Hypertonic stress as novel modulator of 1α,25$(OH)_2D_3$-induced osteoclastogenesis in osteoblast and osteoclast

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Abstract

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The bone remodeling is dependent on the balance between bone-forming osteoblasts and bone-resorbing osteoclasts. During the bone resorption, activated osteoclasts induce extracellular Ca^{2+}/PO_4^{2-} concentration to reach up to 40mM and degrade organic materials in the extracellular fluid around bone cells. In preliminary experiment, it was found that the hypertonic osmolality inhibited 1α, 25$(OH)_2D_3$-induced osteoclastogenesis using TRAP staining. In hypertonic conditions, the expression of RANKL and its mRNA were decreased in dose-dependent manner, but no change of the expression of OPG and M-CSF was seen using RT-PCR and ELISA assay. To demonstrate the effect of

hypertonic osmolality on bone metabolism at the molecular level. This study investigated Ca^{2+}-regulated signaling and the change of intracellular Ca^{2+} concentration by hypertonic stress. Hypertonicity decreased the expression of calmodulin-sensitive protein kinase (CaMK Ⅱ, Ⅳ) with dependent manner of the activity of L-type VSCCs and inhibited increase of intracellular Ca^{2+} concentration in the osteoblast. To know the mechanism of how RANKL specifically effects on oteoclast differentiation in hypertonic condition, we took screening approach to RANKL signal molecules including MAPK(ERK, JNK, p38), NF-κB, and NFATc1. The results show that hypertonic osmolaliry blocked the activation of MAPK activity, and NF-κB. Also hypertonic osmolaliry inhibited NFATc1 expression and RANKL-induced Ca^{2+} ocillation in BMMs. Conclusively, hypertonic osmolaliry inhibited RANKL expression via decrease of CaMKⅡ activity in osteoblast and blocked the activation of MAPK, NF-κB, and NFATc1 in RANKL-induced osteoclast differentiation signaling. The decrease of RANKL-induced Ca^{2+} ocillation by hypertonic stress induced the inhibition of NFATc1. Hypertonic osmolaliry may be a novel candidate for the regulation of bone metabolism through bone resorption.

Key word : 1α, $25(OH)_2D_3$-induced osteoclastogenesis, RANKL, NFATc1, Ca^{2+} signal, CaMKⅡ(Ⅳ)

I. Introduction

Bone confers multiple mechanical and metabolic functions to the skeleton and undergoes a process termed remodelling that involves bone-resorption and bone-formarion, but this mechanism is not fully understood when one goes up or down and the other usually follows. The bone remodeling is dependent on the balance involved the coordinate regulation and interaction of the component cell types: bone- forming osteoblasts and bone-resorbing osteoclasts. The function of osteoblasts and osteoclasts are intimately linked. The osteoblast lineage synthesize and secrete molecules that in turn initiate and control osteoclast differentiation.[8,13,16] The osetoclast is a tissue-specific macrophage polykaryon to be created by the differentiation of monocyte/macrophage precursors cells at or near the bone surface.[30] For the differentiation of osteoclast progenitor cells into mature osteoclasts, a cell-to-cell interaction between osteoclast precursors and osteoblastic/stromal cells have been demonstrated to be necessary.[17] The essential signaling molecules of the cell-to-cell interaction include RANKL (receptor activator of NF-κB ligand, also known as OPGL and TRANCE) and M-CSF (macrophage colony stimulating factor).

M-CSF, which is imperative for macrophage maturation, binds to its receptor, c-Fms, on early ostoeclast, thereby proceding signals required for their survival and proliferation. RANKL is

also important in osteoclast differentiation. RANKL is a membrane-bound protein of the tumor necrosis factor ligand family that is expressed on the oteoblast cell surface and has been shown to play a major role in osteoclast differentiation along with M-CSF.[16] RANKL binds to its receptor RANK [21] on hematopoietic cells and initiates a cascade of signaling events that leads to osteoclast differentiation.[23] Furthermore, osteoblast cells also secrete a glysoprotein called osteoprotegerin (OPG) [27], a soluble "decoy" receptor of RANKL and prevents its interaction with the cognate receptor RANK[3,33,34]. OPG has been shown to be a potent inhibitor of osteoclast differentiation, survival and function *in vitro* and bone resorption *in vivo* [1,5,12].

Bone resorption leads to the localized degradation of fully mineralized bone matrix, including removal of both inorganic and organic matrix components [24]. The degradation of mineral part of matrix precedes the degradation of organic matrix, which mostly takes place in extracellular resorption lacunae [15]. During osteoclastic bone resorption, crystal hydroxyapatite is dissolved into free Ca^{2+}/PO_4^{2-} ions in the resorptive hemivacuole where it can reach concentration as high as 40 mM [26,36]. High extracellular Ca^{2+} results in the inhibition of bone resorption [37,38] and induction of osteoclast apoptosis [20]. On the other hand, when bone is actively resorbed, it might be possible that high osmolality is provided due to the high concentration of Ca^{2+}/PO_4^{2-} and the degraded organic materials in the extracellular fluid around bone

cells. Under these high osmotic conditions, the functions of bone cells could be changed through the cell shrinkage. It can be assumed that there are two pathways to arrest osteoclastogenesis; one is regulated by high concentration of Ca^{2+}, which induces apoptosis of osteoclst . The other one is regulated in response to hypertonic stress caused by degraded orgamic and mineral materials, which affect bone cells metabolism. We hypothesized that the hypertonic stress in bone could be one of the regulating factors for bone remodeling. This study use sucrose to make hypertonic condition and apply 1α, 25-dihydroxyvitamin D_3 (1α, 25$(OH)_2D_3$) to stimulate osteoclast formation. To investigate the effect of hypertonic stress on bone metabolism, it is necessary of study at the molecular level of osteoblast and osteoclast., The hypothesis suggested that hypertonic stress might concern 1α, 25$(OH)_2D_3$- induced osteoclastogenesis in bone cell; osteoblast and osteoclast.

To demonstrate whether hypertonic stress affect the osteoclastogenesis, The author applied hypertonic stress to an osteoblast/stromal cells culture system. Resorption is regulated through OPG and RANKL expression by osteoblastic cells and is altered by vatious osteotropic factors that alter plasma membrane permeability to Ca^{2+}, including 1α, 25$(OH)_2D_3$ or ionophores [14]. 1α, 25$(OH)_2D_3$ activates rapid, plasma membrane-initiated signaling events and longer-term nuclear receptor-mediated pathways in osteoblastic cells by different receptor pathways [10.11]. 1α,

$25(OH)_2D_3$ activation of the plasma membrane signaling system changes the functional properties of voltage-sensitive Ca^{2+} channels (VSCCs) and alters the expression and activity of protein kinases [6,22,25,32]. Application of 1α, $25(OH)_2D_3$ increases plasma membrane permeability to Ca^{2+} within milliseconds by shifting the threshold of activation toward the resting potential and increase the mean open time of L-type VSCC [18]. Spontaneous and hormonally regulated opening of Ca^{2+} channels leads to localized elevations of intracellular Ca^{2+} that directly control that release of secretory vesicles [2]. Ca^{2+} influx also directly or indirectly influences the expression and activity of intracellular protein kinases, including protein kinase A (PKA), Ca^{2+}/ calmodulin-dependent protein kiase (CaMK), and MAPK [7,9,19,30,35].This study investigated the role of the intracellular Ca^{2+} influx and expression of RANKL and OPG in osteoblastic cells under hypertonic condition and also tried to find the potential interaction between 1α, $25(OH)_2D_3$ and VSCC-regulated Ca^{2+} dependent signaling and CaMK.

To demonstrate whether hypertonic stress affect the RANKL induced osteoclastogenesis, we applied hypertonic stress to a bone marrow macrophage cells cultrure system. Activation of RANK by its RANKL leads to the expression of osteoclast specific genes during differentiation, the activation of resorption by mature osteoclasts, and their survival. The key prelimiminary step in RANK signaling is the binding of TNFR- associated

cytoplasmic factors (TRAFs) to specific domains within the cytoplasmic domain of RANK. TRAF2, -5, and -6 have all been shown to bind to RANK . Especially, activation of TRAF6 can be induced by RANK signaling cascades. RANK signaling is mediated by cytoplasmic factors that activate down-stream signaling cascades mediated by protein kinase which are induced during osteoclastogenesis and activation ; NF-κB (IKK), c-Jun N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK) and Src pathway. The NF-κB and MAPK (mitogen-activated protein kinase; JNK, p38, and ERK) is required for osteoclast differentiation. However, Scr protein is required for osteoclast survival, cytoskeletal rearrangements and motility (Boyle et al., 2003). Recently, nuclear factor of activated T cell (NFAT)-2 (or NFATc1) is also involved in mediating key signals induced by RANKL-RANK signaling [28]. RANKL stimulation results in the induction of Ca^{2+} oscillation, which would contribute to the sustained activation of NFATc1 via a calcineurin- dependent mechainsm. While the activated NFATc1 induces a number of genes involved in cell differentiation, NFATc1 also acts on its own gene to amplify the NRATc1-mediated transcriptional program. However, RANKL-induced Ca^{2+} oscillation initiates as late as 24h following the ligand stimulation, preceded by induction and activation of c-Fos and NF-κB, respectively (Takayanagi et al., 2002). To identify the espression of RANKL- induced NFATc1 and change

of Ca^{2+} oscillation in responce to hypertonic stress, Western blotting and Ca^{2+} flow cytometry were done in BMMS with presense of RANKL and hypertonic stress

II. Materials and Methods

1. Materials

Routine cell culture media was obtained from GIBCO/BRL(Grand Island, NY). The Tartrate-Resistant Acid Phosphatase Staining Kit was purchased from the Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Trizol was purchased from Invitrogen Corp. (Carlsbad, CA), and the ICR mice were from Koathech. Co., Ltd. (Kyung-gi-Do, Korea). Sucrose (α-D Glucopyranosyl β-D-fructofuranoside; Saccharose; cane sugar) and 1α, 25-dihydroxyvitamin D_3 (1α, 25$(OH)_2D_3$) were also purchased from the Sigma Chemical Co., Ltd. (St. Louis, MO, USA). All other chemicals were of reagent grade. The osmolalities of all media and solutions were measured with a FISKE ONE-TEN Osmometer (FISKE ASSOCIATES, USA).

