Hypertonic stress down-regulates 1α,25- dihydroxyvitaminD$_3$-induced osteoclastogenesis via TonEBP in co-culture system

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Abstract

Hypertonic stress down-regulates 1α,25-dihydroxyvitaminD$_3$-induced osteoclastogenesis via TonEBP in co-culture system

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Bone remodeling is a process controlled by the action of two major bone cell-the bone forming osteoblast and the bone resorbing osteoclast. In the process of osteoclastogenesis, stromal cells and osteoblast express RANKL, and M-CSF, which are up-regulated by the osteoclastogenetic molecules and OPG, which is decoy receptor of RANKL. During the bone resorption by activated osteoclasts, extracellular Ca^{2+}/PO_4^{2-} concentration becomes to be increased as high as 40 mM. In this study, we demonstrated the effects of hypertonicity on osteoclastogenesis in osteoblast-osteoclast co-culture system for the purpose of understanding at the molecular level of hypertonicity on bone metabolism.

Hypertonic stress down-regulates 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$)-induced osteoclastogenesis in co-culture system as well as RAW 264.7 cell (osteoclast cell line). Hypertonicity (sucrose 25, 50 mM) inhibits the number of tartrate-resistant acid phosphatase (TRAP) positive multinucleated cells induced by 10 nM 1α,25$(OH)_2D_3$. In order to investigate

the mechanism by which hypertonicity inhibits osteoclastogenesis, the mRNA expression of receptor activator of nuclear factor NF-κB ligand (RANKL) and osteoprotegerin (OPG) were analyzed by RT-PCR and ELISA. In hypertonic condition, RANKL mRNA and RANKL protein expression were decreased in a dose-dependent manner, while the change in OPG mRNA and OPG protein were not occurred in significantly. Hypertonic stress inhibits osteoclast differentiation by reducing RANKL/OPG ratio in osteoblastic cells. We tested the acts as a signal for various cellular functions in osteoclastogenesis using the western blot in RAW 264.7 cell. But hypertonicity did not affect RANKL-RANK mediated signaling of osteoclast differentiation such as MAP Kinase and NF-κB. In these results, hypertonicity may affect RANKL synthesis pathway in osteoblastic cell.

Furthermore, TonEBP (tonicity-responsive enhancer binding protein), which plays a central role in protecting renal medullary cells from the stress of high osmolality, expressed in osteoblastic cell. The shut-down of TonEBP in the osteoblastic cells induced the expression of RANKL mRNA as well as TonEBP overexpression suppressed the expression of RANKL. These findings indicated that TonEBP gene had an effect on the regulation of RANKL expression and that TonEBP might be involved in the process of $1\alpha,25(OH)_2D_3$ inducing osteoclastogenesis in terms of signal transduction pathway.

Further studies are needed to clarify the exact target and inhibition mechanism of TonEBP in osteoblastic cells.

Key words : Hypertonicity, Osteoclastogenesis, RANKL, OPG, TonEBP, $1\alpha,25(OH)_2D_3$

I. Introduction

In general, bone remodeling is regulated by the activity of bone-forming osteoblasts and bone-resorbing osteoclasts. Osteoblasts stem from mesenchymal stem cells, whereas osteoclasts arise by the differentiation of osteoclast precursors of hematopoietic stem cells. Osteoblasts and osteoclasts are required not only for skeletal development, but also for mineral homeostasis and the normal remodeling of bone in adults.[18] Both osteoblasts and osteoclasts are regulated by a variety of hormones such as parathyroid hormone (PTH), interleukin-1 (IL-1), interleukin-6 (IL-6), estrogen and prostagrandin E_2 (PGE_2), and local factors like fluid movement, and located Ca ion.[2, 4, 13, 14] Theoretically, an imbalance between bone formation and bone resorption causes metabolic bone diseases like osteopetrosis and osteoporosis.[1] Therefore, osteoblasts and osteoclasts are known to be closely related during the process of remodeling of bone.[23, 24, 28] A certain kinds of signaling molecules, such as, RANKL, osteoprotegerin (OPG) and macrophage colony stimulating factor (M-CSF), expressed by osteoblasts, are involved in osteoclastogenesis and in osteoclast development.[28] For instance, when osteoblasts/stromal cells are stimulated by osteotropic factors such as parathyroid hormone, RANKL is expressed and induces the differentiation of osteoclast progenitors by binding to the receptor activator of NF-κB (RANK; also known as ODF receptor).[9] In addition, M-CSF is known to be essential for macrophages to be transformed into osteoclasts, while OPG, a decoy receptor of RANKL, is participated in the regulation of osteoclastogenesis.[14] Specifically, OPG, as a member of the tumor necrosis factor receptor (TNFR) family, inhibits the osteoclastogenesis stimulated by $1\alpha,25(OH)_2D_3$, PTH, or IL-11.[24] Consequently, it is believed that RANKL, M-CSF and OPG, which are expressed by osteoblasts, are associated with osteoclastogenesis, and that osteoblasts may play a major role in the bone remodeling process. RANKL binds and signals via a membrane-bound TNF receptor super family member named TRANCE/RANK.[24] RANKL also bind a naturally occurring soluble receptor antagonist named osteoprotegerin.[29, 35] RANK mediates the osteoclastic activity associated with RANKL. However OPG as both a monomer and disulfide-linked homodimer, abrogates the osteoclast effects

associated with RANKL by binding the both membrane attached-RANK and soluble RANK.[7, 8, 24, 28]

During the bone resorption, activated osteoclasts induced extracellular Ca^{2+}/ PO_4^{2-} concentration to rise between 8 – 40 mM.[10] In microenvironment around bone surface, accumulation of high Ca^{2+} / PO_4^{2-} induce apoptosis of osteoclast and may change the function of osteoblast in the response to shrinkage.[29]

On the whole, hypertonicity stresses animal cells because the ensuing osmotic efflux of water shrinks the cells and concentrations their contents. In the kidney, tubular cells adapt to the hypertonicity by accumulating compatible osmolytes that are small organic solutes, such as betaine, inositol, taurine, sorbitol, and glycerophosphorylcholine.[10, 16]

