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타액선 중탄산이온 수송체 및 자가면역항체에 관한 연구

Roles of sodium bicarbonate cotransporters in salivary glands and autoantibodies in Sjögren’s syndrome

2018 년 2 월

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남 궁 은
Primary Sjögren’s syndrome is a systemic autoimmune disease that impairs the structural integrity and function of the salivary and lachrymal glands, leading to dry mouth and eyes. According to previous studies, the disease is multifactorial, showing many extraglandular features such as arthritis, myalgia, pulmonary disease, vasculitis, lymphoma, renal tubular acidosis, poor dentition, leukopenia, and bicarbonate (HCO$_3^-$) secretion. This study aims to investigate Sjögren’s syndrome-associated leukopenia and the physiological mechanisms of salivary sodium bicarbonate cotransporters (NBCs) associated with HCO$_3^-$ secretion. Sjögren’s syndrome patients have low salivary HCO$_3^-$ concentration and buffer capacity in comparison to healthy patients. Salivary HCO$_3^-$ buffers H$^+$ ions
produced from oral bacterial fermentation, so decreased salivary HCO$_3^-$ secretion accelerates tooth decay and further compromises poor dentition. However, only a few studies have reported the roles and regulatory mechanisms of HCO$_3^-$ secretion and intracellular pH regulation in salivary gland epithelial cells (SGECs).

Therefore, chapter I mainly investigates intracellular pH (pH$_i$) regulation and regulatory mechanisms of two different types of NBCs—electroneutral (NBCn1) and electrogenic NBC (NBCe1)—in human submandibular glands (hSMGs) and HSG cells (isolated from human submandibular gland intercalated duct cells). Intracellular pH and the pH$_i$ recovery rate from cell acidification induced by an NH$_4$Cl pulse were recorded. Subcellular localization and protein phosphorylation were determined using immunohistochemistry and co-immunoprecipitation techniques. I determined that NBCn1 is expressed on the basolateral side of acinar cells and the apical side of duct cells, while NBCe1 is exclusively expressed on the apical membrane of duct cells. The pH$_i$ recovery rate in hSMG acinar cells, which only express NBCn1, was not affected by pre-incubation with 5 µM PP2 (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo [3,4-d]pyrimidine), an Src tyrosine kinase inhibitor. However, in HSG cells, which express both NBCe1 and NBCn1, the pH$_i$ recovery rate was inhibited by PP2. The apparent difference in regulatory mechanisms for NBCn1 and NBCe1 was evaluated by artificial overexpression of NBCn1 or NBCe1 in HSG cells, which revealed that the pH$_i$ recovery rate was only inhibited by PP2 in cells overexpressing NBCe1. Furthermore, only NBCe1 was significantly phosphorylated and translocated by NH$_4$Cl, which was inhibited by PP2.
In chapter II, a possible mechanism of Sjögren’s syndrome-associated leukopenia is suggested and examined. Patients with primary Sjögren’s syndrome have been shown to have serum autoantibodies that react with muscarinic acetylcholine type 3 receptors (M3R). Interestingly, leukopenia has been reported to be significantly more common in primary Sjögren’s syndrome patients who have anti-M3R-autoantibodies in their sera. However, the pathophysiological relationship between leukopenia and Sjögren’s syndrome is yet to be established. I hypothesized that anti-M3R autoantibodies would have effects on M3R and MHC I expression in T cells, which may lead to the death of T cells. Purified IgG antibodies were isolated from the serum of healthy individuals and primary Sjögren’s syndrome patients. Jurkat cell line was used to represent T lymphocytes. In situ immunofluorescence confocal microscopy was used to confirm the binding reactivity of primary Sjögren’s syndrome IgG antibodies to M3R. Co-immunoprecipitation and immunofluorescence results suggested a direct interaction between M3R and MHC I. Co-internalization of M3R and MHC I was observed when Jurkat cells were exposed to the primary Sjögren’s syndrome IgG, but this co-internalization was prevented by the presence of exogenous interferon gamma (IFN-γ). Primary Sjögren’s syndrome IgG itself did not affect the viability of Jurkat cells, but Jurkat cells exposed to primary Sjögren’s syndrome IgG underwent significant cell death when co-cultured with primary Natural Killer cells.

Taken together, I suggest that both NBCn1 and NBCe1-B play a role in pH\textsubscript{i} regulation in hSMG acinar and duct cells. NBCe1-B is phosphorylated by Src tyrosine kinase and translocates to the plasma membrane, whereas NBCn1 is not regulated by Src kinase. Further, anti-M3R autoantibodies in primary Sjögren’s
syndrome induced downregulation of plasma membrane M3R and MHC I molecules in leukocytes, followed by NK cell-mediated cell death. These physiological and immunological approaches may help to understand the pathophysiology of Sjögren’s syndrome-associated diseases or phenotypes.

**Keywords:** Sodium bicarbonate cotransporters (NBC), human submandibular gland (hSMG), HSG cells, Src-family tyrosine kinase, Sjögren’s syndrome, leukopenia, anti-muscarinic 3 receptor (M3R) autoantibody, Natural Killer cells

**Student Number:** 2011-23823
Chapter I. Regulatory mechanisms of sodium bicarbonate cotransporters in human submandibular glands

1. Introduction .................................................................................. 6
2. Materials and Methods ................................................................. 8
3. Results ........................................................................................ 15
4. Discussion ................................................................................... 32
Chapter II. The effects of autoantibodies on the function of T cells in Sjögren’s syndrome patients

1. Introduction ............................................................................. 31

2. Materials and Methods .......................................................... 44

3. Results ..................................................................................... 48

4. Discussion ............................................................................... 61

Conclusions ................................................................................. 65

References .................................................................................. 67

Abstract in Korean ................................................................. 79
List of Figures

Chapter I

Figure 1. Intracellular pH calibration from BCECF-loaded HSG cells… 12
Figure 2. NBCe1 and NBCn1 are expressed in human submandibular
gland (hSMG) and HSG cells……………………………………17
Figure 3. Src tyrosine kinase does not affect pH$_i$ recovery of hSMG
acinar cells……………………………21
Figure 4. pH$_i$ recovery of HSG cells is inhibited by PP2………………….24
Figure 5. Transfected NBCe1-B is affected by PP2…………….27
Figure 6. PP2 inhibits tyrosine phosphorylation and translocation of
   NBCe1-B, but NBCn1, in HSG cells…………………………..30
Figure 7. Role and location of NBCe1-B and NBCn1 in human
   submandibular glands………………………………………34
Figure 8. Schematic model of NBCe1-B and NBCn1 regulation by Src
   kinase…………………………………38

Chapter II

Figure 9. Detection of anti-M3R autoantibodies in patients with pSS by
dual immunolabeling of the mouse submandibular gland…… 49
Figure 10. Effects of pSS IgG on M3R and MHC I membrane expression
   in Jurkat cells and on Jurkat cell viability…………………..53
Figure 11. Effect of IFN-γ on pSS IgG-induced downregulation of M3R
   and MHC I………………………………………………56
Figure 12. Effects of pSS IgG on NK-cell mediated lysis…………………59
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Intracellular pH</td>
</tr>
<tr>
<td>NHE</td>
<td>Na&lt;sup&gt;+&lt;/sup&gt;-H&lt;sup&gt;+&lt;/sup&gt; exchanger</td>
</tr>
<tr>
<td>NBC</td>
<td>Na&lt;sup&gt;+&lt;/sup&gt;-HCO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt; cotransporter</td>
</tr>
<tr>
<td>NBCe1</td>
<td>Electrogenic NBC</td>
</tr>
<tr>
<td>NBCn1</td>
<td>Electroneutral NBC</td>
</tr>
<tr>
<td>hSMGs</td>
<td>Human submandibular glands</td>
</tr>
<tr>
<td>EIPA</td>
<td>5-(N-ethyl-N-isopropyl) amiloride</td>
</tr>
<tr>
<td>DIDS</td>
<td>4,4-diisothiocyanostilbene-2,2-disulfonylic acid</td>
</tr>
<tr>
<td>PP2</td>
<td>4-amino-5-(4-chlorophenyl)-7-(dimethylthethyl)pyrazolo[3,4-d]pyrimidine</td>
</tr>
<tr>
<td>AQP5</td>
<td>Aquaporin 5</td>
</tr>
<tr>
<td>BBS</td>
<td>Bicarbonate buffered solution</td>
</tr>
<tr>
<td>CCh</td>
<td>Carbachol</td>
</tr>
<tr>
<td>pSS</td>
<td>Primary Sjögren’s syndrome</td>
</tr>
<tr>
<td>M3R</td>
<td>Muscarinic acetylcholine type 3 receptors</td>
</tr>
<tr>
<td>MHC I</td>
<td>Major histocompatibility complex I</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural Killer cell</td>
</tr>
</tbody>
</table>
General Introduction

Primary Sjögren’s syndrome (pSS) is a systemic autoimmune disease characterized by lymphocyte infiltration into the salivary and lachrymal glands, which causes dry mouth and eyes (Chinthamani et al., 2012). Aside from sicca symptoms, pSS also represents extraglandular organ manifestations such as vasculitis, renal malfunction, lymphoma, anemia, poor dentition, leukopenia, and bicarbonate (HCO$_3^-$) secretion (Almstahl and Wikstrom, 2003; Kassan and Moutsopoulos, 2004; Kovacs et al., 2005).

