Calcium Mediated WNK pathway activation induced by hypotonicity in the HSG cell line

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Calcium Mediated WNK pathway activation induced by hypotonicity in the HSG cell line

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그리고 석사졸업때 축하해주시고 박사졸업식에도 함께 하자며 약속하셨던
어머님이 함께 하지 못하시고 작년에 하늘로 가셨지만 아버님과 어머님이
지금 제 모습을 보시고 많이 대견하시고 자랑스러워 하실 것 같습니다.
그리고 항상 곁에서 힘이 되준 사랑스런 명진이와 우리 도연, 대훈이 와도
이 기쁨과 행복을 함께 하고 싶습니다.

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ABSTRACT

Calcium Mediated WNK pathway activation induced by hypotonicity in the HSG cell line

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(Directed by Professor Jong-Hoon Choi)

Wnk kinase maintains cell volume, regulating various transporters such as sodium-chloride cotransporter, potassium-chloride cotransporter, and sodium-potassium-chloride cotransporter 1 (NKCC1) through the phosphorylation of oxidative stress responsive kinase 1 (OSR1) and STE20/SPS1-related

proline/alanine-rich kinase (SPAK). However, the activating mechanism of Wnk kinase in specific tissues and specific conditions is broadly unclear. In the present study, I used a human salivary gland (HSG) cell line as a model and showed that Ca^{2+} may have a role in regulating Wnk kinase in the HSG cell line. Through this study, I found that the HSG cell line expressed molecules participating in the WNK-OSR1-NKCC pathway, such as Wnk1, Wnk4, OSR1, SPAK, and NKCC1. The HSG cell line showed an intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increase in response to hypotonic stimulation, and the response was synchronized with the phosphorylation of OSR1. Interestingly, when I inhibited the hypotonically induced $[Ca^{2+}]_i$ increase with nonspecific Ca^{2+} channel blockers such as 2-aminoethoxydiphenyl borate, gadolinium, and lanthanium, the phosphorylated OSR1 level was also diminished. Moreover, a cyclopiazonic acid-induced passive $[Ca^{2+}]_i$ elevation was evoked by the phosphorylation of OSR1, and the amount of phosphorylated OSR1 decreased when the cells were treated with BAPTA, a Ca^{2+} chelator. Finally, through that process, NKCC1 activity also decreased to maintain the cell volume in the HSG cell line. These results indicate that Ca^{2+} may regulate the WNK-OSR1 pathway and NKCC1 activity in the HSG cell line. This is the first demonstration that indicates upstream Ca^{2+} regulation of the WNK-OSR1 pathway in intact cells.

Key words: Ca^{2+} signaling, OSR1, WNK, NKCC, Salivary gland

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I. INTRODUCTION

Wnk, with no lysine, means that there is a lack of a conserved lysine residue in subcatalytic domain II, which is required for ATP binding [1]. Wnk kinases (WNKs) are serine/threonine protein kinases and are well conserved in many species from fungi to mammals [1]. Previous work by another group revealed that four types of

WNKs are expressed in humans: Wnk1, Wnk2, Wnk3, and Wnk4 [2]. After the finding that mutations in Wnk1 and Wnk4 cause the disease pseudohypoaldosteronism type II (PHA II, OMIM no. 145260), many subsequent studies were followed [3]. PHA II involves hypertension, with increased NaCl reabsorption and impaired K^+ and H^+ excretion [3]. Those clinical symptoms of PHA II indicate that WNKs perform roles in the kidney [4]. Consequently, early Wnk studies focused on transporters or channels located in the kidney and found that Wnk4 regulates the renal outer medullary potassium channel [5], the sodium-chloride cotransporter (NCC) [6,7], the sodium-potassium-chloride cotransporter (NKCC) [6-8], and claudin [9,10] which is a component of tight junctions. Thereafter, it was found that activated Wnk1 phosphorylates oxidative stress responsive kinase 1 (OSR1) and STE20/SPS1-related proline/alanine-rich kinase (SPAK) and that activated OSR1/SPAK phosphorylates the N-terminus of NKCC, which is required for activation [7,8]. WNK is activated via autophosphorylation and regulated by the presence of an autoinhibitory domain [11]. Moreover, the Wnk autoinhibitory domain is conserved in all of the WNKs [11], and the autoinhibitory domain of Wnk1 restrains not only the autophosphorylation of Wnk1 but also that of Wnk2 and Wnk4 [12].

Although the presence of autophosphorylation/autoinhibition was discovered early, the regulating mechanism of WNKs is still poorly understood [13-15]. Several mechanisms regulating WNKs have been revealed [13]. One is ubiquitination

through scaffolding proteins such as CUL3, KLHL2, and KLHL3 [16-22]. Another regulating system is phosphorylation modulated by phosphatidylinositol 3-kinase/Akt (PIP3K/Akt) [23] and apoptosis signal-regulating kinase 3 (ASK3) [24,25]. The activation of WNKs in low Cl^- conditions is the most direct regulation of WNKs [13]. Although the presence of SPAK/OSR1 phosphorylation induced by low Cl^- was revealed early [26], it remains a mystery how WNKs sense the low Cl^-, and the possibility of there being another Cl^- sensor that regulates WNKs still exists. Recently, the direct binding of Cl^- to a catalytic domain of Wnk1 was revealed by crystallography [27]. Nonetheless, an upstream regulator of WNKs is still not enough to explain all of the regulatory machinery.

Ca^{2+} is a well-known second messenger that mediates various cell functions, such as a epithelial secretion, locomotion, fertilization, development, differentiation and learning [28]. From the various role of Ca^{2+}, it is well known that hypotonicity induces the elevation of intracellular Ca^{2+} levels through the Ca^{2+} channels located in the plasma membrane. In addition, Ca^{2+}-activated Cl^- channels are well defined in the salivary glands.

In summary, intracellular Cl^- concentration is related with Wnk, and intracellular Cl^- leaks through Ca^{2+} activated-Cl^- channel. Moreover, hypotonic stress could induce Wnk activation and intracellular Ca^{2+} increase. Therefore, I hypothesized that Ca^{2+} might regulate the WNK-OSR1 pathway. To investigate this hypothesis, I used human salivary gland (HSG) cell line as a model system.

HSG cell line originated from the irradiated human salivary gland tissues[29], and it has been used widely as a model for studying the molecular mechanisms of the salivary gland cells[30,31]. In here, through the sequential experiments, I found that alterations of the intracellular Ca^{2+} level determined the phosphorylation of OSR1 and the activity of NKCC1 in a HSG cell line.