TonEBP (Tonicity-responsive enhancer binding protein) is responsible for transcriptional activation of the genes encoding the regulation of compatible osmolyte transporter in consult with survival of kidney cells in hypertonicity ; sodium/myo-inositol co-transporter (SMIT), the sodium/ chloride/ betaine co-transporter (BGT-1), and aldose reductase (AR). TonEBP is a member of the Rel family of transcriptional activators, distinct from other members NF-κB and NFAT.[3, 5, 21] Apart from some knowledge of the general functions of hypertonicity in a kidney as well as other the tissue, there are no experimental evidences whether the hypertonic stress is related to osteoclastogenesis at the cellular level.[10, 21] With respect to osteoclastogenesis, we have focused on the effect of hypertonicity on the osteoblast and osteoclast. Understanding of hypertonicity on bone metabolism is necessary at the molecular level of osteoblast and osteoclast. Therefore, we hypothesized that hypertonicity might concern $1\alpha,25(OH)_2D_3$–induced osteoclastogenesis and bone metabolism with respect to RANKL and OPG on the osteoblast. To see whether hypertonic stress (sucrose) can affect the osteoclastogenesis, we applied it to an osteoblast/stromal cell co-culture system (for $1\alpha,25(OH)_2D_3$–induced osteoclastogenesis) and RAW 264.7 cells (for RANKL-mediated osteoclastogenesis). We tested pit formation on bone slices for the purpose to ascertain whether hypertonicity acts on the process of the osteoclast activity. Also we compared not only RANKL and OPG mRNA expression profiles

but also secreted soluble RANKL and OPG protein in osteoblasts. To rule out the possibility that sucrose causes non-physiological cell damage, an MTT viability test was performed. To see the hypertonicity acts as a signal for various cellular functions in osteoclastogenesis, we investigated the change of phosphorylated mitogen activated protein (MAP) kinase and NF-κB in RAW 264.7 cell. So, we suggested that hypertonicity affected RANKL synthesis pathway in osteoblastic cell. Also, we confirmed that hypertonic stress led TonEBP to expression in osteoblastic cells. Finally, To investigate the role of TonEBP, osteoblastic cells were transfected with RNA interference of TonEBP, mouse wild and dominant negative TonEBP in osteoblastic cells. We expect that hypertonicity regulates $1\alpha,25(OH)_2D_3$–induced osteoclastogenesis via TonEBP by effecting the RANKL synthesis signal pathway.

II. Materials and Methods

1. Materials

Routine cell culture media were obtained from GIBCO/BRL (Grand Island, NY). The Tartrate-Resistant Acid Phosphatase Staining Kit and sucrose were purchased from the Sigma Chemical Co., Ltd. (St. Louis, MO). Trizol was purchased from Invitrogen Corp. (Carlsbad, CA), and the ICR mice were from Samtacho Co., Ltd. (Seoul, Korea). All other chemicals were of the highest grade commercially available. TonEBP and Yc-1 dominant-negative TonEBP genes were approved by Dr. Sang-do Lee (Chungnam national university college of medicine).

2. *In vitro* osteoclast formation assay

The osteoclast formation assay was carried out as previously reported by Choi *et al*. (2001). Briefly, the osteoblasts were isolated from 1 - 2 day old newborn mice. Three-ten calvariae were digested in 10 ㎖ of an enzyme solution containing 0.2 % collagenase (Wako, Japan) and 0.1 % dispase (GIBCO/BRL, USA) for 20 minutes at 37°C in a shaking water bath. The supernatant was discarded and 10 ml of the enzyme solution was added. After shaking at 37°C for 20 minutes, the supernatant was collected carefully and transferred to a new tube. This digestion of calvariae by collagenase and dispase was repeated three times. The collected supernatant (30 ml) was placed in a centrifuge at 1,500 rpm for 5 minutes, and to collect the osteoblastic cells. Cells were resuspended in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) and cultured to confluence in 100 mm culture dishes at a concentration of 1×10^5 cells/dish. The cells were then detached from the culture dishes using trypsin-EDTA, suspended in α-MEM with 10% FBS and used for the co-culture as osteoblastic cells.

Femoral and tibiae bone marrow cells were collected from 4-week-old mice. The tibiae and femora were removed and dissected free of adhering tissues. The bone ends were removed and the marrow cavities flushed by slowly injecting

media at one end using a 25-gauge needle. The calvaria and bone marrow cells collected were washed and used in the co-culture. Mouse calvarial cells (1×10^4 cells/well) were co-cultured with bone marrow cells (1×10^5 cells/well) in α-MEM containing 10% FBS in 48-well plates (Corning Inc., Corning, NY). The culture volume was made up to 400 μl per well with α-MEM supplemented with 10% FBS, in the presence of $1\alpha,25(OH)_2D_3$ (10 nM), without or with sucrose (25, 50 or 100 mM). All cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in air. After incubation for 4 days, the cells were subjected to tartrate-resistant acid phosphatase (TRAP, an osteoclast marker enzyme) staining. We counted TRAP positive multinucleated (more than three) cells as osteoclast. *In vitro* formation assay of osteoclast was repeated four times.

3. Viability test

The MTT (3-[4, 5-dimethylthiazol-2-yl-]-2, 5-diphenyltetrazolium 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. MTT was dissolved in phosphate-buffered saline (PBS) at 5 mg/ml and filtered to remove any insoluble residue. MTT solution was added directly to the assay plates. The cells were subsequently incubated for an additional 4 hours at 37°C. The purple formazan crystals that formed were dissolved in DMSO (dimethyl solfoxide), and the plates were read on a spectrophotometer at 570 nm.

4. Pit formation assay

Osteoblastic cells obtained from the calvariae of newborn ICR mouse and bone marrow cells obtained from the tibiae and femora of male ICR mouse were cocultured in α-MEM in calcium phosphate apatite-coated 24-well plate, OAAS (Osteoclast activity assay substrate ; Oscotec, korea) according to the method of Eijiro *et al*. and Youngnim *et al*. with a slight modification.[6, 34] Briefly, the osteoblastic cells and bone marrow cells were resuspended in complete α-MEM

medium and plated into a calcium phosphate apatite-coated plate, OAAS at 2×10^5 cells/0.8 ml/well and 2×10^6 cells/0.8 ml/well, respectively. The cells were cultured for 4 days at 37° C in a humidified 5% CO_2 atmosphere. Then the cells treated with 10 nM 1α,25$(OH)_2D_3$ and sucrose by different concentration (25, 50 100, 150, and 200 mM) respectively. Cultures were maintained for 4 days. The medium in each well was replaced with the respective fresh completive medium and 1α,25$(OH)_2D_3$ and sucrose. The experiments were performed four times. After termination of culture, attached cells were removed from the plate by abrasion with 4% sodium hypochloride solution (Sigma). Images of pit were gathered with a digital camera attached to a microscope at ×100 magnification, and total areas of resorption pits were analyzed by the Image Meta Morph program. The data were presented as means ±SD of four times sample.