In primary Sjögren’s syndrome, salivary buffer capacity and concentration of HCO$_3^-$ secretions were lower than in healthy patients (Almstahl and Wikstrom, 2003). Salivary HCO$_3^-$ secretion buffers acids (H$^+$) produced by oral bacteria. Thus, decreased salivary HCO$_3^-$ secretion in Sjögren’s syndrome accelerates tooth decay and further compromises poor dentition (Dinour et al., 2004).

In epithelial cells, especially salivary glands, fluid and HCO$_3^-$ secretion are essential. Abnormal fluid and HCO$_3^-$ secretions cause epithelial diseases, such as Sjögren’s syndrome, cystic fibrosis, and other epithelial inflammatory and autoimmune diseases (Lee et al., 2012). HCO$_3^-$ is an important ion that functions as a buffer to maintain an optimal pH, as well as regulating intracellular pH (pH$_i$). An imbalance of pH causes several epithelial diseases (Kurtz, 2014), and an aberrant HCO$_3^-$ secretion to the oral cavity leads to severe dental carries (Catalan et al., 2011). Thus, sodium bicarbonate cotransporters (NBCs) are important salivary gland proteins playing a putative role in HCO$_3^-$ secretion and intracellular pH regulation.
NBCs are classified into electrogenic and electroneutral according to their net transport activity: electrogenic type and electroneutral type (Boron et al., 2009). For example, in the renal cells, NBCe1-A mediates HCO$_3^-$ efflux from cells with 1Na$^+$ to 3HCO$_3^-$ stoichiometry (Yoshitomi et al., 1985). In other epithelial cells, NBCe1-B mediates HCO$_3^-$ uptake into cells with 1Na$^+$ to 2HCO$_3^-$ stoichiometry (Romero et al., 2013). Meanwhile, NBCn1 and NBCn2 have an apparent stoichiometry of 1:1 (Chen et al., 2011). NBCe1s are divided into NBCe1-A, -B, and -C by their sequence structure. NBCe1-B and NBCe1-C are identical except for their C-terminal domains, which are 46 and 61 amino acids in length, respectively (Bevensee et al., 2000). NBCe1-A is the shortest of the three NBCe1 variants, and is identical to NBCe1-B except for a unique 41 N-terminal amino acid sequence, which replaces the 85 amino acids of the N-terminus of NBCe1-B (Abuladze et al., 1998). In the human submandibular gland, two variants of NBCs are expressed: NBCe1 and NBCn1 (Lee et al., 2012; Melvin et al., 2005; Parker and Boron, 2013). However, the role of NBCs in pH regulation and locations of each isotype of NBCs in hSMG remain elusive.

Protein phosphorylation is involved in numerous cellular processes via the addition of phosphate group onto serine, threonine, or tyrosine residues on the specific protein. Phosphorylation by Src family tyrosine kinase controls various channels and receptors, such as CFTR (Billet et al., 2013), potassium channels (Bae et al., 2014), and NMDA receptors (Salter and Kalia, 2004). NBCe1 activity was increased by interaction of non-receptor proline-rich tyrosine kinase 2 and Src family tyrosine kinases (Espiritu et al., 2002). However, regulatory mechanisms of specific isotypes of NBCs in hSMG have to date remained poorly understood.
Patients with primary Sjögren’s syndrome have been shown to have serum autoantibodies directed against organ-specific or tissue-specific autoantigens, such as ribonucleoproteins Ro (SS-A/ Ro) and La (SS-B/La) (Iwasaki et al., 2003), carbonic anhydrase (Kino-Ohsaki et al., 1996), and M3R (Kovacs et al., 2005). Since muscarinic type 3 receptor (M3R) is endogenously expressed in salivary glands and plays a key role in salivary fluid production in humans, anti-M3R autoantibodies have been shown to functionally inhibit salivary secretion (Jin et al., 2012a).

Leukopenia is a decrease in the number of white blood cells found in the blood, which places individuals at increased risk of infection. Leukopenia is significantly more common in primary Sjögren’s syndrome patients who have anti-M3R-autoantibodies in their sera (Kovacs et al., 2005). Indeed, patients with systemic autoimmune diseases have been reported to have reduced numbers of peripheral blood lymphocyte cells compared with healthy patients (Shibatomi et al., 2001). However, the pathophysiological relationship between leukopenia and Sjögren’s syndrome has not been studied.

MHC I has critical roles in the innate and adaptive immune response and regulation, and it serves as an inhibitory ligand of immunoglobulin-like receptors on NK cells. NK cell activation is inhibited by expressed MHC I ligand, and thus MHC I prevents self-killing of host cells. According to previous studies, pSS autoantibodies induce M3R and MHC I downregulation from the plasma membrane in primary submandibular gland cells (Kim et al., 2015) and in HeLa cells (Scarselli and Donaldson, 2009).

Despite significant research, the correlation of Sjögren’s syndrome with leukopenia
and regulatory mechanisms of HCO$_3^-$ secretion remains unexplained. This study aims to investigate Sjögren’s syndrome-associated leukopenia and the physiological mechanisms of salivary sodium bicarbonate cotransporters associated with HCO$_3^-$ secretion.

A key purpose of this thesis is to shed light on the roles of sodium bicarbonate cotransporters in salivary glands and Sjögren’s syndrome autoantibodies. To this end, the following are addressed in the two subsequent chapters.

- To identify the expression and cellular localization of specific isoforms of NBCe1 and NBCn1 in the human submandibular gland.
- To demonstrate the regulatory mechanisms of NBCe1 and NBCn1.
- To investigate M3R and MHC I expression and their association with the pathogenesis of pSS-related leukopenia.
CHAPTER I

Regulatory mechanisms of sodium bicarbonate cotransporters in human submandibular glands
Introduction

The ability to maintain intracellular pH (pH$_i$) homeostasis is critical, and dysregulated pH$_i$ is connected with several diseases (Gorbatenko et al., 2014). Furthermore, pH$_i$ can influence various metabolic reactions and vascular functions (Casey et al., 2010; Schulz and Munzel, 2011). There are two major types of proteins that regulate pH$_i$, namely, Na$^+$-H$^+$ exchangers (NHEs) and Na$^+$-HCO$_3^-$ cotransporters (NBCs). Moreover, bicarbonate ion (HCO$_3^-$), which functions as a buffer that provides optimal pH, is one of the crucial ions in epithelial cells and fluctuations in the concentration of HCO$_3^-$ in final fluids is associated with several epithelial diseases (Kurtz, 2014). HCO$_3^-$ ions in saliva are also protective against enamel erosion under low pH conditions (Catalan et al., 2011). Thus, bicarbonate transporter is an important protein in the epithelia, especially salivary glands.

NBCs are classified into either electrogenic (NBCe1) or electroneutral (NBCn1) types according to their net transport activity (Boron et al., 2009). Human submandibular glands (hSMGs) express two NBC variants, namely NBCe1-B and NBCn1, which were originally cloned as pancreatic and Cl$^-$-independent electroneutral NBCs, respectively (Lee et al., 2012; Melvin et al., 2005; Parker and Boron, 2013). NBCe1s are further divided into NBCe1-A, -B and -C. NBCe1-A and NBCe1-B are identical except for their N-terminal domains, which are 41 and 85 amino acids in length, respectively (Abuladze et al., 1998). NBCe1-C is the longest of the three NBCe1 variants, and is identical to NBCe1-B except for a unique 61 C-terminal amino acid sequence, which replaces the 46 amino acids of
the C-terminus of NBCe1-B (Bevensee et al., 2000). Despite significant research, the role of NBCs in pH regulation as well as the regulatory mechanisms and subcellular localizations of the different NBCs isotypes in hSMG remain elusive. Protein phosphorylation, a common mechanism of protein regulation, is mediated via the addition of phosphate groups onto serine, threonine, or tyrosine residues. In addition to NBCs, CFTR (Billet et al., 2013) and neuronal channels such as potassium channels (Bae et al., 2014) and NMDA receptors (Salter and Kalia, 2004) are controlled by Src family tyrosine kinases (SFK). In renal epithelial cells, non-receptor tyrosine kinase proline-rich tyrosine kinase 2 (Pyk2) increases the activity of NBCe1 by autophosphorylation and interactions with Pyk2-Src family kinases (Espiritu et al., 2002). However, the role and regulatory mechanisms of the specific isotypes of NBCe1 and NBCn1 in hSMG remain poorly understood. Interestingly, I found that the Src kinase inhibitor PP2 alters pHᵢ regulation in an HSG cell line originating from hSMG duct cells.

