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Glechoma hederacea Suppresses RANKL-mediated Osteoclastogenesis

APPENDIX

MATERIALS & METHODS

Cell Culture and Reagents

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and incubated in 5% CO_2. The day before transfection, cells were seeded at 80% confluence in 35-mm dishes. Cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. Vehicle vector (p3xFLAG-CMV-7.1) and hOrai1 (1 μg/well) were diluted in Opti-MEM and then incubated for 20 min before cells were treated. The transfected cells were re-plated onto a cover glass the following day and then used as samples for $[Ca^{2+}]_i$ measurement. hOrai1 was a generous gift from Dr. Dong Min Shin (Yonsei University, Seoul, South Korea). Briefly, full-length Orai1 was subcloned into the p3xFLAG-CMV-7.1 (Sigma-Aldrich) vector with NotI(5′) and SalI(3′). Soluble recombinant mouse receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) were purchased from KOMAbiotech (Seoul, Korea). U73122, U73343, pAb β-actin, nicardipine, and nifedipine were purchased from Sigma-Aldrich. Fura2/AM and pAb phosphor-PLC γ1 were obtained from Tef Labs (Austin, TX, USA) and Cell Signaling (Danvers, MA, USA), respectively. mAb NFATc1 and mAb total-PLCγ1 were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

In vitro Osteoclast Formation

Murine bone marrow-derived macrophages (BMMs) were prepared from the femur and tibia of 4- to 6-week-old mice, as previously described (Kim *et al.*, 2010). Briefly, bone marrow was flushed out with culture media (αMEM) and collected. After removal of red blood cells, whole-marrow cells were plated on non-coated petri dishes in the presence of M-CSF (10 ng/mL). The following day, BMMs were collected and then seeded on designated culture dishes for each experiment. Henceforth, M-CSF was supplemented in culture media at a concentration of 30 ng/mL. To generate osteoclasts, BMMs were treated with RANKL (50 ng/mL) for indicated times. To evaluate the formation of multi-nucleated cells (MNCs) and tartrate-resistant acidic phosphatase (TRAP) activity, BMMs were plated on 24-well culture dishes at a density of 1.2 x 10^5 cells *per* well. Cytochemical staining for TRAP expression was performed with the leukocyte acid phosphate assay kit (Sigma-Aldrich), following the manufacturer's procedures. For measurement of total TRAP activity, p-nitrophenyl phosphate (Sigma-Aldrich) substrate was added to the culture media containing whole lysates of BMMs. Optical density was measured at an absorbance of 405 nm.

Cell Viability Assay

Cell viability following treatment with *Glechoma hederacea* ethanol extract (GHE) was determined with the EZ-Cytox Enhanced Cell Viability Assay Kit (ITSbio, Seoul, Korea), as previously described (Gu *et al.*, 2013). Briefly, BMMs were prepared as described in "*In vitro* osteoclast formation" and were then plated in 96-well plates at a density of 1 x 10^4 cells *per* well. GHE was then added to each well at indicated concentrations. Following incubation with GHE at specified lengths of time, EZ-Cytox reagents were added and cells were incubated for an additional 4 hr at 37 ºC. Optical density was measured in a microplate reader at 450 nm (Tecan, Männedorf, Switzerland).

Appendix Table. Nucleotide Sequences of the Primers Used for Real-time PCR

Genes	Primers
Cacna1A	Forward 5′-TTCCTCTACTATGCAGAATTCATTTTCT-3′
	Reverse 5′-CCGAGCCCGTACATTTTTATAAA-3′
Cacna1B	Forward 5′-GGCATTTGCGTTCTCAGGAT-3′
	Reverse 5′-CGCAGGACTCTCAGAGACTTGA-3′
Cacna1C	Forward 5′-GGGAGCCACGGTGAATCA-3′
	Reverse 5′-GCAGTACTCGGCTTCTTCACTCA-3′
Cacna1D	Forward 5′-GGTGTAAGGCCCCAGAAACAC-3′
	Reverse 5′-GCGACGGACGCAAAGGT-3′
Cacna1E	Forward 5′-TTGGTGCATTGGTAGCATTTG-3′
	Reverse 5′-TAGTCTTGATATCCCTCCCCTTGT-3′
Cacna1F	Forward 5′-TCGAACCCAGTCCTTTTACCA-3′
	Reverse 5′-CGACATGTGCCCGTTTACG-3′
Cacna1G	Forward 5′-CACTGGGTGCAGATCCTAGCT-3′
	Reverse 5′-TGGCTACTTACGCCCCTTTG-3′
Cacna1H	Forward 5′-CTGAGCATGGGTGTTGAGTATCA-3′
	Reverse 5′-GGTGAACACGATGTTGCTTATCTC-3′
Cacna1S	Forward 5′-CCTCACCCCAGGACGAGTT-3′
	Reverse 5′-CCTTAGCCTCCGGGTCGTA-3′
Orai1	Forward 5′-CATGGTAGCGATGGTGGAAG-3′
	Reverse 5′-GTTGCTCACAGCCTCGATGT-3′
GAPDH	Forward 5′-TGCCAGCCTCGTCCCGTAGAC-3′
	Reverse 5′-CCTCACCCCATTTGATGTTAG-3′

