Hypo-osmolality Increases RANKL Expression by Activating Ca^{2+} Entry via TRPM3 and TRPV4 in Osteoblastic Cells

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Directed by Professor Heung-Kyu Son

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Abstract

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(Directed by Prof. Heung-Kyu Son, D.D.S., Ph. D.)

Bone is continually remodeled by osteoblasts and osteoclasts. 1α,25$(OH)_2D_3$ activates osteoclastogenesis by releasing molecules from osteoblast, osteoprotegerin and receptor activator of NF-κB ligand (RANKL). RANKL, a tumor necrosis factor family member, plays an essential role in osteoclastic differentiation to precursors of osteoclast and in osteoclast activation and cell survival. On the other hand, bone remodeling is also regulated by mechanical stresses such as fluid

shear stress, or hypo-osmotic pressure, which induce increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in osteoblasts and change the function of osteoblasts. However, the mechanism of osmolality-induced Ca^{2+} response and effect of osmolality on RANKL expression in osteoblastic cells are not known. In the present work, RANKL expression and Ca^{2+} signaling with the changes of osmotic pressure and the type of Ca^{2+} channel regulated by osmolality in osteoblastic cells were investigated using RT-PCR, $[Ca^{2+}]_i$ measurement, and RNA interference.

Hyper-osmolality reduced $1\alpha,25(OH)_2D_3$–induced RANKL expression, and hypo-osmolality itself increased RANKL expression. $1\alpha,25(OH)_2D_3$–induced $[Ca^{2+}]_i$ increases were blocked by hyper-osmolality. In contrast, $[Ca^{2+}]_i$ was increases by hypo-osmolality, and then it was a reversible response. Removal of extracellular Ca^{2+}, or treatment with Gd^{3+}, a non-specific blocker of plasma Ca^{2+} channel, inhibited hypo-osmolality-induced $[Ca^{2+}]_i$ increases, suggesting that hypo-osmolality-induced $[Ca^{2+}]_i$ increases are evoked from Ca^{2+} influx across the plasma membrane. Ruthenium red, an inhibitor of TRPV4, and 2-APB, an inhibitor of TRPM3 partially inhibited hypo-osmolality-induced $[Ca^{2+}]_i$ increases, respectively. In addition, knockdown of

TRPM3 and TRPV4 using siRNA inhibited hypo-osmolality-induced $[Ca^{2+}]_i$ increases, respectively. Moreover, expressions of TRPM3 and TRPV4 were increased by hypo-osmolality, not by hyper-osmolality. These results indicated that hypo-osmolality increases RANKL expression by activating Ca^{2+} entry via TRPM3 and TRPV4 in osteoblastic cells.

Key words: Hypo-osmolality, RANKL, Calcium Signaling, TRPM3, TRPV4, Osteoblasts

I. Introduction

The functions of bones include maintaining blood calcium levels, providing mechanical support to soft tissues and serving as liver for muscle action, supporting haematopoiesis, and housing the brain and spinal cord (Harada *et al*, 2003). Bone is continuously destroyed (resorbed) and rebuilt at about 1 to 2 million microscopic sites per adult skeleton (Rodan *et al*, 2000). This balance is achieved bone remodeling. Bone remodeling involves the bone resorption of bone by osteoclasts and the formation of bone matrix by osteoblasts, and is a predominant metabolic process regulating bone structure and function during adult life (Chambers, 2000; Teitelbaum, 2000). During imbalance of remodeling, it can result in gross perturbations in skeletal structure and function.

Most adult skeletal disease are due to excess osteoclastic activity, leading to an imbalance in bone remodeling which induce debilitating loss of bone mass (Chambers, 2000). Such diseases would include osteoporosis, Paget's disease, inflammatory bone disorders of

rheumatoid arthritis, periodontal disease, and metastatic cancers (Rodan *et al*, 2000). These diseases of bone and cartilage are responsible for a large portion of healthcare expenditures in developed countries. To develop therapeutics for treatment of bone diseases, we need for a better understanding of the biology of osteoclasts and osteoblasts (DeWitt, 2003).

Osteoclasts are multinucleated giant cells of hematopoietic origin and are formed *in vitro* from osteoclast progenitors by co-culturing with osteoblasts/stromal (Ito *et al*, 2004). The close contact between osteoblast/stromal cells and bone marrow cells (BMCs) was essential for osteoclastogenesis. Osteoblast is regulated osteoclast differentiation through secretion of three critical factors; OPG (osteoprotegerin), RANKL (receptor activator of NF-κB ligand, also known as OPGL and TRANCE), and M-CSF (macrophage colony stimulating factor). RANKL and M-CSF are essential and sufficient to promote osteoclastogenesis through mechanism of cell-to-cell interaction (Suda *et al*, 1992; Boyle *et al*, Simonet *et al*, 2003; Feng, 2005).

M-CSF binding receptor c-fms acts on proliferation and on survival of osteoclast precursors (Roux *et al*, 2000), and RANKL acts on

differentiation into mature osteoclast, enhancement of the activity of mature osteoclasts, and inhibition of osteoclast apoptosis (Jimi, *et al*, 1999; Teitelbaum *et al*, 2003; Ito *et al*, 2004). RANKL, a member of the tumour necrosis factor superfamily, is interacts with its receptor RANK (receptor activator of NF-κB) on macrophages and mature osteoclasts. It is first cloned as a regulatory factor of T cells and dendritic cells at 1997, and is considered a novel member of tumor necrosis factor ligand superfamily (Ito *et al*, 2004). RANKL containing of 317 amino-acid peptides consists of two distinct forms such as a 45-kDa membrane-associated form (mRANKL) and a 32-kDa soluble form (sRANKL), which derived from proteolytic cleavage of the membrane-associated form (Lacey *et al*, 1998). OPG (osteoprotegerin) is known to be a “decoy” receptor that competes with RANK for RANKL (Simonet *et al*, 1997; Teitelbaum *et al*, 2003), and inhibits osteoclast differentiation and activation. It is synthesized as resulting in a mature protein of 380-amino acids (Ito *et al*, 2004). OPG lacks transmembrane and cytoplasmic domains and is secreted as a soluble protein. During osteoclast differentiation, RANKL-RANK signaling leads to recruitment of downstream signaling pathway factors that control various function such as a maturation of osteoclast, activation of resorption by mature

osteoclast and their survival (Boyle *et al*, 2003; Lee *et al*, 2003). In these reason, expression of RANKL and OPG is therefore coordinated to regulate bone resorption and density positively. In fact, osteoblasts require stimulating hormones to upregulate RANKL expression such as parathyroid hormone (PTH), 1, 25-dihydroxyvitamin D_3 (1α, $25(OH)_2D_3$), prostaglandin E_2 (PGE_2), interleukin (IL)-11, IL-1β, or tumor necrosis factor (TNF)-α. Among these, 1α, $25(OH)_2D_3$, biologically active form of vitamin D_3, affect RANKL gene expression both directly and indirectly (Kitazawa *et al*, 1999). In addition, 1α, $25(OH)_2D_3$ treatment of osteoblastic cells activates intracellular Ca^{2+} responses, which is induced RANKL expression (Bergh *et al*, 2004; Farach-Carson *et al*, 2004). Moreover, intracellular Ca^{2+} increase also is induced by mechanical stress.

Mechanical stimulation, such as shear stress, membrane stretch, or hypo-osmotic swelling, induces a change in the shape of osteoblasts and an increase intracellular Ca^{2+} response (Miller *et al*, 1976). This mechanical stress plays a major role in regulating bone remodeling (Bowler *et al*, 2001). Recently, hypo-osmotic stress induced mechanical perturbation plays a major role in regulating bone turnover, which is induced increase of intracellular Ca^{2+} concentration in

osteoblastic cells by cell swelling (Yamaguchi *et al*, 1989; Weskamp *et al*, 2000; Romanello *et al*, 2005). According to Takani *et al* (2000), increase of intracellular Ca^{2+} level is induced elevation of RANKL expression (Takami *et al*, 2000). Therefore, I suggest that osmotic pressure regulating osteoblast cell volume has an effect on change of intracellular Ca^{2+} concentration.