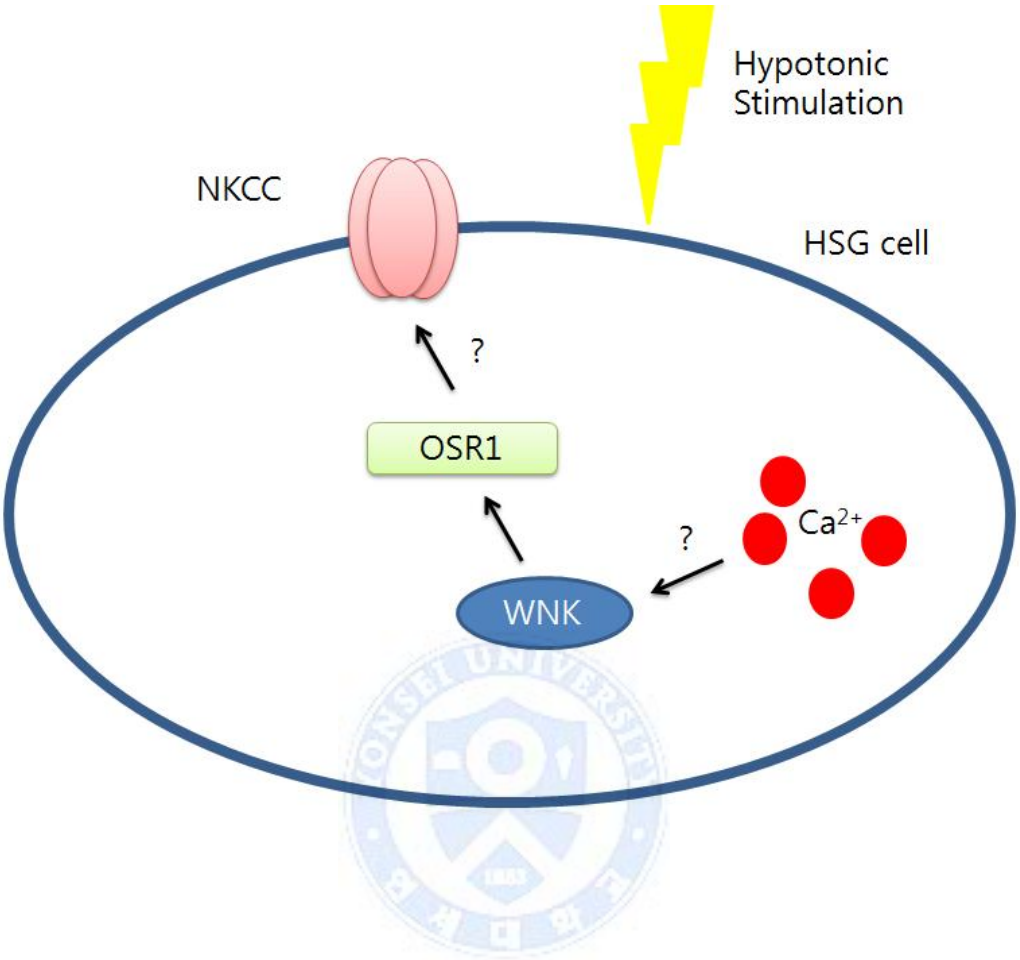

Figure 1. **Postulated model of hypotonic stimulated HSG cell line.** Increased Ca^{2+} induced by a hypotonic stimulation may regulate the WNK pathway. This process would be related with NKCC, and volume regulation of the HSG cell.

II. MATERIALS AND METHODS

1. HSG cell line and culture conditions

HSG cell-line was a gift from Dr. Kyungpyo Park in Seoul National University. HSG cell-line was maintained with high-glucose Dulbecco's Modified Eagle's medium with 10% FBS (Gibco, CA, USA) and 1X Antibiotic-Antimycotic (Gibco, CA, USA) in 95% CO_2, 5% O_2, 37˚C incubator. Passage number of HSG cell line used in experiment was 12-25. Subculture performed when cells are confluent. In details, culture media removed after treatment of Trypsin/EDTA for 2min in the cell incubator. All suspended cell was collected and centrifuge in 100g for 5min. Cells that used in the calcium imaging and NKCC1 activity measurement were cultured on the 24mm X 24mm size of cover glass.

2. Isolation of salivary gland acinar cell

ICR mice were purchased from (KOATEC, Korea). All experiments were performed on adult male ICR mice (6-8 weeks of age) that were maintained on a 12h day/night cycle with normal mouse chow and water provided ad libitum. The animal studies were performed after receiving approval of the Institutional Animal Care and Use Committee (IACUC) in Yonsei University (IACUC approval no. 2014-0067). Mice were sacrificed by cervical dislocation under CO2 anesthesia.

The cells were prepared from the parotid gland and submandibular gland of ICR mice by limited collagenase digestion as previously described [32]. Following isolation, the acinar cells were suspended in an extracellular physiologic salt solution (PSS), the composition of which was as follows (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, and 10 Glucose, adjusted to pH 7.4 with 5 N NaOH and to 310 mOsm with 5 M NaCl.

3. Reverse-transcriptional PCR

Total RNA was extracted from isolated pancreatic acinar cells using Trizol reagent (Sigma-Aldrich) according to the manufacturer's instructions. RT-PCR was performed using a SuperScript III RT kit (Sigma-Aldrich, USA) and oligo-dTs (Fermentas, MA, USA). For PCR analysis of the following primers were used (Table 1).

PCR reaction was performed using EmeraldAmp GT PCR Master Mix (Takara, Shiga, Japan). PCR condition was initiated by a 5min incubation of the samples at 94˚C, preceded by 30(Wnk1, Wnk4), 35(OSR1, SPAK, NKCC1, GAPDH) cycles of 30sec at 94 ˚C, 30sec for annealing (Wnk1 57 ˚C, Wnk4 60 ˚C, OSR1 58 ˚C, SPAK 59 ˚C, NKCC58 ˚C, GAPDH 58 ˚C), and 30sec at 72 ˚C. After 30(Wnk1, Wnk4), 35(OSR1, SPAK, NKCC1, GAPDH) cycles, samples were incubated for 10 min at 72 ˚C for complete extension. No reverse transcription control was performed using the same protocol, except for using reverse transcriptase.

Table 1. Primers for reverse-transcriptional PCR

Primer	Sequence (5′-3′)
hWNK1-F[a]	AATCCAGTGCTTCCCAGACA
hWNK1-R[a]	TCTGTTGGCTGCTCACTCGAGATT
hWNK4-F[b]	AGCTGCGTAAAGCAAGGGAATTGG
hWNK4-R[b]	TTTGTCCCATCCCTTCTCCCACAT
hOSR1-F[c]	AGGTTCCAGTGGGCGTCTTCATAA
hOSR1-R[c]	AGGCCAGCAGAAATGAGTTCCTGA
hSPAK-F[d]	ATTCAAGCCATGAGTCAGTGCAGC
hSPAK-R[d]	GCGCTGCTCCTGTTGCTAATTCAA
hNKCC1-F[e]	AAGCAGTCCTTGTTCCTATGGCCT
hNKCC1-R[e]	GCAATGCAGCCCACCAGTTAATGA
hGAPDH-F[f]	CGGAGTCAACGGATTTGGTCGTAT
hGAPDH-R[f]	AGCCTTCTCCATGGTGGTGAAGAC

[a] These primers were used to amplify the Wnk1 coding sequence region.

[b] These primers were used to amplify the Wnk4 coding sequence region.

[c] These primers were used to amplify the Osr1 coding sequence region.