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

RT-PCR experiment consists of RNA isolation, cDNA polymerization, PCR amplification. The expressions of RANKL, OPG, M-CSF and β-actin were evaluated by RT-PCR using total RNA isolated from murine osteoblastic cells. Total RNA was isolated using Trizol reagent, chloroform, isoprophylethanol. The primers used were: for RANKL (750 bp), 5'-ATCAGAAGACAGCACTCACT-3'(forward), 5'-ATCTAGGACATCCATGCT AATGTTC-3'(reverse); for OPG (636bp), 5'-TGAGTGTGAGGAAGGGCGTTAC-3'(forward) 5'-TTCCTCGTTC TCTCAATCTC-3' (reverse) ; for β-actin (366 bp), 5'-GGACTCCTATGGTG GGTGACGAGG-3' (forward), and 5'-GGGAGAGCATAGCCCTCGTAGAT-3' (reverse); for M-CSF (395bp), 5'-CATGACAAGGCCTGCGTCCGA-3' (forward), and 5'-AAGCTCTGGCAGGTGCTCCTG-3' (reverse1), 5'-GCCGCCTCCACC TGTAGAACA-3' (reverse2)

Relative RT-PCR was performed to measure gene expression of RANKL, OPG, and β-actin mRNAs. Polymerase chain reactions were performed on a T gradient 96 PCR machine (Biometra Co., Gottingen, Germany) using ~100 ng of cDNA (complementary DNA), 5 pmoles each oligonucleotide primer, 200 μM of each

dNTP, 1 unit of Taq Polymerase (Applierd Biosystems, CA, USA) and 10× Taq polymerase buffer in a 50 μℓ volume. The PCR program initially started with a 95°C denaturation for 5 min, followed by 25 to 38 cycles of 95°C /1 min, T_a /1 min, 72°C/1 min (T_a, annealing temperature; 45.3°C for RANKL, 47.9°C for OPG, and 58°C for β-actin and M-CSF). Linear amplification range for each gene was tested on the adjusted cDNA. The less expressed transcripts of RANKL and OPG required 35 cycles of PCR for detection. For M-CSF and β-actin, 30 and 25 cycles of PCR was performed, respectively. 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.

6. 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 2000pg/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.

7. Western blot

Protein extracts were prepared from osteoblastic cells, MC3T3-E1 cells and RAW 264.7 cells as follows. These cells were washed with ice-cold PBS and then lysed by adding Tris-HCl, NaCl, and EDTA buffer (1% NP-40, 10 mM of Tris-HCl [pH 7.8], 150 mM NaCl, 1 mM EDTA), 2 mM Na_3VO_4, 10 mM NaF, and 10 μℓ/mℓ aprotinin, 10 μℓ/mℓ leupeptine, and 10 μℓ/mℓ PMSF. The lysates were clarified by centrifugation at 15,000rpm for 20 min. An equal amount of protein was subjected 10% sodium deoxyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred electrophoretically onto a nitrocellulose membrane. The membrane was incubated in a blocking solution containing 5% non-fat milk at room temperature for 1hour and washed 1x TBS (Tris-buffered saline) solution containing 0.1% of Tween-20. The membrane was probed with 1:1000 diluted of anti-phospholyration MAP kinase (New England Biolabs.) and TonEBP at 4°C for overnight. The same membrane followed by secondary antibody coupled with horseradish peroxidase-linked goat anti-rabbit IgG antibody (Santa Cruz Biotechnology). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) western blotting detection reagents (Amersham Pharmacia Biotech., Alington Heights, IL, USA) following the procedure recommended by the supplier.

8. RNA interference

8.1. Cloning of small interfering RNA

siSTRIKE™ U6 hairpin cloning systems kit (Promega, Madison, USA)

were used to synthesize 21-nucleotied single-stranded RNA . Briefly, two hairpin oligonucleo-tides are annealed to form a double-stranded DNA fragment for inserting it into the psiSTRIKE™ vectors. For assistance with hairpin oligonucleotide design visit the siRNA Designer at : **www.promega.com/ techserv/tools.** The primers used were: for TonEBP (56 bp), 5'-ACCGAGTTCCTGAGATCTTAAATTCAAGAGATTGATCTC AGGAACTCTTTTTC-3' (anti-sense), 5'-TGCAGAAAAAGAGTTCCTG AATCTTAAATCTCTTGAATTTAAGATCTCAGGAACT-3' (sense).

For cloning the psiSTRIKE™ vectors inserting a hairpin oligonucleotides, the annealing reaction was performed at 90℃ for 3 min followed by incubating at 37℃ for 15 min using 1 μg/μℓ each oligonucleotide.

8.2. Ligation of small interfering RNA into the psiSTRIKE™ vectors

The annealed hairpin oligonucleotides were diluted at the final concentraion of 4ng/ml. For ligating hairpin insert to the psiSTRIKE™ vectors, psiSTRIKE™ vectors and diluted hairpin oligonucleotides were incubated at the room temperature for 1hr using the T4 DNA ligase.

8.3. Transformation of *E.coli* with psiSTRIKE™ vectors

To obtain a reasonable number of the ligation of fragments with a hairpin, performed transformation reaction using the *EOS*-1 min Competent cells (Yeastern Biontech. Co. Taipei, Taiwan.). Recombinant plasmid DNA was isolated.

8.4. Confirmation of recombinant plasmid DNA

The recombinant plasmid DNA was digested with restriction enzyme *Pst*I yielded 3655bp and 958bp. This construct was 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.

