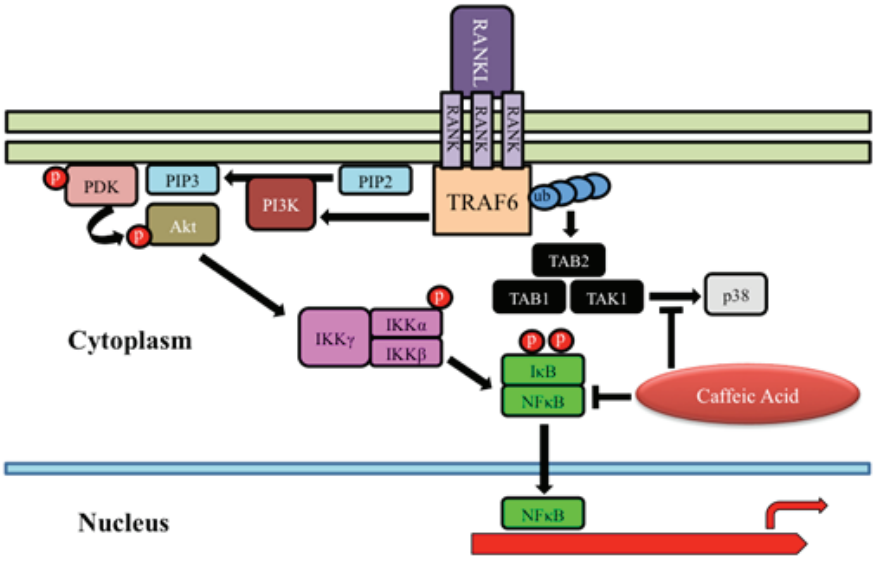

Figure 3. Signaling pathway of RANKL-induced osteoclastogenesis in RAW-D cells.

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