2. Preparation of primary osteoblastic cells

Primary osteoblastic cells were isolated from calvariae of ICR newborn mouse (Koathech Inc., Kyung-gi-Do, Korea) for 1 day by a previously reported method [Takahashi *et al.*, 1988; Choi, B. K. *et al.*, 2001] with a slight modification. This calvariae were digested four times for 20 min, each time with an enzyme solution containing 0.1% collagenase (Sigma Chemical Co., Ltd., St. Louis, MO, USA) and 0.1% dispase (GIBCO-BRL, Life Technologies, Grand Island, NY, USA) in shaking water bath at

37℃. The first digestion was discarded, and then after second digestion was collected carefully. This digestion was centrifuged 1,100 rpm for 5 min and was transferred to a fresh α-minimum essential medium (α-MEM) (GIBCO-BRL) containing 10% fetal bovine serum (GIBCO-BRL, Life Technologies), 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotricin B(GIBCO-BRL). The osteoblastic cells were cultured in 100 mm culture dishes for 4 days at 37℃ in humidified atmosphere containing 5% CO_2 in air.

3. Preparation of bone marrow macrophage cells

Bone marrow macrophage cells (BMMs) were isolated from mice long bones. The tibiae and femora of 4-week-old ICR mouse were separated and dissected free of adhering tissues. Both edges of these bones were removed so as to inject new media. After gathering of that solution, it was seeded on 100 mm dishes and cultured for 1~2 days in α-MEM containing 10% FBS, 30ng/ml M-CSF (KOMA Biotech. Inc., Kyung-gi-Do, Korea). The non-adhesive cells were moved to new dishes and cultured for few days in presence of 50ng/ml RANKL (KOMA Biotech. Inc., Kyung-gi-Do, Korea) and 50ng/ml M-CSF.

4. Co-culture of mouse BMMs and osteoblasts

Each preparation of BMMs (1×10^5) and osteoblasts (1×10^4) were seeded on each well of 48-well plates and cultured for 6

days including 10^{-8} M 1α, $25(OH)_2D_3$. After incubation for 6 days, the cells were subjected to tartrate-resistant acid phosphatase (TRAP, an osteoclast marker enzyme [Minkin C., 1982]) staining. using an acid phophatase kit (Sigma Chemical Co., Ltd.). TRAP positive multinucleated (more than three) cell was counted as osteoclast. *In vitro* formation assay of osteoclast was repeated four times.

5. Assessment of cell viability

Cell viability was determined by the conventional MTT assay [Mosmann T., 1983; Choi, B. K., *et al.*, 2001] with slight modification. The MTT (3-[4, 5-dimethylthiazol-2-yl-]-2, 5-di-phenyltetrazolium bromide) test is based on the principle that tetrazolium salts are reduced by reducing mitochondrial enzymes (succinate, and dehydrogenase), which allows the toxicity of viable cells and the level of cellular differentiation to be measured. The MTT was dissolved in phosphate-buffered saline (PBS) at 5 mg/ml and filtered to remove any insoluble residue. The MTT solution was added directly to the assay plates. The cells were subsequently incubated for an additional 4 hours at 37℃. The purple formazan crystals that formed were dissolved in dimethyl solfoxide (DMSO) at room temperature for 20 minutes and was diluted with suitable volume of DMSO in 96-well plates. The optical density of the formazan solution was measured on a spectrophotometer at 570 nm.

6. RT-PCR (Reverse Transcriptase-Polymer Chain Reaction)

The expressions of RANKL, OPG, M-CSF and -actin were evaluated by RT-PCR using total RNA isolated from murine osteoblastic cells. Primary osteoblastic cells were seeded in 35 mm culture dishes (1×10^4 cells/dish) and grown to 2 days with 10^{-8} M 1α, $25(OH)_2D_3$ and sucrose. Total RNA was isolated using Trizol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacture's instructions. The concentration of the RNA obtained was determined by measuring the absorbance at 260 and 280 nm. RT-PCR for RANKL, OPG, and β-actin mRNA was carried out with a commercial two-step RNA-PCR kit (ELPIS bio.). To synthesize for the cDNA, each reaction tube contained 1 μg of mRNA, 1μl of Random octamers (40 μM), 8μl DEPC treated water, 2μl DTT (100 mM), and 8μl RT & GoTM MasterMix 2.5×C and reacted 5 min at 95℃, and 60 min at 45℃. Polymerase chain reactions were performed on a thermal Cycler 96 PCR machine (BIO-RAD, MyCyclear TM Thermical Cycler, USA) using 1 ㎍ of cDNA (complementary DNA), 2 pmoles each oligonucleotide primer, 200 M of each dNTP, 5μl of Taq & GoTM MasterMix 5×C and 10× Taq polymerase in a 20 μl volume. The primers used were: for RANKL (750 bp), 5′-ATCAGAAGACAGCACTCACT -3′(forward), 5′-ATCTAGGACATCCATGCTAATGTTC-3′ (reverse); for OPG (636bp), 5′-TGAGTGTGAGGAAGGGCGTTAC-

3′(forward) 5′-TTCCTCGTTCTCTCAATCTC-3′ (reverse) ; for β-actin (366 bp), 5′-GGACTCCTATGGTGGGTGACGAGG-3′ (forward), and 5′-GGGAGAGCATAGCCCTCGTAGAT-3′(reverse); for M-CSF (395 bp), 5′-CATGACAAGGCCTGCGTCCGA-3′ (forward), and 5′-AAGCTCTGGCAGGTGCTCCTG-3′ (reverse 1), 5′-GCCGCCTCCACCTGTAGAACA- 3′ (reverse 2). The PCR program initially started with a 95℃ denaturation for 5 min, followed by 35 cycles of 95℃ /1 min, T_a /1 min, 72℃/1 min (T_a, annealing temperature; 48℃ for RANKL, 50℃ for OPG, and 55℃ for β-actin and M-CSF). The PCR samples were electrophoresed on 1.5 % agarose gels in TAE (Tris-acetate-EDTA electrophoresis) buffer. The gels were stained with ethidium bromide [10 μg/ml] and photographed on top of a 280 nm UV light box. The quantity and base pair size of the PCR generated DNA fragments were estimated relative to DNA ladder standards. Densitometry values were measured at each cycle sampling using the TINA software (University of Manchester, Manchester. U.K.). RT-PCR values are presented as a ratio of the specified gene′s signal in the selected linear amplification cycle divided by the β-actin positive control signal.

7. ELISA (Enzyme Linked Immuno-Solbent Assay)

Quantikine M murine Mouse RANK Ligand kit and OPG kit (R & D systems Inc., Minneapolis, IN) were used to analyze RANKL and OPG protein. Briefly, mouse soluble RANKL

standard was diluted in Calibrator Diluent RD6-12 solution to make final concentration of 0, 31.2, 62.5, 125, 250, 500, 1000, and 2000 pg/ml. Assay Diluent RD1W, standards, and samples (50 µl each) were added to each well and incubated for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed. Each well was aspirated and washed, repeating the process four times for a total of five washes. Mouse RANKL conjugate (100 µl) was added to each well and incubated for 2 hours at room temperature. Washing was repeated as described above. Substrate solution (100 µl) was added to each well and incubated for 30 minutes at room temperature in dark room. Stop Solution (100 µl) was added to each well and mixed by gentle tapping. Then the enzyme reaction yields a blue product that turns yellow. The Intensity of the color of each well was determined within 30 minutes, using a microplate reader at 450 nm.

