Hypotonic Stress Induces RANKL via Transient Receptor Potential Melastatin 3 (TRPM3) and Vanilloid 4 (TRPV4) in Human PDL Cells

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Abstract

Bone remodeling occurs in response to various types of mechanical stress. The periodontal ligament (PDL) plays an important role in mechanical stress-mediated alveolar bone remodeling. However, the underlying mechanism at the cellular level has not been extensively studied. In this study, we investigated the effect of shear stress on the expression of bone remodeling factors, including receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL) and osteoprotegerin (OPG), as well as its upstream signaling pathway in primary human PDL cells. We applied hypotonic stress to reproduce shear stress to PDL cells. Hypotonic stress induced the messenger RNA (mRNA) and protein expression of RANKL but not OPG. It also increased intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Extracellular Ca^{2+} depletion and nonspecific plasma membrane Ca^{2+} channel blockers completely inhibited the increase in both $[\text{Ca}^{2+}]_i$ and RANKL mRNA expression. We identified the expression and activation of transient receptor potential melastatin 3 (TRPM3) and vanilloid 4 (TRPV4) channels in PDL cells. Pregnenolone sulfate (PS) and 4 α -phorbol 12, 13-didecanoate (4 α -PDD), which are agonists of TRPM3 and TRPV4, augmented Ca^{2+} influx and RANKL mRNA expression. Both pharmacological (2-aminoethoxydiphenyl borate [2-APB], ruthenium red [RR], ononetin [Ono], and HC 067047 [HC]) and genetic (small interfering RNA [siRNA]) inhibitors of TRPM3 and TRPV4 reduced the hypotonic stress-mediated increase in $[\text{Ca}^{2+}]_i$ and RANKL mRNA expression. Our study shows that hypotonic stress induced RANKL mRNA expression via TRPM3- and TRPV4-mediated extracellular Ca^{2+} influx and RANKL expression. This signaling pathway in PDL cells may play a critical role in mechanical stress-mediated alveolar bone remodeling.

Keywords: bone remodeling/regeneration, mechanotransduction, cell signaling, osmotic stress, ion channels, periodontal ligament

Introduction

The periodontal ligament (PDL) attaches a tooth to the alveolar bone. It supports the tooth and allows it to withstand the mechanical stress that occurs during chewing and continuous orthodontic tooth movement. Mechanical stress induces a change in the shape of cells (Malek and Izumo 1996) and subsequently activates alveolar bone remodeling (Kanzaki et al. 2006; Maeda et al. 2007; Nakao et al. 2007).

Bone metabolism is mainly regulated by complex signaling pathways that are mediated by 2 major cytokines: receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL) and osteoprotegerin (OPG). RANKL is expressed in osteoblasts and coordinates bone resorption and synthesis by stimulating osteoclasts. OPG abolishes RANKL/RANK signaling by acting as a decoy receptor. Thus, the balance between RANKL and OPG (RANKL/OPG ratio) is important for the regulation of bone remodeling (Boyle

et al. 2003). PDL cells have osteoblast-like properties and mediate alveolar bone remodeling (Basdra and Komposch 1997). These cells also modulate osteoclast formation by producing RANKL and OPG in co-culture with bone marrow cells (Kanzaki et al. 2001). In addition, PDL cells produce RANKL and OPG in response to mechanical stress, such as compression and tension force (Kanzaki et al. 2006;

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Maeda et al. 2007; Nakao et al. 2007). Recently, Pavasant and Yongchaitrakul (2011) demonstrated that mechanical stimuli induces adenosine triphosphate (ATP) release, which stimulates RANKL expression through the cyclooxygenase/prostaglandin E₂ (PGE₂)-dependent pathway in human PDL cells.