Recently, the novel player regulating Ca^{2+} entry in mammalian cells have been found in the still-growing family of so-called 'transient receptor potential (TRP)' cation channels. TRP channels comprise a superfamily of non-selective cation channels with at least seven subfamilies, such as TRPC, TRPV, TRPM, TRPA, TRPN, TRPP, and TRPML (Takami *et al*, 2004). Of these, high sequence similarity in the region of the pore-forming domains is the classic TRPC, the vanilloid-like TRPV, and the melastatin-like TRPM subfamilies (Harteneck *et al*, 2000; Montell *et al*, 2002). In particular, TRPM3 (TRP melastatin 3) and TRPV4 (TRP vanilloid 4) have been characterized as cation channels activated by extracellular hypo-osmolarity (Strotmann *et al*, 2000; Grimm *et al*, 2003). It has been demonstrated that intracellular Ca^{2+} level by osmotic pressure is regulated through TRPM3 and TRPV4 (Oberwinkler *et al*, 2007; Plant *et al*, 2007).

TRPM3 is the last identified member of the TRPM subfamily and is most closely related to TRPM1. TRPM3 channels comprise of six splice variants, and could be detected in human kidney and brain (Grimm *et al*, 2003; Lee *et al*, 2003), and show constitutive activity of short pore region by increase of intracellular Ca^{2+} concentration (Oberwinkler *et al*, 2007; Plant *et al*, 2007). TRPM3 activity is reduced by the application of hyper-osmotic solutions, whereas directly enhanced intracellular Ca^{2+} concentrations were measured upon extracellular applications of hypo-osmotic solutions (Harteneck *et al*, 2007). Furthermore, TRPM3 generates constitutively active indirectly by D-*erythro*-sphingosine as well as ceramides and sphingosine 1-phosphate (S1P). Especially, sphingosine 1-phosphate (S1P) is induced RANKL expression in osteoblastic cells through COX2 signaling (Ryu *et al*, 2006). In these facts, TRPM3 can regulate intracellular Ca^{2+} concentration by change of cell volume induced hypo-/or hyper- osmotic stress in osteoblastic cells.

TRPV (TRP vanilloid) 4 is a non-selective cation channel subunit expressed in a wide variety of tissues (Plant *et al*, 2007). TRPV4 can be activated by a wide range of stimuli including physical (cell swelling, heat, mechanical stimulation) and chemical stimuli (endocannabinoids,

arachidonic acid, and, 4α-phorbol esters). In particular, the reduction of the basal Ca^{2+} concentration by the application of hyper-osmotic solution shows that TRPV4 is inhibited by the hyper-osmolarity, whereas hypo-osmotic solutions activate TRPV4 (Harteneck *et al*, 2007). The mechanism of sensitivity to changes in osmolarity was initially unclear, but did not seem to involve membrane stretch or changes in intracellular ionic strength, latter being the link between swelling and signaling pathways activating volume-sensitive anion channels (Plant *et al*, 2007). In these reason, I suggest that change of osmolarity is regulated RANKL expression in osteoblastic cells by intracellular Ca^{2+} response through TRPM3 or TRPV4 as well as voltage-sensitive Ca^{2+} channels.

Here I investigated that hypo-osmotic solution increases RANKL expression in osteoblastic cells by intracellular Ca^{2+} response through TRPM3 and TRPV4. I found RANKL expression is increased by hypo-osmotic pressure, which induces increase of intracellular Ca^{2+} concentration by extracellular Ca^{2+} influx. This Ca^{2+} influx is evoked by activity of TRPM3 and TRPV4 in hypo-osmotic solution. My studies identify osmotic pressure as new candidates in RANKL expression in osteoblastic cells.

II. Materials and Methods

1. Materials

The routine cell culture media and the dispase were obtained from GIBCO/BRL. The 1α, 25$(OH)_2D_3$, sucrose, TRAP staining kit, collagenase, and most of the chemicals were purchased from Sigma Chemical Co.. The trizol was obtained from Invitrogen Corp.. 2-acetixymethyl ester derivative Fura-2/AM was obtained from Teflabs Inc. and Pluronic acid and thapsigargin were purchased from Molecular Probes. siSTRIKE^{TM} vector was purchased from Promega. 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

Briefly, primary osteoblastic cells were isolated from calvariae of 1-day-old ICR newborn mouse (KOATEC, Korea) by a previously

reported method (Takahashi, *et al*, 1988) with a slight modification. These calvariae were digested with an enzyme solution containing 0.1% collagenase and 0.1% dispase for 10 min in shaking water bath at 37℃. The first digestion was discarded, and then osteoblastic cells were collected from second digestion to fourth. These cells were cultured to a fresh α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS), and 1% antibiotic-antimycotic (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B) in 100 mm dishes for 4 days at 37℃ in humidified atmosphere containing 5% CO_2 in air.

3. RT-PCR (Reverse Transcriptase –Polymer Chain Reaction)

3.1. RNA Isolation

Primary osteoblastic cells were seeded in 35 mm culture dishes (1×10^4 cells/dish) in α-MEM with 10% FBS. After 2 days, the cells were treated with 10^{-8} M 1α, $25(OH)_2D_3$, 50 mM sucrose, and 160 mOsm α-MEM for 48 hrs. Then, these cells collected and total RNA was extracted using a TRIzol reagent (Invitrogen Corp.) according to the manufacturer's instructions. The concentration of RNA obtained

was determined by measuring the absorbance at 260 and 280 nm.

3.2. RT-PCR

That total RNAs (1 μg) isolated from each sample were used as templates for the cDNA synthesis. AccuPower® RT PreMix (BIONEER, Korea) reaction tubes mixed 1μg of total RNA and 100 pmol oligo dT_{18} and filled up the 20$\mu \ell$ reaction volume with DEPC treated water. Reverse transcription was performed as 5 min at 95℃, and 60 min at 42℃ in a Mycycler™ (BIO-RAD, USA). Polymerase chain reactions were performed using HiPi RCP PreMix (1unit/4$\mu \ell$ HiPi™ Thermostable DNA polymerase in 250 mM Tris-HCl (pH 9.0), 80 mM $(NH_4)_2SO_4$, 10% DMSO, 8.75 mM $MgCl_2$, 0.05% bromophenol blue, 12% glycerol, and stabilizer (Elpis, Korea) with 1μg of cDNA, 2 pmol of each oligonucleotide primer (table 1) and sterile water in a 20 $\mu \ell$ volume.