8. Whole cell protein extraction and Western blotting

The osteoblasts and BMMs were washed with chilled 1X DPBS, and resuspended in lysis buffer (10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1% NP-40, 1 mM EDTA, and 0.1% SDS). Protease and phosphatase inhibitors were added to the lysis buffer (2 mM Na_3VO_4, 10 mM NaF, and 10 µl/ml aprotinin, 10 µl/ml leupeptine, and 10 µl/ml PMSF). The samples were then centrifuged at 12,000 rpm for 20 min at 4℃, and the supernatant

protein concentration was measured by the BCA protein assay. Protein extracts were loaded (50 μg/lane) onto a sodium deoxyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system (BIO-RAD laboratories, Hercules, CA.). Following electrophoresis, proteins were transferred to a nitrocellulose membrane using a Bio-Rad wet transfer system. Protein transfer efficiency and size determination were verified using pre-stained protein markers (Intron Co., Korea). Membrane were then blocked with 5% non-fat milk for an hour at room temperature and subsequently incubated for overnight at 4℃ with diluted antibodies directed against MAPK (ERK, JNK, and p38 : Cell signaling, UK), NF-κB p65, NFATc1, phospho-α-CaMKⅡ and β-actin (Santa cruz Biotechnology, CA). After washes, this bolt incubated with the horseradish peroxidase-conjugated secondary antibodies and detected using an enhanced chemiluminescence detection kit (ECL, Amersham Phamacia Biotech, Inc., Alington Heights, IL, USA).

9. Immunofluorescence staining

BMMs were cultured on cover slips for 48h treated 50μg/ml RANKL, 50μg/ml M-CSF and sucrose. Cells were then washed with cold-1X DPBS and fixed with 4% paraformaldehyde for 20 min at 4℃ and treated with 0.2% Triton X-100 for 10 min at 4℃. Samples were sequentially incubated in blocking solution (0.1% gelatine, 1% BSA, 0.01% Sodium Azide, and 5% goat

serum) for an hour. The mouse- anti-NFATc1 monoclonal antibody was 1:100 diluted in blocking solution and applied to the samples for overnight incubation at 4℃. Fluorescence isothiocyanate-conjugated (FITC) secondary antibody (Jackson Immunoresearch Inaboratories Inc., Boltimore, USA) was then used for an hour. At the least 10 randomly selected microscopic fields were examined using X 20 objectives. Photo were taken with a LEICA, MZ FL III digital camera.

10. Intracellular Ca^{2+} measurement

For Ca^{2+} measurement, cells grown onto glass cover slips (22 mm × 22 mm) were incubated in PSS (138 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM Glucose, 10 mM HEPES, and 1.5 mM $CaCl_2$) involved 5μM of the 2-acetixymethyl ester derivative (membrane permeable) Fura-2/AM (Teflabs Inc., Austin, USA) and 0.05% Pluronic F127 (Invitrogen Corp., Carlsbad, CA) for 40 min at room temperature. Unloading dye was washed out. For fluorescence measurement, the coverslips containing dye-loaded cells were then mounted on the stage of an inverted microscope (Nikon, Japan) with microfluorometer (MetaFlour system) and maintained at 25~30 ℃. The excitation wavelength was switched over 340 and 380 mM. Emitted cellular fluorescence was collected at 510 mM and ratios from short and long wavelength signals were obtained (R=340/380) at 2 seconds interval using CCD camera (Photon Technology International Inc., Lawrenceville, NJ),

thus marking the measurement independent of variations in cellular dye content, dye leakage or photobleaching. Background fluorescence was subtracted from the raw signals at each excitation wavelength before calculating the fluorescence ratio.

11. Data analysis and statistics

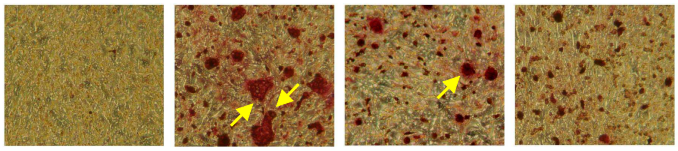
All experiments were reproduced at least three times. The results are expressed as the mean ± SEM. The statistical significances of differences between the groups were determined using the two-tailed Student *t* test. A *p* value of less than 0.05 was considered to be statistically significant.

Ⅲ. Results

1. Inhibition of 1α, 25(OH)$_2$D$_3$-induced osteoclast formation by hypertonic stress in co-culture system.

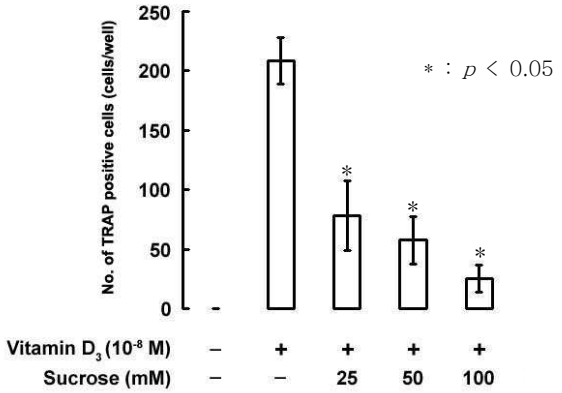
Osteoclastogenesis was induced by 1α,25(OH)$_2$D$_3$ in co-culture system with osteoblastic cells and bone marrow/macrophage cells. To clarify the role of hypertonic stress on bone metabolism, sucrose as hypertonic stresses (25, 50, 100, 150, and 200 mM) was added to co-cultures and incubated at 37℃ for 6 days to investigate the differentiation of osteoclast. When 10^{-8} M of 1 α,25(OH)$_2$D$_3$ was added to the co-culture, TRAP positive multinucleated cells were formed, whereas no TRAP positive cells were detected in media only. In the presence of sucrose, 1 α,25(OH)$_2$D$_3$ -induced osteoclast differentiation was inhibited (Figure. 1A). The addition of 50 mM of sucrose reduced the number of TRAP positive multinucleated cell up to about 60% (Figure. 1B). However, it might be possible that sucrose causes cell damage directly without interrupting the normal maturation of osteoclasts. To rule out this possibility, viability test was done by MTT test. As shown in Figure. 1C, sucrose did not show a toxic effect when treated at up to 100 mM. These results suggest that the effect of sucrose on formation of osteoclast is caused by hypertonic stress, not by the toxic effect of sucrose upon the cells.

A.



Control | Vitamin D_3 (10^{-8} M) | Vitamin D_3 (10^{-8} M) + Sucrose 25 mM | Vitamin D_3 (10^{-8} M) + Sucrose 50 mM

B.



C.

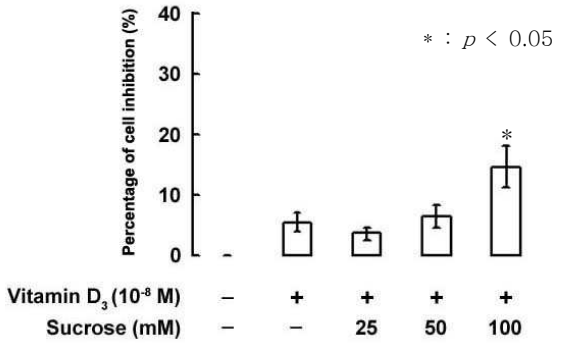


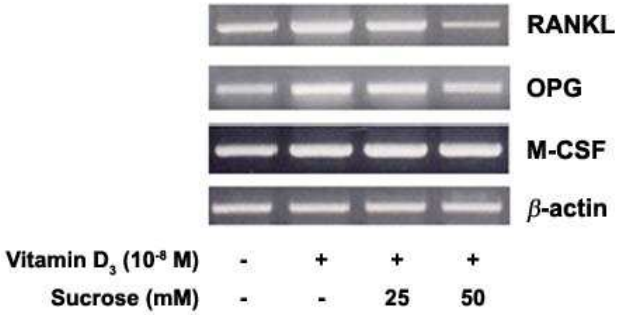
Figure 1. Hypertonic stress inhibited 1α,25(OH)$_2$D$_3$-induced osteoclast differentiation at dose-dependent concentration in co-culture system. (A) In the presence of sucrose, 10^{-8} M of 1α,25(OH)$_2$D$_3$ induced osteoclast differenciation was reduced (× 200). The yellow arrow indicates osteoclast cells. (B) TRAP-positive multinucleated cell containing three or more nuclei was counted as osteoclast. (C) MTT test result. One hundred mM of sucrose showed remarkable a high cell inhibition. The statistical significance of differences between the groups was determined using the two-tailed Student *t* test. In all statistical tests, a p value < 0.05 was considered to be statistically significant. * , significantly different ($p < 0.05$) in compared to 10^{-8} M of 1 α,25(OH)$_2$D$_3$ group. Each data was shown in mean ± SEM of four cultures.

2. Effects of hypertonic stress on RANKL and OPG expression in mouse calvarial osteoblasts.

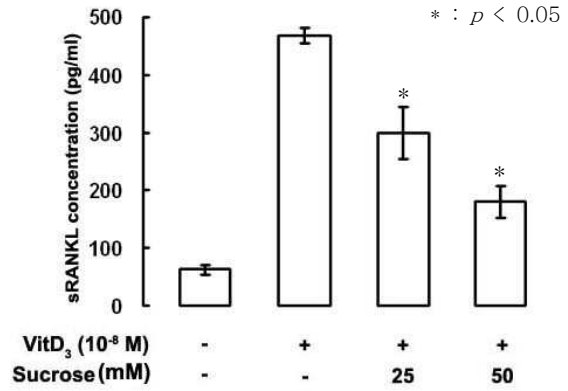
As shown in Figure 2A, the expressions of RANKL, OPG, and M-CSF mRNA in osteoblasts were monitored by RT-PCR in the presence and absence of sucrose. The β-actin mRNA was used for standardization for total RNA amounts which remaines unchanged. As the concentration of sucrose in the cell culture medium increased, the expression of 1α,25 $(OH)_2D_3$-induced RANKL mRNA was decreased with dose dependent manner . On the other hand, the expression of OPG and M-CSF mRNA were not changed . These findings indicate that sucrose (25, 50 mM) inhibits the formation of osteoclst by down-regulating the expression of RANKL.