[d] These primers were used to amplify the Spak coding sequence region.

[e] These primers were used to amplify the NKCC1 coding sequence region.

[f] These primers were used to amplify the GAPDH coding sequence region.

4. Measurement of intracellular calcium

Cultured on 24 X 24mm coverglass, with density of 1.5 X 10^6 cells/ml with 48h were loaded with 4 M Fura-2/AM and 0.05% Pluronic acid F-127 for 30 min in PSS at room temperature. Fura-2/AM fluorescence was measured at an excitation wavelengths of 340/380 nm, and emission was measured at 510 nm (ratio=F340/F380) using an imaging system (Molecular Devices, CA, USA). The emitted fluorescence was monitored using a CCD camera (CoolSNAP HQ, AZ, USA) attached to an inverted microscope. Fluorescence images were obtained at 2 sec intervals. All data were analyzed using the MetaFluor software (Molecular Devices, Downingtown, PA, USA).

5. Immunoblotting

Protein extracts were prepared from HSG cell-line and ICR salivary glands as follows. Cells were lysed in a buffer containing (in mM): 150 NaCl, 10 Tris (pH 7.8 with HCl), 1 EDTA, 1% NP-40, 0.1% SDS, and a protease inhibitor mixture ($2Na_3VO_4$, 10NaF, 10 g/ml leupeptin, and 10 g/ml phenylmethylsulfonyl fluoride). The samples were probed overnight with 1:2,000 dilutions of antibodies against p-OSR1, OSR1 at 4˚C and then separated by SDS-PAGE.

6. Measurement of intracellular pH (pH_i) and NKCC activity

pH_i was measured using pH sensitive fluorescent dye, BCECF-AM. Cells were loaded with 2.5 M BCECF-AM in PSS for 30min at room temperature. The fluorescence at excitation wavelength of 490nm and 440nm was recorded using a CCD camera (CoolSNAP HQ, AZ, USA). Fluorescence images were obtained at 2 sec intervals. All data were analyzed using the MetaFluor software (Molecular Devices, Downingtown, PA, USA).

Na^+-K^+-$2Cl^-$ cotransporter(NKCC) activity was measured from the pH_i decrease induced by the intracellular uptake of NH_4^+ using the methods of Evans and Turner [33] with modifications. Adding NH_4Cl 20 mM in the extracellular solution induce robust increase of pH_i due to diffusion of NH_3. Thereafter, pH_i is getting decreased by influx of NH_4^+, because NH_4^+ acts as a surrogate of K^+ in the NKCC. To discriminate only the NKCC activity, 100 M bumetanide applied. Slope inclination was measured from 1 min to 3 min after solution change.

7. Other materials and antibodies

Rabbit polyclonal p-OSR1 antibody was purchased from Millipore (Cat#07-2273). Rabbit polyclonal OSR1 antibody was purchased from Cell signaling (#3729). Cyclopiazonic acid was from Enzo life science (Farmingdale, NY, USA). 2-Aminoethoxydiphenyl borate was from Tocris (Bristol, BS11 0QL, UK).

Trypsin/EDTA, Fetal Bovine Serum, and 100X Antibiotic-Antimycotic was from Gibco (Carlsbad, CA, USA). BCECF-AM, BAPTA-AM, and Pluronic acid F-127 was from Life technologies (Carlsbad, CA, USA). Fura2-AM was from Teflabs (Austin, TX, USA). NaCl, NH4Cl was from Duksan (Ansan, Gyeonggi-do, Korea). KCl, EGTA, Glucose, and HEPES was from Amresco (Solon, OH, USA). Collagenase P was from Roche (Indiana polis, IN, USA). All other chemicals include $MgCl^2$, $CaCl^2$, Trypsin inhibitor, sodium pyruvate, gadolinium, lanthanum, and ruthenium red was from Sigma-Aldrich (St. Louis, MO, USA).

8. Data analysis and statistics

Densitometry performed with the scanned film images or digital photo images using the ImageJ (NIH, USA). All error bars are expressed as the mean ± SEM. The statistical significance of differences between groups was determined using the Student's t-test. In statistical tests, *p*-values less than 0.05 were considered significant.

III. RESULTS

1. Wnk kinase expression in the human salivary gland cell line

The expression of WNKs in the HSG cell line was previously unknown. Therefore, I first investigated WNK and its substrates, OSR1 and SPAK. Reverse-transcription PCR was performed, and I confirmed the mRNA expression of Wnk1, Wnk4, OSR1, SPAK, and NKCC1 in an HSG cell line (Fig. 2). The Wnk4 expression level was lower than the Wnk1 expression level (Fig. 2).

2. Wnk kinase activation in the human salivary gland cell line

The next step was the measurement of OSR1 phosphorylation at serine 325 by immunoblotting. The phosphorylation of OSR1 at serine 325 to form p-OSR1, the activated form of OSR1, is mediated by WNK. Hence, the elevation of p-OSR1 levels is considered indirect evidence showing the activation of WNK, because OSR1 is a main substrate of WNK. I observed an increase in the p-OSR1 level after 15min of hypotonic (215 mOsm) stimulation at 37˚C (Fig. 3A). The hypotonic stimulation was induced using a hypotonic solution (HS) that reduced only the NaCl content of a physiologic salt solution (PSS).

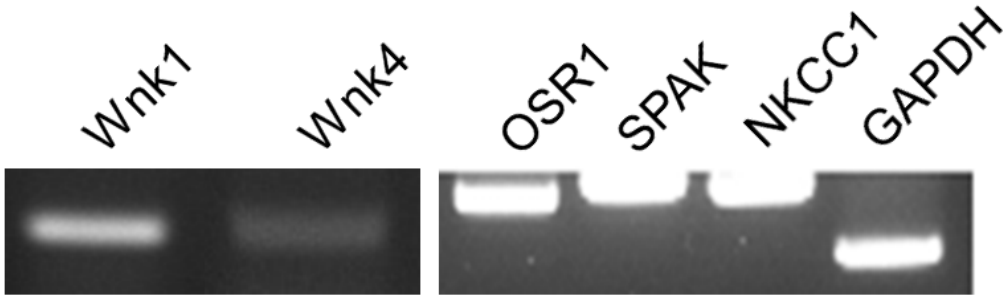

Figure 2. **Expression of Wnk and other associated protein.** Extracted total RNA converted to cDNA by using oligo-dT based reverse transcription. Following PCR procedure performed using specific primers for each gene. PCR amplicon was loaded in the agarose gel with UV-sensitive dye and the expression of Wnk1, Wnk4, OSR1, SPAK, and NKCC1 was confirmed at mRNA level. GAPDH expression was used as a positive control.