In the present study, I studied the expression of NBCn1 and NBCe1-B in hSMG and HSG cells. I also examined whether NBCn1 in hSMG cells plays a role in pHᵢ regulation and investigated its regulatory mechanism via tyrosine phosphorylation compared with NBCe1-B.
Materials and Methods

Source of Human Submandibular glands (hSMGs)

Human submandibular glands (hSMGs) were obtained from patients who underwent resection of their submandibular gland as part of their treatment for oral tumors. The patient group included both males and females ranging from 37 to 82 years of age. Tissues were kept in cold physiological saline while transporting the dissected gland from the hospital to the laboratory for analysis. Some of the tissues were fixed with 4% paraformaldehyde for immunohistochemistry studies while the remainder were prepared for physiological experiments. All patients gave written informed consent for participation in this study. The collection and use of human tissue was performed according to ethical guidelines and the study protocol was approved by the Institutional Review Board of Seoul National University Dental Hospital (CRI11023G).

hSMG acinar cell preparation

hSMG acinar cells were prepared as described previously (Jin et al., 2012a). Briefly, after trimming fat and connective tissues, tissues were minced with scissors in an ice-cold Ca\(^{2+}\)-free incubation solution containing 130 mM NaCl, 4.5 mM KCl, 1 mM NaH\(_2\)PO\(_4\)·2H\(_2\)O, 1 mM MgCl\(_2\), 10 mM D-glucose, and 10 mM HEPES at pH7.4. The minced tissue was then incubated for 60 min at 37°C in a Ca\(^{2+}\)-free incubation solution containing 2 mg/mL trypsin inhibitor (Sigma, St. Louis, MO, USA), 0.04 mg/mL collagenase P (Worthington, Lakewood, UK), and
1% BSA. During the incubation period, the tissue was mechanically dissociated by repeated pipetting with different sizes of 1 mL pipet tips at 20 min intervals. The isolated acinar cells and cell clusters were then filtered through a 200 µm nylon mesh to remove large debris and harvested by centrifugation.

**Cell culture**

HSG cells, which were isolated from human submandibular gland intercalated duct cells (Shirasuna et al., 1981), were cultured in Dulbecco’s modified Eagle’s medium (Welgene, Republic of Korea) supplemented with 10% fetal bovine serum (Welgene) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA) at 37°C in a 5% CO₂ atmosphere.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Total RNA was extracted from hSMG acinar cells obtained from the hSMG acinar cell preparation, whole hSMG, and HSG cells using Trizol (Invitrogen, Carlsbad, CA, USA). Reverse transcription reactions were performed using 1 µg total RNA to generate cDNA (Invitrogen). PCR was performed with 1 µL of cDNA and specific primers (Table 1). The cycling parameters were as follows: 32 cycles of denaturation at 95°C for 30s, annealing for 30s, and extension at 72°C for 30s, followed by a final extension at 72°C for 10 min. Products from RT-PCR reactions were sequenced to confirm their identity.
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers</th>
<th>Length</th>
<th>GenBank no.</th>
</tr>
</thead>
</table>
| NBCe1-A     | F: ACCTTGGGGAGAGAGGAAGA  
R: TCCTTCCACTCCATCTCCTG | 214 bp | NM_003759.3 |
| NBCe1-B/C   | F: TGGAGGATGAAGCTGTCCTG  
R: TGCAGCAGGAGAGATGAGAG | 266 bp | NM_001134742.1 / NM_001098484.2 |
| NBCe1-A/B/C | F: AGCATGACCTCAGCTTCCTG  
R: CAGCATGATGTTGCGGCTTC | 253 bp | NM_003759.3 / NM_001098484.2 |
|             |         | 156 bp | NM_001134742.1 |
| NBCn1       | F: CCCAGTCTGCTCTGGAAAC  
R: ACCCTGTAAGGAGACACAGCA | 234 bp | NM_003615.4 |
| AQP5        | F: TCCATTGGCCTGTCTTCAC  
R: CACTCAGGCTCAGGAGTTG | 211 bp | NM_001651.3 |
| GAPDH       | F: CATCACTGCCACCCAGAAGA  
R: GTCAAAGGGTGAGGAGTGGG | 349 bp | NM_001289745.1 |

**Table 1.** List of DNA primers sequences designed for RT-PCR
**pH<sub>i</sub> measurements**

Intracellular pH (pH<sub>i</sub>) measurements were performed as described previously (Bae et al., 2013). HSG cells were loaded with 2 µM BCECF-AM (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C in DMEM medium (10% FBS and 1% penicillin/streptomycin) and washed twice with PBS. Isolated hSMG acinar cells were incubated with 2 µM BCECF-AM for 30 min at room temperature in normal HEPES solution with 0.2% BSA and washed twice with normal HEPES. The standard HCO<sub>3</sub>−-buffered solution consisted of 10 mM D-glucose, 10 mM HEPES, 115 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 25 mM NaHCO<sub>3</sub>, and equilibrated with 95% O<sub>2</sub> + 5% CO<sub>2</sub> gas. For intracellular acidification, cells were perfused with 20 mM NH<sub>4</sub>Cl in a Na<sup>+</sup>-free bath solution. In Na<sup>+</sup>-free HCO<sub>3</sub>−-buffered solutions, NaCl, and/or NaHCO<sub>3</sub> were replaced with equimolar concentrations of N-methyl-D-glucamine Cl and choline-HCO<sub>3</sub>−, respectively. All solutions were adjusted to pH 7.4 at 37°C. pH<sub>i</sub> recordings were obtained using a MetaFlour imaging system and BCECF-AM was excited at 440 and 490 nm with an emission wavelength of 530 nm. The resulting excitation/emission ratios were converted into pH values using a nigericin-based calibration technique. The figure 1 shows calibration curves. Specifically, cells were perfused with calibration solutions containing 10 mM NaCl, 130 mM KCl, 0.8 mM MgCl<sub>2</sub>, 20 mM HEPES, and 0.005 mM nigericin corresponding to pH 6.2, 6.6, 7.0, 7.4, and 7.8.
Figure 1. Intracellular pH calibration from BCECF-loaded HSG cells.

(A) Fluorescence ratio (490/440 nm) changes during exposure to nigericin-containing solutions at pH 6.2, 6.6, 7.0, 7.4, and 7.8. (B) Dependence of fluorescence ratio on intracellular pH (n = 16).
Immunofluorescence

hSMG tissues were cut into small pieces, fixed with 4% paraformaldehyde for at least 48 hours at 4°C, and embedded in paraffin. After microdissecting the block into 10 µm thick sections, the tissues were deparaffinized with Histo-Clear II and rehydrated with a graded series of ethanol (100%, 90%, 80%, and 70%). Sections were incubated in pepsin antigen retrieval solution for 15 minutes at 37°C and permeabilized in PBS containing 0.1% Triton X-100. After permeabilization, the tissues were covered with a blocking solution consisting of PBS with 20% normal donkey serum for 1 h at room temperature. Next, the cells were stained with either rabbit anti-NBCe1 (ab78326, Abcam) or rabbit anti-NBCn1 (ab82335, Abcam) followed by Alexa Flour 468 donkey anti-rabbit IgG (Invitrogen) at a dilution of 1:400 to detect NBCe1 and NBCn1, respectively. Finally, tissues were mounted with Vectashield mounting medium (Vector Laboratories) and visualized using a laser scanning confocal microscope (LSM 700, Carl Zeiss).

HSG cells were grown on cover-glass bottom dishes, fixed in ice-cold methanol for 15 minutes at -20°C, and then washed in ice-cold PBS three times for 5 minutes. The samples were then incubated in PBS containing 1% Triton X-100 for 10 minutes at room temperature, followed by washing in PBS three times and re-incubating in blocking solution consisting of PBS with 10% normal donkey serum for 1 hour at room temperature. The primary and secondary antibodies as well as methods for detection and visualization were the same as those described for hSMG tissues.
Plasmid Construction, Transfection, and Co-immunoprecipitation

NBCe1-B was cloned in pFlag-CMV-2 vector for expression in HSG cells. The NBCn1 construct in pcDNA3.1 was a gift from Dr. Jeong Hee Hong and Dr. Shmuel Muallem. pFlag-CMV-2-NBCe1-B, pcDNA3.1-NBCn1, and empty vectors were transfected into HSG cells using Lipofectamine 2000 (Invitrogen). Co-immunoprecipitation experiments were performed as described previously (Bae et al., 2013).

Reagents

5-(N-ethyl-N-isopropyl) amiloride (EIPA) and 4,4-diisothiocyanostilbene-2,2-disulfonic acid (DIDS) were obtained from Sigma-Aldrich.

Statistical analysis

Data are presented as the mean ± SEM and n is the number of experimental repeats with different hSMG sample preparations and HSG cultures. Differences between means were evaluated by ANOVA followed by post-hoc test for determining statistical analysis where appropriate. Statistical significance of experiments with multiple comparisons was assessed by analysis of variance; $p < 0.05$ was considered significant. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. 
Results

Expression of NBCe1 and NBCn1 in human submandibular glands (hSMGs) and an HSG cell line

I used RT-PCR to investigate the expression of various isotypes of sodium bicarbonate cotransporters (NBCs) in human submandibular glands (hSMGs) and HSG cells originating from hSMG ducts. Three types of primers were designed for NBCe1, one to detect NBCe1-A only at 214 bp, another to detect both NBCe1-B and -C at 266 bp, and the other to detect each of the NBCe1 isoforms (NBCe1-A/B/C, Table 1) at 253 bp and 156 bp corresponding to NBCe1-A/B and NBCe1-C, respectively. The NBCe1 mRNA transcripts, except NBCe1-A, were expressed in whole hSMGs and HSG cell lines, but appeared as a faint band in hSMG acini (Fig. 2A). With respect to the NBCe1-A/B/C primer, only the 253 bp product was detected, indicating that only NBCe1-B, but not NBCe1-C, was expressed at the mRNA level in hSMG duct cells and HSG cells. NBCe1-B/C mRNA transcripts were also detected, indicating that NBCe1-B is expressed at the mRNA level in both hSMG ducts and HSG cells. These findings were consistent with the results of previous study (Bae et al., 2013). On the other hand, NBCn1 mRNA transcripts were detected in hSMG acinar cells, whole hSMGs, and the HSG cell line. In these assays, AQP5 was used as an acinar cell marker (Gresz et al., 2001).