Western Blot Analysis

Isolated BMMs were plated on 60-mm dishes at a density of 1 x 10^6 cells. Following incubation under stated conditions, each sample underwent lysis in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors, and collected whole-cell lysates were cleared by centrifugation at 14,000 x *g* for 10 min at 4ºC. Protein expression was evaluated in the resulting supernatants. Proteins in total lysates were separated by size by SDS-PAGE and transferred onto PVDF membranes. Membranes were incubated with mAb NFATc1 (1:1,000), pAb phospho-PLCγ1 (1:1,000), mAb total-PLCγ1 (1:1,000), and mAb β-actin (1:2,000) overnight, and immunoreactive proteins were detected by means of an ECL detection system on the following day.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined with the Ca^{2+}-sensitive fluorescence dye Fura2, as described previously (Kim *et al.*, 2010). Briefly, isolated BMMs were plated on cover glass at approximately 80% confluence (6 x 10^5 cells/35-mm dish) and cultured in αMEM medium. After 2 days of RANKL stimulation, cells plated on cover glass were used as samples for $[Ca^{2+}]_i$ measurement. Cells were transferred from culture medium containing RANKL and M-CSF to HEPES buffer containing 10 mmol/L HEPES, 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, and 10 mmol/L glucose, adjusted to pH 7.4 and 310 mOsm, and loaded with Fura-2 fluorescent Ca^{2+} indicator for 50 min at room temperature and placed in a chamber connected to a perfusion system. Cells were briefly washed out with regular HEPES buffer and continuously perfused with HEPES buffer excluding RANKL and M-CSF. Each of the indicated compounds was diluted in HEPES buffer or Ca^{2+}-free HEPES buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, and 10 mmol/L glucose, adjusted to pH 7.4 and 310 mOsm) and perfused for a designated length of time. Under continuous perfusion with regular HEPES buffer (37ºC), intracellular fluorescence was excited at dual wavelengths (340, 380 nm), and emitted fluorescence (510 nm) was captured with a CCD camera. Captured images were digitized and analyzed with MetaFluor software (Molecular Devices, LLC, Sunnyvale, CA, USA), with data expressed as a ratio of fluorescence intensities (F_{340} / F_{380}).

Real-time Quantitative PCR

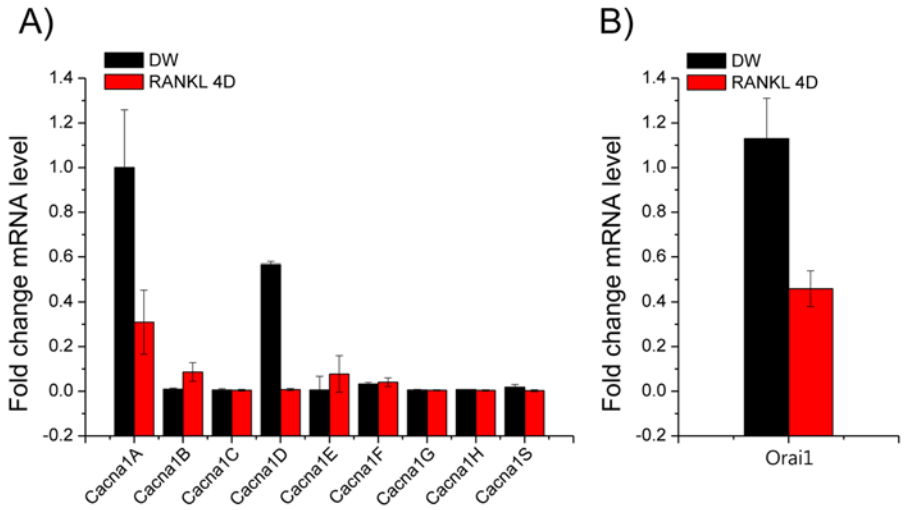
Cultured BMMs, as described in "*In vitro* osteoclast formation", were maintained in RANKL-containing culture media for 4 days. As negative control, the same volume of DW was treated. Following incubation, total RNA was extracted with Trizol (Invitrogen). A 1-μg quantity of the total RNA was transcribed to first-strand cDNA with random hexamers. Real-time PCR was performed with the VeriQuest SYBR Green qPCR master mix (Affymetrix, Santa Clara, CA, USA) and StepOnePlus Real-Time PCR systems (Applied Biosystems, Foster City, CA, USA). Results were normalized to GAPDH (housekeeping gene). Relative quantitation was performed by the comparative $\Delta\ \Delta C_t$ method, according to the manufacturer's instructions. Primers used in this study are listed in the Appendix Table.