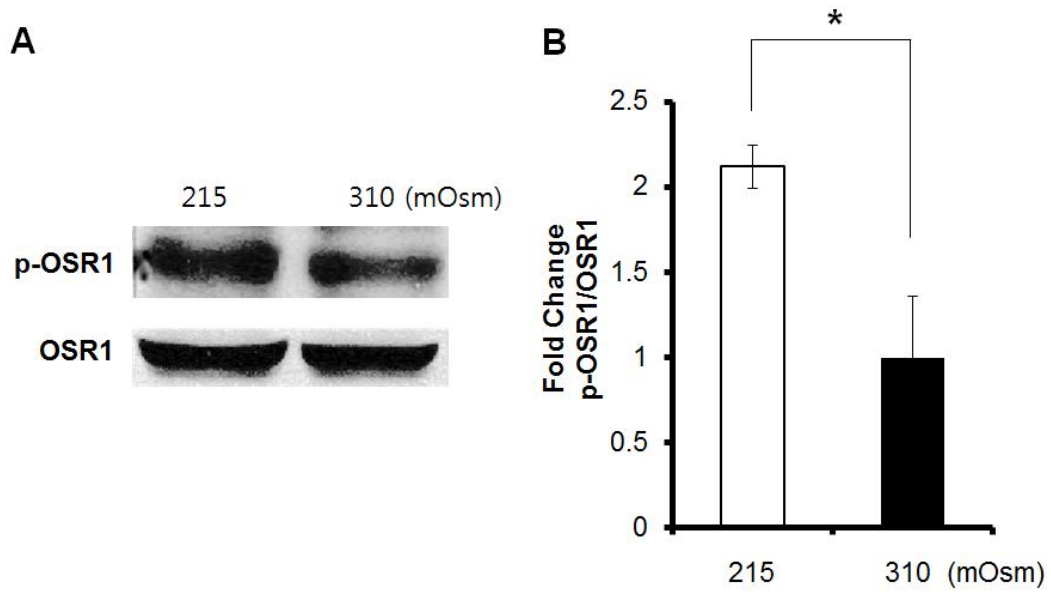

Figure 3. **Phosphorylation of OSR1 by hypotonic stimulation in HSG cell line.** A hypotonic solution (215 mOsm) induced an increase in phosphorylated OSR1 (p-OSR1) in the HSG cell line (A). The p-OSR1 level in the HSG cell line was normalized with the total OSR1 (B). HSG cell line n=3, * $p < 0.05$.

3. Isolated acinar cell from mouse model showed Wnk kinase activation

To confirmed our *in vitro* HSG cell line model is suitable for the exploring the WNK-OSR1 pathway, I observed a p-OSR1 increase in isolated parotid gland acini and also in submandibular gland acini from an ICR mouse (Fig. 4). Both of the tissue shows increased p-OSR1 intensity in the hypotonic stimulation, as same as HSG cell line. These data suggest that the HSG cell line can imitate hypotonically induced OSR1 activation.

4. Ca^{2+} signaling evoked by hypotonic stress in the HSG cell line

I hypothesized that Ca^{2+} signaling may regulate the WNK-OSR1-pathway in the HSG cell line. To investigate this hypothesis, I checked Ca^{2+} signaling pattern induced with hypotonic solution in the HSG cell line. Extracellular HS treatment induced an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and was abolished with the depletion of Ca^{2+} ions in the extracellular solution (Fig. 5), suggesting that the increased Ca^{2+} signal originated from the outside of the cell.

5. 4α-PDD induced Ca^{2+} signaling in the HSG cell line.

To further exploring the characteristics of the Ca^{2+} signaling pattern represented in the HSG cell line. I treated specific TRPV4 agonist, 10 M 4α-phorbol 12, 13-didecanoate (4α-PDD), to cultured HSG cell line. When I added 4α-PDD to the isotonic PSS (310 mOsm), I observed similar patterns of $[Ca^{2+}]_i$ increase (Fig. 6).

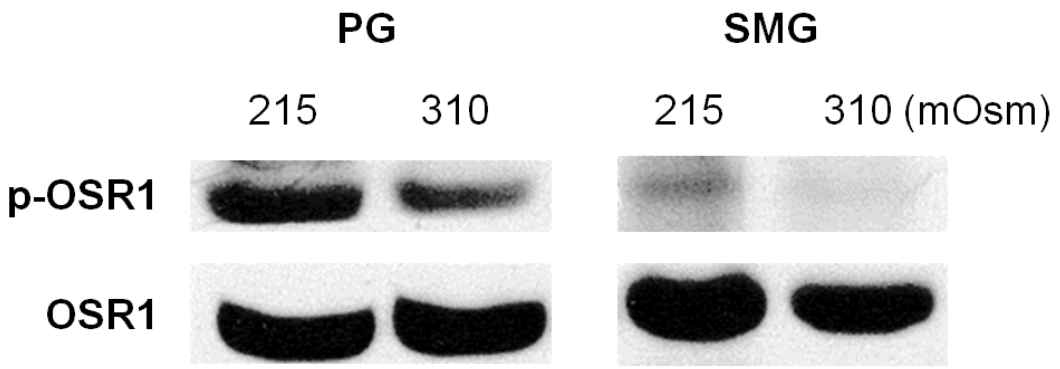

Figure 4. **Phosphorylation of OSR1 by hypotonic stimulation in isolated mouse cell.** A hypotonic solution (215 mOsm) induced an increase in phosphorylated OSR1 (p-OSR1) in a parotid gland (PG) and a submandibular gland (SMG) from ICR mouse salivary gland acinar cell. n=1.

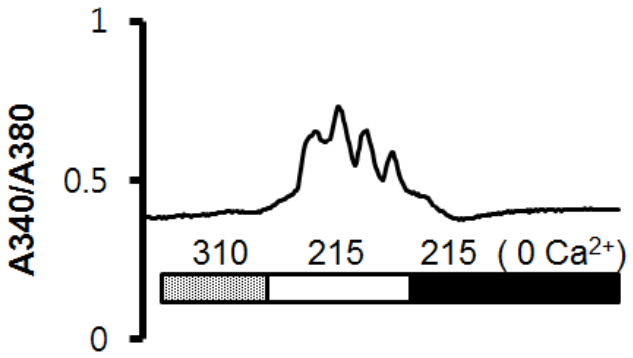

Figure 5. **Hypotonic stress-induced intracellular Ca^{2+} increase.** The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was shown as the Fura2 intensity ratio of fluorescence intensity in 340nm wavelength (A340) over that of 380nm wavelength (A380). A $[Ca^{2+}]_i$ increase was observed when the cells were treated with a hypotonic solution(215mOsm, *white bar*), and the increased $[Ca^{2+}]_i$ was diminished after the removal of extracellular Ca^{2+}(*black bar*), even though hypotonic stimulation still remained. This graph is representative trace.