Table1. Synthetic oligonucleotide primers used for reverse transcription-polymerase chain reaction (RT-PCR)

Target gene	Primer sequence	T_a
RANKL (750 bp)	(forward) : 5'-ATCAGAAGACAGCACTCACT-3' (reverse) : 5'-ATCTAGGACATCCATGCTAATGTTC-3'	54℃
TRPM3 (366 bp)	(forward) : 5'-TCACAGTCTCACCGTTCATA-3' (reverse) : 5'-ACATATTCATGGCCTACCTG-3'	56℃
TRPV4 (365 bp)	(forward) : 5'-AGAACACCAAGTTTGTCACC-3' (reverse) : 5'-GAAGAATACACAGGCCCATA-3'	56℃
β-actin (366 bp)	(forward) : 5'-GGACTCCTATGGTGGGTGACGAGG-3' (reverse) : 5'-GGGAGAGCATAGCCCTCGTAGAT-3'	58℃

4. Intracellular Ca^{2+} concentration measurement

For Ca^{2+} measurement, osteoblastic cells were seeded (1×10^5) on cover glass (22 mm × 22 mm) in a 35 mm dishes and cultured in α-MEM containing 10% FBS. Cells in physiological salt solution (PSS; 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM Glucose, 10 mM HEPES, 1.5 mM $CaCl_2$, pH7.4, and 310 mOsm) were incubated with 5μM of the 2-acetixymethyl ester derivative Fura-2/AM (membrane permeable) and 0.05% Pluronic F127 for 40 min at room temperature. Unloading dye was washed out with PSS. For fluorescence measurement, the

adherent cells on the cover glasses were placed on the bottom of a perfusion chamber, and the cells were perfused continuously with pre-warmed (37℃) PSS. Various osmotic solution and each of chemicals diluted in PSS were delivered to cells and fura-2 fluorescence was measured using excitation wavelengths of 340 and 380 nm, and emission wavelength was measured at 510nm (Ratio = F340/F380). Various osmotic solutions are involved hyper-osmotic (addition of 50 mM sucrose in PSS, 385 mOsm) and hypo-osmotic (80 mM NaCl in PSS, 215 mOsm) solution. The emitted cellular fluorescence was monitored using CCD camera (Photon Technology International Inc., Lawrenceville, NJ) attached to an inverted microscope (Nikon, Japan). These monitored images were digitized and analyzed through MetaFluor software.

5. RNA interference

5.1. Cloning of samll interfering RNA

siSTRIKE^{TM}U6 hairpin cloning systems kit (Promega, Madison, USA) were used to synthesize 19-nucleotied single-stranded RNA. Briefly, two hairpin oligonucleotides are annealed to form a double-

stranded DNA fragment for inserting it into the siSTRIKE vector. For assistance with hairpin oligonucleotide design visit the siRNA Designer at: www.promega.com/techserv/tools.

The primers used were: for TRPM3 (56 bp), 5'-ACCGCAGTGCATAG AGGAATATTTCAAGAGAATATTCCTCTATGCACTGCTTTTTC-3' (sense), 5'-TGCAGAAAAAGCAGTGCATAGAGGAATATTCTCTTGA AATATTCCTCTATGCACTG-3' (anti-sense). For TRPV4 (56 bp), 5'-ACCGGACCCTGGCAAGAGTGAAATCTTTCAAGAGAAGATTTCAC TCTTGCCAGGGTCCTTTTTC-3' (sense), 5'-TGCAGAAAAAGGACC CTGGCAAGAGTGAAATCTTCTCTTGAAAGATTTCACTCTTGCCA GGGTC-3' (anti-sense) (Bioneer Oligo Synthesis, Korea).

For cloning the siSTRIKE vectors inserting hairpin oligonucleotides, the annealing reaction was performed at 90℃ for 3 minutes followed by incubating at 37℃ for 15 minutes using 2 $\mu g/\mu \ell$ each oligonucleotide.

5.2. Manufacture of Recombinant plasmid DNA

To make recombinant plasmid DNA, siSTRIKE vectors and annealing hairpin oligonucleotides were incubated at room temperature for 1 hour using T4 DNA ligase, and then performed

transformation. The recombinant plasmid DNA was digested with restriction enzyme *Pst*I. This is confirmed by electrophoresis on 1.5% agarose gels in TAE buffer. The gels were stained with ethidium bromide (10 μg/ml) and photographed on top of a 280 nm UV light box.

6. Transfection experiments

The calvarial osteoblastic cells that were isolated from 1-day-old newborn ICR mice were seeded at 60-70% confluence in culture dishes in α-MEM containing 10% FBS and 1% antibioticin-antimycoticin. Before experiment, osteoblastic cells changed the α-MEM antibiotics and FBS-free medium. Transfection of recombinant plasmid DNA (1㎍/㎖) (final concentration) was carried out using lipofectamine™2000 (Invitrogen Corp., Carlsbad, CA) for 4 hours. After then, cells changed with α-MEM containing 10% FBS and antibioticin - antimycoticin medium. Stable-transfected cells were selected with 0.2 ㎎/㎖ of G418-neomycin (GENETICIN®, Invitrogen Corp.).

7. Data analysis and statistics

All experiments were reproduced at least three times. The results are expressed as the mean ± SE. 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

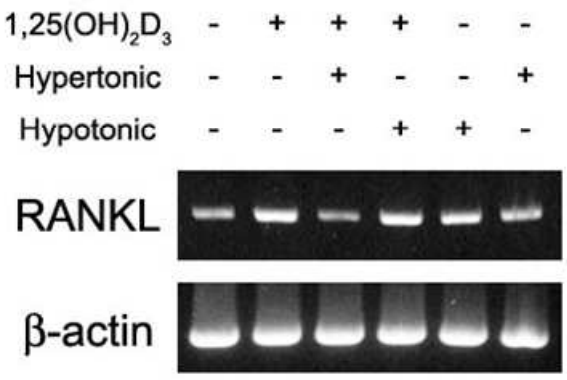
1. Extracellular osmotic pressure regulates 1α,25(OH)$_2$D$_3$-induced RANKL expression in osteoblastic cells.

I established that addition of hyper-osmotic pressure inhibits osteoclast formation by down-regulating the 1α,25(OH)$_2$D$_3$–induced osteoclastogenesis in co-culture system (Jeong *et al*, 2005). It is suggested that hyper-osmotic stress was inhibited expression of essential regulated factor to osteoclast differentiation in osteoblastic cells, such as RANKL, M-CSF, and OPG.

To clarify the role of osmotic pressure on 1α,25(OH)$_2$D$_3$ –induced RANKL expression, I analyzed expression levels of RANKL during stimulation of osmotic pressure using RT-PCR. As shown in Figure 1, RANKL mRNA expression was decreased by hyper-osmotic stress, but was increased by hypo-osmotic pressure. The *β*-actin mRNA was used standardization for total RNA amount which remains unchanged.

This result indicates that RANKL expression was regulated in response to extracellular osmotic pressure, which lead to decrease at hyper-osmolality and increase at hypo-osmolarity.

A.


1,25(OH)2D3 - + + + - -
Hypertonic - - + - - +
Hypotonic - - - + + -
RANKL
β-actin

B.

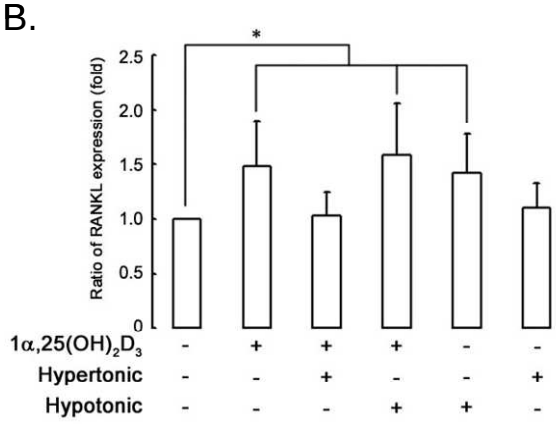

*
Ratio of RANKL expression (fold)
2.5
2.0
1.5
1.0
0.5
0
1α,25(OH)2D3 - + + + - -
Hypertonic - - + - - +
Hypotonic - - - + + -

Figure. 1. The Effect of 1α,25$(OH)_2D_3$–induced RANKL expression in response to osmolality changes in osteoblastic cells.

Osteoblastic cells were cultured in presence of 1α,25$(OH)_2D_3$, 50 mM sucrose as hyper-osmotic solution (385 mOsm) and hypo-osmotic solution (215 mOsm) for 48 hrs. (A), the expression levels of RANKL mRNA in osteoblastic cells were monitored by RT-PCR. The PCR products (750bp for RANKL, and 366bp for *β*-actin) were visualized in a 1.5% agarose gel stained with ethidium bromide. (B), the density of each band was measured by Meta Morph Program. The values were normalized against control. Results represents mean ± SEM (n = 3 ; *p < 0.05 versus control).