To confirm the protein expression and localization of NBCe1-B and NBCn1 in hSMGs and HSG cells, immunostaining was performed with NBCe1 and NBCn1 antibodies. NBCe1 was strongly expressed on the apical side of all hSMG duct
cells (Fig. 2B), whereas NBCn1 was expressed on the basal side of acinar cells (white arrows, Fig. 2C) and on the lateral and possibly basal membrane of duct cells (yellow arrow, Fig. 2C). NBCe1 and NBCn1 were diffusely located in both the cytosol and membrane in HSG cells (Figs. 2D and 2E). Taken together, these data demonstrated that NBCe1-B is expressed in hSMG ducts and HSG cells, whereas NBCn1 is expressed in the acinar and duct cells of hSMGs and HSG cells.
### Table A

<table>
<thead>
<tr>
<th>Gene</th>
<th>hSMG (bp)</th>
<th>Acini (bp)</th>
<th>Whole hSMG (bp)</th>
<th>HSG (bp)</th>
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</thead>
<tbody>
<tr>
<td>NBCe1-A</td>
<td>214</td>
<td></td>
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<tr>
<td>NBCe1-B/C</td>
<td>266</td>
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<tr>
<td>NBCe1-A/B/C</td>
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<td>156</td>
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<tr>
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<tr>
<td>GAPDH</td>
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</tbody>
</table>

### Images

**B**
![Image B](image1.png)

**C**
![Image C](image2.png)

**D**
![Image D](image3.png)

**E**
![Image E](image4.png)
Figure 2. NBCe1 and NBCn1 are expressed in human submandibular gland (hSMG) and HSG cells.

(A) NBCe1 and NBCn1 mRNA transcripts in hSMG and HSG cells. Aquaporin 5 (AQP5) was used as a marker for acinar cells. (B and C) hSMG tissue sections were stained with NBCe1, NBCn1, and AQP5 antibodies. (Bar = 50 µm). AQP5 was used as a marker for acinar cells. White and yellow arrows indicate acinar cells and duct cells, respectively. NBCe1-B is expressed in human submandibular gland (hSMG) duct cells, whereas NBCn1 is expressed in acinar (white arrow) and duct cells (yellow arrow). (D and E) HSG cells were stained with antibodies for NBCe1 and NBCn1. (Bar = 20 µm).
Intracellular pH (pH\textsubscript{i}) regulation and the effect of Src tyrosine kinase in hSMG acini and HSG cells

To evaluate the activity of NBCs on pH\textsubscript{i} recovery, Na\textsuperscript{+}-H\textsuperscript{+} exchanger (NHE) activity should be blocked, as NHEs also regulate intracellular pH. I first examined NHE activity on Na\textsuperscript{+}-dependent pH\textsubscript{i} recovery from cell acidification induced by an NH\textsubscript{4}\textsuperscript{+} pulse in hSMG acinar cells in a HEPES buffered solution (Fig. 3A). The pH\textsubscript{i} recovery rate mediated by NHE was inhibited by 5-(N-ethyl-N-isopropyl) amiloride (EIPA) in a concentration dependent manner. In addition, the pH\textsubscript{i} recovery rate of 0.228 ± 0.010 pH units/min (n = 6) in resting states was completely inhibited by 25 µM EIPA, a specific NHE inhibitor (n = 6, Fig. 3B).

I measured pH\textsubscript{i} in an HCO\textsubscript{3}\textsuperscript{-}-buffered solution (BBS) to investigate NBCs activity. The pH\textsubscript{i} of the unstimulated cell was 7.36 ± 0.05 (n = 16) in BBS. When the cell was exposed to NH\textsubscript{4}Cl, the pH\textsubscript{i} was increased to 9.16 ± 0.13 (n = 16), and then the cell was acidified to pH 6.31 ± 0.04 (n = 16). As shown in Fig. 3C, pre-incubation of cells for 20 min with 5 µM PP2, a Src tyrosine kinase inhibitor, had little effect on pH\textsubscript{i} recovery in hSMG acinar cells in BBS. Further, the pH\textsubscript{i} recovery rate (grey lines, 1.263 ± 0.142 pH units/min, n = 12) was not significantly different from that of control cells (black lines, 1.277 ± 0.166 pH units/min, n = 12). Continued recovery of pH\textsubscript{i} was observed in the presence of 25 µM EIPA (0.207 ± 0.018 pH units/min, n = 9, Fig. 3D), indicating that remnant pH\textsubscript{i} recovery was mediated by NBCn1. In addition, the pH\textsubscript{i} recovery rate of PP2 pretreated hSMG acinar cells (grey lines, 0.228 ± 0.018 pH units/min, n = 8) was not significantly different from control cells. The pH\textsubscript{i} recovery mediated by NBCn1 was further inhibited by 25 µM EIPA and 4,4-diisothiocyanostilbene-2,2-disulfonic acid (DIDS) (0.087 ±
0.011 pH units/min, n = 8, Fig. 3E), and was completely blocked in the presence of high concentrations of EIPA and DIDS (n = 8, Fig. 3F). Together, these data suggested that NBCn1 regulates intracellular pH in hSMG acinar cells, but is not affected by Src tyrosine kinase. Fig. 3G summarizes the results of these experiments.
Figure 3. Src tyrosine kinase does not affect pH$_i$ recovery of hSMG acinar cells.

(A and B) The intracellular pH recovery patterns of hSMG acinar cells in the absence or presence of several concentrations of EIPA in HEPES-buffered solution (HBS) were measured and the pH$_i$ recovery rates were summarized. (C-F) The pH$_i$ recovery patterns of hSMG acinar cells following an NH$_4^+$-pulse (blank bar) were recorded in a bicarbonate-buffered bath solution (BBS). The cells were pretreated for 20 min with 5 µM PP2, a Src tyrosine kinase inhibitor (grey trace) or incubated in normal BBS (black trace). The effects of treatment with EIPA and DIDS are shown using horizontal bars. (G) Summary of pH$_i$ recovery rates. The data are presented as the mean ± SEM.
In addition to hSMG acinar cells, I also studied the activities of NBCs in an HSG cell line. Specifically, I first confirmed the effective concentration of EIPA needed to block NHE activity in HSG cells by measuring its effect on the rate of pH<sub>i</sub> recovery in a HEPES buffered solution (Fig. 4A). I found that the pH<sub>i</sub> recovery rate of 0.042 ± 0.004 pH units/min (n = 5) was completely inhibited by 25 µM EIPA (n = 6, Fig. 4B).

I next measured pH<sub>i</sub> in BBS to examine the effect of PP2 on NBCe1-B and NBCn1 activities in HSG cells using the same technique as for hSMG acinar cells. The pH<sub>i</sub> recovery rate in HSG cells (0.377 ± 0.043 pH units/min, n = 9, Fig. 4C, black line) was less than that of hSMG acinar cells (Fig. 3C), and was decreased by ~50% upon pre-incubation with 5 µM PP2  (0.183 ± 0.016 pH units/min, n = 9, Fig. 4C, grey line). When the cells were exposed to 25 µM EIPA to inhibit the NHE activity, the pH<sub>i</sub> recovery rate of PP2 pretreated HSG cells (0.069 ± 0.004 pH units/min, n = 10, Fig. 4D, grey line) was significantly different from control treated cells (0.136 ± 0.006 pH units/min, n = 9, Fig. 4D, black line). In addition, the pH<sub>i</sub> recovery by NBCe1-B and NBCn1 was inhibited by 25 µM EIPA supplemented with DIDS (0.037 ± 0.008 pH units/min, n = 7, Fig. 4E) and was completely blocked by a combination of DIDS and 200 µM EIPA (n = 5, Fig. 4F). The pH<sub>i</sub> recovery rates of HSG cells are summarized in Fig. 4G.
Figure 4. pH$_i$ recovery of HSG cells is inhibited by PP2.