9. Transfection experiments

The osteoblasts that were isolated from 1 - 2 day-old newborn mice were seeded at 70% confluence in 35 mm dishes. Cells were resuspended in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS). The day before transfection, osteoblastic cells changed the α-MEM antibiotics-free medium. Trasfection of recombinant DNA (TonEBP, DN-TonEBP, and siRNA of TonEBP) was carried out using lipofectamine^{TM} 2000 (Invitrogen Corp., Carlsbad, CA) for 24hr.

10. Data analysis and statistics

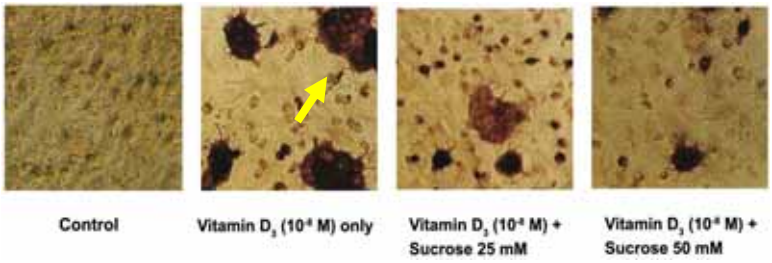
The results are expressed as the mean ± S.E. The statistical significances of differences between the groups were determined using the Kruskal-Wallis and Bonferroni's test. In statistical tests, the p value < 0.05 was considered to be significant.

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 osteoblastic cells/ bone marrow co-culture. To clarify the role of hypertonicity on bone metabolism, 25, 50, 100, 150, and 200 mM of sucrose as hypertonic stresses were added to co-cultures and incubated at 37°C for 4 days to investigate osteoclast differentiation. When 10 nM of 1α,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, 1α,25(OH)$_2$D$_3$-induced osteoclast differentiation was reduced (Fig. 1A). In detail, the addition of 25, 50, 100, 150, and 200 mM of sucrose reduced the number of TRAP positive multinucleated cells up to about 50% in 50 mM sucrose (Fig. 1. blue panel). However, it might be possible that sucrose causes cell damage directly without interrupting the normal maturation of osteoclasts. To rule out this possibility, we used a viability test. As shown in Fig. 1 yellow panel, sucrose did not show a toxic effect when treated at up to 100 mM. These results suggest that the effect of sucrose on osteoclast formation was caused by its some effect of osteoclastogenesis or changing of activity, not by its direct toxic effect upon the cells.

A.



B.

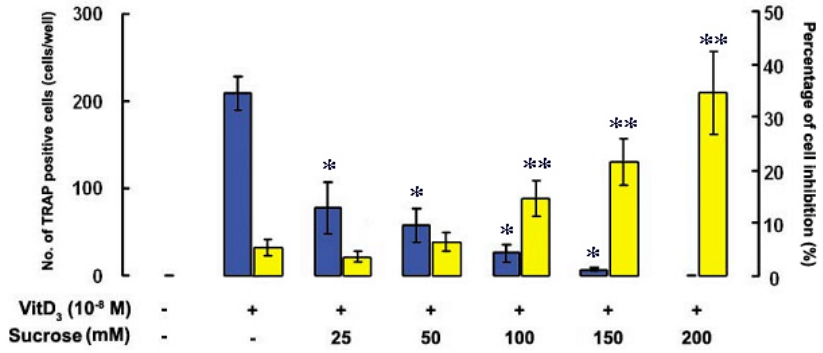
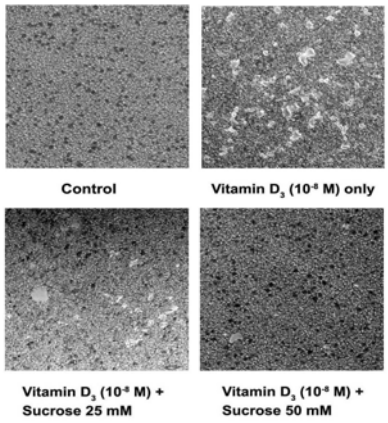


Fig. 1. Hypertonic stress inhibited 1α,25(OH)$_2$D$_3$-induced osteoclast differentiation at dose-dependent concentration in co-culture system. (A) In the presence of sucrose, 10 nM 1α,25(OH)$_2$D$_3$ –induced osteoclast differenciation was reduced (×200). The yellow arrow indicated osteoclast cells. (B) TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts (blue panel) and MTT test (yellow panel). One hundred mM of sucrose showed remarkable a high cell inhibition. The statistical significance of differences between the groups was determined using the one-way ANOVA test. In all statistical tests, a p value < 0.05 was considered to be statistically significant. . * (TRAP staining) and ** (MTT assay) ; Insignificantly different (p < 0.05). Each data was shown in mean ±SE of four cultures.

2. Effects of hypertonic stress on resorption pit formation

We measured bone lacuna resorbed at each bone slice. There was no resorption lacuna on OAAS (osteoclast activity assay substrate) plates in the absence of $1\alpha,25(OH)_2D_3$-induction. On the other hand, $1\alpha,25(OH)_2D_3$ (10 nM) induced a number of bone resorption pits and we could find multiple lacuna, which was used as a control. But in the experimental group, resorption pits were incrementally decreased as the function of hypertonicity up to 50 mM sucrose (Fig. 2). As show in Fig. 2, total resorption area was also decreased by the addition of sucrose.

A.



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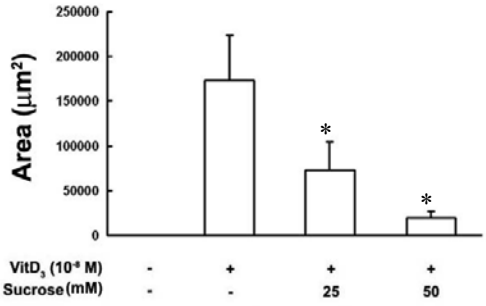


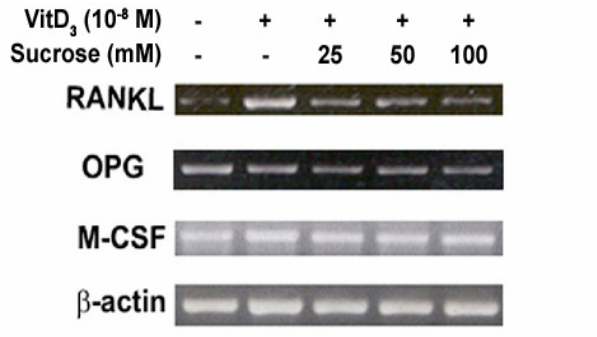
Fig. 2. Hypertonicity inhibits 1α,25(OH)$_2$D$_3$-induced pit formation. (A) The resorbed lacunae on the OAAS (osteoclast activity assay substrate) plates were photographed the microscope (×100). (B) Total resorption area per well was measured by image analyzer and graphed. * ; Insignificantly different ($p < 0.05$) in compared to control.