RANKL and OPG were also analyzed with ELISA using anti-RANKL and anti-OPG antibodies. RANKL was decreased with the increase of sucrose concentration (Figure 2B). However, the addition of sucrose had no effect on the OPG quantitatively which is consistent with OPG mRNA data (Figure 2C). Consequently, the addition of sucrose inhibited RANKL mRNA and soluble RANKL, and led to altered osteoclastogenesis. In addition, such change of RANKL mRNA and soluble RANKL was dependent on the sucrose concentration.

A.


RANKL
OPG
M-CSF
β-actin
Vitamin D3 (10-8 M) - + + +
Sucrose (mM) - - 25 50

B.


* : p < 0.05
sRANKL concentration (pg/ml)
500
400
300
200
100
0
*
*
VitD3 (10-8 M) - + + +
Sucrose (mM) - - 25 50

C.

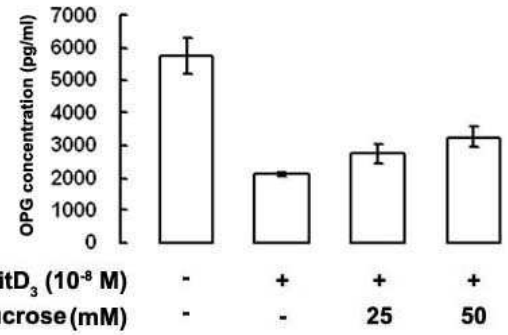

OPG concentration (pg/ml)
7000
6000
5000
4000
3000
2000
1000
0
VitD3 (10-8 M) - + + +
Sucrose (mM) - - 25 50

Figure 2. Hypertonic stress caused change in RANKL expression. (A) Various concentrations of sucrose were added to the mouse calvarial osteoblasts culture with 10 nM 1α,25$(OH)_2D_3$. After incubation for 2 days, total RNA was extracted, and the expression of RANKL, OPG, and M-CSF mRNAs were analyzed by RT-PCR. (B) The protein analysis using ELISA showed that sucrose inhibited the expression of soluble RANKL in osteoblasts. It was statistically significant. (C) On the other hand, OPG level was increased slightly in osteoblasts stimulated by sucrose, but it was not statistically significant. * , significantly different ($p < 0.05$) in compared to 10^{-8} M 1α,25$(OH)_2D_3$ group. The results were expressed as the means ±SEM of four experiments.

3. Identification of suppression of transient 1α,25(OH)$_2$D$_3$ -induced [Ca^{2+}]$_i$ in response to hypertonic stress in calvarial osteoblast.

From Figure 2 results, this study established that addition of sucrose inhibits osteoclast formation by down-regulating the expression of RANKL. In osteoblast, RANKL and OPG regulated Ca^{2+} influx across the plasma membrane. Application of 1α,25(OH)$_2$D$_3$ increases plasma membrane permeability to Ca^{2+} within milliseconds by shifting the threshold of activation toward the resting potential and increasing the mean open time of the L-type VSCC (Joel et al., 2004). In this reason, It can be assumed that hypertonic stress decreases the expression of RANKL in response to block the intracellular Ca^{2+} level in osteoblast.

To determine whether the hypertonic stress blocks 1 α,25(OH)$_2$D$_3$ -induced Ca^{2+} influx in osteoblast, The study measured intracellular Ca^{2-} level. As shown in Figure 3A, stimulation of calvarial osteoblastic cells with 10^{-8} M 1 α,25(OH)$_2$D$_3$ was raised transient [Ca^{2+}]$_i$ rapidly. However, the addition of 50 mM sucrose did not raise [Ca^{2+}]$_i$ (Figure 3B). As the addition of sucrose blocked 1α,25(OH)$_2$D$_3$-induced [Ca^{2+}]$_i$, hypertonic stress inhibited RANKL expression.

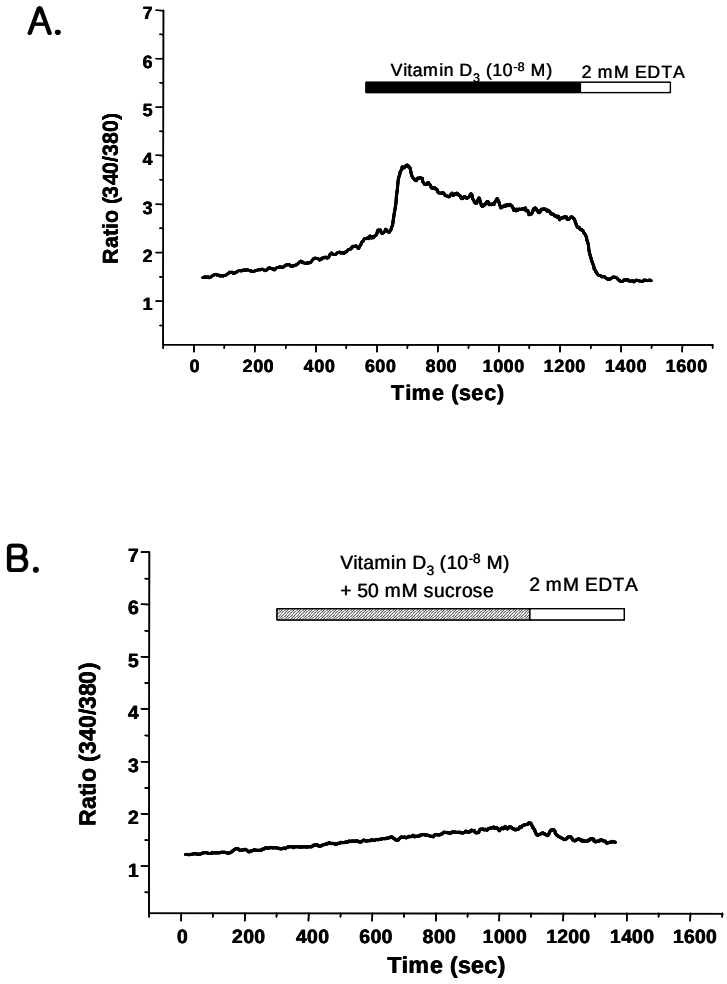


Figure 3. Intracellular Ca^{2+} level was changed by hypertonic stress in osteoblastic cells. After isolated osteoblasts for 4 days, osteoblasts (3 x 10^5 cell) were cultured onto glass cover slips at 37℃. Fura-2 loaded oseoblasts were incubated at room temperature for an hour. (A) Cells were treated with 10^{-8} M 1α,25$(OH)_2D_3$. Increase of $[Ca^{2+}]_i$ occurred rapidly at once. (B) In the presence of sucrose, $[Ca^{2+}]_i$ induced by 10^{-8} M of 1α,25$(OH)_2D_3$ was blocked. Note that hypertonic stress suppressed Ca^{2+} responses to 1α,25$(OH)_2D_3$.

4. Expression of Ca^{2+}/Calmoduline-dependent kinase II (CaMK II) in calvarial osteoblastic cells under the hypertonic condition.

Alternation in local intracellular Ca^{2+} levels regulates activity of several second messenger through activation or inactivation of protein kinase, including protein kinase K (PKA), Ca^{2+}/ calmoduline-dependent protein kinase (CaMK), and MAPK. Especially, Ca^{2+}/CaMK pathway is important and it regulates secretion of RANKL and OPG in osteoblast. Ca^{2+} influx occurring in response to 1α,25$(OH)_2D_3$ rapidly leads to inactivation of the osteoblast L-type VSCC by phosphorylated CaMKII in whole cell recordings (Bergh et al., 2004). This study suggested that inhibition of Ca^{2+} influx by hypertonic stress might decrease phosphorylation of CaMKII. It was decided to investigate whether phosphorylated CaMKII were inhibited by sucrose or not . In the result, activation form of CaMKII was increased in response to treatment 1α,25$(OH)_2D_3$ for 48 hr, and 72 hr, on the other hand, phosphorylated CaMKII was not increased with both 1α,25$(OH)_2D_3$ and 50 mM sucrose (Figure. 4). It shows that hypertonic stress might affect RANKL synthesis pathway with suppression of Ca^{2+} influx by inactivation of CaMKII in osteoblastic cells.