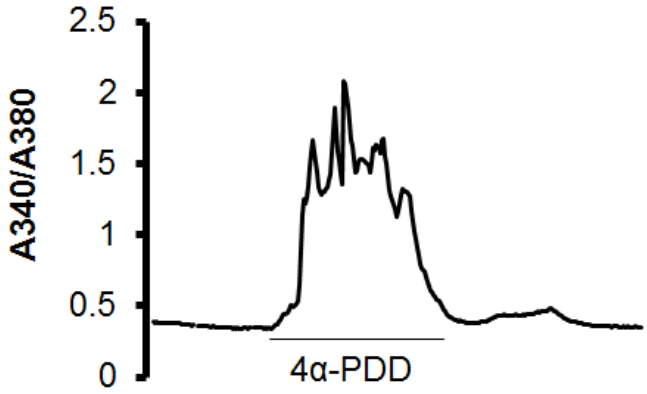

Figure 6. **4α-PDD induced intracellular Ca^{2+} increase.** The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was shown as the Fura2 intensity ratio of fluorescence intensity in 340nm wavelength (A340) over that of 380nm wavelength (A380). A $[Ca^{2+}]_i$ increase was observed when the cells were treated with a 10μM 4α-PDD (*underlined*) evoked an increase in the $[Ca^{2+}]_i$. This graph is representative trace.

6. Hypotonicity induced Ca^{2+} signaling inhibited by calcium channel inhibitors.

To define how the Ca^{2+} influx was mediated by extracellular Ca^{2+} ions, I used 10 M ruthenium red (RR), 100 M 2-aminoethoxydiphenyl borate (2APB), 10 M gadolinium (Gd^{3+}), and 10 M lanthanum (La^{3+}) as blockers. After 5min of pre-incubation with 2APB, Gd^{3+}, and La^{3+}, hypotonic stimulation did not evoke an elevation in the $[Ca^{2+}]_i$. However, RR could not inhibit the $[Ca^{2+}]_i$ increase (Fig. 7C).

7. Reduced OSR1 phosphorylation observed with the calcium channel inhibitors.

In agreement with the Ca^{2+} signaling pattern, the p-OSR1 level was reduced following a 20min pre-incubation with the blockers 2APB, Gd^{3+}, and La^{3+} (Fig. 8A). RR could not inhibit the hypotonic-induced $[Ca^{2+}]_i$ increase in the HSG cell line (Fig. 7C), and the p-OSR1 level was increased in the RR pre-incubation only (Fig. 8A).

8. Chelating intracellular Ca^{2+} increase with BAPTA attenuated OSR1 phosphorylation.

1, 2-bis (o-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid (BAPTA) is a high-affinity Ca^{2+} chelator that acts rapidly. When I loaded 25 M BAPTA-AM,

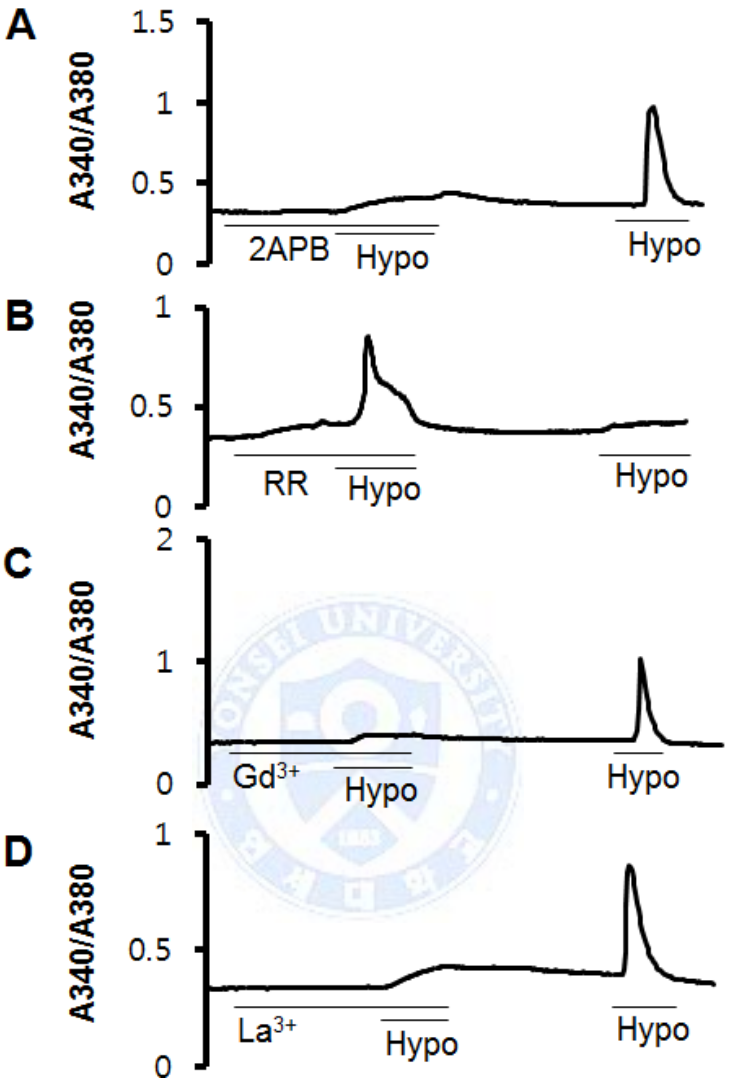

Figure 7. Various calcium channel inhibiting drugs attenuated the intracellular Ca^{2+} increase. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was shown as the Fura2 intensity ratio of fluorescence intensity in 340nm wavelength (A340) over that of 380nm wavelength (A380). Pretreatment with 100μM 2-

aminoethoxydiphenyl borate (2-APB), 10μM gadolinium (Gd^{3+}), and 10μM lanthanium (La^{3+}) reduced the $[Ca^{2+}]_i$ induced by the hypotonic stimulation, and after washout of the blockers, the hypotonic stress-induced $[Ca^{2+}]_i$ was recapitulated (A, C, D). However, Pretreatment with 10μM ruthenium red (RR) could not inhibits the calcium increase by following hypotonic stimulation (B). All the graphs are representative trace.

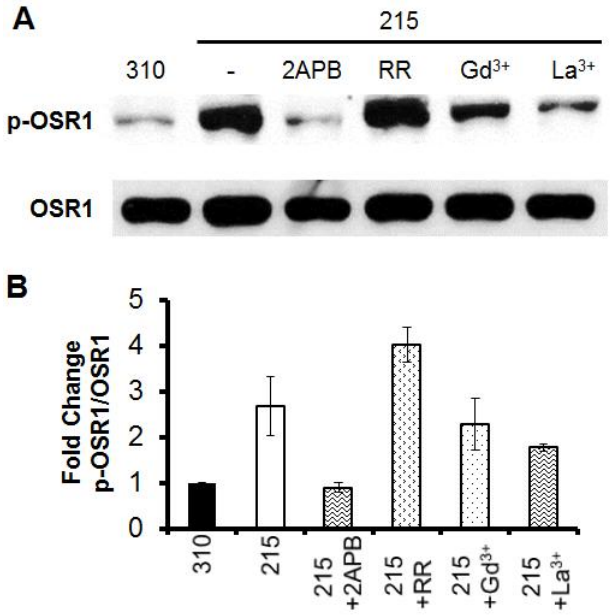

Figure 8. **Phosphorylation of OSR1 decreased by calcium channel inhibiting drugs.** Before hypotonic stimulation(215mOsm), each of the blockers was incubated for 20 min at 37˚C, and then the cells were treated with the hypotonic solution for 10 min (A). 100μM 2-aminoethoxydiphenyl borate (2-APB), 10μM gadolinium (Gd^{3+}), and 10μM lanthanium (La^{3+}), and 10μM ruthenium red (RR). The p-OSR1 level in the HSG cell line was normalized with the total OSR1 (B). n=3

which is an acetomethoxy ester conjugate of BAPTA, 20 min prior to the hypotonic stimulation, the p-OSR1 level was reduced compared with that in a control after 10 min of hypo-osmotic stress (Fig. 9A). The fold change of the p-OSR1/total OSR1 intensity was 2.88 ± 0.15 in 215 mOsm, 1.51 ± 0.02 in 215 mOsm + BAPTA, and 1.06 ± 0.01 in 310 mOsm + BAPTA (Fig. 9B). This result indicates that p-OSR1 may be regulated by upstream Ca^{2+} ions.