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

As shown in Fig. 3, the expressions of RANKL and OPG mRNA in osteoblasts were monitored by RT-PCR in the presence and absence of sucrose. As the sucrose concentrations in the cell culture medium were increased, the $1\alpha,25(OH)_2D_3$-induced expression of RANKL mRNA was down-regulated (Fig. 3). RANKL mRNA expression in osteoblasts was inversely proportionally to sucrose concentration. On the other hand, the expression of OPG mRNA was not changed regardless of sucrose concentration. These findings indicate that addition of sucrose (25, 50, and 100 mM) inhibits osteoclast formation by down-regulating the expression of RANKL.

RANKL and OPG proteins were also analyzed with ELISA using anti-RANKL and anti-OPG antibodies. RANKL protein was decreased with the increase of sucrose concentration (Fig. 4. dark blue panel). On the other hand, the addition of sucrose did not change the OPG protein quantitatively which is consistent with OPG mRNA data (Fig. 4. purple panel). Consequently, the addition of sucrose inhibited RANKL mRNA and soluble RANKL protein, and led to altered osteoclastogenesis. In addition, such changes of signaling molecules were dependent on the sucrose concentration.

A.



B.

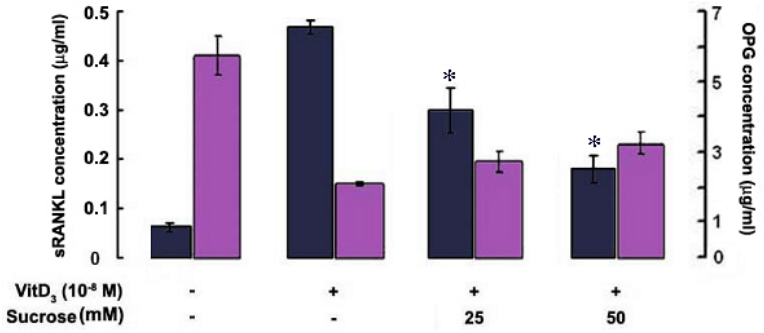
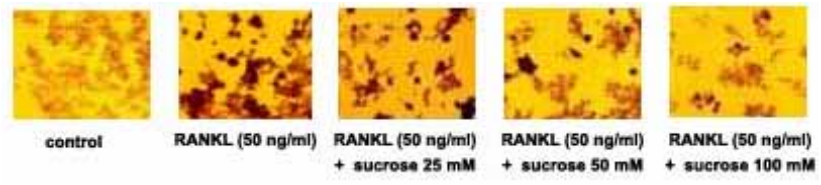


Fig. 3. Hypertonic stress caused changes in RANKL and OPG expression. (A) Various concentrations of sucrose were added to the mouse calvarial osteoblasts culture with 10 nM 1α,25$(OH)_2D_3$. After incubation for 4 days, total RNA was then extracted from osteoblasts, and the expression of RANKL and OPG mRNAs was analyzed by RT-PCR products for RANKL, OPG, and M-CSF were 750bp, 636bp, and 395bp, respectively. (B) Protein analysis using ELISA showed that addition of sucrose inhibited the expression of soluble RANKL (dark blue panel). On the other hand OPG level were increased slightly in osteoblasts stimulated by sucrose (purple panel), but it was not statistically significant. * (RANKL) and ** (OPG) ; Insignificantly different ($p < 0.05$). The results were expressed as the means ±SE of four experiments.

4. Inhibition of soluble RANKL-induced osteoclast formation by hypertonicity in RAW 264.7 cells

Osteoclastogenesis was induced by sRANKL in RAW 264.7 cell culture. To clarify the role of hypertonicity on osteoclast formation, the sucrose (25, 50, 100, 150, and 200 mM) were added to cultures and incubated at 37°C for 4 days. When 50 ng/ml of sRANKL was added to RAW264.7 cell culture, TRAP positive multinucleated cells were formed, whereas no TRAP positive cells were detected in media only (Fig. 4A). In the presence of sucrose, sRANKL-induced osteoclast formation was reduced (Fig. 4B yellow panel). However, it might be possible that hypertonic stress causes cell damage directly without interrupting the normal maturation of osteoclasts. To rule out this possibility, we used a viability test. As shown in Fig. 4B sky blue panel, lypertonic stress did not show a toxic effect when treated up to 100 mM sucrose.

A.



B.

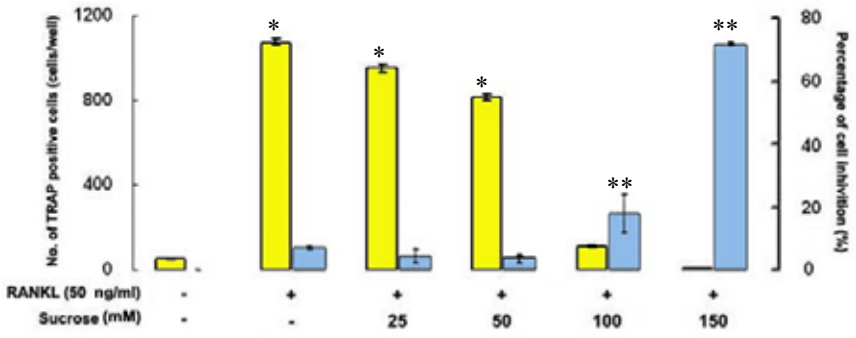


Fig. 4. Hypertonicity inhibits soluble RANKL-induced osteoclastogenesis. (A) In the presence of hypertonic stress (in addition of sucrose), soluble RANKL (sRANKL) -induced osteoclast differentiation was reduced (×200). (B) TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts (yellow panel) and MTT test (sky blue panel). The statistical significance of differences between the groups was determined using the one-way ANOVA test. In all statistical tests, a p value ‹ 0.05 was considered to be statistically significant. . * (TRAP) and ** (MTT assay) ; Insignificantly different ($p < 0.05$). Each data was shown in mean ±SE of four cultures.