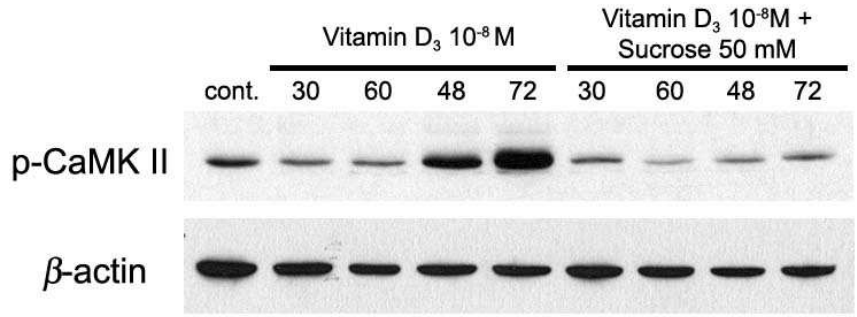


Figure 4. Hypertonic stress suppressed phosphorylation of 1α,25$(OH)_2D_3$-induced CaMKII in osteoblastic cells. Osteoblastic cells from nowbone mice calvariae were cultured 4 days and then treated with 10^{-8} M 1α, 25$(OH)_2D_3$ and/ or sucrose. After 4 days, the expression of activated CaMKII was analyzed using western blot. Anti-β-actin used for standardization for total protein amounts.

5. Inhibition of soluble RANKL-induced osteoclast formation by hypertonic stress in bone marrow macrophage cells.

Osteoclastogenesis was induced by soluble RANKL (sRANKL) in bone marrow macrophage (BMMs) cell culture. To clarify the role of hypertonic stress on osteoclast formation, RANKL and sucrose were added to cultures and incubated at 37℃ for 5 days. When 50 ng/ml of sRANKL was added to BMMs cell culture. TRAP positive multinucleated cells were formed, whereas no TRAP positive cell was detected in media only (Figure. 5A). In the presence of sucrose, sRANKL-induced osteoclast formation was reduced (Figure. 5B). However, it might be possible that hypertonic stress causes cell damage directly without interrupting the normal maturation of osteoclasts.

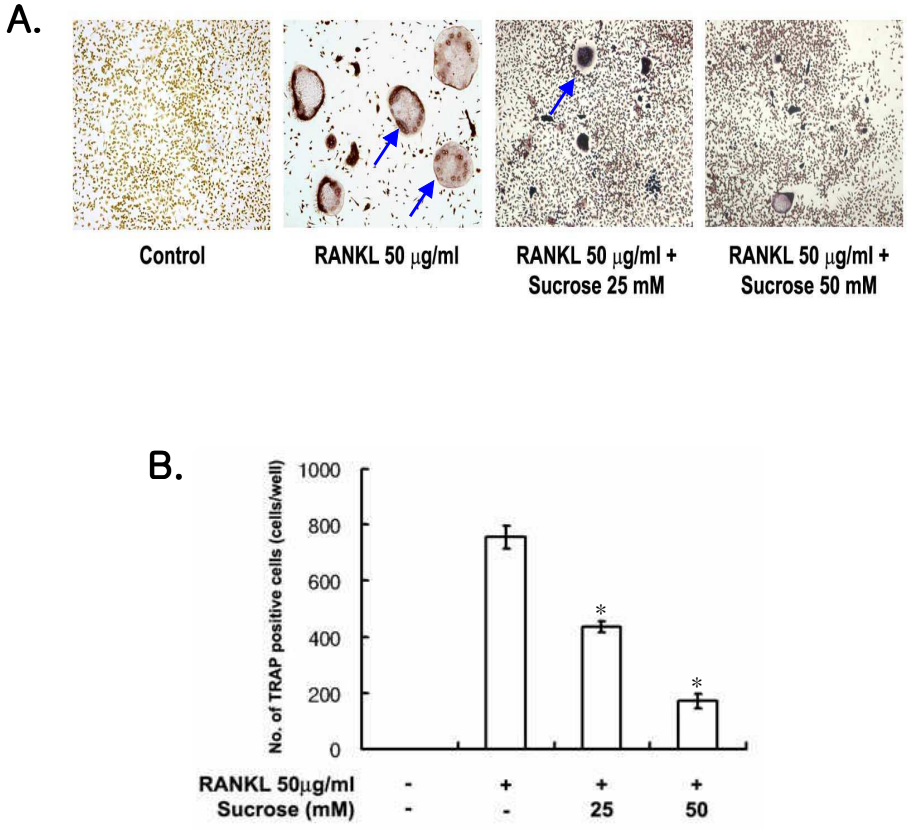
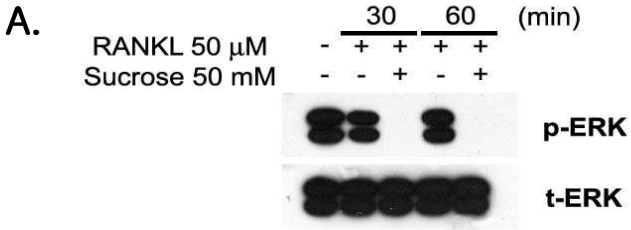
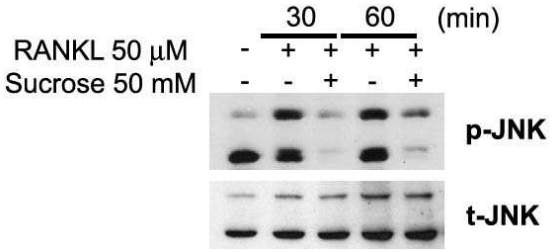


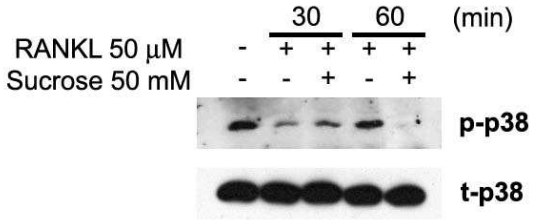
Figure. 5. Hypertonic stress inhibited sRANKL-induced osteoclastogenesis. (A) In the presence of hypertonic stress (in addition of sucrose), sRANKL-induced osteoclast differentiation was reduced (× 200). (B) TRAP-positive multinucleated cell containing three or more nuclei was counted as osteoclasts. The statistical significance of differences between the groups was determined using the two-tailed Student *t* test. In all statistical tests, a *p* value < 0.05 was considered to be statistically significant. * : significantly different. Each data was shown in mean ± SEM of four cultures.

6. Expression of MAPK and NF-κB in response to hypertonic stress in BMMs cells

It was demonstrated hypertonic stress is linked to the sRANKL-induced osteoclastogenesis in BMMs cell. In general, mitogen activated protein kinase (MAPK : ERK, JNK, and p38) and NF-κB are preferentially activated by cytokines and cellular stress, and play a key role in regulating the activity of various transcriptional factors. This study suggested that the hypertonic stress might act as a signal for various cellular functions in osteoclastogenesis. The phosphorylated MAPK were examined to investigate whether those kinases were inhibited by sucrose or not in BMMs. As a result, activatied MAPK by treatment with 50 μg/ml RANKL were inhibited in response to 50 mM sucrose (Figure. 6A). Also sucrose had a effect of the inhibition of NF-κB expression (Figure. 6B). It was shown that hypertonic stress might affect the expression of transcription factors which are associate with sRANKL- induced osteoclastogenesis.


A.
30
60
(min)
RANKL 50 μM - + + + +
Sucrose 50 mM - - + - +
p-ERK
t-ERK


30
60
(min)
RANKL 50 μM - + + + +
Sucrose 50 mM - - + - +
p-JNK
t-JNK


30
60
(min)
RANKL 50 μM - + + + +
Sucrose 50 mM - - + - +
p-p38
t-p38

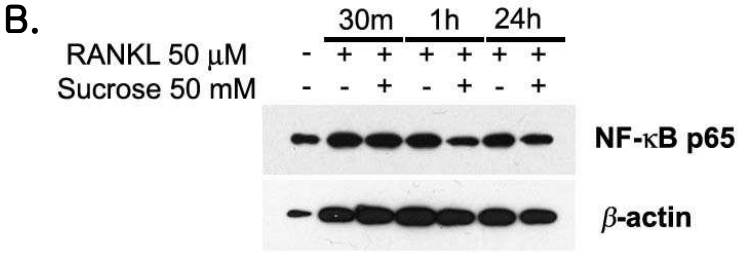

B.
30m
1h
24h
RANKL 50 μM - + + + + + +
Sucrose 50 mM - - + - + - +
NF-κB p65
β-actin

Figure. 6. Hypertonic stress affects the activation of MAPK and NF-κB in BMMs cells. (A) BMM cells (2 x 10^6cells) were treated with 50ng/ml sRANKL and 50ng/ml M-CSF in presence with 50 mM of sucrose for the indicated periods of time. After cells are lysed, Cell lysates were then subjected to Western blot analysis with a polyclonal antibody against phosphorylated MAPK (phospho-ERK, phospho-JNK, and phospho-p38) and nonphosphorylated MAPK (ERK, JNK, and p38). (B) Western blot analysis with a monoclonal antibody against NF-κB p65.