Mechanosensitive ion channels are directly activated by various stimuli, such as touch, pressure, sound, stretch, and osmolality, and they transduce the activity into electrical signals (Xiao and Xu 2010). Recent studies have shown that PDL cells express several mechanically gated channels, including K_{2p} channels (TREK-1, TWIK-1, TWIK-2, TRAAK, KCNK7, TASK-1, TASK-2, TASK-3, and TASK-4) (Ohara et al. 2006; Saeki et al. 2007) and transient receptor potential (TRP) channels (TRPV1, TRPV4, and TRPA1) (Sooampon et al. 2013; Tsutsumi et al. 2013). TRP channels are nonselective cation channels, most of which are Ca²⁺ permeable. In mammals, they are divided into several subfamilies: canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), ankyrin (TRPA), polycystin (TRPP), and mucolipin (TRPML) (Jin et al. 2011). Among them, TRPM3 and TRPV4 are activated by extracellular hypo-osmolality accompanied by the increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i) (Grimm et al. 2003; Wu et al. 2007). However, the effects of hypotonic stress on signal transmission and cellular response in PDL cells have not been reported.

In this study, we examined the role of PDL cells in alveolar bone remodeling and focused on the regulatory mechanism involving RANKL. We also identified the critical effect of TRP channels, especially TRPM3 and TRPV4, on increasing [Ca²⁺]_i in primary human PDL cells.

Materials and Methods

Reagents

Fura-2/AM was a product of Teflabs (Austin, TX, USA). Ionomycin, ruthenium red (RR), ononetin (Ono), and HC 067047 (HC) were obtained from Tocris (Bristol, UK). Penicillin-streptomycin, fetal bovine serum (FBS), and Superscript III were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals unless otherwise mentioned were purchased from Sigma (St. Louis, MO, USA). Stock solutions of all chemicals were prepared in distilled water, except 2-aminoethoxydiphenyl borate (2-APB) in ethanol and 4α-phorbordidecanoate (4α-PDD), ionomycin (Iono), Ono, HC, and fura-2/AM in DMSO.

PDL Cell Culture

All experimental protocols were reviewed and approved by the Research Ethics Committee of Yonsei University College of Dentistry and Dental Hospital. Informed consent

was obtained from all volunteers according to the requirements of the institutional review board. The PDL tissues were isolated from healthy donors' premolars extracted for orthodontic reasons at Yonsei University Dental Hospital. The PDL cells were obtained from the tissues located in the middle of the tooth root by scraping, mincing, and then incubating for 40 min at 37°C in a humidified atmosphere composed of 5% CO₂/95% air. Immortalized human PDL (IPDL) cells were kindly provided by Dr. Eun-Chul Kim at Kyung Hee University College of Dentistry (Pi et al. 2007). Both human primary and immortalized PDL cells were maintained in α-minimal essential medium (α-MEM; GIBCO, Grand Island, NY, USA) containing 10% FBS with antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). When a cell density of 80% confluence was reached, cells were washed with PBS and treated with trypsin/ethylenediaminetetraacetic acid (EDTA) for 1 min and then transferred to new culture dishes. Primary PDL cells at 4 to 7 passages were used for all experiments.

Reverse Transcription–Polymerase Chain Reaction

After reagents treatment, total RNA was extracted from primary PDL cells using the Trizol reagent (Invitrogen), and then complementary DNA (cDNA) synthesis was performed using either AccuPower RT PreMix (BIONEER, Daejeon, Korea) or Superscript III (Invitrogen). Samples without RTase served to verify the absence of genomic DNA. cDNAs were amplified by polymerase chain reaction (PCR) with HiPi Thermostable DNA polymerase (Elpis, Pusan, Korea) and the following primers (BIONEER): TRPM3 (5'-CACCTGATGACCAAGGAATG-3' and 5'-CTTGTGTTTATCTTCTGGAGTG-3'), TRPV4 (5'-GAGGAGTTTCGAGAGCCATCTACG-3' and 5'-CCGTCAGGTAGTTGACAATGTGGG-3'), RANKL (5'-CCAGCATCAAAATCCCAAGTTC-3' and 5'-CTCCCACTGGCAGGTAAATACG-3'), OPG (5'-GTCTCCTGCTAACTCAGAA-3' and 5'-AAGACACTAAGCCAGTTAGG-3'), and GAPDH (5'-GTCGGAGTCAACGGATT-3' and 5'-GCCATGGGTGGAATCATA-3'). PCR was performed under the following conditions: 94°C for 5 min, 94°C for 30 s, 30 s for annealing step (temperatures varied upon primers), 72°C for 30 s, and followed by 72°C for 10 min after cycles were finished. The respective annealing temperature and cycles were 60°C and 35 cycles for TRPM3, 61°C and 35 cycles for TRPV4, 58°C and 35 cycles for RANKL, and 56°C, 30 cycles for OPG and GAPDH. The products were visualized on 1.5% agarose gels.