9. Phosphorylation of OSR1 observed with the release of intracellular Ca^{2+}.

Cyclopiazonic acid (CPA) inhibits the sarcoendoplasmic reticulum Ca^{2+} ATPase, which is an active Ca^{2+} transporter located in endoplasmic reticulum(ER). Therefore, CPA treatment to the intact cell induces a passive release of Ca^{2+} from ER, which is the major intracellular Ca^{2+} store organelle. To explore the role of Ca^{2+} from different source, I checked p-OSR1 level in the HSG with 25 M of CPA treatment for 30min. The p-OSR1 level was also increased in CPA treatment group (Fig. 10), however the amount of increasing.

10. Time course of phosphorylation of OSR1.

The other issue in the phosphorylation of OSR1 is the sustaining of the p-OSR1. To figure it out, I used 10 M 4α-PDD to the HSG cell line in the isotonic

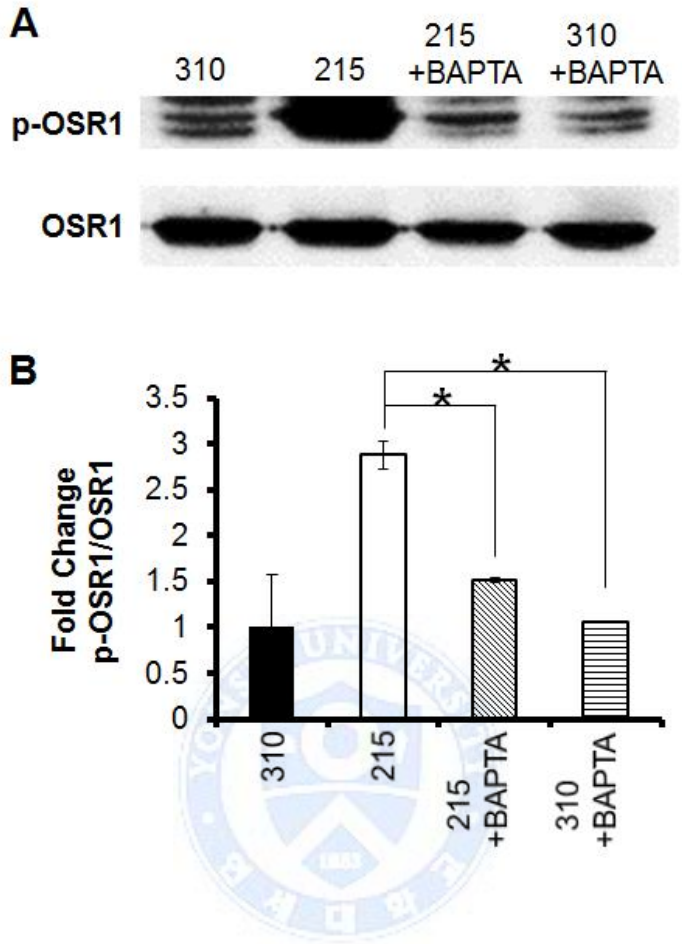

Figure 9. **Phosphorylation of OSR1 inhibited by treatment of calcium chelator.** Pretreatment with 25 M BAPTA/AM for 20 min followed by treatment with a hypotonic solution (215 mOsm) for 10 min (A). The fold change of the p-OSR1/total OSR1 between control and BAPTA-loaded cells was statistically significant in the hypotonic stimulation. n=3, * $p < 0.05$.

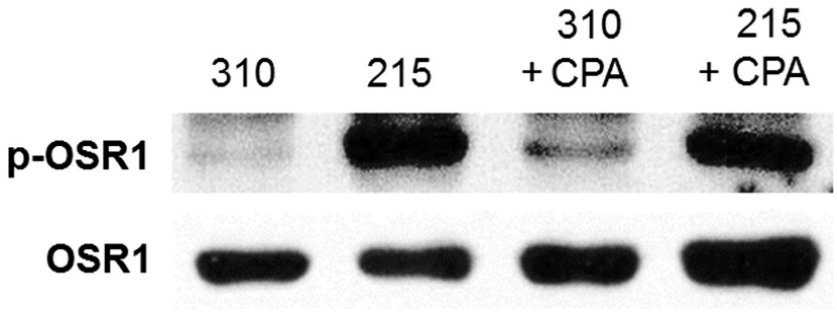

Figure 10. **Phosphorylation of OSR1 increased by Ca^{2+} release from internal store.** Cyclopiazonic acid (CPA), inhibitor of sarcoendoplasmic reticulum ATPase, induces release of Ca^{2+} from ER which is a major intracellular Ca^{2+} store. 25μM of CPA treatment induced phosphorylation of OSR1 without hypotonic stimulation. n=3.

(310mOsm) solution. Induce p-OSR1 level was increased maximum in 10min after treatment and diminished gradually time passes on. After 1hr, the intensity of p-OSR1 was lower than the initiation of the treatment (Fig. 11). This data suggest that WNK-OSR1 pathway activation is no longer prolonged than 1hr, and diminished the signal through un-investigated mechanism.

11. NKCC1 activity reduced under hypotonic stress.

The activation of OSR1 could affect the various transporters in the plasma membrane, such as the NCC, the K^+-Cl^- cotransporter (KCC), and the NKCC. In the present study, I measured NKCC activity using pH changes in the HSG cells. The traces in Fig. 4A/B/C show the BCECF ratio of emitting wave intensity in the excitation wavelengths 490 nm and 440 nm. Thus, the value of the BCECF ratio indicates the intracellular pH (pH_i). When 20mM NH4Cl was added to the extracellular solution, with the substitution of NaCl to maintain the osmolarity, the influx of NH_3 cause pH increase. After that, through NKCC1 activity, which carries NH_4^+ as a surrogate of K^+, the pH in the solution acidified. Bumetanide (100 M) can inhibit NKCC activity. Therefore, the difference in the slope inclination between the NH_4Cl treatment and the bumetanide treatment was assessed to measure the NKCC1 activity by quantifying the acidification rate. The greatest slope change was with 100 M bumetanide in the isotonic solution (310 mOsm; Fig. 12A). After pre-incubation in HS (215 mOsm) for 5min, the fold