5. Expression of MAP kinase and NF-κB in response to hypertonicity in RAW 264.7 cells

We have demonstrated how hypertonic stress is linked to the RANKL mRNA and protein expression in osteoblastic cell. In general, mitogen activated protein (MAP) kinase (ERK, JNK, and p38) and NF-κB are preferentially activated by cytokines and cellular stress and plays a key role in regulating the activity of various transcriptional factors. The phosphorylated-MAP kinases were examined to investigate whether those kinases were inhibited by sucrose or not in RAW264.7 cells. As a result, although MAP kinases were activated by treatment with RANKL, they were not inhibited when 50 mM sucrose was added because the maximal concentration to inhibited soluble RANKL protein expression (Fig. 5A). On the other hand, the addition of sucrose did not affect the expression of NF-κB (Fig. 5B).

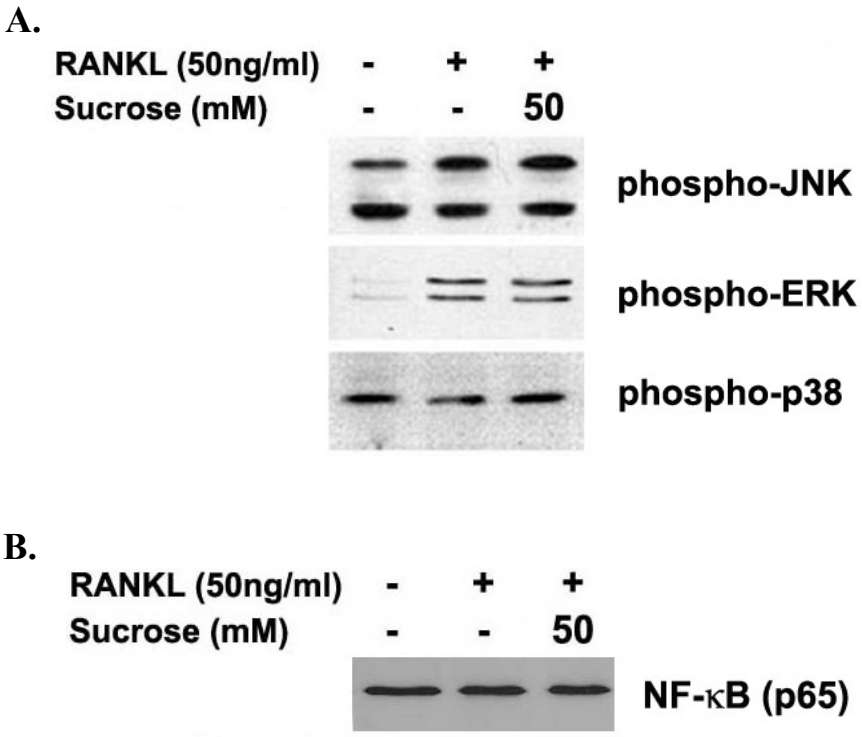


Fig. 5. Hypertonic stress has no effect on the activation of MAP kinases and NF-κB in RAW 264.7 cells. (A) RAW 264.7 cells (2 x 10^6cells/culture) were treated with 50ng/ml sRANKL and 50 mM of sucrose for the indicated periods of time, and the cell lysed. Cell lysates were then subjected to Western blot analysis with a polyclonal antibody against phosphorylated MAP kinase. (B) Western blot analysis with a polyclonal antibody against NF-κB.

6. Expression of TonEBP in bone cell on hypertonic condition

In the Kidney cell, TonEBP (Tonicity-responsive enhancer element binding protein) that is responsible for transcriptional activation of the genes encoding the regulation of compatible osmolyte transporter is stimulated by hypertonicity.

We ascertained whether TonEBP is expressed in the bone cells. Western analyses have shown that osteoblastic cells and osteoblast cell (MC3T3-E1) express TonEBP. It was shown that TonEBP by the hypertonic challenge might be involved in the process of $1\alpha,25(OH)_2D_3$ induced osteoclastogenesis in terms of signal transduction pathway.

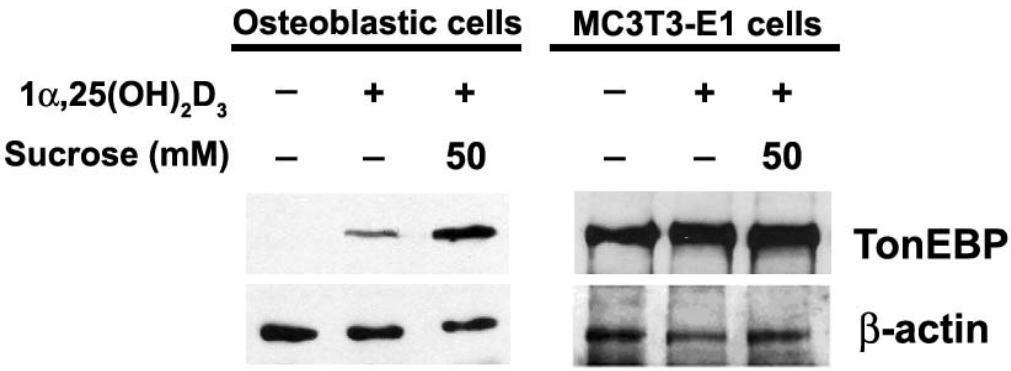


Fig. 6. Hypertonicity regulated TonEBP expression in the osteoblastic cells. Osteoblastic cells and MC3T3-E1 cells (2 x 10^6 cells/culture) were treated with 10 nM $1\alpha,25(OH)_2D_3$ and 50 mM of sucrose for the indicated periods of time, and the cell lysed. Cell lysates were then subjected to Western blot analysis with a polyclonal antibody against TonEBP.