Western Blot

Whole cells were lysed with 100 μl RIPA buffer (Bio-solution, Suwon, Korea), including proteinase inhibitor cocktail (Roche, Lewes, UK), and 50 μg protein was separated by

8% to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The proteins were transferred to a PVDF membrane (Whatman, Clifton, NJ, USA). After blocking with 5% skimmed milk (BD–Difco, Le Pont de Claix, France) in Tris buffered saline with Tween 20 (TBST; 20 Tris–HCl [pH 7.6], 137 NaCl, and 0.1% Tween 20 [in mM]) at room temperature for 1 h, the blots were incubated in primary antibodies against RANKL (1:1000), OPG (1:1000), and β -actin (1:40,000) in 5% skimmed milk and TRPM3 (1:2000) and TRPV4 (1:2000) in 1% bovine serum albumin (BSA). After a series of three washes with TBST, the blots were incubated in goat anti–rabbit antibody (1:10,000) in 5% skimmed milk. The blots were washed and detected by a mixture of luminol/enhancer solution and stable peroxide solution (Thermo Scientific, Rockford, IL, USA).

Intracellular Ca^{2+} Concentration ($[\text{Ca}^{2+}]_i$) Measurement

$[\text{Ca}^{2+}]_i$ was measured as described previously (Son et al. 2009). In brief, human PDL cells were loaded with 5 μM of the acetoxymethyl-ester form of fura-2 (fura-2/AM) and 0.05% Pluronic F-127 for 50 min in physiological saline solution (PSS; 140 NaCl, 5 KCl, 1 MgCl_2 , 10 HEPES, 1 CaCl_2 , 10 glucose [in mM], 310 mOsm, pH 7.4) at room temperature. Fluorescence intensity was measured at excitation wavelengths (340/380 nm), and images were obtained at 2-s intervals. All data were analyzed with MetaFluor software (Molecular Devices, Downingtown, PA, USA). For hypotonic stress, PSS was replaced with a hypotonic solution (80 mM NaCl, 215 mOsm).

Whole-Cell Voltage Clamping

Whole-cell voltage clamp recordings were performed by the perforated patch-clamp method at room temperature. Currents were recorded in a MultiClamp 700B amplifier, subsequently digitized at a sampling rate of 10 kHz, and analyzed with pCLAMP10 software (Axon Instruments, Union City, CA, USA). The pipette resistance varied between 3 and 5 MO. Whole-cell currents were elicited by voltage ramps from -100 mV to $+100$ mV (400-ms duration) applied every 10 s from a holding potential of 0 mV. The bath solution contained (in mM) the following: 140 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 HEPES, and 10 glucose, adjusted to pH 7.4 with NaOH. The internal solution for recording TRPV4 currents contained (in mM) the following: 140 KCl, 5 EGTA, and 10 HEPES, adjusted to pH 7.4 with KOH (Güler et al. 2002). The internal solution for recording TRPM3 currents contained (in mM) the following: 140 CsCl, 5 MgCl_2 , 10 BAPTA, and 10 HEPES, adjusted to pH 7.2 with CsOH, and the bath solution was changed to a K^+ -free external solution (Grimm et al. 2003).