7. The effect of hypertonic stress on the expression of NFATc1 in BMMs.

The NFATc1 may represent a master regulator of RANKL-induced osteoclast differentiation, switching on the transcriptional program for the terminal differentiation of osteoclast, wherein the autoamplication of NFATc1 intimately participates in sustaining the program (Takayanagi et al., 2004). It was expected hypertonic stress might inhibite the expression of NFATc1 and decrease osteoclast formation in BMMs .

To identify the hypothesis, The author examined the expression and cellular localization of the NFATc1 protein in RANKL-stimulated BMMs. The NFATc1 protein expression level was increased in RANKL-stimulated BMMs for 48h. However, when BMMs were treated with 50 mM sucrose and RANKL for 48h, NFATc1 protein expression level was decreased (Figure 7A). As shown in Figure 7B, immunofluorescence straining revealed that the increased NFATc1 protein by RANKL was translocated from cytosol to nuclear at 48h, but was not detectable in BMMs after 50 mM sucrose and RANKL stimulation at 48h. Consistent with this results, hypertonic stress reduced osteoclastogenesis by inhibition of NFATc1 protein level.

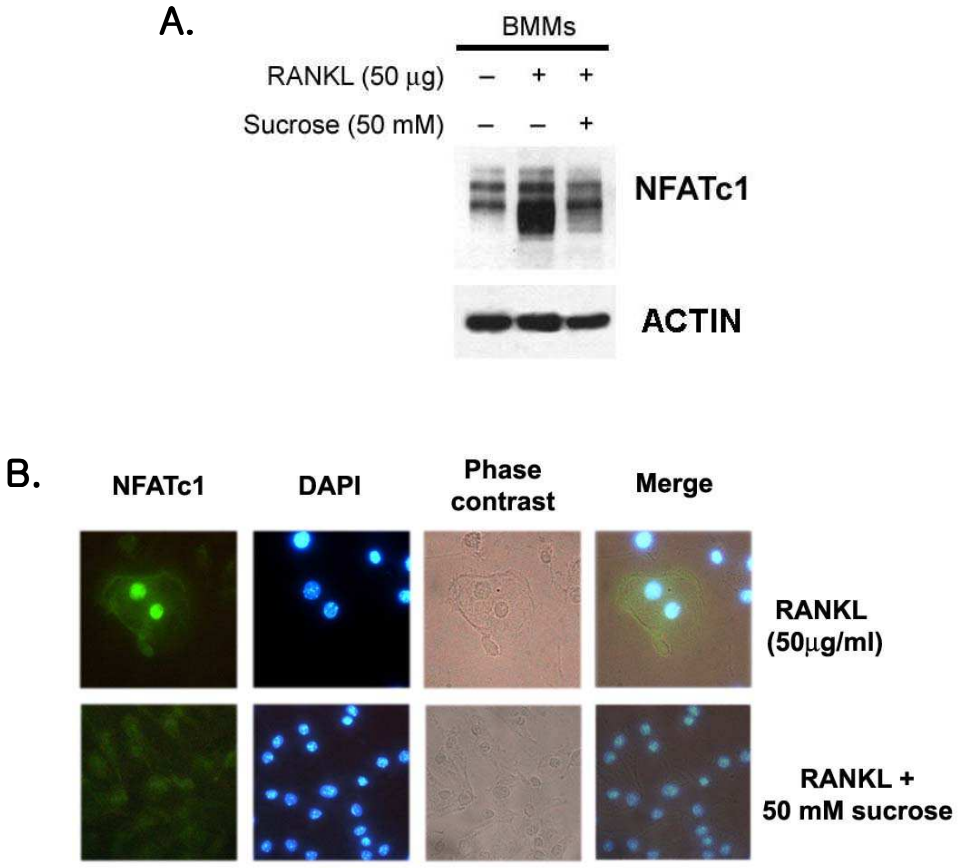


Figure. 7. Expression of NFATc1 protein during osteoclast differentiation in presence of hypertonic stress. (A) BMMs (2 x 10^6cells/culture) were treated with 50 μg/ml sRANKL and 50 mM of sucrose for 2 days, and cells were lysed. Cell lysates were then subjected to Western blot analysis with a monoclonal antibody against NFATc1. (D) Immunofluorscense staining of NFATc1 protein in BMMs stimulated with RANKL/M-CSF and hypertonic stress.

8. The effect of hypertonic stress on induction of Ca^{2+} oscillation in RANKL-stimulated BMMs.

The activities of transcription factors of the NFAT family are regulated by the Ca^{2+}/ calmoduline-dependent calcineurin, a serine/threonine phosphatase, which allows nuclear translocation of these transcription fators (Takayanagi et al., 2004). Since NFATc1 induced by RANKL undergoes efficient nuclear translocation, one may envisage the Ca^{2+} - dependent activation of calcineurin by RANKL. A sustained Ca^{2+} oscillation was observed in BMMs stimulated by RANKL (Figure. 8A). The RANKL-induced Ca^{2+} oscillation initiated as late as 24hr after RANKL stimulation, that is, when NFATc1 induction becames notable; the oscillation was sustained thereafter, provide that RANKL was present (Hiroshj et al., 2004). However, hypertonic stress blocked the RANKL- induced Ca^{2+} oscillation (Figure 8B). This observation suggests that RANKL-induced Ca^{2+} oscillation is necessary for the sustained NFATc1 activation during osteoclastogenesis and the hypertonic stress blocks RANKL-induced Ca^{2+} oscillation by inhibition of NFATc1 protein expression.

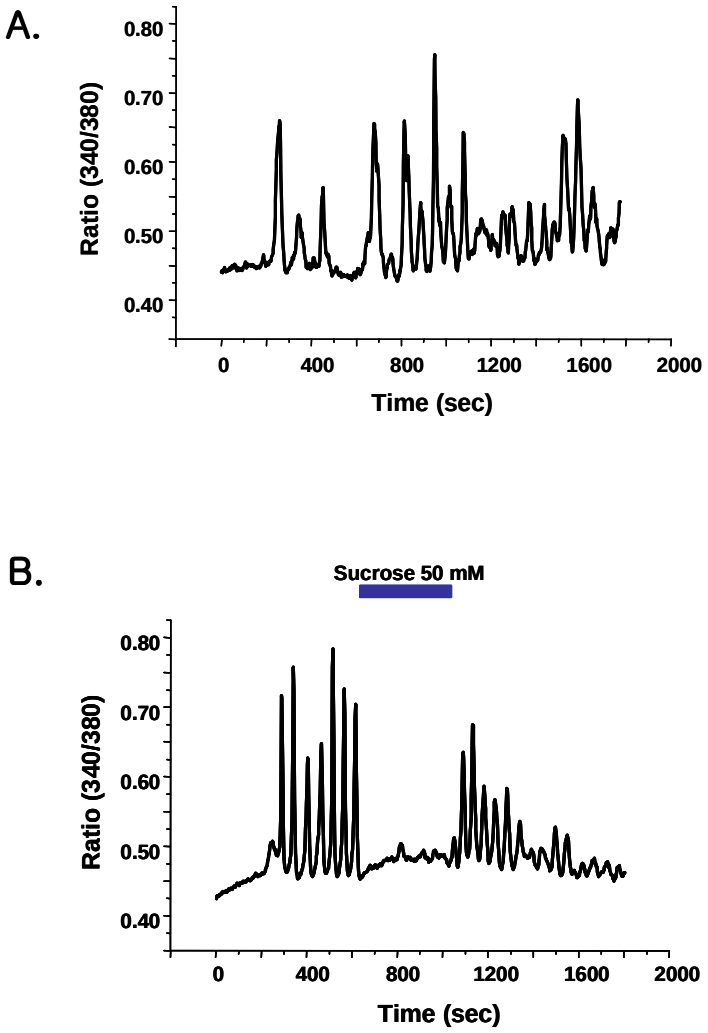


Fig. 8. Inhibition of RANKL-induced Ca^{2+} signaling in response to hypertonic stress in BMMs. (A) Ca^{2+} oscillation was observed in RANKL-treated BMMs which were incubated with RANKL in the presence of M-CSF for 48h. (B) RANKL-induced Ca^{2+} oscillation were inhibited by hypertonic stress.

Ⅳ. Discussion

Bone resorption leads to the localized degradation of fully mineralized bone matrix, including removal of both inorganic and organic matrix components [24]. The degradation of mineral part of matrix precedes the degradation of organic matrix, which mostly takes place in extracellular resorption lacunae [15]. During osteoclastic bone resorption, crystal hydeoxyapatite is dissolved into free Ca^{2+}/POas high as 40 mM [36]. High extracellular Ca^{2+} results in the inhibition of bone resorption [37,38] and induction of osteoclast apoptosis (Lorger et al., 2000). On the other hand, when bone is actively resorbed, it might be expected that high osmolality is provided due to the high concentration of Ca^{2+}/PO_4^{2-} and the degraded organic materials in the extracellular fluid around bone cells. Under these high osmotic conditions, the function of bone cells could be changed through the cell shrinkage. It is possible that there are two pathways in arrest of osteoclastogenesis; one is regulated by high concentration of Ca^{2+}, which induces osteoclst apoptosis. The other one is regulated in response to hypertonic stress caused by degraded orgamic and mineral materials, which affects bone cells metabolism. We hypothesized that the hypertonic stress in bone cell could be one of the regulating factors for bone remodeling. This study use sucrose to make hypertonic condition and apply 1α,25 $(OH)_2D_3$ to stimulate osteoclast formation.