2. 1α,25(OH)$_2$D$_3$ –induced [Ca^{2+}]$_i$ increases are inhibited by hyper-osmolality.

From Figure 1 results, I found that hyper-osmotic pressure inhibits the expression of RANKL in osteoblastic cells. Recently, it was reported that RANKL and OPG expression and secretion regulated Ca^{2+} influx across the plasma membrane. According to Takami *et al* (Takami *et al*, 2000), the increase of RANKL expression was mediated high intracellular Ca^{2+} level in osteoblastic cells. Moreover, 1α,25(OH)$_2$D$_3$–induced OPG and RANKL expression were regulated by Ca^{2+} influx (Farach-Carson *et al*, 2004). In this reason, I could be assumed that hyper-osmolality decreases the expression of RANKL as inhibition of intracellular Ca^{2+} level in osteoblastic cells. However, most of high intracellular Ca^{2+} level, which was reported until now, occurred in a 1α,25(OH)$_2$D$_3$, and induced increase of OPG expression in osteoblastic cells. Therefore, I needed to confirm that hyper-osmolalitic pressure is regulated the 1α,25(OH)$_2$D$_3$–induced intracellular Ca^{2+} influx.

When osteoblastic cells expose to 10^{-8} M 1α,25(OH)$_2$D$_3$, $[Ca^{2+}]_i$ elevated transiently by $F_{340/380}$ = 1.5±0.3 (Fig. 2. A). In contrast, during

the addition of hyper-osmolalitic solution (50 mM sucrose), $[Ca^{2+}]_i$ did not effect on 10^{-8} M $1\alpha,25(OH)_2D_3$ (Fig. 2. B).

These results suggest that the hyper-osmolalitic pressure is inhibited RANKL expression by blocking of $1\alpha,25(OH)_2D_3$–induced Ca^{2+} influx in osteoblastic cells.

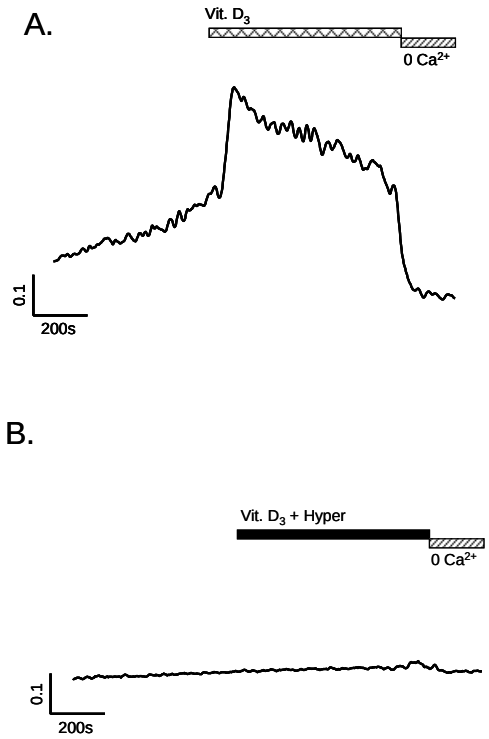


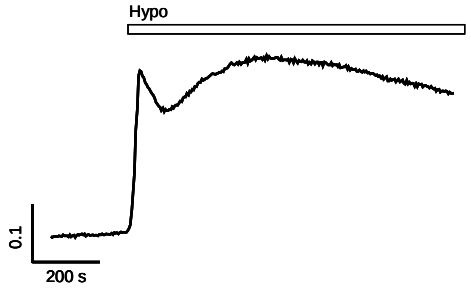
Figure 2. $[Ca^{2+}]_i$ change in response to hyper-osmolalitic pressure.

Osteoblastic cells were seeded (1×10^5) on cover glass (22 mm × 22 mm) in a 35 mm dishes for 2days before experiment and loaded with fura-2/AM at room temperature for an hour. The physiological solution was allowed to flow on the cover glass for 10 min, and then the buffer including 10^{-8} M 1α,25$(OH)_2D_3$ (A) and/or 50 mM sucrose (B) were perfused for 10 min.

3. Hypo-osmolality increases $[Ca^{2+}]_i$.

As previously stated, I conformed that increase of $1\alpha,25(OH)_2D_3$-induced intracellular Ca^{2+} was inhibited by hyper-osmotic stress in osteoblastic cells. Moreover, it is reported that hypo-osmotic pressure induced Ca^{2+} influx in osateoblastic cells (Romanello *et al*, 2005). On the basis of these results, I suggested that intracellular Ca^{2+} could be regulated by osmotic pressure (hyper-/or hypo-osmotic stress) in osteoblastic cells. To find out that whatever changes the intracellular Ca^{2+} concentration by hypo-osmotic pressure, I measured intracellular Ca^{2+} level into the hypo-osmotic solution in osteoblastic cells. As expected, I identified that hypo-osmotic stress was increased intracellular Ca^{2+} level ($F_{340/380}$ = 0.27 ± 0.04, *n* = 16, Fig. 3-A) in opposition to hyper-osmotic stress induced $[Ca^{2+}]_i$. Although intracellular Ca^{2+} level was blocked by hypertonic stress, it had reproductive application of $[Ca^{2+}]_i$ by hypotonic stress as shown in Fig. 3-B. Moreover, it was reversible to increase of hypo-osmotic stress-induced intracellular Ca^{2+} level and was not significantly different from that second application of the hypotonic solution (Fig. 3-C). Therefore, increase of intracellular Ca^{2+} concentration by hypotonic stress dose not desensitize.

A.



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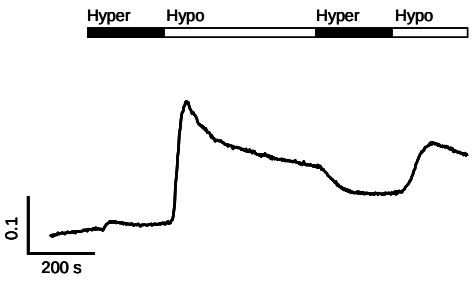

C.

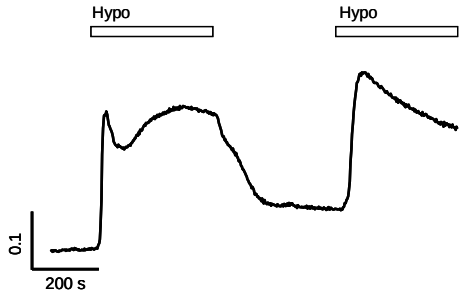

Figure 3. Hypo-osmolality-induced $[Ca^{2+}]_i$ increases

(A) Stimulation with hypotonic solution (215 mOsm) for 8.3 min was evoked increase intracellular Ca^{2+} concentration in osteoblastic cells. (B) Hypotonic stress induced-increase of intracellular Ca^{2+} concentration was inhibited in hypertonic solution. (C) Application of hypotonic solution repeatedly produced an increase of intracellular Ca^{2+} level.

4. Hypo-osmolality-induced $[Ca^{2+}]_i$ increases are dependent on extracellular Ca^{2+} entry.

I investigated whether $[Ca^{2+}]_i$ increase induced by hypo-osmotic stress was due to extracellular Ca^{2+} influx. I tested the response to hypo-osmotic stress in presence of Ca^{2+} channel modulation in osteoblastic cells. First, when $[Ca^{2+}]_i$ increased by hypo-osmotic pressure, I treated Ca^{2+} free hypo-osmotic solution into osteoblastic cells. Increase of $[Ca^{2+}]_i$ by hypo-osmotic solution was completely inhibited by removal extracellular Ca^{2+} in hypo-osmotic solution (Fig. 4A). After treatment with Ca^{2+} free normal osmotic solution, $[Ca^{2+}]_i$ response was not affected by Ca^{2+} free hypo-osmotic solution (Fig. 4B). To confirm this results, I measured $[Ca^{2+}]_i$ by gadolinium instead of Ca^{2+} free solutions. Gd^{3+} is a nonspecific blocker of stretch-activated ion channels in many cells (Tsuzuki *et al*, 2000). Pretreatment with 1uM Gd^{3+} had no effect on hypotonic stress-induced $[Ca^{2+}]_i$ increase as shown in Fig. 4C. Conversely, increase of $[Ca^{2+}]_i$ by pretreatment of hypo-osmotic solution was inhibited by 1uM Gd^{3+} in hypotonic solution (Fig. 4D).