Small Interfering RNA Transfection

When a cell density of 70% confluence was reached, small interfering RNA (siRNA) duplexes specific for human TRPM3, TRPV4, or negative control (BIONEER) were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Statistical Analysis

Data were expressed as means $\pm$ SEM. Statistical significance was analyzed by paired or unpaired Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

Results

Hypotonic Stress Increases RANKL Expression and $[\text{Ca}^{2+}]_i$ in Primary Human PDL Cells

To evaluate the underlying mechanisms of mechanical stress–mediated bone remodeling, we examined the effect of hypotonic stress on RANKL and OPG messenger RNA (mRNA) and protein expression. Based on the previous study (Luckprom et al. 2010), ATP was used as a positive control. The basal mRNA and protein levels of RANKL were lower than OPG in primary human PDL cells. Expression of RANKL mRNA and protein was significantly enhanced after 12 h of treatment with hypotonic solution (215 mOsm). In contrast, expression of OPG mRNA and protein was unchanged during the treatment of hypotonic solution (Fig. 1A–C).

The increase in $[\text{Ca}^{2+}]_i$ by thapsigargin (Tg) augmented RANKL but not OPG mRNA expression (Fig. 1D). Thus, we investigated the effect of hypotonic stress on $[\text{Ca}^{2+}]_i$ in PDL cells. As shown in Figure 1E, hypotonic stress evoked an increase in $[\text{Ca}^{2+}]_i$. The repetitive application of the hypotonic solution resulted in the reduction of the hypotonic stress–induced increase in $[\text{Ca}^{2+}]_i$ (Fig. 1F, G).

Ca^{2+} Uptake Is Responsible for the Hypotonic Stress–Induced Increases in $[\text{Ca}^{2+}]_i$ and RANKL Expression

To identify the source of $[\text{Ca}^{2+}]_i$ elevation, we first examined the influx of Ca^{2+} from the external medium by removing extracellular Ca^{2+} . The Ca^{2+} -free solution suppressed the hypotonic stress–induced increase in $[\text{Ca}^{2+}]_i$ (Fig. 2A). Gadolinium (Gd^{3+}) and lanthanum (La^{3+}), which are non-specific plasma membrane Ca^{2+} channel blockers, also completely abolished the increase in $[\text{Ca}^{2+}]_i$ by hypotonic stress (Fig. 2B).

To directly examine whether the increase in $[\text{Ca}^{2+}]_i$ can influence RANKL mRNA expression, we exposed PDL

Figure 1. Effect of hypotonic stress on receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL) messenger RNA (mRNA) expression and Ca^{2+} signaling in primary human PDL cells. (A–C) Increase in the expression of RANKL but not osteoprotegerin (OPG) by hypotonic stress. (A) Cells were treated with the hypotonic solution (Hypo; 215 mOsm) or adenosine triphosphate (100 μ M) for 12 h. The mRNA levels of RANKL and OPG were analyzed by reverse transcription–polymerase chain reaction (RT-PCR). (B) The levels of RANKL and OPG mRNA were quantified after the value was normalized to GAPDH ($n = 10$). (C) Cells were treated with the hypotonic solution for 24 h. The protein levels of RANKL and OPG were analyzed by Western blot. (D) Increase in RANKL, but not OPG, mRNA expression by thapsigargin (Tg). Cells were treated with Tg (1 μ M) for the indicated time. RANKL and OPG mRNA levels were analyzed by RT-PCR. (E–G) Increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by hypotonic stress. After hypotonic stress was applied to fura-2/AM-stained cells once (E) or repetitively (F), the fluorescence intensity was measured at excitation wavelengths of 340 and 380 nm. (G) Summary of the effect of repetitive hypotonic solution application on $[Ca^{2+}]_i$ ($n = 8$). C, control; Hypo, hypotonic solution. The asterisks denote statistically significant differences between the compared values: *** $P < 0.001$.