7. Effect of TonEBP on the expression of RANKL mRNA in the osteoblastic cells.

To silence the expression of TonEBP by RNA interference, we designed small interfering RNA (siRNA) duplex. The osteoblastic cells were transfected with RNA interference of TonEBP, mouse TonEBP gene and dominant negative TonEBP gene. After osteoblastic cells were changed with isotonic medium containing $1\alpha,25(OH)_2D_3$ (10^{-8} M) for 2 days, we analysed the expression of RANKL mRNA in osteoblastic cells by RT-PCR.

Overexpression of TonEBP led to decrease the expression of RANKL mRNA. Furthermore, the RANKL mRNA was increased silencing of TonEBP and dominant negative TonEBP in osteoblastic cells (Fig. 7). These findings suggest that TonEBP gene may be involved in the regulation of RANKL expression at the upstream level.

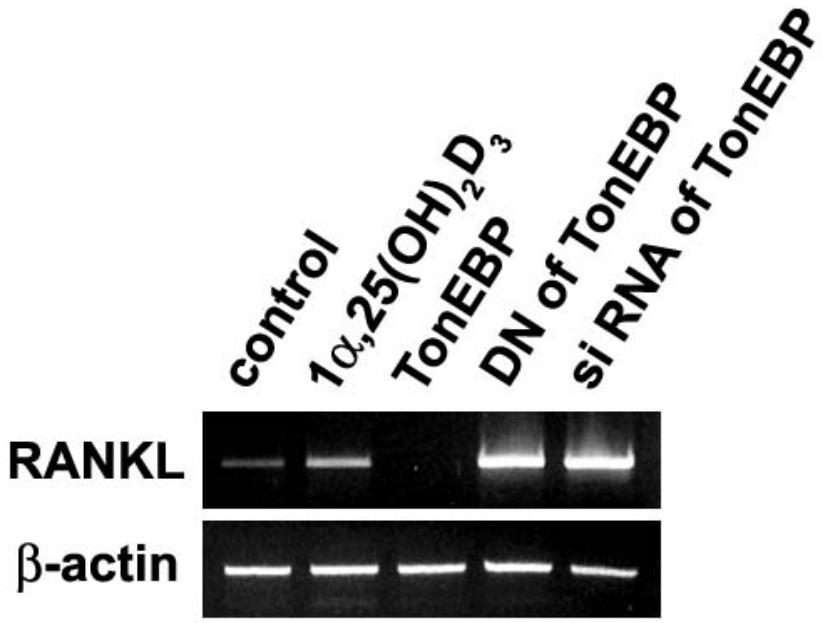


Fig. 7. Transfected osteoblastic cells changed the RANKL mRNA expression. Osteoblastic cells were transfected with RNA interference of TonEBP (siRNA of TonEBP), mouse TonEBP gene and dominant negative TonEBP (DN of TonEBP) gene. Cells were treated with $1\alpha,25(OH)_2D_3$ (10^{-8} M) for 2 days before mRNA isolation. The expression of RANKL (750bp) and β-actin (366bp) mRNAs was analyzed by RT-PCR.

IV. Discussion

During the mineralization phase, unusually high Ca^{2+} concentrations are generated in this compartment, and osteoblasts are exposed to Ca^{2+} concentration as high as 40 mM.[10] Such extracellular Ca^{2+} concentration alters the normal Ca^{2+} homeostasis and signaling, leading to a rapid rise in the cytosolic Ca^{2+} level, resulting in a dramatic reduction of bone resorption. More recently, it is reported that osteoclasts happen to apoptosis at the end of the bone resorbing process and the apoptotic change in osteoclast may potentially be a critical factor in the regulation of bone resorption. [11, 15] The increase of the extracellular Ca^{2+} means that osteoblasts and osteoclasts become to be in the hypertonic condition in microenvironment. [12, 20, 25, 36]

We hypothesized that released-Ca^{2+} and -PO_4^{2-} to extracellular fluid might affect osmotically on osteoclastogenesis. In other words, if extracellular Ca^{2+} / PO_4^{2-} concentration would be changed, osteoblasts could recognize extracellular Ca^{2+} / PO_4^{2-} concentration and control osteoclastogenesis. In this study, we demonstrated the effects of hypertonicity on osteoclastogenesis in osteoblast-osteoclast co-culture system for the purpose of understanding at the molecular level of hypertonicity on bone metabolism.

At the first time, this finding confirms that the hypertonicity acts as a modulator which might be involved in $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis. Interestingly, we found that hypertonicity inhibited $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis in co-culture system. According to increase of the hypertonic stress, the number of TRAP positive multinucleated cells was reduced up to about 65 %, compared with control in co-culture system (Fig. 1). Nevertheless, it could be argued that the inhibition of osteoclastogenesis by sucrose might be caused not due to the physiological intervention of sucrose in the normal process of osteoclastogenesis but due to the cell damage. To rule out the possibility that sucrose causes non-physiological cell damage, an MTT viability test was performed. The test showed that sucrose under 50 mM did not exert any harmful effect upon the cells in this co-culture system. It was the effects of hypertonic stress without a toxic effect upon the cells to reduction of the formation of TRAP

positive cells. Furthermore, hypertonicity inhibited 1α,25$(OH)_2D_3$-induced pit formation using the bone slice (Fig. 2). The area of resorption in OAAS plates was remarkably decreased by influence of hypertonic stress. We suggested that hypertonicity plays a role as a down-regulated modulator in 1α,25$(OH)_2D_3$-induced osteoclastogenesis.

Since osteoclast differentiation was mediated by several factors, such as RANKL, OPG and M-CSF [2,7, 11,12], we used an osteoblast/stromal cell in co-culture system to evaluate whether the mRNA expression profiles of RANKL and OPG induced by 1α,25$(OH)_2D_3$ were changed or not. In the result, the expression of RANKL mRNA was down-regulation upon increasing the hypertonic stress, and the expression of OPG mRNA was not changed significantly (Fig. 3). In addition, the expression of sRANKL protein was decreased with hypertonic stress in the process of 1α,25$(OH)_2D_3$-induced osteoclastogenesis, being in consistency with the decrease in RANKL mRNA expression (Fig. 4). On the other hand, OPG protein was slightly increased with hypertonic stress, but it was not statistically significant (Fig. 4). On the basis of such findings, inhibition mechanism of osteoclastogenesis by hypertonicity might be associated with modulating RANKL and OPG expression in osteoblasts.