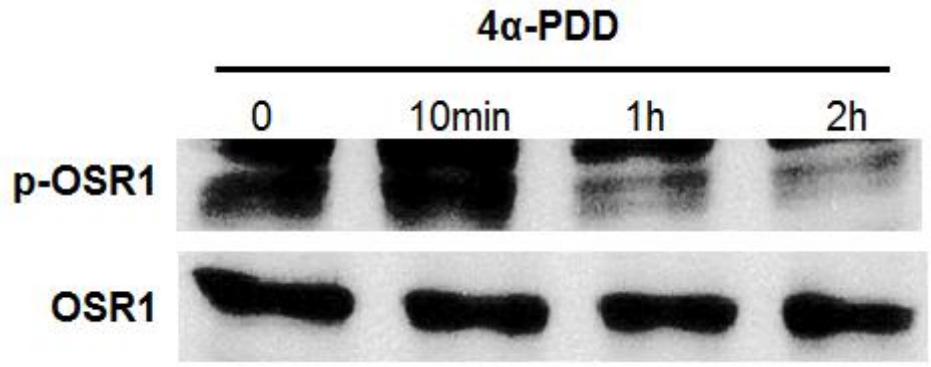

Figure 11. **Time-dependent manner of p-OSR1 after treatment of TRPV4 agonist.** Up-regulated p-OSR1 was observed with treatment of 10 M 4α-PDD applied in an isotonic solution (310 mOsm). Most significant phosphorylation of OSR1 was shown in 10min after stimulation, and decreased gradually.

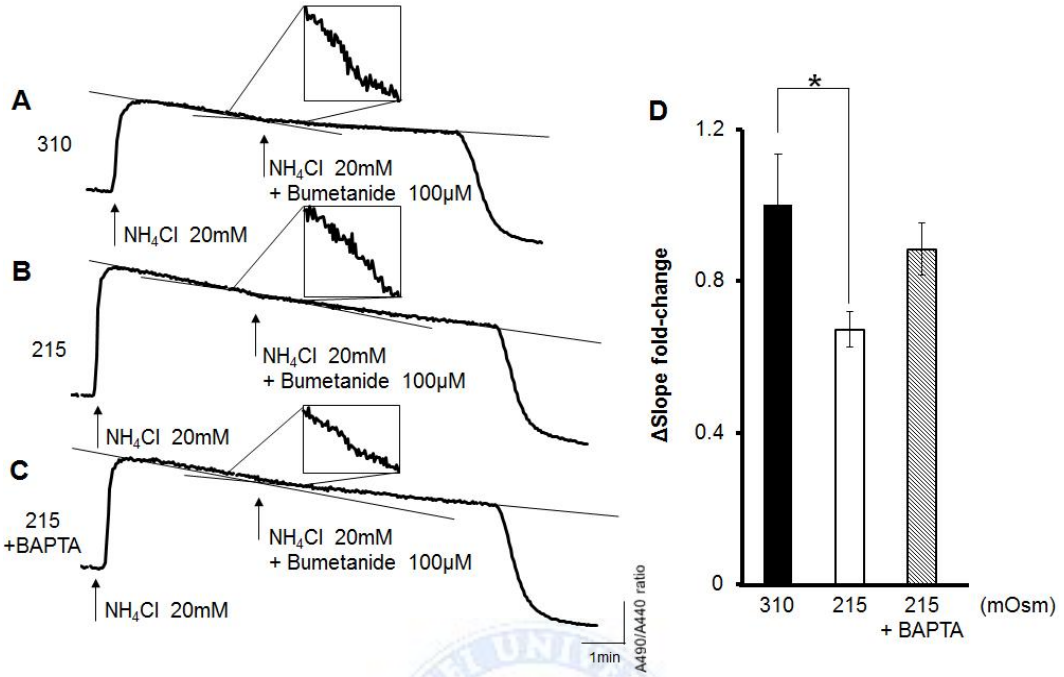

Figure 12. **Down-regulation of NKCC activity is induced by hypotonic stress in a HSG cell line.** Intracellular pH indicated by the BCECF signal ratio. A decline in the rate after bumetanide (100 M) treatment was decreased in an isotonic solution (310 mOsm) (A); however, after hypotonic stimulation (215 mOsm), the degree of the decline in the rate was reduced (B). Chelating $[Ca^{2+}]_i$ with 25 M BAPTA-AM treatment restored the bumetanide-sensitive modification of the BCECF ratio (C). The narrow *black line* represents a trend line indicating the slope of the pH declination (A, B, C). The alteration of the slope by treatment by 100 M bumetanide is emphasized in the *box*. The shifting slope of the declination was expressed by the fold change (D). All the traces are representative graphs, with n=25–35 cells in each experiment. * $p < 0.05$

change in the slope was 0.67 ± 0.05 (Fig. 12B), which was statistically significant (Fig. 12D). After pre-incubation with 25 M BAPTA-AM in HS, the decreased fold change in the slope was restored (Fig. 12C/D).

IV. DISCUSSION

In the present study, I found that the phosphorylation of OSR1 is regulated by the $[Ca^{2+}]_i$. This was a unique finding of our study. In other articles, it was shown by using a kinase assay that Wnk4 kinase activity increased depending on the Ca^{2+} concentration in vitro, and that effect was diminished by the substitution of acidic amino acid residues such as E562 and D564 in Wnk4 [34]. Tacrolimus, which is a calcineurin inhibitor, induced Wnk3, Wnk4, and SPAK expression in mouse kidney [35]. Those previous studies support our findings that p-OSR1 is regulated by the $[Ca^{2+}]_i$.

The other issue in this study is that a source of increased Ca^{2+} triggered the phosphorylation of OSR1. When I removed the external Ca^{2+} from the hypotonic solution (Fig. 5), the $[Ca^{2+}]_i$ was reduced to the baseline. That result shows that the increased $[Ca^{2+}]_i$ in the HSG cell line originated from the influx of external calcium. 2APB, an inhibitor of Ca^{2+} channels [36,37], was used to block the influx of Ca^{2+}. 2APB also inhibits the IP_3 induced Ca^{2+} increase. However, even though 2APB acts as an inhibitor of either IP_3 induced Ca^{2+} increase or nonselective cation channel, the outcome of 2APB treatment is an inhibition of $[Ca^{2+}]_i$ increase. In the results pretreatment with 2APB inhibited the elevation of the $[Ca^{2+}]_i$ and reduced the level of p-OSR1 induced by hypotonic stress in the HSG cell line (Fig. 7/8). Furthermore, nonselective cation channel blockers, like Gd^{3+} and La^{3+}, also

inhibited the $[Ca^{2+}]_i$ and the p-OSR1 level under hypotonic stress in the HSG cell line (Fig. 7/8). Those results indicate external Ca^{2+} influx mediated by Ca^{2+} channels sensitive to 2APB, Gd^{3+}, and La^{3+} in the HSG cell line. Interestingly, treatment with 4α-PDD, which is a selective activator of TRPV4, displayed Ca^{2+} signaling patterns similar to those displayed by hypotonic stimulation [38,39]; TRPV4 is a well-known Ca^{2+} permeable channel activated by hypotonic stress [40]. Thus, a TRPV4-induced $[Ca^{2+}]_i$ appears to be one potent channel that mediates the OSR1 pathway in the HSG cell line, but that hypothesis requires more experiments to be confirmed. Likewise, another TRP channels, such as TRPC3, and TRPM4 also involved hypotonicity stimulated Ca^{2+} influx in the HSG cell line, but I have not included in this paper. It was unexpected that RR could not inhibit the $[Ca^{2+}]_i$ and the p-OSR1 level. That result suggests that RR-resistant Ca^{2+} channels play a role in the HSG cell line. However, to solve that remaining question about RR, another investigation is required.