Figure 2. Effect of the hypotonic stress–induced increase in $[Ca^{2+}]_i$ on receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL) messenger RNA (mRNA) expression. (A, B) Extracellular Ca^{2+} depletion and nonspecific plasma membrane Ca^{2+} channel blockers abolished the hypotonic stress–induced increase in $[Ca^{2+}]_i$. The change in $[Ca^{2+}]_i$ in response to the hypotonic solution was measured after the application of a Ca^{2+} -free solution (A) or pretreatment with gadolinium (Gd^{3+} : 100 μ M) or lanthanum (La^{3+} : 100 μ M) for 4 min (B). (C, D) Nonspecific plasma membrane Ca^{2+} channel blockers inhibited the effects of hypotonic stress on RANKL, but not osteoprotegerin (OPG), mRNA expression. Cells were pretreated with Gd^{3+} (10 μ M), La^{3+} (100 μ M), or the intracellular Ca^{2+} -selective chelator, BAPTA-AM (5 μ M), for 30 min and incubated with or without the hypotonic solution for 12 h. (C) The mRNA levels of RANKL and OPG were determined by reverse transcription–polymerase chain reaction (RT-PCR). (D) The mRNA expression levels of RANKL and OPG were quantified after normalized to GAPDH ($n = 4$). C, control; Hypo, hypotonic solution; $-Ca^{2+}$, Ca^{2+} -free solution. The asterisks denote statistically significant differences between the compared values: * $P < 0.05$, ** $P < 0.01$.

Figure 3. Role of TRPM3 and TRPV4 in the Ca^{2+} influx and receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL) messenger RNA (mRNA) expression. (**A, B**) TRPM3 and TRPV4 were expressed in periodontal ligament (PDL) cells. The mRNA and protein expression of TRPM3 and TRPV4 were examined using reverse transcription–polymerase chain reaction (RT-PCR) and Western blot, respectively. (**C, D**) Effect of TRPM3 and TRPV4 activation on $[\text{Ca}^{2+}]_i$. $[\text{Ca}^{2+}]_i$ was measured after the treatment with the indicated concentration of pregnenolone sulfate (PS) (**C**) or 4 α -phorbol 12, 13-didecanoate (4 α -PDD) (**D**). (**E, F**) Increase of RANKL, but not osteoprotegerin (OPG), mRNA expression by TRPM3 or TRPV4 activators. Cells were treated with PS (50 μM) (**E**) or 4 α -PDD (10 μM) (**F**) for 12 h. The mRNA levels of RANKL and OPG were determined by RT-PCR. (**G, H**) Induction of TRPM3 and TRPV4 expression by hypotonic stress. (**G**) Expression of TRPM3 and TRPV4 mRNA was analyzed by RT-PCR with RNA isolated from cells treated with hypotonic solution for 12 h. (**H**) Expression of TRPM3 and TRPV4 protein was analyzed by Western blot with protein isolated from cells treated with hypotonic solution for 24 h. (**I–L**) TRPM3 and TRPV4 were activated by hypotonic stress. After the application of hypotonic stress, current–voltage relations of TRPM3 (**I, J**) and TRPV4 (**K, L**) were determined by whole-cell patch clamp (–100 to +100 mV in 400-ms intervals; $V_h = 0$ mV) in immortalized human PDL cells ($n = 3$). C, control; Hypo, hypotonic solution.

cells to hypotonic stress, along with Gd^{3+} , La^{3+} , and the intracellular Ca^{2+} -selective chelator, BAPTA-AM. As shown in Figure 2C, hypotonic stress–induced RANKL mRNA expression is markedly repressed by Gd^{3+} , La^{3+} , and BAPTA-AM, whereas OPG mRNA expression is not changed. Quantification of the mRNA level showed that the Ca^{2+} channel blockers and the Ca^{2+} chelator significantly reduced the mRNA expression of RANKL but not OPG (Fig. 2D).