Measurement of Osteoclast Function (pit assay)

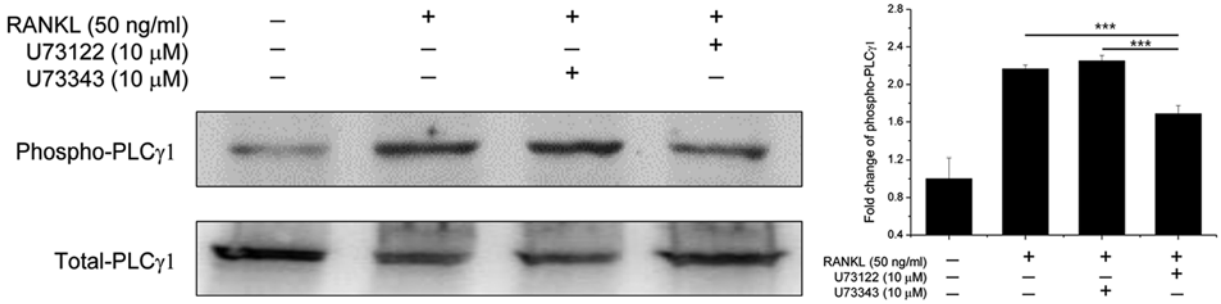
Isolated BMMs were seeded onto an Osteo assay plate (Corning, NY, USA) coated with a proprietary hydroxyapatite mineral surface. BMMs were maintained for 5 days under indicated conditions. Following incubation, cells were washed out with sodium hypochlorite solution. Pits formed on the surface were measured and calculated with ImageJ software (NIH, Bethesda, MD, USA).

Abbreviations

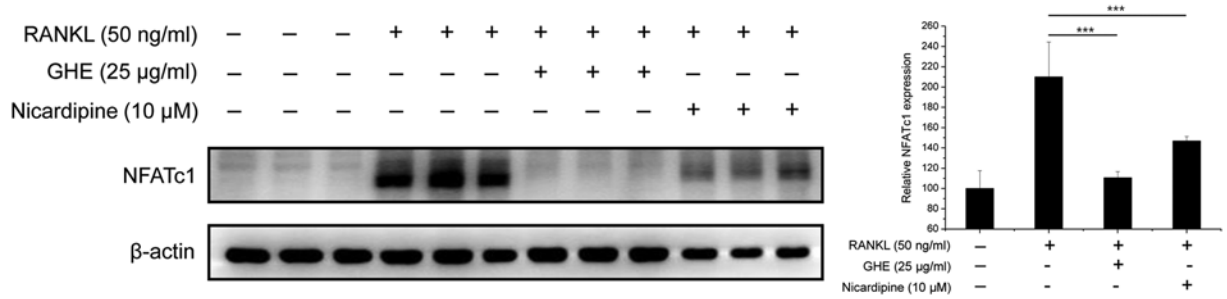
$[Ca^{2+}]_i$, concentration of intracellular Ca^{2+}; $[K^+]_e$, extracellular K^+ concentration; α-MEM, minimum essential medium α; AP, activator protein; BMM, bone-marrow-derived macrophages; CaMKIV, Ca^{2+}/calmodulin-dependent protein kinase IV; COX2, cytochrome c oxidase subunit II; DAP12, DNAX-activating protein 12; EGTA, ethylene glycol tetraacetic acid; ER, endoplasmic reticulum; FcRγ, Fc receptor common γ subunit; GH, *Glechoma hederacea*; GHE, *Glechoma hederacea* ethanol extract; IFNγ, interferon γ; IL, interleukin; iNOS, inducible nitric oxide synthase; IP_3, inositol 1,4,5-triphosphate; IP_3R, inositol 1,4,5-triphosphate receptor; ITAM, immunoreceptor tyrosine-based activation motif; LPS, lipopolysaccharides; M-CSF, macrophage colony-stimulating factor; MAPK, mitogen-activated protein kinase;