Inhibition of the 1α,25(OH)$_2$D$_3$-induced osteoclast formation by hypertonic stress

The bone remodeling is dependent on the balance including the coordinate regulation and interaction of the component cell types: bone-forming osteoblasts and bone-resorbing osteoclasts. For the differentiation of osteoclast progenitor cells into mature osteoclasts, a cell-to-cell interaction between osteoclast precursors and osteoblastic/stromal cells has been demonstrated to be necessary [17]. The essential signaling molecules of the cell-to-cell interaction include RANKL, M-CSF, and OPG. That are secreted in osteoblastic cells. RANKL is a membrane-bound protein of the tumor necrosis factor ligand family that has been shown to play a major role in osteoclast differentiation along with M-CSF [16]. RANKL binds to its receptor RANK on hematopoietic cells and initiates a cascade of signaling events that leads to osteoclast differentiation [23]. Furthermore, osteoblast cells also secrete an osteoprotegerin (OPG)[27], a soluble "decoy" receptor of RANKL and prevents its interaction with the cognate receptor RANK[3]. OPG has been shown to be a potent inhibitor of osteoclast differentiation, survival and function *in vitro* and bone resorption *in vivo* [1,5,12,13]. we suggested that hypertonic stress will might concern 1α, 25(OH)$_2$D$_3$-induced osteoclastogenesis in bone cell; osteoblast and osteoclast. Firstly, to clarify the role of hypertonic stress on bone metabolism, sucrose as hypertonic

stresses was added to co-cultures in presence with 10^{-8} M of 1α,25$(OH)_2D_3$. After 6 days, in the presence of sucrose, 1α,25 $(OH)_2D_3$-induced osteoclast differentiation was inhibited and the number of TRAP positive multinucleated cells decreased up to about 60% in 50 mM sucrose (Figure. 1B). To identify the possibility of hypertonic toxicity, viability test was done. The sucrose did not show a toxic effect when treated at up to 100 mM (Figure. 2C). It says that the effect of sucrose on osteoclast formation was caused by hypertonic stress, not by direct toxic effect upon the cells. These results suggest that hypertonic stress may inhibit 1α,25 $(OH)_2D_3$-induced osteoclast differentiation by itself.

Decrease of the 1α,25 $(OH)_2D_3$-induced RANKL expression by hypertonic stress in osteoblast

Resorption is regulated through OPG and RANKL expression by osteoblastic cells and is affected by vatious osteotropic factors that alter plasma membrane permeability to Ca^{2+}, including 1α, 25$(OH)_2D_3$ or ionophores [4]. Moreover, 1α, 25$(OH)_2D_3$ activation of the plasma membrane signaling system changes the functional properties of voltage-sensitive Ca^{2+} channels (VSCCs) and alters the expression and activity of protein kinases [6,22,25,32]. Application of 1α, 25$(OH)_2D_3$ increases plasma membrane permeability to Ca^{2+} within milliseconds by shifting the threshold of activation toward the resting potential and increasing the mean open time of

L-type VSCC [18]. Spontaneous and hormonally regulated opening of Ca^{2+} channels leads to localized elevations of intracellular Ca^{2+} that directly control [1]. Ca^{2+} influx also directly or indirectly influences the expression and activity of Ca^{2+}/ calmodulin-dependent protein kiase (CaMK) [9,18,30,35]. For that reason, RANKL expression might be inhibited by hypertonic stress in osteoblastic cells accompany with intracellular Ca^{2+} influx change. As shown in Figure 2A, the experiment identified the expressions of RANKL, OPG, and M-CSF in osteoblasts in the presence and absence of sucrose using RT-PCR and ELISA. The 1α,25 $(OH)_2D_3$-induced RANKL expression was decreased with dose-dependent manner in sucrose. On the other hand, the expression of OPG and M-CSF were not changed regardless of sucrose concentration (Figure 2). Consequently, the addition of sucrose inhibits osteoclast formation by down-regulating the expression of RANKL in osteoblastic cells. From this results, It can be suggested that hypertonic stress decreases the expression of RANKL in response to block of the intracellular Ca^{2+} level in osteoblast. To determine whether the hypertonic stress blocks 1 α,25$(OH)_2D_3$-induced Ca^{2+} influx in osteoblast, the experiment measured intracellular Ca^{2-} level. As shown in Figure 3A, the stimulation of 1α,25$(OH)_2D_3$ raised transient $[Ca^{2+}]_i$ rapidly in osteiblastic cells. However, the addition of 50 mM sucrose suppressed transient $[Ca^{2+}]_i$ (Figure 3B). It is possible that alternation in local intracellular Ca^{2+} levels by hypertonic stress

may regulate the inactivation of the osteoblast L-type VSCC with phosphorylated CaMKII in osteoblastic cells. To identify such a possibility, It was decided to investigate whether phosphorylated CaMKII were inhibited or not by sucrose in osteoblasts. As result, activation of CaMKII was increased in response to treatment 1α,25$(OH)_2D_3$ for 48 h, and 72 h, but in the same time, phosphorylated CaMKII was not increased with 1 α,25$(OH)_2D_3$ and 50 mM sucrose (Figure. 4). It shows that hypertonic stress inhibites RANKL expression via suppression of Ca^{2+} influx with inactivated CaMKII in osteoblastic cells.

Inhibition of the RANKL-induced osteoclast differentiation by hypertonic stress

It was expected that hypertonic stress might affect the RANKL- induced osteoclast differentiation in BMMs as well as in osteoblsat. To clarify the role of hypertonic stress on osteoclast formation, sucrose was added to BMMs cultures for 5 days. When 50 ng/ml of sRANKL was added to BMMs cell culture, TRAP positive multinucleated cells were formed, whereas no TRAP positive cells were detected in media only (Figure. 5A). In the presence of sucrose, sRANKL-induced osteoclast formation was reduced (Figure. 5B).

The decrease of RANKL-induced osteoclast differentiation factors in response to hypertonic stress

Activation of RANK by its RANKL leads to the expression of osteoclast specific genes during differentiation[29]. RANK activates six key signaling pathway in osteosclast: NF-κB, JNK, ERK, p38, Src, and NFATc1. However, initiation of RANK signaling is the binding of TRAF6. The activation of TRAF6 can be induced by RANK signaling cascades. Scr protein is required for osteoclast survival, cytoskeletal rearrangements and motility (Boyle et al., 2003). The NF-κB and MAPK (JNK, p38, and ERK) is required for osteoclast differentiation. To know the way how hypertonic stress, which is thought to act as a signal for various cellular functions in osteoclastogenesis, is linked to the RANKL-induced osteoclastogenesis in BMMs cell. The experiment investigate whether those kinases were inhibited or nor by sucrose in BMMs. As a result, activatied MAPK by treatment with 50 μg/ml RANKL were inhibited in response to 50 mM sucrose (Figure. 6A). Also sucrose had a effect of inhibition of NF-κB expression (Figure. 6B). It can be said that hypertonic stress might affect the expression of transcription factors associated with sRANKL- induced osteoclastogenesis.

The decrease of NFATc1 expression by hypertonic stress in BMMs

NFATc1 is also involved in mediating key signals induced by RANKL-RANK signaling[28]. The NFATc1 may represent a master regulator of RANKL-induced osteoclast differentiation, switching on the transcriptional program for the terminal differentiation of osteoclast. The activated NFATc1 induces its own gene to amplify. The experiment expected that hypertonic stress might inhibite the expression of NFATc1 and then result in inhibition of osteoclast formation in BMMs. To identify this hypothesis, the expression and cellular localization of the NFATc1 protein were studided in RANKL-stimulated BMMs. The expression of NFATc1 protein increased in RANKL- stimulated BMMs for 48h. However, when treated with 50 mM sucrose and RANKL in BMMs for 48h, NFATc1 protein expression level was decreased (Figure 6A). As shown in Figure 6B, immunofluorescence straining revealed that the increased NFATc1 protein by RANKL was translocated from cytosol to nuclear at 48h, but it was not detectable in BMMs after 50 mM sucrose and RANKL stimulation at 48h. Considering this results, hypertonic stress may affect osteoclastogenesis by inhibition of NFATc1 protein

Blocking Ca^{2+} oscillation via the decrease of NFATc1 expression by hypertonic stress

The activities of transcription factors of the NFAT family are regulated by the Ca^{2+}/calmoduline-dependent calcineurin, a serine/threonine phosphatase, which allows nuclear translocation of these transcription fators [28]. Since NFATc1 induced by RANKL undergoes efficient nuclear translocation, one may envisage the Ca^{2+}-dependent activation of calcineurin by RANKL. A sustained Ca^{2+} oscillation was observed in BMMs stimulated by RANKL (Figure. 8A). Interestingly, The RANKL-induced Ca^{2+} oscillation initiated as late as 24h after RANKL stimulation, that is, when NFATc1 induction became notable; the oscillation was sustained thereafter, provide that RANKL was present [28]. However, hypertonic stress blocked the RANKL- induced Ca^{2+} oscillation (Figure 8B). This observation suggests that RANKL-induced Ca^{2+} oscillation is necessary for the sustained NFATc1 activation during osteoclastogenesis, and the hypertonic stress blocks RANKL-induced Ca^{2+} oscillation and causes the inhibition of NFATc1 protein expression.