These findings indicate that the main source for hypotonic stress-induced $[Ca^{2+}]_i$ increase is influx of Ca^{2+} from extracellular space and exclude any involvement of intracellular Ca^{2+} store in osteoblastic cells.

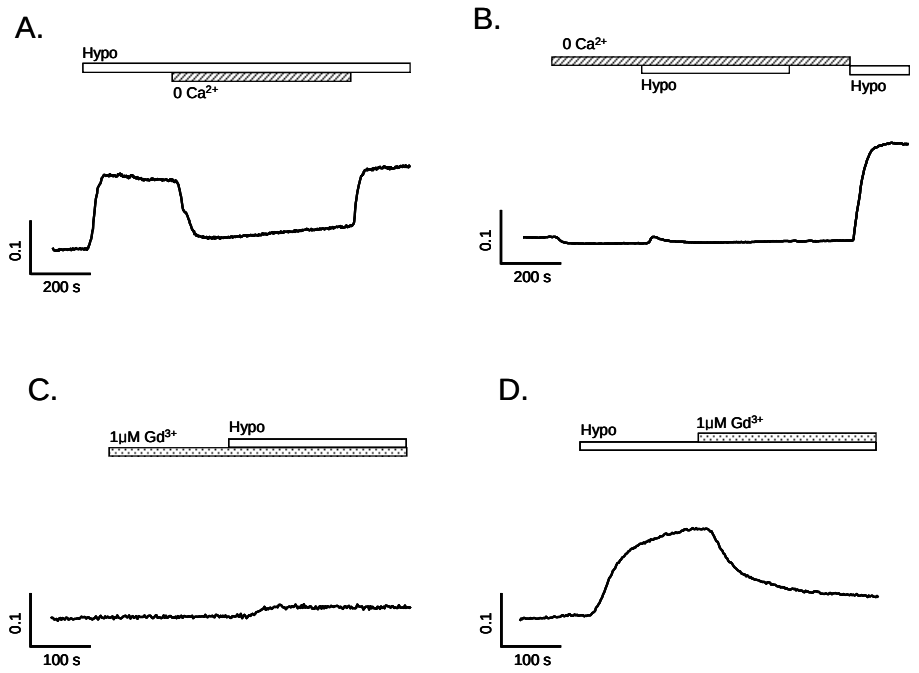

Figure 4. Characterization of hypo-osmolality-induced $[Ca^{2+}]_i$ increases

Osteoblastic cells were measured by described previously. When cells are showing increase intracellular Ca^{2+} concentration by hypotonic solution, first, 1μM Gd^{3+} was treated respectively (C-D). Second, extracellular Ca^{2+} was removed by exchanging PSS for Ca^{2+} free solution (A-B).

5. Hypo-osmolality-induced $[Ca^{2+}]_i$ increases are from Ca^{2+} influx via TRPM3 or TRPV4.

I confirmed that increase of $[Ca^{2+}]_i$ by hypo-osmotic stress is dependent on extracellular Ca^{2+} influx through Ca^{2+} channel. It is known this process occurs by L-type voltage-sensitive Ca^{2+} channel (Bergh *et al*, 2004; Farach-Carson *et al*, 2004) in osteoblastic cells. However, L-type voltage-sensitive Ca^{2+} channel is activated by $1\alpha,25(OH)_2D_3$, not hypo-osmotic pressure. I suggested that intracellular Ca^{2+} response by osmotic stress is involved in other Ca^{2+} channel. Recently, TRPV4 and TRPM3 has been known novel proteins mediating extracellular Ca^{2+} entry in cells upon application of hypotonic solutions (Strotmann *et al*, 2000; Grimm *et al*, 2003). In order to confirm that whether TRPM3 and TRPV4 are directly involved in increase of $[Ca^{2+}]_i$ by hypo-osmotic solution, I checked the responsiveness of their blocker, 2-aminoethoxydiphenyl borate (2-APB)(Xu *et al*, 2005) and ruthenium red (RR). First, I exposed to hypotonic solution with 1 μM ruthenium red (RR). The ruthenium red completely inhibited TRPV4-mediated Ca^{2+} signaling but did not affect response to hypo-osmotic induced Ca^{2+} influx in TRPM3 expressing cells (Grimm *et al*, 2003). The increase of $[Ca^{2+}]_i$ -induced hypo-

osmotic solution was suppressed by addition of 1μM RR containing hypo-osmotic solution (Fig. 5A-a). Therefore, in side-by-side experiment, I measured Ca^{2+} response in TRPM3 inhibitor treated osteoblastic cells. TRPM3 activity can be blocked by Gd^{3+} ions and by 2-APB at a concentration of 100 μM (Oberwinkler *et al*, 2007; Plant *et al*, 2007). In previous results, Gd^{3+} was inhibited hypo-osmotic stress-induced Ca^{2+} influx. Also, 100 μM 2-APB was inhibited increase of $[Ca^{2+}]_i$ in response to hypo-osmotic solution (Fig. 5A-b). After pre-treatment with RR or 2-APB in osteoblastic cells, hypo-osmolality-induced $[Ca^{2+}]_i$ was reduced as shown Fig 5B-a and b. From these I suggest that hypo-osmotic stress induced $[Ca^{2+}]_i$ responses are appeared by TRPV4-/ and TRPM3- mediated Ca^{2+} signals.

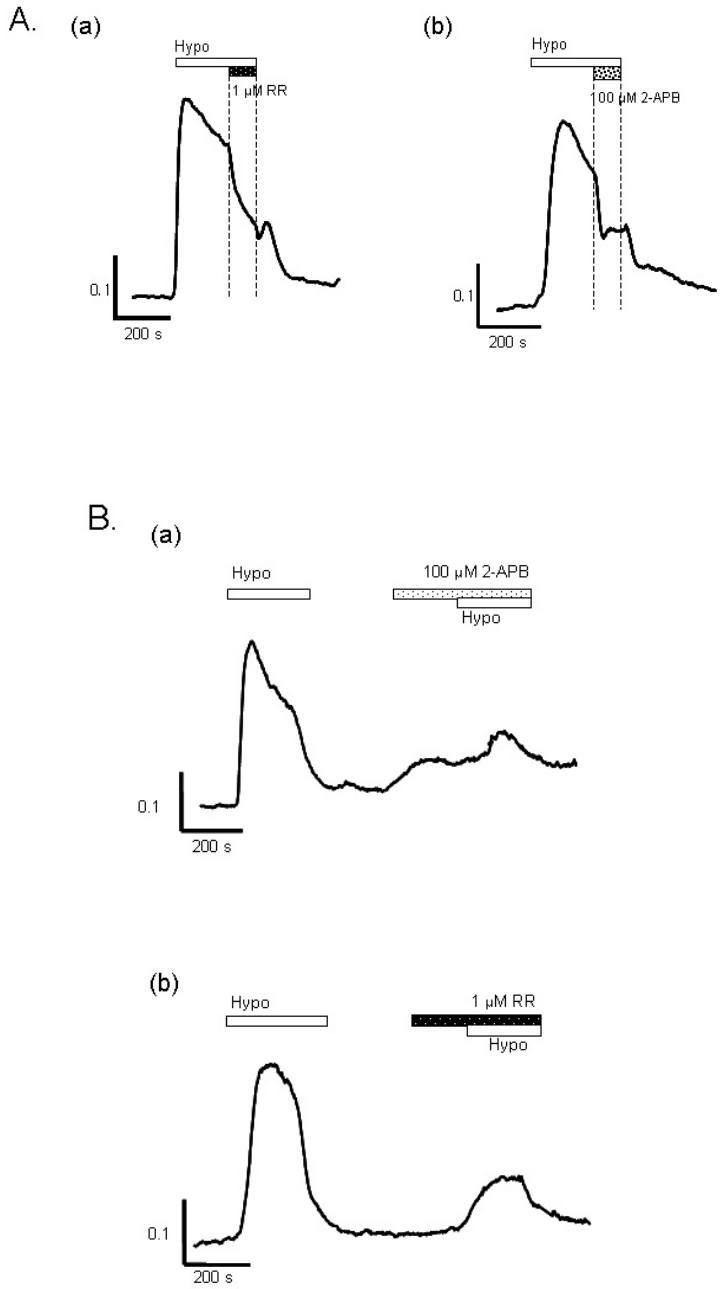
A.
(a)
Hypo
1 μM RR
0.1
200 s
(b)
Hypo
100 μM 2-APB
0.1
200 s
B.
(a)
Hypo
100 μM 2-APB
Hypo
0.1
200 s
(b)
Hypo
1 μM RR
Hypo
0.1
200 s