TRPM3 and TRPV4 Are Involved in the Ca^{2+} Influx and RANKL Expression in Human PDL Cells

Among the TRP family, TRPM3 and TRPV4 are directly gated by mechanical stress, such as hypotonic cell swelling and membrane stretch (Grimm et al. 2003; Wu et al. 2007). To determine whether TRPM3 and TRPV4 play a role in the

hypotonic stress–induced increase in $[\text{Ca}^{2+}]_i$, we first examined their expression in PDL cells. As shown in Figure 3A, B, mRNA and protein expression of both TRPM3 and TRPV4 are detected. To assess the functional activities of these channels, PDL cells were exposed to PS and 4 α -PDD, which are agonists of TRPM3 and TRPV4, respectively. Measurements of $[\text{Ca}^{2+}]_i$ indicated that both PS and 4 α -PDD enhanced the increases in $[\text{Ca}^{2+}]_i$ in PDL cells in a dose-dependent manner (Fig. 3C, D). In addition, the mRNA level of RANKL but not OPG was enhanced by activation of TRPM3 or TRPV4 upon the treatment of their agonists (Fig. 3E, F).

To determine whether TRPM3 and TRPV4 play a role in the hypotonic stress–induced increases in $[\text{Ca}^{2+}]_i$, we first examined their expression and activation. As shown in Figure 3G, H, mRNA and protein levels of both TRPM3 and TRPV4 were significantly enhanced after the treatment of PDL cells with the hypotonic solution. In addition, we

Figure 4. Role of TRPM3 and TRPV4 in the hypotonic stress-induced increase in $[Ca^{2+}]_i$ and receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL) messenger RNA (mRNA) expression. (A, B) Effect of 2-aminoethoxydiphenyl borate (2-APB) and ruthenium red (RR) on hypotonic stress-mediated $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured after pretreatment with 2-APB (75 μ M) or RR (10 μ M) for 4 min and quantified as peak value ($n = 3$). (C, D) Prevention of hypotonic stress-induced RANKL mRNA expression after the treatment of nonspecific transient receptor potential (TRP) channel blockers. (C) Cells were pretreated with 2-APB (75 μ M) or RR (10 μ M) for 30 min and incubated with or without the hypotonic solution for 12 h. The mRNA levels of RANKL and osteoprotegerin (OPG) were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). (D) The levels of RANKL and OPG mRNA were quantified after being normalized to GAPDH ($n = 4$). (E, F) Effect of ononetin (Ono) and HC 067047 (HC) on hypotonic stress-mediated $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured after pretreatment with Ono (10 μ M) or HC (10 μ M) for 4 min and quantified as peak value ($n = 10$). (G, H) Prevention of hypotonic stress-induced RANKL mRNA expression after the treatment of specific TRPM3 or TRPV4 blockers. (G) Cells were pretreated with Ono (10 μ M) or HC (10 μ M) for 30 min and incubated with or without the hypotonic solution for 12 h. The mRNA levels of RANKL and OPG were analyzed by RT-PCR. (H) The levels of RANKL and OPG mRNA were quantified after being normalized to GAPDH ($n = 3$). C, control; Hypo, hypotonic solution; Iono, ionomycin. The asterisks denote statistically significant differences between the compared values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

performed whole-cell patch-clamp with IPDL cells. The hypotonic solution augmented the currents produced by both channels. Quantifying the amplitude of channel currents showed that the hypotonic stress-activated current of TRPM3 was higher than that of TRPV4 (Fig. 3I–L).

Hypotonic Stress-Induced Increases in $[Ca^{2+}]_i$ and RANKL Expression Occur via TRPM3 and TRPV4