(A and B) The pH$_i$ of HSG cells in HBS was obtained in the absence or presence of 5, 10, or 25 µM EIPA and the results were summarized (C-F) pH$_i$ measurements were performed using HSG cells in BBS, and the effects of pre-treatment with 5 µM PP2 for 20 min were evaluated (grey trace). (G) Summary of pH$_i$ recovery rates in HSG cells. The data are presented as the mean ± SEM (error bars) (*, P < 0.05; ***, P < 0.001).
Overexpression of NBCe1-B and NBCn1 in HSG cells

To establish definitively whether Src kinase modulate the activity of NBCe1-B or NBCn1, HSG cells overexpressing either NBCe1-B or NBCn1 were generated and the subsequent effect on intracellular pH was measured. Overexpression of NBCe1-B and NBCn1 was confirmed via immunocytochemistry (Figs. 5A and 5B). Overexpression of NBCe1-B increased the pHᵢ recovery rate (1.419 ± 0.235 pH units/min, n = 12, Fig. 5C, black trace), but this was suppressed by PP2 (0.474 ± 0.048 pH units/min, n = 12, red trace). Moreover, addition of 25 µM EIPA to NBCe1-B overexpressing cells shifted the pHᵢ recovery rate to 0.690 ± 0.060 pH units/min (n = 11), which was decreased to 0.159 ± 0.021 pH units/min (n = 11) upon pre-treatment with PP2, suggesting that NBCe1-B is affected by PP2.

Overexpression of NBCn1 in HSG cells also increased the pHᵢ recovery rate (0.493 ± 0.067 pH units/min, n = 10, Fig. 5D, black trace) compared with control cells (0.377 ± 0.043 pH units/min, dotted trace), indicating that NBCn1 regulates pHᵢ similar to NBCe1-B. On the other hand, the pHᵢ recovery rate induced by NBCn1 overexpression was not significantly decreased by PP2 (0.381 ± 0.073 pH units/min, n = 8, red trace). Likewise, upon incubation with 25 µM EIPA, a pHᵢ recovery rate of 0.218 ± 0.012 pH units/min (n = 3) was noted, which decreased to 0.162 ± 0.027 pH units/min (n = 4) in the presence of PP2; however, this difference was not significant, suggesting that NBCn1 is not regulated by Src kinase. The pHᵢ recovery rates of HSG cells overexpressing NBCe1-B and NBCn1 are summarized in Figs. 5E and 5F. The above results were consistent with the shown in Figs. 3 and 4, indicating that Src kinase regulates the activity of NBCe1-B only, and not that of NBCn1.
**C** (NBCe1-B overexpressed HSG)

NH$_4^+$ - Na$^+$

**D** (NBCn1 overexpressed HSG)

**E** NBCe1-B overexpressed HSG pH$_i$ recovery rate (unit/min)

**F** NBCn1 overexpressed HSG pH$_i$ recovery rate (unit/min)
Figure 5. Transfected NBCe1-B is affected by PP2.

(A and B) Flag-NBCe1-B and NBCn1 were transfected into HSG cells and overexpression was confirmed by immunofluorescence assay (Bar = 20 µm). (C and D) pH, recovery rates were recorded in HSG cells overexpressing NBCe1-B or NBCn1. Horizontal bars indicate all applications. (E and F) Graphical summary of pH, recovery rates. The data are presented as the mean ± SEM (error bars) (*, P < 0.05; ***, P < 0.001).
NBCe1-B tyrosine residue phosphorylation and NBCe1-B translocation by Src kinase

I next hypothesized that Src tyrosine kinase may affect NBC activity by phosphorylating tyrosine residues on NBCe1-B or NBCn1. To investigate this possibility, I performed co-immunoprecipitation assays using HSG cells. The degree of NBCe1-B tyrosine phosphorylation was significantly increased by NH$_4$Cl, but was suppressed by PP2 (n = 4, Figs. 6A and 6C). Contrary to NBCe1-B, tyrosine on NBCn1 was not affected either by NH$_4$Cl or PP2, indicating that Src tyrosine kinase has no effect on NBCn1 phosphorylation (n = 4, Figs. 6B and 6D). Carbachol (CCh) was used as a positive control to stimulate NBCs. Immunocytochemistry analysis also showed that the NBCe1-B, which was located at the plasma membrane by NH$_4$Cl, remained in the cytosol when Src tyrosine kinase was inhibited by PP2 pre-treatment (Fig. 6E). On the other hand, NBCn1 was constitutively expressed on the plasma membrane side. Consistent with my result thus far, these findings suggested that Src tyrosine kinase influences only the activity of NBCe1-B, and not that of NBCn1.
A
IP: NB Ce1
IB: p-Tyr
Input: NB Ce1
PP2  
NH\textsubscript{4}Cl  
CCh
-  -  +  -  
-  +  +  -  
-  -  -  +  

B
IP: NBCn1
IB: p-Tyr
Input: NBCn1
PP2  
NH\textsubscript{4}Cl  
CCh
-  -  +  -  
-  +  +  -  
-  -  -  +  

C
Tyrosine-phosphorylated NB Ce1, % of control
PP2  
NH\textsubscript{4}Cl  
CCh
-  -  +  -  
-  +  +  -  
-  -  -  +  

D
Tyrosine-phosphorylated NBCn1, % of control
PP2  
NH\textsubscript{4}Cl  
CCh
-  -  +  -  
-  +  +  -  
-  -  -  +  

E
Control  NH\textsubscript{4}Cl  PP2 / NH\textsubscript{4}Cl

NB Ce1 / DAPI

NBCn1 / DAPI
Figure 6. PP2 inhibits tyrosine phosphorylation and translocation of NBCe1-B, but NBCn1, in HSG cells.

(A and B) Cell lysates were subjected to immunoprecipitation with NBCe1 and NBCn1 antibodies and evaluated by Western blotting with a phosphotyrosine antibody. The cells were pre-incubated in 20 mM of NH₄Cl for 2 mins, in 5 µM of PP2 for 20 mins, and in 50 µM of CCh for 5 mins. The input control comprised 5% of the lysates. (C and D) Phosphorylated NBCe1 and NBCn1 were quantified based on protein band intensities. The data are shown as the mean ± SEM (error bars) (n = 4; *, P < 0.05). (E) Locations of NBCe1 and NBCn1 in response of ammonium pulse in the presence or absence of PP2 were confirmed using immunocytochemistry (Bar = 20 µm).
Discussion

In the present study, I identified the expression and cellular localization of specific isoforms of NBCe1 and NBCn1 in human submandibular glands (hSMG) by RT-PCR and immunohistochemistry. Intracellular pH measurement and co-immunoprecipitation studies confirmed the activities of NBCs, especially NBCe1-B and NBCn1 in hSMG acinar and HSG cells. In addition, my data demonstrated that only NBCe1-B activity is regulated by Src tyrosine kinase.

I first asked which NBC isoforms are expressed in hSMGs. NBC expression was previously demonstrated in an HSG cell line originating from hSMG ducts. I confirmed that all of the NBC isoforms in hSMG ducts were expressed in an identical pattern in HSG cells. Specifically, RT-PCR and immunofluorescence studies were used to determine the isoforms of NBCe1 or NBCn1 expressed in hSMGs and HSG cells and their cellular localization. The immunohistochemical studies demonstrated that NBCn1 was expressed at the basolateral membrane of acinar cells in hSMGs (Fig. 2 and 7). Consistent with this data, NBCn1 is also expressed on the basolateral side of the ParC5 rat parotid acinar cell line (Perry et al., 2008; Perry et al., 2009), suggesting that NBCn1 may be involved in HCO$_3^-$ influx and pH$_i$ regulation in acinar cells as well as in other secretory epithelial cells (Kwon et al., 2003; Praetorius et al., 2004; Pushkin et al., 1999), including the murine duodenum (Chen et al., 2012). In this experiments, NBCn1 appeared to function as a major pH$_i$ regulator in hSMG acinar cells. Specifically, decreased pH$_i$ in hSMG acinar cells evoked by an ammonium pulse recovered rapidly to
prestimulus levels, which was inhibited by high concentrations of EIPA, an inhibitor of NBCn1 (Luo et al., 2001; Pushkin et al., 1999) (Fig. 3). On the other hand, NBCn1, referred to as NBC3 (GeneBank accession no. 047033) in Ref. 28, was expressed on the luminal side of hSMG duct cells, which was consistent with previous work on human salivary glands (Gresz et al., 2002; Roussa, 2001). Indeed, apical NBCn1 likely functions as an intracellular pH regulator and HCO$_3^-$ salvage mechanism to maintain acidic saliva in human salivary glands in a resting state (Luo et al., 2001; Park et al., 2002b).
Figure 7. Role and location of NBCe1-B and NBCn1 in human submandibular glands.

The diagram indicates a generic acinus (orange) and duct (yellow) for the salivary gland. In the acinar cell, NBCn1 activity regulates intracellular pH and could support secretion of transepithelial fluid and ions by submandibular gland acinar cells. NBCn1 in the acinar cells aids formation of a HCO$_3^-$-rich saliva. In duct cells, NBCe1-B and NBCn1 support intracellular pH regulation and HCO$_3^-$ salvage mechanisms by absorbing HCO$_3^-$ to maintain acidic saliva in human submandibular gland duct cells.
In rat parotid glands, NBCe1-B is expressed at the basolateral membrane of acinar cells (Roussa et al., 1999), and is also located at the basolateral side of human parotid acinar cells as an acid extruder for intracellular pH regulation and HCO₃⁻ ion absorption from the basolateral side (Park et al., 2002a). However, unlike cells of the parotid gland, NBCe1-B is expressed on the apical membrane of hSMG duct cells as shown in Fig. 2B, suggesting that NBCe1-B may also function as an HCO₃⁻ salvage mechanism in resting state cells, similar to NBCn1.