Figure. 5. Effect of ruthenium red or 2-APB on hypo-osmolality-induced $[Ca^{2+}]_i$ increases

Osteoblastic cells were cultured by described previously. (A), the application of hypo-osmotic stress is partially inhibited by ruthenium red at a concentration of 1 μM (a) and 2-APB of 100 μM (b). (B), Hypo-osmolality-induced effect was reduced by pre-treatment with the intracellular Ca^{2+} reuptake inhibitor 1 μM RR (a) or 100 μM 2-APB (b).

6. TRPM3 and TRPV4 regulates hypo-osmolality-induced $[Ca^{2+}]_i$ increases.

In an earlier experiment, I found that increase of hypotonic stress-induced $[Ca^{2+}]_i$ was made extracellular Ca^{2+} influx via TRPM3 and TRPV4. Thus, I demonstrated that both TRPM3 and TRPV4 might be immediately involved in hypotonic stress-induced $[Ca^{2+}]_i$ increase. First, I confirmed TRPM3 down-regulation in osteoblastic cells by siRNA transfection (Fig. 6A). Transfected osteoblastic cells with TRPM3 siRNA (red line) were induced to inhibit intracellular Ca^{2+} concentration in response to hypotonic solutions compared with normal osteoblastic cells (black line) (Fig 6B). These data indicate that TRPM3 activity involves intracellular Ca^{2+} influx by hypotonic solution in osteoblastic cells. Using the same method, I utilized the calvarial osteoblastic cells transfected with the TRPV4 siRNA (Fig 6C). The silence of TRPV4 in osteoblastic cells caused a blocking Ca^{2+} response in hypo-osmotic solution (Fig 6D). From the above results, it could be speculated that the TRPM3 and TRPV4 genes may be capable of regulating the Ca^{2+} influx by hypo-osmotic pressure, which in turn leads to regulation of RANKL expression in osteoblastic cells.

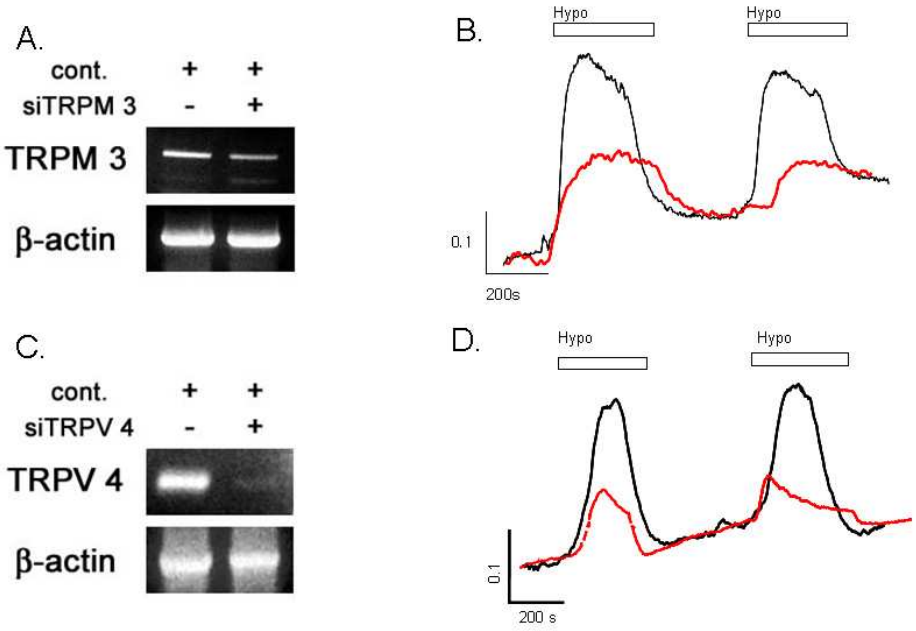

Figure 6. Effect of knockdown of TRPM3 or TRPV4 channels on hypo-osmolality-induced $[Ca^{2+}]_i$ increases.

(A) and (C), Osteoblastic cells were transfected with siRNA of TRPM3 or TRPV4 and selected by G418 treatment. The expression levels of TRPM3 and TRPV4 in osteoblastic cells were monitored by RT-PCR. (B) and (D), Intracellular Ca^{2+} increase by hypotonic stress was blocked in knockdown of TRPM3 or TRPV4. The red lines indicated down-regulating TRPM3 or TRPV4 and black lines are normal osteoblastic cells.

7. Extracellular osmolality regulates TRPM3 and TRPV4 expression.

I analyzed expression level of TRPM3 or TRPV4 during extracellular hypo- or hyper-osmotic condition in osteoblastic cells. Osteoblastic cells were cultured in the present with vitamin D_3 plus hypo- or hyper-osmotic solution for 48 hours. As expected, TRPM3 expression level was increased by hypotonic condition with or without vitamin D_3 rather than vitamin D_3 only. In constant, it was reduced by hypertonic solution (Fig. 7A, and B). Like the preceding, TRPV4 was shown to be regulated by hypo- or hyper- osmotic solution in a same manner (Fig 7C, and D). Both TRPM3 and TRPV4 expression were enhanced by hypo-osmotic solution, but were inhibited by hyper-osmotic solution in osteoblastic cells. These results suggest that TRPM3 and TRPV4 is a main channel to regulate $[Ca^{2+}]_i$ by osmolarity change and also indicate possible osmolarity-mediated modulation on RANKL expression pathway in osteoblastic cells.