The next question is whether the Ca^{2+} performed as an upstream regulator or as a downstream messenger of OSR1 phosphorylation. Because BAPTA is a rapid and high-affinity chelator of Ca^{2+}, it is regarded as a highly efficient chelator that blocks the action of Ca^{2+} in the cell [41]. When the $[Ca^{2+}]_i$ was chelated by BAPTA during the hypotonic stimulation of the HSG cell line, the p-OSR1 level was decreased (Fig. 9). That suggests that Ca^{2+} is an upstream regulator of p-OSR1. Ca^{2+} is more important than the mechanical membrane stretching induced by the

HS. It is inferred from the facts that the $[Ca^{2+}]_i$ increases with 4α-PDD (Fig. 11), and CPA (Fig. 10) also resulted in increased p-OSR1 levels. However, the CPA induced less p-OSR1 than did the hypotonic stimulation. That could be explained by the localization of the WNKs near the plasma membrane [1].

Although, Ca^{2+} regulates OSR1 through phosphorylation, it is still unclear whether OSR1 is directly or indirectly regulated by the WNKs. Ca^{2+} was required to show the kinase activity of Wnk4. In the intact cell, however, remaining kinase activity appeared at very low Ca^{2+} concentrations, below 1nM [34]. Moreover, it is well known that WNKs exhibit autoinhibition and autophosphorylation [11,12], and role of low Cl^- in the activation of WNKs was revealed by crystallography [27]. Furthermore, it is well known that Ca^{2+}-activated Cl^- channels, like TMEM16A, are expressed in the apical membranes of salivary gland acinar cells [42]. Therefore, I assumed that TMEM16A activated by an elevated $[Ca^{2+}]_i$ contributes to the local depletion of Cl^- at the microdomain level, and that the depletion of Cl^- leads to the activation of the Wnk-OSR1 pathway. However, more investigations will be required to test that model.

The popular effectors of activated OSR1 are the NCC, the KCC, and the NKCC [13-15]. Furthermore, the NKCC is the essential molecule that maintains Cl- homeostasis in salivary gland acinar cells [42]. For that reason, I investigated the alteration of NKCC activity in the HSG cell line (Fig. 12). After hypotonic stimulation, NKCC activity was reduced in the HSG cell line and was

recapitulated by chelating Ca^{2+} through BAPTA pretreatment (Fig. 12). It is still controversial whether activated OSR1 contributes to the activation or to the inactivation of NKCC, depending on the cell type [25,43]. In terms of volume homeostasis, I think that the inhibition of NKCC requires cells to decrease the intracellular ion osmolarity to maintain the cell volume.

In conclusion, I found that Ca^{2+} regulates the WNK-OSR1-NKCC pathway. This is the first study to demonstrate the Ca^{2+}-mediated WNK-OSR1-NKCC pathway in an intact HSG cell line. I hope that these results will help to clarify the regulatory mechanism of the WNK-OSR1 pathway and further our understanding of the mechanism of salivary secretion.

V. CONCLUSION

The present study support the calcium ion regulates the WNK-OSR1-NKCC pathway in the human salivary gland (HSG) cell line. The activity of NKCC was reduced with the calcium increase and OSR1 phosphorylation.

1. HSG cell line was expressed functional Wnk, OSR1, and NKCC.
2. Activation of WNK-OSR1 pathway was inhibited by intracellular Ca^{2+} chelation.
3. Only an intracellular Ca^{2+} increase evoked WNK-OSR1 activation without cell volume increase.
4. Treatment of hypotonic solution activated WNK-OSR1 pathway, and inhibited NKCC activity.

Therefore, calcium ion is a major regulator that controls the WNK-OSR1-NKCC pathway. This finding might provide new insights to the drug discovery to treat the xerostomia patient.

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국문요약

사람 타액선 세포주에서 저삼투성용액에 의해 유도된 칼슘 매개 WNK 신호전달과정의 활성화

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구상균

Wnk 인산화효소는 다양한 세포막 수송체들을 조절하여 세포의 부피를 유지하는 역할을 한다. Wnk 인산화효소에 의해 조절되는 공동수송체로는 나트륨-염소 이온 공동수송체와 칼륨-염소 이온 공동수송체, 그리고 나트륨-칼륨-염소이온 공동수송체 제1형 (NKCC1) 등이 있다. 이들 공동수송체는 Wnk 인산화효소가 OSR1과 SPAK를 인산화하고, 이에 의해 활성화된 OSR1과 SPAK이 하부에 있는 공동수송체들을 인산화 시킴으로써 활성을 조절한다고 알려져 있다. 하지만, Wnk 인산화효소가 특정 조직에 따라서, 또한 특정 조건에 따라

서 어떤 과정을 통해 활성화되고, 조절기능을 수행하는지는 대체적로 잘 알려지지 않았다. 본 연구에서는 사람 타액선 세포주를 모델로 이용하여, 칼슘이온에 의한 Wnk 인산화효소의 조절 기전을 보였다. 우선 Wnk1, Wnk4, OSR1, SPAK, 그리고 NKCC1 등과 같은 WNK-OSR1-NKCC 신호전달과정에 관여하는 분자들이 발현됨을 확인하였다. 또한, 저삼투성 용액을 사람타액선 세포주에 처리하면, 세포내부의 칼슘이온농도가 증가하는 것과 동시에 OSR1에 인산화가 일어나는 것을 확인하였다. 흥미로운 점은 2APB, Gd^{3+}, La^{3+}와 같은 비특이적 칼슘통로 저해제를 전처리하면 저삼투성 용액에 의한 칼슘농도의 증가와 OSR1의 인산화가 모두 사라지는 것을 확인하였다. 그리고, CPA를 이용하여 수동적으로 세포 내부의 칼슘 이온 농도를 증가시킨 경우에도 OSR1의 인산화가 증가하는 것을 확인하였고, 칼슘 킬레이터인 BAPTA를 처리하면 OSR1의 인산화가 줄어드는 것을 확인하였다. 이런 과정을 통하여, NKCC1 활성도가 감소되는 것 또한 확인하였다. 이런 결과는 칼슘이온이 WNK-OSR1 신호전달과정을 통해 NKCC1을 조절할 수 있음을 보여준다, 이런 결과는 살아있는 세포에서 WNK-OSR1 신호전달과정이 칼슘 이온에 의해 조절될 수 있다는 최초의 보고이다.

중심되는 말: 칼슘신호전달, OSR1, WNK, NKCC, 타액선