Resting saliva, in which submandibular and sublingual glands play a dominant role, contain low HCO₃⁻ concentrations (Dawes and Dong, 1995; Roussa, 2011). NBCe1 was expressed on the apical membrane of hSMG ducts (Fig. 2B), and appeared to play a salvage role by absorbing HCO₃⁻. On the other hand, stimulated saliva in which the parotid glands play a dominant role contains high concentrations of HCO₃⁻. Indeed, in the human parotid glands, NBCe1 is also expressed at the basolateral membrane in acinar cells (Park et al., 2002a), which may enable parotid glands to accumulate more HCO₃⁻.

Although the specific mechanisms of NBCs activities were not fully identified in this study, several studies have investigated the signaling molecules that regulate the activities of NBCs such as Ca²⁺ (Muller-Berger et al., 2001), cAMP (Bachmann et al., 2003), PKA (Gross et al., 2003), and PKC (Perry et al., 2007). In addition, a few other studies have looked into whether CO₂-induced renal NBC activity is modulated by Src kinase (Ruiz et al., 1998; Ruiz et al., 1999). The activity of Src kinase is increased by CO₂, low intracellular pH, and metabolic acidosis (Bernardo et al., 2003; Yamaji et al., 1997); however, there is currently no evidence regarding which specific NBC isoform is regulated by Src kinase and whether Src kinase...
directly phosphorylates tyrosine residues on NBCs or if it is a part of an upstream signaling cascade. Thus, in this study, I examined whether PP2, a Src kinase inhibitor, can inhibit pH$_i$ recovery mediated by two different types of NBCs. My data demonstrated that the activity of NBCe1-B is regulated by Src kinase in hSMG acinar and HSG cells, while that of NBCn1 is not. pH$_i$ recovery was not affected by PP2 in hSMG acinar cells in which only NBCn1 was expressed (Fig. 3C). On the other hand, when HSG cells containing both NBCe1-B and NBCn1 were exposed to PP2, the pH$_i$ recovery rate was significantly decreased (Fig. 4C), indicating that NBCe1-B might be modulated by Src kinase. In the presence of 25 µM EIPA, the pH$_i$ recovery rate was affected by PP2 pre-treatment when both NBCe1 and NBCn1 were functionally expressed (Fig. 4D). As shown in Fig. 3, expression of NBCe1-B differs between hSMG acinar cells and HSG cells, in that only hSMG acinar cells express NBCn1, which is not affected by PP2. Indeed, because the HSG cell line only expresses NBCe1-B, I was able to confirm that only NBCe1-B is regulated by Src tyrosine kinase, while NBCn1 is not. The data in Figs. 4C and 4D demonstrate a significant regulation of EIPA-sensitive pH$_i$ recovery, which is regulated by NHE in HSG cells, suggesting this process is influenced by Src tyrosine kinase. The result was further confirmed by the overexpression of either NBCn1 or NBCe1-B in HSG cells. Specifically, the pH$_i$ recovery rate in cells overexpressing NBCn1 was not changed by PP2 (Fig. 5D), while overexpression of NBCe1-B led to a decreased pH$_i$ recovery rate following pre-treatment with PP2 (Fig. 5C). Taken together, these data indicate that only NBCe1-B is modulated by Src tyrosine kinase (Fig. 5C).
In the present study, I noted that phosphorylation of NBCe1 tyrosine residues by an NH₄Cl pulse was suppressed by PP2 using co-immunoprecipitation experiments (Fig. 6A), and that only phosphorylated NBCe1 was able to translocate from the cytoplasm to the plasma membrane (Fig. 6E). However, NH₄Cl did not affect the phosphorylation status of NBCn1 tyrosine residues, nor was the phospho-tyrosine of NBCn1 dephosphorylated by PP2 (Fig. 6B). Carbachol (CCh) was used as a positive control in the experiments shown in Fig. 6, since CCh stimulates NBCs via ERKs and a PKC-dependent pathway, which is independent of Src (Lin et al., 2008). A schematic model summarizing the regulatory mechanisms of NBCe1-B and NBCn1 is presented in Fig. 8.
Figure 8. Schematic model of NBCe1-B and NBCn1 regulation by Src kinase.

NBCe1-B and NBCn1 mediate intracellular pH in an HSG cell line and human submandibular glands, especially on the apical side of duct cells. NBCe1-B is phosphorylated by Src kinase and translocates to the plasma membrane, whereas the NBCn1 is not regulated by Src kinase. The effect of Src kinase is inhibited by PP2. Arrows indicate activation and bars indicate inhibition.
It was investigated the relationship between NBC and Src kinase from several previous studies in renal cells (Ruiz et al., 1998; Ruiz et al., 1999). In these studies, the NBC was identified as NBCe1-A, which is known to be expressed renal cells. Moreover, these studies only showed differences in activities of NBC by Src kinase. In the present study, I not only characterized the effects of Src kinase on NBC activity, but also the phosphorylation status of tyrosine residues on activated NBCs due to Src kinase activity. In addition, I was concerned as to why NBCn1 did not associate with Src kinase, and also which molecule is responsible for NBCn1 activation. I hypothesized that another kinase may be responsible for NBCn1 activation, since it has an abundance of putative serine phosphorylation sites compared with tyrosine phosphorylation sites (analyzed by NetPhos 2.0, http://www.cbs.dtu.dk). Moreover, differences in membrane trafficking regulation of electrogenic type and electroneutral type NBC have been observed (Perry et al., 2008).

In conclusion, I confirmed that electrogenic NBCe1-B and electroneutral NBCn1 are expressed in hSMGs. I found that NBCe1 is localized to the apical membrane of duct cells while NBCn1 is expressed on the basolateral side of acinar cells and the apical side of duct cells. I also demonstrated that NBCe1-B is modulated and phosphorylated by Src kinase, whereas NBCn1 is not regulated by Src kinase. Taken together, these results suggest that \( \text{Na}^{+}\)-HCO\(_3\)^{-} cotransporters, especially NBCe1-B and NBCn1, play an important role in hSMG pH\(_i\) regulation and are regulated by different mechanisms. Future work will focus on the molecules that regulate NBCn1 activity and their respective mechanisms of action.
CHAPTER II

The effects of autoantibodies on the function of T cells in Sjögren’s syndrome patients
Introduction

Primary Sjögren’s syndrome (pSS) is a systemic autoimmune disease that impairs the structural integrity and function of the salivary and lacrimal glands (Chinthamani et al., 2012). Lymphocyte infiltration into exocrine glands, which is commonly observed in pSS patients, ultimately causes salivary and lacrimal gland dysfunctions (Emamian et al., 2009; Garcia-Carrasco et al., 2006). In addition, patients with pSS show various extraglandular organ manifestations such as vasculitis, renal malfunction, lymphoma, anemia, and leukopenia (Kassan and Moutsopoulos, 2004; Kovacs et al., 2005). Generally, serum from patients with pSS contains several autoantibodies, including anti-nuclear autoantibodies (e.g. anti-SS-A/Ro and anti-SS-B/La antibodies) (Nakken et al., 2001), carbonic anhydrase (Kino-Ohsaki et al., 1996), and muscarinic acetylcholine type 3 receptors (M3R) antibodies (Park et al., 2011; Park et al., 2013). A cohort study conducted by Kovacs et al. reported that 90% of all patients with pSS had anti-M3R autoantibodies. Moreover, patients with pSS who have serum anti-M3R autoantibodies showed a significantly higher frequency of leukopenia compared to patients without anti-M3R autoantibodies (Kovacs et al., 2005). Leukopenia is a condition characterized by low white blood cell count (less than 4000 cells/mm3) (Ing, 1984). This condition is frequently observed in systemic lupus erythematosus, which is an autoimmune disease, and usually occurs concurrently with lymphopenia and/or neutropenia (Carli et al., 2015). However, the pathophysiological relationship between Sjögren’s syndrome and leukopenia has not previously been studied.
Various autoantibodies existing in the serum of pSS patients are considered to play a key role in the development and progression of pSS. It was previously reported that pSS autoantibodies induce M3R and MHC I downregulation from the plasma membrane in primary human submandibular gland cells via clathrin-independent endocytosis, which may be associated with subsequent pathogenesis of salivary gland of pSS patients (Kim et al., 2015). M3R is a G protein-coupled receptor expressed in several organs such as exocrine glands and parasympathetic nerves. This receptor regulates the contraction and secretion of smooth muscle cells and exocrine glands. It is known that lymphocytes also express most of the cholinergic receptors as expressed in other tissues and that the immune function of T- and B-lymphocytes is also regulated via muscarinic acetylcholine receptors (Kawashima and Fujii, 2000). MHC I is ubiquitously distributed on the plasma membrane of all somatic cells and has several critical roles in the innate and adaptive immune response and regulation. Most importantly, MHC I serves as an inhibitory ligand of immunoglobulin-like receptors on NK cells, thus inhibiting NK cell activation, and thereby preventing self-killing of host cells. Therefore, cells with decreased plasma membrane expression of MHC I are at a greater risk of targeting and damage by NK cells. MHC I expression can be upregulated by interferon gamma (IFN-γ) or downregulated under several pathological conditions. Interestingly, although M3R and MHC I are unrelated, they were found to be constitutively cointernalized via a clathrin-independent pathway in HeLa cells (Scarselli and Donaldson, 2009).