To determine the specific activation of TRPM3 and TRPV4 upon hypotonic stress, we first used 2-APB and RR, which are known to inhibit several TRP channels, including TRPM3 and TRPV4. Both 2-APB and RR partially inhibited the hypotonic stress-induced elevation of $[Ca^{2+}]_i$ (Fig. 4A). Quantifying peak ratios of Ca^{2+} transients by the blockers showed the possibility that TRP channels, including TRPM3 and TRPV4 channels, participated in the hypotonic stress-induced increase in $[Ca^{2+}]_i$ (Fig. 4B). In addition, both 2-APB and RR prevented the induction of RANKL but not OPG mRNA expression (Fig. 4C, D). Ononetin (Ono) and HC 067047 (HC) are suggested as potent and selective antagonists for TRPM3 (Straub et al. 2013) and TRPV4 (Everaerts et al. 2010), respectively. Therefore, we treated PDL cells with Ono and HC and measured the effect of TRPM3 and TRPV4 on the $[Ca^{2+}]_i$, RANKL, and OPG mRNA expression. The inhibition of TRPM3 or TRPV4 by Ono and HC, respectively, repressed the hypotonic stress-mediated increase in $[Ca^{2+}]_i$ and RANKL mRNA induction. However, no changes in OPG expression were observed during the treatment (Fig. 4E–H).

To further verify the functional effect of TRPM3 and TRPV4 on the Ca^{2+} influx and RANKL expression, we silenced both channels with siRNA and assessed $[Ca^{2+}]_i$ and RANKL and OPG mRNA expression. Upon

knockdown with specific siRNA for TRPM3 or TRPV4, their mRNA levels were completely suppressed (Fig. 5A). The PS- or 4 α -PDD-mediated increase in $[Ca^{2+}]_i$ was also inhibited by TRPM3 or TRPV4 knockdown (Fig. 5B–D). In addition, the hypotonic stress–induced increase in $[Ca^{2+}]_i$ was partially inhibited by TRPM3 or TRPV4 silencing (Fig. 5E–G). The suppression of TRPM3 and TRPV4 also abolished the hypotonic stress–induced RANKL mRNA expression (Fig. 5H, I).

Discussion

It has been suggested that the cyclo-oxygenase/PGE₂- and ATP-dependent pathways are important for alveolar bone homeostasis through RANKL induction (Pavasant and Yongchaitrakul 2011). Our findings indicate that the increase in $[Ca^{2+}]_i$ via TRPM3 and TRPV4 upregulates RANKL expression in PDL cells, thus revealing a new mechanism. Although the application of tensile force upregulates OPG expression (Tsuji et al. 2004; Kanzaki et al. 2006), we were unable to detect any changes in OPG in PDL cells. These results suggest that even in the same cell type, each mechanical stress may induce a different mechanism of bone remodeling.

The biting force causes shear stress between the interfaces of PDL cells, the alveolar bone, and the tooth. Thus, PDL cells are exposed to constant shear stress during mastication (Komatsu and Chiba 1993; Bergomi et al. 2010). We used hypotonic stress to mimic shear stress, although PDL cells are rarely exposed to hypotonic stress under physiological conditions. However, many studies have indicated that hypotonic cell swelling shares similar characteristics with shear stress. For example, both stresses change cell shape and evoke $[Ca^{2+}]_i$ elevation (Shen et al.

Figure 5. Effect of TRPM3 and TRPV4 knockdown with small interfering RNA (siRNA) on the hypotonic stress–induced increase in $[Ca^{2+}]_i$ and receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL) messenger RNA (mRNA) expression. **(A)** Inhibition of TRPM3 and TRPV4 with siRNA. The reduced mRNA expression of TRPM3 and TRPV4 was measured by reverse transcription–polymerase chain reaction (RT-PCR) in periodontal ligament (PDL) cells. **(B–D)** Knockdown of TRPM3 or TRPV4 prevented TRPM3- or TRPV4-mediated Ca^{2+} influx. $[Ca^{2+}]_i$ was measured after treatment with pregnenolone sulfate (PS) (B) or 4 α -phorbol 12, 13-didecanoate (4 α -PDD) (C) and quantified as peak value (D; $n = 5$) in the siRNA-transfected PDL cells. **(E–G)** Effect of TRPM3 and TRPV4 knockdown on hypotonic stress–mediated increase in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured after TRPM3 and TRPV4 siRNA transfection and quantified as peak value ($n = 4$). **(H, I)** Suppression of hypotonic stress–induced RANKL mRNA expression by TRPM3 and TRPV4 knockdown. **(H)** Cells were transfected with siRNA of TRPM3 and TRPV4 and incubated with or without the hypotonic solution for 12 h. The mRNA levels of RANKL and osteoprotegerin (OPG) were analyzed by RT-PCR. **(I)** The levels of RANKL and OPG mRNA were quantified after being normalized to GAPDH ($n = 4$). C, control; NC (negative control), control siRNA; Hypo, hypotonic solution; siTRPM3, TRPM3 siRNA; siTRPV4, TRPV4 siRNA. The asterisks denote statistically significant differences between the compared values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