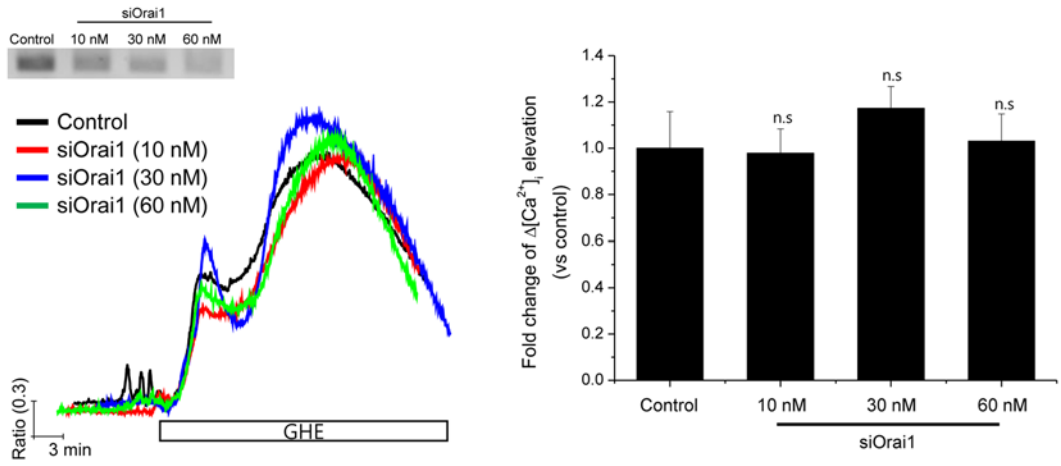
Appendix Figure 1. Endogenous expression of α-subunits of VGCCs and Orai1. Total RNA was extracted from cultured BMMs that were stimulated with RANKL (50 ng/mL) and then maintained for 4 days. DW-treated BMMs were used as the control group and presented as DW. The expression of α-subunits of VGCCs **(A)** and Orai1 **(B)** was evaluated by real-time PCR. Results were normalized with GAPDH and described as fold change of mRNA levels. Data are expressed as the mean ± SD and are representative of at least 3 independent experiments.



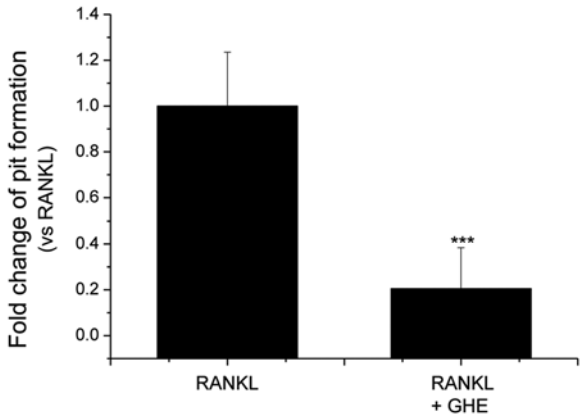
Appendix Figure 2. Validation of the inhibitory effects of U compounds on PLCγ1 activation. BMMs were cultured under indicated conditions for 2 days. Total proteins were collected and used to validate inhibitory effects of U compounds on phosphorylation of PLC γ1. Data are expressed as the mean ± SD and are representative of at least 3 independent experiments.



Appendix Figure 3. Suppression of RANKL-mediated NFATc1 expression by nicardipine. BMMs were prepared from 3 different mice and then cultured under indicated conditions for 4 days as described in "*In vitro* osteoclast formation". Total protein was collected and used to detect NFATc1 expression. Data are presented as relative value compared with the control group, which was treated with only vehicles of RANKL, GHE, and nicardipine (DW, EtOH, and DMSO), and are expressed as the mean ± SD.



Appendix Figure 4. Effects of knock-down of Orai1 in GHE-mediated $[Ca^{2+}]_i$ elevation. Small interfering RNA (synthetic dsRNA) of Orai1 (NM_175423; sense 5′-GUCAAGUUCUUACCUCUCA-3′, antisense 5′-UGAGAGGUAAGAACUUGAC-3′) was delivered into BMMs with Lipofectamine 2000 and then incubated for the next 2 days. Following incubation, RANKL was treated and GHE-mediated $[Ca^{2+}]_i$ was measured after 2 days of RANKL stimulation. The maximal $[Ca^{2+}]_i$ levels reached following GHE are presented as relative values compared with those in the control group transfected with scrambled siRNA and are expressed as the mean ± SD. The efficacy of the siRNA was determined by RT-PCR.



Appendix Figure 5. Effects of GHE on osteoclast function. Isolated BMMs were plated on hydroxyapatite-coated culture dishes and cultured for 5 days under the indicated conditions. Data were normalized to the total pits in the control group treated with RANKL and EtOH (vehicle of GHE) and are expressed as the mean ± SD.

NF-κB, nuclear factor-κB; MITF, microphthalmia-associated transcription factor; MNC, multinucleated cells; NFATc1, nuclear factor of activated T-cells; NO, nitric oxide; PLCγ, phospholipase C γ; PMCA, plasma membrane Ca^{2+} ATPase; RANK, receptor activator of nuclear factor κ-B; RANKL, receptor activator of nuclear factor κ-B ligand; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; SOCC, store-operated Ca^{2+} channels; TNFα, tumor necrosis factor α; TRAF, tumor necrosis factor receptor-associated factor; TRAP, tartrate-resistant acidic phosphatase; and VGCC, voltage-gated Ca^{2+} channels.

APPENDIX REFERENCES

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