Based on these studies, I hypothesized that the high leukopenia frequency observed in patients with pSS who have M3R autoantibodies might be connected to the ability of these autoantibodies to induce M3R and MHC I internalization in the
affected host cells. To test this hypothesis, here I investigated M3R and MHC I expression in human Jurkat T cells, which dominantly express M3R rather than the other muscarinic receptor subtypes (Alea et al., 2011), and their association with the pathogenesis of pSS-related leukopenia.
Materials and methods

Patients with pSS and human IgG preparation

pSS patients from the Rheumatology Clinic at Seoul National University Hospital were recruited for this study. The patients with pSS (9 women and 1 man) ranged in age from 37 to 71 years (mean 56.2 ± 3.4 years). As controls, 6 healthy patients (all female; population who are not specifically diagnosed as primary Sjögren’s syndrome) ranging in age from 40 to 59 years (mean 47.3 ± 3.5 years) were recruited. Written consents of agreement to participation were obtained from all patients. As described previous study, Immunoglobulin G (IgG) was purified from the serum samples provided by the pSS patients (Jin et al., 2012b).

Cell culture

Jurkat T cells were purchased from Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI-1649 medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Hyclone), 1% penicillin/streptomycin (Gibco, Carlsbad, CA), and 1 mM sodium pyruvate (Gibco) at 37 °C in a humidified atmosphere with 5% carbon dioxide. Jurkat cells were pre-incubated for 24 hours with control IgG (0.1 mg/mL), pSS IgG (0.1 mg/mL), carbachol (1 mM), or muscarinic acetylcholine type 3 receptors (M3R) antibody (abcam, ab154835) in serum-free RPMI-1649 at 37 °C. The cells were incubated for 1 hour with 20 µM of nigericin (InvivoGen, San Diega, CA) to induce cell death. NK-92 cells (a gift of Dr. Hun Sik Kim) were cultured in RPMI-1649 medium supplemented with 10% FBS, 1%
penicillin/streptomycin, 1mM sodium pyruvate, and 200U/mL recombinant human IL-2 (Peprotech).

**Immunofluorescence**

For dual immunostaining assays, mouse submandibular glands were fixed with 4% paraformaldehyde and embedded in paraffin as described previously (Namkoong et al., 2015). The paraffin block was cut into 10 µm thick sections by microdissection. The sections were deparaffinized and rehydrated, after which antigen retrieval with pepsin solution (0.5% in 5 mM HCl) was performed. After permeabilization and blocking, the sections were incubated with IgG antibodies from control healthy patients or patients with pSS or commercialized human IgG (0150-01, Southern Biotech, Birmingham, AL) for 1 hour at 37 °C. For dual staining, rabbit anti-M3R antibodies (Abcam, Cambridge, UK) with Alexa Fluor 568-conjugated donkey anti-rabbit antibodies (Invitrogen, Carlsbad, CA) and FITC-conjugated goat anti-human IgG antibodies (Sigma-Aldrich) were used.

For immunocytochemistry, Jurkat cells were incubated on poly-L-lysine-coated slides for 5 minutes, enabling the cells to adhere to the slides. The cells were then fixed in 2% paraformaldehyde for 10 minutes at room temperature, after which they were washed in PBS three times for 5 minutes. The samples were incubated in blocking solution consisting of PBS with 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) and 10% normal goat serum (Jackson ImmunoResearch) for 1 hour at room temperature. The cells were then stained with rabbit anti-M3R and mouse anti-MHC I antibodies (Abcam), followed by Alexa
Fluor 488-conjugated goat anti-rabbit antibodies and Alexa Fluor 594-conjugated donkey anti-mouse antibodies at a dilution of 1:400.

**Cell Counting Kit-8 (CCK-8) viability assay**

The Cell Counting Kit-8 assay (Dojindo Molecular Technologies, Rockville, MD) was used to determine cell viability. Briefly, cells were plated in 96-well plates (10^6 cells/well), after which 10 µL of CCK-8 solution was added to each well. The plates were incubated for 2 hours in an incubator, after which the absorbance of each well at 450 nm was measured using a multi-detection microplate reader (Synergy 2, BioTek, VT).

**Fluorescence-activated cell sorting (FACS)**

Jurkat cells were pretreated with control IgG, pSS IgG alone, pSS IgG with interferon gamma (IFN-γ; Sigma-Aldrich, I3265), or carbachol in serum-free RPMI-1649 for 24 hours. Pretreated cells were fixed at room temperature with 2% paraformaldehyde for 15 min. After washing with FACS buffer (ice-cold PBS with 10% FBS), the fixed cells were stained with anti-mAChR M3 antibodies (M3R; sc-7474, Santa Cruz Biotechnology, Santa Cruz, CA) or PE-conjugated mouse anti-human HLA-ABC antibodies (MHC I; BD Biosciences, Franklin Lakes, NJ) for 1 hour at 4 °C. For M3R detection, cells were washed and stained with Alexa Fluor 488-conjugated donkey anti-goat IgG antibody (Inivitrogen) for 1 hour. Finally, cells were washed and analyzed on a FACSCalibur flow cytometer (BD Biosciences).
For detection of NK cell-mediated lysis, Jurkat and NK-92 cells were co-cultured. Before co-culture with NK-92 cells, in the first, Jurkat target cells were labeled with 10 μM of 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE; Sigma-Aldrich, 21888) for 5 min, after which they were washed in PBS three times. Cells were adjusted to $1 \times 10^6$ cells, then incubated with pSS IgG or commercialized human IgG in serum-free RPMI media. After 6 hours, $2.5 \times 10^6$ cells of NK-92 were added into each pre-treated Jurkat cells. After 18 hours of incubation at 37 °C, for detection of NK cell-mediated lysis, cells were labeled with 7-Aminoactinomycin D (7-AAD; Sigma-Aldrich, A9400) for 5 min. After washing, cells were fixed at room temperature with 2% paraformaldehyde for 10 min. Finally, cells were washed and analyzed on a FACS Aria III (BD Biosciences).

**Co-immunoprecipitation**

Co-immunoprecipitation of MHC I and M3R was performed as previously described (Kim et al., 2015). Mouse anti-MHC I monoclonal antibodies (W6/32) (Santa Cruz Biotechnology) and rabbit anti-M3R antibodies (Abcam) were used at optimized dilutions.

**Statistical analysis**

Data are presented as means ± SEM (error bars) and n represents the number of independent experiments. Unpaired t-test followed by the post-hoc test was used to determine the statistical significance of differences between groups. P values < 0.05 were considered significant. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Results

Immunolabeling of anti-M3R autoantibodies in patients with pSS

As in previous study, a dual immunostaining method was used to determine whether anti-M3R autoantibodies, in purified whole immunoglobulin G (IgG) fractions from serum from primary Sjögren’s syndrome (pSS) patients (hereafter referred to as pSS IgG), bind directly to human M3R. Mouse submandibular gland tissue sections were incubated with commercialized human IgG antibodies, control IgG antibodies from healthy control patients, or pSS IgG antibodies for duration of 1 hour, after which they were stained with anti-M3R autoantibodies and human IgG antibodies. To omit any influence from nonspecific signals resulting from human IgG cross-reactivity with antigens present in the mouse gland, the whole IgG signal from commercialized human IgG was subtracted from each experimental signal (Fig. 9B). The observed co-localization of M3R (red) and whole IgG (green) demonstrated that anti-M3R autoantibodies were present in pSS IgG (Fig. 9D), whereas they were not present in control IgG (Fig. 9C). Using ZEN 2010 software, the colocalization coefficient of pSS IgG was determined to be 0.666 ± 0.047 (n = 10), which was significantly higher than the coefficient for control IgG (0.213 ± 0.049, n = 3) (Fig. 9E).
Co-localization coefficient

**Ctrl IgG**

**pSS IgG**

**Commercialized IgG**

**M3R / hIgG**

**Control IgG**

**pSS IgG**

![Image of immunohistochemistry results](A) (M3R / hIgG)

![Image of immunohistochemistry results](B) (Commercialized IgG)

![Image of immunohistochemistry results](C) (Control IgG)

![Image of immunohistochemistry results](D) (pSS IgG)

![Graph showing co-localization coefficient](E)

**E Co-localization coefficient**

***

Ctrl IgG

pSS IgG
Figure 9. Detection of anti-M3R autoantibodies in patients with pSS by dual immunolabeling of the mouse submandibular gland.

(A) Negative control. Tissues incubated with commercialized human immunoglobulin G (IgG; B), control (ctrl) IgG from healthy control patients (C, n = 3), and Sjögren’s syndrome (pSS) patients IgG (D, n = 10). M3R (red), IgG (green), and nuclei (blue) were stained. Scale bar = 20 µm. (E) Colocalization coefficients. Each experiment consisted of six biological replicates.
pSS IgG downregulates M3R and MHC I expression in Jurkat cells

It was previously demonstrated that the anti-M3R autoantibodies in pSS IgG are responsible for the internalization of M3R and MHC I from the plasma membrane to the cytosol in HSG cells (Kim et al., 2015). Thus, I confirmed the interaction between M3R and MHC I in human T lymphocytes using Jurkat cells (an immortalized human T lymphocyte cell line) via co-immunoprecipitation. As shown in Figure 10A, M3R and MHC I interact in the resting state; the degree of interaction slightly increased with pSS IgG stimulation.