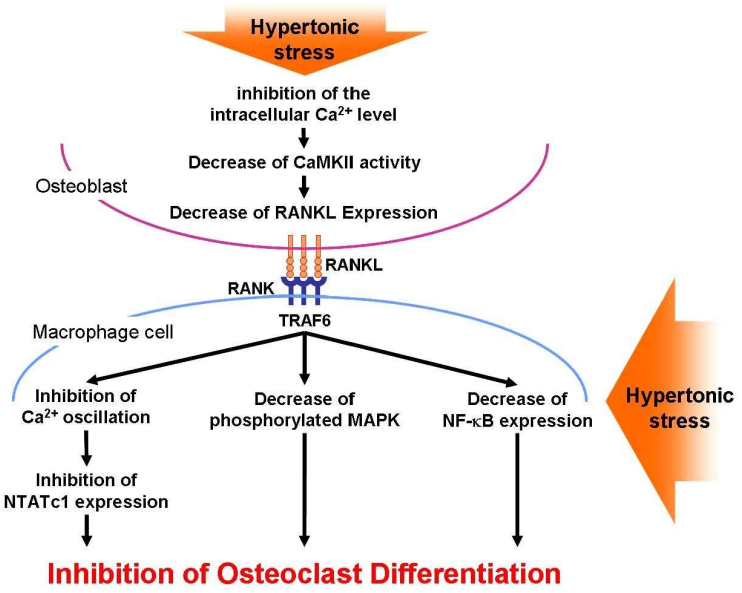


Figure 9. Experimental scheme for regulation mechanism of hypertonic stress on 1α,25(OH)$_2$D$_3$-induced osteoclastogenesis.

Taken all together, these findings reveal that hypertonic stress may be novel candidate for the regulation of bone resorption. In osteoblast, hypertonic stress decreased RANKL expression by blocking of 1α,25(OH)$_2$D$_3$-induced Ca^{2+} influx via CaMKII. On the other hands, various RANKL-induced osteoclast differentiation factor were inhibited in response to hypertonic stress in BMMs. Also, hypertonic stress blocked RANKL-induced Ca^{2+} oscillation via NFATc1.

Further studies are need to know the details of intracellular Ca^{2+} signal pathway affeted by hypertonic stress in osteoblast and osteoclast.

Ⅴ. Conclusion

This study provided the first evidence that Hypertonic stress inhibited osteoclast formation in co-culture system. In osteoblast, the expression of RANKL mRNA and protein were down-regulated upon the increase of hypertonic stress, but the expression of OPG mRNA and protein were not changed significantly and also hypertonic stress blocked 1α,25 $(OH)_2D_3$-induced Ca^{2+} influx and decreased the expression of phosphorylated CaMKII in osteoblst. In case of BMMS, hypertonic stress inhibited the RANKL-mediated osteoclast formation and various RANKL-induced osteoclast differentiation factors were inhibited in response to hypertonic stress in BMMs. Also, hypertonic stress blocked RANKL-induced Ca^{2+} oscillation and inhibited both expression and translocation of NFATc1

This results show that The hypertonic stress affects osteoclastogenesis in osteoclast and osteoblast. In this regards, it can be said that hypertonic stress is a novel candidate for the regulation of bone resorption.

Ⅵ. References

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Abstract (in korean)

1α,25(OH)$_2$D$_3$으로 유도되는 뼈파괴세포분화기전에서 hypertonic stress가 뼈모세포와 뼈파괴세포에 미치는 영향.

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김 석 현

뼈개조는 뼈를 형성하는 뼈모세포 (osteoblast)와 뼈흡수를 유발하는 뼈파괴 세포(osteoclast)에 의해 조절된다. 뼈파괴세포분화과정에서 뼈모세포는 뼈파괴세포분화를 촉진하는 인자인 RANKL과 M-CSF를 분비하며, 이와 동시에 RANKL 대한 저해제인 OPG를 분비하여 뼈파괴세포분화를 조절한다. 뼈파괴세포에 의하여 뼈흡수가 일어나면, 분해산물로서 Ca^{2+} 이나 인산염 (PO_4^{2-}), 아교질, osteopontin 등과 같은 뼈무기질과 뼈유기질이 증가하게 되어, 뼈모세포와 뼈파괴세포 주위에 삼투압이 높은 상태의 미세환경 (hypertonic microenvironment)을 유발된다. 이러한 고삼투압 환경자체가

뼈대사 조절에 영향을 미칠수 있을것으로 추측되며 본 실험에서는 높은 삼투압이 뼈파괴세포분화에 미치는 영향과 뼈대사에 미치는 영향을 분석하였다.

뼈모세포와 큰대식세포를 혼합배양하여 10 nM의 1α,25-dihydroxy vitamin D_3 (1α, $25(OH)_2D_3$)을 투여하여 뼈파괴세포분화를 유도한 후, 삼투성 자극으로 sucrose를 농도별 (25, 50, 100, 150, 200 mM)로 처리하였다. TRAP 염색법을 이용하여 핵이 3개 이상으로 융합된 뼈파괴세포의 수를 측정한 결과, sucrose 농도가 증가함에 따라 뼈파괴세포의 수가 감소하였다. 이러한 뼈파괴세포수의 감소는 삼투성 자극으로 인한 결과라는 것을 확인하기 위하여 세포 독성능 검사를 수행한 결과, sucrose 50 mM 이하의 농도에서는 삼투성 자극으로 인한 세포의 독성은 나타나지 않는 것으로 확인하였다. 삼투성 자극으로 인하여 뼈파괴세포분화가 감소하는 기전을 규명하기 위하여 뼈모세포에서 뼈흡수 기전의 중요한 인자인 RANKL과 OPG의 mRNA 와 단백질 발현양을 역전사중합효소연쇄반응법 (RT-PCR)과 효소면역측정법 (ELISA)으로 측정하였다. 그 결과, 삼투성 자극의 농도가 증가함에 따라 RANKL mRNA와 단백질 발현양이 모두 감소하였으나 OPG mRNA와 단백질의 발현양은 변화가 없었다. RANKL 발현은 1α, $25(OH)_2D_3$으로 유도되는 세포내 칼슘 유입이 발생되면서 시작되는데, 이때 삼투성 자극을 투여하게 되면 세포내 칼슘 유입이 일어나지 않는다. 또한, 세포내 칼슘 유입으로 인한 CaMKII의 발현은 삼투성 자극에 의하여 억제되었다. 이는 삼투성 자극이 뼈모세포에서 1α, $25(OH)_2D_3$으로 유도되는 세포내 칼슘유입을 막아 CaMKII의 발현을 억제하고, 이로 인하여 RANKL 발현을 감소시켜 뼈파괴세포분화를 억제한다고 생각할 수 있다.

또한, 삼투성 자극은 RANKL로 인하여 유도되는 뼈파괴세포분화 과정에서도 뼈파괴세포의 수를 감소시켰다. 본 실험에서는 그원인이 RANKL에 의해 시작되는 뼈파괴세포 분화과정에서 필수적 전사인자의 발현이 억제되었기 때문일 것이라 예상하였다. 이에 뼈파괴세포 분화인자인 JNK, ERK, p38, 그리고 NF-κB를 면역검색법(western blotting)으로 발현양을 측정하였다. 그 결과, 삼투성 자극에 의하여 분화인자인 JNK, ERK, p38, 그리고 NF-κB 발현양이 감소하였다. 또한 최근 뼈파괴세포분화의 결정적 인자라고 알려진 NFATc1의 발현양 또한 삼투성 자극으로 인하여 감소하였다. 특히 NFATc1은 RANKL 자극 후 24시간 뒤에 발생되는 Ca^{2+} oscillation과 밀접한 연관성이 있는 것으로 알려져 있으며, 삼투성 자극은 이러한 Ca^{2+} oscillation을 억제하였다. 이러한 결과는 삼투성 자극이 RANKL에 의해 유도되는 Ca^{2+} oscillation을 억제하며, 이로 인한 NFATc1 발현의 억제로 인하여 뼈파괴세포분화를 억제하는 것으로 이해할 수 있다.

이상의 사실로 미루어 삼투성 자극에 의하여 감소된 뼈파괴세포분화는 뼈모세포에서 RANKL 발현의 감소와, 대식세포에서 뼈파괴세포분화인자인 NFATc1의 발현 감소로 인한 복합적인 원인에 의하여 발생되는 결과로 해석된다. 따라서 삼투성 자극은 뼈파괴를 조절할 수 있는 새로운 뼈대사 조절인자로서 뼈대사 기전에서 중요한 요소로 작용할 것으로 예상된다.

핵심되는 말 : 삼투성 자극, 뼈파괴세포분화, 뼈모세포, RANKL, NFATc1, 1α, $25(OH)_2D_3$