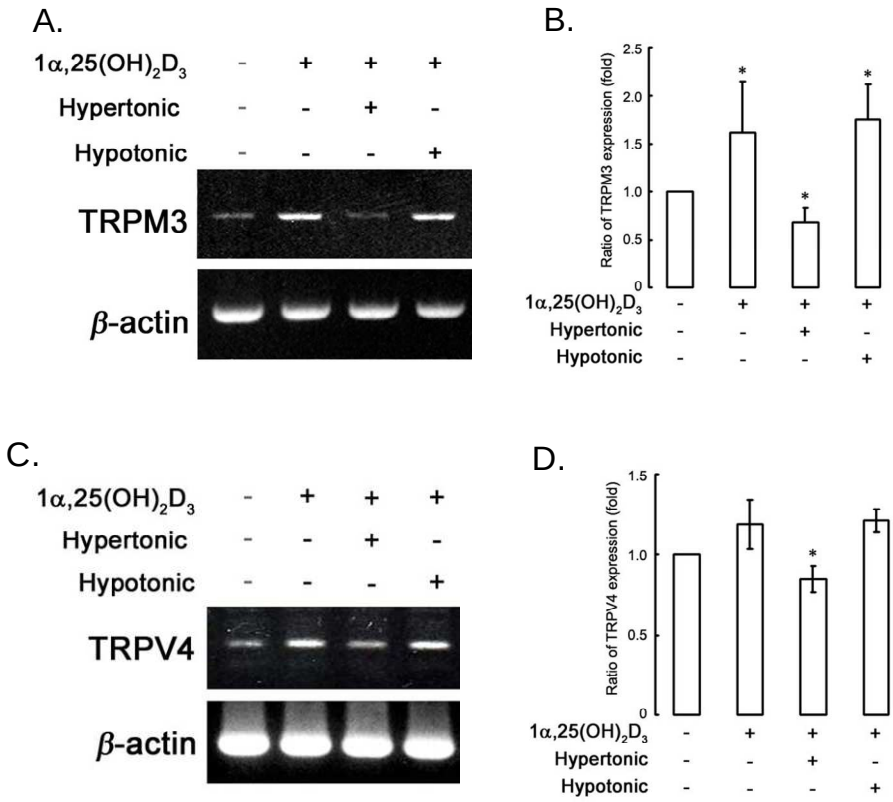
A.
1α,25(OH)2D3 - + + +
Hypertonic - - + -
Hypotonic - - - +
TRPM3
β-actin
B.
Ratio of TRPM3 expression (fold)
2.5
2.0
1.5
1.0
0.5
0
*
*
*
1α,25(OH)2D3 - + + +
Hypertonic - - + -
Hypotonic - - - +
C.
1α,25(OH)2D3 - + + +
Hypertonic - - + -
Hypotonic - - - +
TRPV4
β-actin
D.
Ratio of TRPV4 expression (fold)
1.5
1.0
0.5
0
*
1α,25(OH)2D3 - + + +
Hypertonic - - + -
Hypotonic - - - +

Figure 7. Expression of TRPM3 and TRPV4 by change of osmotic pressure

Osteoblastic cells were cultured by described previously. (A) and (C), Cells were harvested at the 48 hours and mRNA expression levels of TRPM3 or TRPV4 were monitored by RT-PCR (260bp for TRPM3, 360bp for TRPV4, and 366bp for *β*-actin). (B) and (D), TRPM3 and TRPV4 expression level were inhibited by increase of sucrose concentration and were activated by hypo-osmotic solution. Bar represents mean±SEM (n=3; **p < 0.05 versus 1α, 25$(OH)_2D_3$ treatment only).

IV. Discussion

The remodeling of bone consists of a strict coupling of bone resorption and formation that continues throughout life and is necessary not only for skeletal growth but also to maintain normal bone structure (Martin *et al*, 2005). The process begins with bone resorption by osteoclasts followed by new bone formation by osteoblasts. Bone resorption is degradation of mineralized bone matrix, including removal of both inorganic and organic matrix components (Salo *et al*, 1996). If decision for resorption has been made, osteoclasts were secreted proteolytic enzymes, such as a collagenase, lysosomal enzymes, and cysteine proteases, to remove organic matrix and protons to dissolve inorganic matrix as acidification of resorption lacuna. At that time, microenvironment surrounding bone becomes high osmolalitic condition by products of dissolved organic and/or inorganic matrix components. For example, local Ca^{2+}/PO_4^{2-} concentration can rise between as high as 40 mM. High extracellular Ca^{2+}/PO_4^{2-} concentration is reduced bone resorption as well as lead to osteoclast apoptosis and interruption of bone resorption (Zaidi *et al*, 1999; Lorget *et al*, 2000; Xu *et al*, 2005). On the other hand,

mechanical perturbation such as shear stress, or hypo-osmotic pressure, plays a major regulating the specialized function of bone remodeling (Bowler *et al*, 2001) and is induced increase of intracellular Ca^{2+} concentration, which is induced elevation of RANKL expression, in osteoblastic cells by cell swelling (Takami *et al*, 2000; Romanello *et al*, 2005). Hypo- or/and hyper-osmotic pressure is induced change of cell volume; cell shrinkage by hyper-osmotic pressure and cell swelling by hypo-osmotic pressure. Therefore, I suggested that change of extracellular osmotic pressure, hypo- and hyper-osmotic pressure, is induced regulation of osteoclast differentiation. In the present study, I show that osmotic pressure is regulated $[Ca^{2+}]_i$ through the activation of a Ca^{2+} entry pathway, resulting in controlling of RANKL expression on osteoblastic cells.

First, I demonstrated that osmotic pressure has an important role in expression of RANKL in osteoblastic cells. Jeong *et al* recently reported that hyper-osmotic pressure inhibits $1\alpha,25(OH)_2D_3$–induced osteoclasto-genesis in response to regulation of RANKL expression in osteoblastic cells (Jeong *et al*, 2005). However, it is unknown that hypo-osmotic pressure is regulated RANKL expression. To clarify that, expression levels of RANKL is measured under hyper- or hypo-osmotic

pressure using RT-PCR. The vitaminD_3-induced RANKL expression is decreased by hypertonic solution (sucrose 50 mM, 385 mOsm) or is increased by hypotonic solution (215 mOsm). This indicates that osmotic pressure must have important role in regulating function of RANKL expression in osteoblastic cells. The increase of 1α,25$(OH)_2D_3$ –induced RANKL expression is regulated high intracellular Ca^{2+} level by extracellular Ca^{2+} influx in osteoblastic cells (Takami *et al*, 2000; Farach-Carson *et al*, 2004). Stand on this fact; I identified that osmotic pressure regulating RANKL expression is involved in intracellular Ca^{2+} concentration. As expects, increase of 1α,25$(OH)_2D_3$-induced intracellular Ca^{2+} was inhibited by hyper-osmotic stress in osteoblastic cells (Fig.2). I could be assumed that hyper-osmolality decreases the expression of RANKL as inhibition of intracellular Ca^{2+} level in osteoblastic cells. Moreover, intracellular Ca^{2+} level not only elevated by hypo-osmotic stress, which is increased RANKL expression, but also application of $[Ca^{2+}]_i$ has reproductive by hypotonic stress (Fig 3). It does not desensitization. In a chain of results, I found that hypotonic stress-induced RANKL expression is caused by increase of intracellular Ca^{2+} concentration in osteoblastic cells.

Intracellular $[Ca^{2+}]_i$ is set by a dynamic balance between the Ca^{2+}

influx and efflux, and a $[Ca^{2+}]_i$ increase could result from the inhibition of Ca^{2+} efflux or activation of Ca^{2+} influx (Tsuzuki *et al*, 2000). To investigate whether $[Ca^{2+}]_i$ increase induced by hypo-osmotic stress was due to extracellular Ca^{2+} influx, I further examined that the response to hypo-osmotic stress in presence of Ca^{2+} channel modulation in osteoblastic cells. Increase of $[Ca^{2+}]_i$ by hypo-osmotic solution was inhibited by Ca^{2+} free hypo-osmotic solution in hypotonic solution as well as Gd^{3+}, which is a nonspecific blocker of stretch-activated ion channels (Fig 4). Moreover, depletion of intracellular Ca^{2+} store site did not significantly affect $[Ca^{2+}]_i$ increase induced by hypo-osmotic stress. These findings indicate that the $[Ca^{2+}]_i$ increase in the present experiment was due to Ca^{2+} entry via Ca^{2+} influx from extracellular solution, and not due to release from intracellular Ca^{2+} store sites in osteoblastic cells.