How could hypertonicity affect on osteoclast? To investigate for this answer, we tested that hypertonicity inhibited RANKL-induced osteoclastogenesis in RAW 264.7 cell culture. In the same manner, hypertonic stress reduced the number of TRAP positive multinucleated cells without toxic effect for hypertonic stress (Fig. 4). Second, this finding confirms that the hypertonicity acts as a signal for various cellular functions in osteoclastogenesis. Hypertonicity did not affect RANKL-RANK mediated signaling of osteoclast differentiation such as MAP Kinase and NF-κB (Fig. 5). Putting these results together, we suggested that hypertonicity affected RANKL synthesis pathway in osteoblastic cell.

Hypertonic stress is a genotoxic agent. Cells in the kidney adapt to the hypertonicity by accumulating compatible osmolytes, also called organic osmolytes. TonEBP plays a central role in the cellular accumulation of

compatible osmolytes. TonEBP is stimulated by hypertonicity in the kidney.[5, 10] However, TonEBP is expressed in kidney as well as in the other tissue such as embryos, brain, heart, and liver.[21] Is TonEBP expressed by hypertonicity in bone cell? This study showed that high concentration of sucrose led TonEBP to express via cell shrinkage of osteoblast caused by the hypertonicity. The expression of TonEBP increased with the hypertonic stress in bone cells, osteoblastic cells, but was not affected to hypertonicity in MC3T3-E1 cell (Fig. 6). To investigate the role of TonEBP, osteoblastic cells were transfected with RNA interference of TonEBP, mouse wild and dominant negative TonEBP gene in these cells. Silencing and dominant negative of TonEBP in osteoblastic cells induced the expression of RANKL mRNA. Moreover, TonEBP overexpression suppressed the expression of RANKL (Fig. 7). These findings indicated that TonEBP gene had an effect on the regulation of RANKL expression.

Further studies are needed to clarify the exact target and inhibition mechanism of TonEBP in osteoblastic cells.

V. Conclusion

In summary, we have provided the first evidence that hypertonicity inhibited not only osteoclast formation in co-culture system but also osteoclast function (bone resorbtion activity) in $1\alpha,25(OH)_2D_3$– induced / RANKL-RANK mediated osteoclastogenesis. And the expression of RANKL mRNA and soluble RANKL protein were down-regulation upon increasing the hypertonic stress, but the expression of OPG mRNA and OPG protein were not changed significantly. Although hypertonicity inhibited the RANKL-mediated osteoclast formation in RAW 264.7 cells, it affected osteoclast formation not by the RANKL-RANK mediated signal including phosphorylated MAPK and NF-κB. Also hypertonicity allowed TonEBP to be stimulated in osteoblastic cell. The TonEBP acts on effect the regulation of RANKL expression. We expect that TonEBP regulates the RANKL synthesis signal pathway.

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국문초록

Hypertonic stress에 의한 1α,25-dihydroxyvitaminD$_3$ – induced osteoclastogenesis 억제 효과와 TonEBP의 역할

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공 석 희

골개조는 뼈를 형성하는 조골세포와 골흡수를 유발하는 파골세포, 이 두가지의 세포에 의하여 조절된다. 파골세포분화과정에서 조골세포는 파골세포분화를 촉진하는 인자인 RANKL과 M-CSF를 분비하게 되는데, 이에 대한 저해제인 OPG 또한 분비하는 것으로 알려져있다. 파골세포에 의하여 골흡수가 일어나면, 세포질내 Ca^{2+}/PO_4^{2-} 의 농도가 약 40 mM까지 증가하게된다. 본 실험에서는 이렇게 형성된 hypertonicity가 파골세포 분화과정에 미치는 영향과 골대사에 미치는 영향에 대하여 알아보고자 하였다.

1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$)에 의하여 유도되는 파골세포 분화과정에서 hypertonic stress는 osteoblastic 세포와 RAW 264.7 cell (osteoclast cell line)에서 파골세포형성을 억제하였다. 10 nM의 1α,25$(OH)_2D_3$ 을 투여하여 파골세포 분화를 유도한 후에 hypertonicity (sucrose 25, 50 mM)를 각각 투여하여 대조군과 비교 분석하였다. 먼저 TRAP 염색으로 파골세포 수를 측정한 결과 공존 배양계와 RAW 264.7 모두에서 hypertonic stress가 증가함에 비례하여 핵이 3개 이상인 파골세포 수가 점차 감소하였다. 또한 골흡수 능력을 비교한 결과에서도 감소하였다. 세포 독성능 검사 결과 Sucrose 50 mM 이하에서는 독성이 없었다. Hypertonicity가 파골세포억제 기전을 규명하기 위하여 조골세포에서 골흡수 기전의 중요한 골흡수 촉진

인자 (RANKL)과 골흡수 억제 인자 (OPG)의 mRNA양과 최종 생산된 단백질의 양을 각각 RT-PCR과 ELISA의 방법으로 측정하였다. 그 결과 sucrose의 농도가 증가함에 따라 RANKL mRNA 와 RANKL 단백질 모두 점차적으로 감소하였다. 반면에 OPG에는 변화가 없었다. RAW 264.7에서 세포내 신호전달 물질인 MAPK와 NF-kB를 western blot을 이용하여 조사하였으나 변화는 보이지 않았다. 일련의 결과를 통하여 hypertonic stress는 조골세포에서 RANKL을 발현시키는 신호전달기전에 영향을 미치는 것으로 생각된다. 신장에서 세포들이 hypertonic한 상황에 적응할 수 있기 위하여 발현되는 TonEBP (tonicity-responsive enhancer binding protein)가 조골세포에서도 발현되는 것을 확인하였다. 또한, TonEBP 유전자를 과발현시켰을 때 RANKL mRNA 발현이 감소하였으며, shut-down시켰을 때 증가하는 것을 확인하였다.

결론적으로 Hypertonicity는 조골세포에서 TonEBP 유전자를 발현시켜 RANKL의 발현을 조절함으로써 파골세포 형성을 억제하는 중요한 요인으로 생각된다. 더 나아가, 조골세포에서 TonEBP 대사기전에 대한 연구가 더 필요할 것이다.

핵심되는 말 : Hypertonicity, 파골세포분화, RANKL, OPG, TonEBP, $1\alpha,25(OH)_2D_3$