1992; Malek and Izumo 1996; Koyama et al. 2001). Therefore, we can apply hypotonic stress as a replacement of shear stress to PDL cells.

The repetitive application of the hypotonic solution resulted in the partial desensitization during the rise in $[Ca^{2+}]_i$ (Fig. 1F, G). Although the pathway that is involved in the desensitization of TRPM3 and TRPV4 has not been fully elucidated, it can be mediated by at least 3 mechanisms: 1) dephosphorylation of the channels by calcineurin, 2) regulation of channel activities by phosphatidylinositol 4,5-bisphosphate depletion/replenishment, and 3) phosphorylation of the channels by protein kinase C isoforms (Akopian et al. 2007).

Little is known about the physiological role of cytosolic Ca^{2+} transients in human PDL cells. Previous reports showed that bradykinin and histamine induce Ca^{2+} mobilization and then activate PGE_2 expression (Ogata et al. 1995; Niisato et al. 1996). Interestingly, like RANKL, PGE_2 is a critical regulator and stimulator of osteoclast differentiation and maturation (Kanzaki et al. 2001; Pavasant and Yongchaitrakul 2011). Findings from these studies suggest that the elevation of $[Ca^{2+}]_i$ in PDL cells may be a critical mediator of osteoclastic bone resorption.

Consistent with our results, Tsutsumi et al. (2013) found that human PDL cells express TRPV4. They also showed that TRPV4 is slightly upregulated by intermittent mechanical stimulation. Therefore, it would be of interest to determine whether TRPM3 and TRPV4 expression is influenced in our system. According to Sooampon et al. (2013), TRPV1 is present on human PDL cells, and its activation by capsaicin induces the expression of OPG but not RANKL. Consequently, the OPG/RANKL ratio is increased, and this leads to the inhibition osteoclastogenesis. In this study, we also detected TRPV1 in PDL cells; however, capsaicin did not mediate RANKL and OPG mRNA expression (data not shown). It is possible that the variation in human primary PDL cells from different volunteers may cause this inconsistent result. Despite the discrepancy, it would be of great interest to examine if TRPM3, TRPV4, and TRPV1 can act as positive and negative regulators of alveolar bone homeostasis, respectively. To address this possibility, further studies that investigate whether the TRPM3 and TRPV4 response is altered during TRPV1 inhibition, or vice versa, are needed.

In the present study, we show that hypotonic stress induced extracellular Ca^{2+} influx through TRPM3 and TRPV4 and, consequently, increased RANKL mRNA and protein expression in human PDL cells. As a result of enhanced RANKL but not OPG, mRNA, and protein expression, the RANKL/OPG ratio in PDL cells was increased. This may lead to osteoclast differentiation and the activation of bone resorption activity, which then stimulates bone synthesis by osteoblasts. To our knowledge, this is the first report to reveal the interrelation of TRP channels, Ca^{2+} mobilization, and RANKL expression in PDL cells for alveolar bone homeostasis.

Author Contributions

G.Y. Son, Y.M. Yang, contributed to data acquisition, analysis, and interpretation, drafted the manuscript; W.S. Park, contributed to conception, critically revised the manuscript; I. Chang, contributed to design, critically revised the manuscript; D.M. Shin, contributed to conception and design, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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