Recently, it has been characterized two different TRP-homologous cation channels, TRPM3 and TRPV4, as protein mediating Ca^{2+} entry in cells upon extracellular application of hypo-osmotic solutions (Harteneck *et al*, 2007). I hypothesized that TRPM3 and TRPV4 expression correlates with hypo-osmotic stress-induced extracellular Ca^{2+} influx. My data was shown that hypo-osmotic induced Ca^{2+} influx

is partially inhibited in response to ruthenium red (TRPM3 blocker) and 2-APB (TRPV4 inhibitor) (Fig 5) and is evoked by activity of TRPM3 and TRPV4 (Fig 7). To confirm my data, TRPM3 and TRPV4 knock-down osteoblastic cells using siRNA were induced to inhibit intracellular Ca^{2+} concentration in response to hypotonic solutions (Fig 6). Therefore, TRPM3 and TRPV4 activity involves intracellular Ca^{2+} influx by hypotonic solution in osteoblastic cells.

Signal transduction pathways regulating RANKL expression in osteoblasts/stromal cells fall into at least four categories: vitamin D receptor, PKA, calcium/PKC, and gp130 mediated signals (Suda *et al*, 1995; Takami *et al*, 2000). Based on these data presented here, a schematic diagram of RANKL expression modulation by hypo-osmolality is shown in Figure 8. Mechanical stress stimulation, as fluid shear stress, membrane stretch or hypo-osmotic swelling, caused a significant increase in intracellular Ca^{2+} level by TRPM3 and TRPV4. Recently, fluid flow induction of RANKL expression in osteoblasts has been reported previously and said to be PKA and ERK signaling dependent (Mehrotra *et al*, 2006). Accordingly, TRPM3 and TRPV4 induced hypo-osmotic stress might be triggered by activation of down-stream PKA-ERK signals.

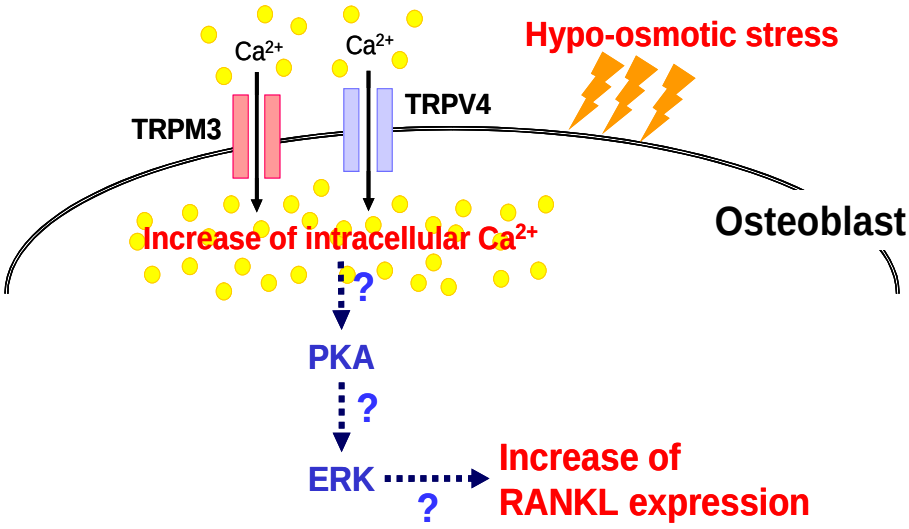

Figure 8. Schematic diagrams for modulation of RANKL expression by hypo-osmolality-induced Ca^{2+} signaling

During hypo-osmotic stimulation, intracellular Ca^{2+} level increased through TRPM3 and TRPV4 channel. Intracellular Ca^{2+} will be also activated downstream signal such as PKA, and ERK pathway, which is increased RANKL expression. In this situation, hypo-osmotic pressure comprises a positive feedback loop for osteoclastogenesis.

V. Conclusion

I suggested that osmotic pressure is regulated RANKL expression by controlling of extracellular Ca^{2+} entry on osteoblastic cells. The results we found out are described bellows.

1. Hypo-osmolality increases RANKL expression in osteoblastic cells.

2. Hypo-osmolality increases $[Ca^{2+}]_i$ and hypo-osmolality-induced $[Ca^{2+}]_i$ increases are evoked from Ca^{2+} influx across the plasma membrane.

3. TRPM3 and TRPV4 are involved in hypo-osmolality-induced $[Ca^{2+}]_i$ increases, respectively.

In conclusion, my studies reveal that hypo-osmotic activation of TRPM3 and TRPV4 play important roles in RANKL expression in osteoblastic cells. This direct action of hypo-osmolality may partly contribute to the regulation of RANKL expression by Ca^{2+} response through TRPM3 and TRPV4. Further investigations will be required to clarify the down-stream signaling of TRPM3 and TRPV4 in order to RANKL expression.

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국문요약

조골세포에서 저삼투압에 의한 TRPM3 와 TRPV4 를 통한 칼슘 유입과 RANKL 발현 증가

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골은 조골세포와 파골세포에 의해 끊임없이 재형성과정을 거친다. 1α,25$(OH)_2D_3$는 조골세포로부터 osteoprotegerin과 receptor activator of NF-κB ligand (RANKL)이라는 주요인자를 분비하여 파골세포 분화과정을 촉진시킨다. 암괴사인자 중 하나인 RANKL은 파골 전구세포의 파골세포로의 분화, 파골세포의 활성화와 세포생존에 주요 역할을 한다. 한편, 골 개조는 fluid shear stress나 저삼투압과 같은

기계적 자극에 의해서도 조절되는데, 이는 조골세포 세포질 내 칼슘이온 농도의 증가와 세포기능에 변화를 일으킨다. 그런데 조골세포에서 삼투성 변화와 같은 기계적 자극에 의한 칼슘농도의 증가 기전과 RANKL 발현의 변화의 연관성 등은 잘 알려져 있지 않다. 이에 본 연구에서는 RT-PCR과 칼슘신호 측정, RNA 간섭 방법 등을 통해 조골세포에서 삼투성 변화시 RANKL 발현량, 세포내 칼슘신호 변화, 그리고 삼투성 변화에 의해 조절되는 칼슘통로 등을 알아보고자 하였다. 고삼투성용액은 1α,25$(OH)_2D_3$-유도성 RANKL 발현을 억제하였으며, 저삼투성 용액은 그 자체만으로 RANKL 발현을 증가시켰다. 1α,25$(OH)_2D_3$-유도성 칼슘농도의 증가는 고삼투성에 의해 억제되었으며, 저삼투성은 자체만으로 세포내 칼슘 농도를 증가시켰으며 이 반응은 가역적이었다. 세포외부의 칼슘제거, 또는 세포막 칼슘통로의 억제제인 gadolinum은 저삼투성 유도성 칼슘 농도의 증가를 각기 봉쇄하여, 저삼투성 유도성 칼슘 농도의 증가는 세포외부로부터의 칼슘 유입에 의한 것 임을 의미한다. TRPV4의 억제제인 ruthenium red나 TRPM3의 억제제인 2-APB는 저삼투성 유도성 칼슘 농도의 증가를 각기 부분적으로 봉쇄하였으며, 또한 TRPV4와 TRPM3의 RNA 간섭은 저삼투성 유도성 칼슘 농도의 증가를 각기 부분적으로 봉쇄하여 저삼투성 유도성 칼슘 농도의 증가기전에 TRPV4와 TRPM3가 관여함을 시사하고 있다. 더욱이, 저삼투성은 TRPV4와 TRPM3의 발현을

증가시켰다. 이상의 결과는 조골세포에서 저삼투압에 의한 TRPM3와 TRPV4 이온통로의 열림 활성화가 세포 외부로부터의 칼슘 유입증가를 통해 RANKL의 발현 증가를 일으킨다는 것을 의미한다.

핵심되는 말: 저삼투성, RANKL, 칼슘신호, TRPM3, TRPV4, 조골세포