Next, I examined the effects of anti-M3R autoantibodies on M3R and MHC I expression in Jurkat cells via FACS and immunocytochemistry. Similar to previous results in HSG cells, M3R expression significantly decreased in Jurkat cells incubated with pSS IgG (Figs. 10B and 10C). MHC I surface expression also decreased significantly in incubation with pSS IgG, whereas incubation with control IgG did not affect MHC I expression (Figs. 10D and 10E). The degree of decrease in M3R and MHC I expression were very similar to each other. Next, the downregulation of M3R and MHC I was further confirmed via immunofluorescence analysis (Fig. 10F). Surface expression of M3R and MHC I was markedly downregulated in cells incubated with pSS IgG, but not in cells incubated with control IgG. M3R antibody was incubated with Jurkat cells to confirm whether the anti-M3R autoantibodies in pSS IgG directly affect the downregulation of M3R and MHC I. Zen 2010 software was next used to calculate the mean relative fluorescence intensities of specific M3R and MHC I surface immunostaining for each cell (Fig. 10G). The membrane to cytosol ratio of fluorescence intensity of M3R were $4.21 \pm 1.11$ (n = 10) and $6.13 \pm 1.22$ (n = 7) in
the control and control IgG treated groups, respectively. However, these values decreased significantly to $1.04 \pm 0.17$ (n = 8) and $1.00 \pm 0.10$ in pSS IgG and M3R antibody treated groups, respectively. The membrane expression of MHC I was also lower in the pSS IgG and M3R antibody groups [$1.25 \pm 0.20$ (n = 8) and $1.00 \pm 0.07$ (n = 9)] compared with the nontreated control group ($4.62 \pm 0.91$, n =10) and the control IgG group ($4.11 \pm 0.86$, n = 7).

Leukopenia has been more commonly observed in patients with pSS who have anti-M3R autoantibodies in comparison to their counterparts without these antibodies. To examine whether the anti-M3R autoantibodies in pSS IgG directly affect the survival of Jurkat cells, I treated Jurkat cells with pSS IgG or control IgG for 24 hours at 37 °C. After that, I used CCK-8 assay kit to determine cell viability. However, no significant differences were observed between control IgG-treated and pSS IgG-treated cells (Fig. 10H).
A

IP: MHCI  
IB: M3R

Input M3R

B

C

D

E

F

G

H

Cell survival (% of control)
Figure 10. Effects of pSS IgG on M3R and MHC I membrane expression in Jurkat cells and on Jurkat cell viability.

Jurkat cells were pre-incubated with 0.1 mg/ml of control IgG or pSS IgG for 24 hours at 37 °C. Carbachol (1 mM), which induces endocytosis of M3R, was used as a positive control. (A) Cell lysates were subjected to immunoprecipitation with anti-MHC I antibodies and evaluated by Western blotting with anti-M3R antibodies. The input comprised 5% of the lysates. (B-E) The membrane expression levels of M3R (B) and MHC I (D) were measured using FACS analysis. Normalized FACS intensities of M3R (C) and MHC I (E) are shown. (F and G) The surface expression levels of M3R and MHC I were confirmed by immunofluorescence (F); mean intensities are shown in G. Scale bar = 10 µm. (H) The viability of Jurkat cells after pSS IgG treatment was quantitated by the CCK-8 assay. The nigericin (20 µM) was used to induce cell death. Each experiment consisted of four biological replicates.
Effect of IFN-γ on pSS IgG-induced downregulation of M3R and MHC I

The downregulation of M3R by pSS IgG mirrored the decrease in MHC I expression after pSS IgG treatment (Fig. 10). Stimulation with IFN-γ is known to upregulate the level of MHC I in the plasma membrane. Moreover, global proteome analysis performed with Jurkat cells exposed to control IgG or pSS IgG has shown significant decrease of IFN-γ expression in the Jurkat cells exposed to pSS IgG. Thus, I examined whether pSS IgG-induced downregulation of MHC I is rescued by IFN-γ stimulation. Jurkat cells were incubated with control IgG, pSS IgG alone, or pSS IgG with IFN-γ. The decrease in M3R expression resulting from pSS IgG incubation was significantly rescued by IFN-γ stimulation (Fig. 11A and 11B). As expected, MHC I expression also increased with IFN-γ stimulation, even in the presence of pSS IgG (Fig. 11C and 11D). These data indicate that M3R interacts with MHC I and that IFN-γ rescues pSS IgG-induced downregulation of M3R and MHC I, leading to increased plasma membrane expression of MHC I.
Figure 11. Effect of IFN-γ on pSS IgG-induced downregulation of M3R and MHC I.

(A-D) FACS histograms showing membrane expression of M3R (A) and MHC I (C) in pretreated Jurkat cells are shown. Jurkat cells were preincubated with control IgG (0.1 mg/ml), pSS IgG (0.1 mg/ml), carbachol (1mM), or IFN-γ (10 ng/ml) for 24 hours at 37 °C. Normalized FACS intensities are also shown (B and D). Each experimental consisted of six biological replicates.
pSS IgG induces NK cell mediated Jurkat cell lysis

To test the effect of pSS IgG on NK cell-mediated lysis, NK-92 cells and Jurkat cells were co-cultured. Jurkat cells were stained by CFSE and incubated with control IgG or pSS IgG for 6 hours. After incubation, NK-92 cells were added into each pre-treated Jurkat cell culture for 18 hours to examined lysed Jurkat cells. CFSE-positive and 7-AAD co-positive cells indicate lysed Jurkat cells (Fig. 12A and 12B, red dots in P1 area of Q1+Q2). 7-AAD positive Jurkat cell population was significantly greater in the presence of pSS IgG (red trace in Fig. 12C and Fig. 12D) than in the presence of control IgG (black trace in Fig. 12C and 12D). Next, the relative fold changes of 7-AADD positive Jurkat cells in different groups were quantified by dividing each group’s % parent (P2 area in Fig 12C; set as a point where the % total 7-AAD positive Jurkat cells of untreated group is less than 2%) value over the % parent value of the untreated group. Jurkat cells treated either with control IgG or pSS IgG showed significantly increased cell death compared to the untreated group, which indicated that the Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) activity of NK cells was normal. However, an approximately 1.5 fold increase in % lysed Jurkat cells was observed in pSS IgG group compared to the control IgG group (n=6, p=0.0047) (Fig. 12D). Although I found this difference in pSS IgG group and control IgG group under identical conditions – NK cell number, Jurkat cell number, and culture condition – I wanted to make it clear whether the cell lysis caused by pSS IgG was due to M3R/MHC I co-internalization or increased ADCC activity. Thus I next examined the relative fold changes of 7-AAD positive NK cells (7-AAD+/CFSE- population) using the same analysis method used for Jurkat cells. Interestingly % 7-AAD positive NK cell
population was also significantly higher in the pSS IgG treated group than in the control IgG group (n=5, p=0.0061) (Fig. 12E), as observed in the Jurkat cell experiment. Therefore it is likely that this effect is not due to change in ADCC activity but to M3R/MHC I co-internalization.
Figure 12. Effects of pSS IgG on NK-cell mediated lysis.

(A-B) Representative FACS profiles showing number of CFSE positive Jurkat cells and 7-AAD positive Jurkat and NK-92 cells. The CSFE stained Jurkat cells were pre-treated with control IgG (A) or pSS IgG (B) for 6 hours, after which co-cultured with NK-92 cells for 18 hours. (C-D) FACS histogram showing the percentage of 7-AAD positive cells in CFSE positive Jurkat cells (C). Black and red traces indicate pre-treated Jurkat cells with control IgG and pSS IgG, respectively. 7-AAD positive Jurkat cell (D) and NK cell (E) fold changes were quantified. Each experiment consisted of six biological replicates.
Discussion

Autoantibodies against M3R in pSS have been reported as inhibitors of cholinergic function in several tissues which express M3R (Park et al., 2011; Waterman et al., 2000). These autoantibodies induce downregulation of membrane-localized M3R through both clathrin-dependent and independent endocytic pathways in salivary gland epithelial cells (Jin et al., 2012a; Kim et al., 2015). However, the effects of pSS autoantibodies on circulating peripheral leukocytes were not previously reported. Therefore this is the first report to provide evidence that pSS autoantibody-mediated co-internalization of M3R and MHC I in leukocytes could play a key role in the leukopenia observed in patients with pSS.

My data confirm that pSS IgG antibodies have a high affinity to membrane-bound M3R and that the binding of pSS IgG elicits co-internalization of M3R and MHC I in Jurkat cells (Fig. 10). Based on the co-immunoprecipitation and immunofluorescence data it is highly probable that M3R and MHC I are physically interacting, thus MHC I may be passively “dragged” into the cytosol by the M3R internalization process. However, future examination is necessary to know more details of M3R/MHC I interaction mechanisms or interacting sites. Another possible cause for the decrease in MHC I expression in Jurkat cells exposed to pSS patient serum is decreased expression of IFN-γ. I carried out global proteome analysis of Jurkat cells exposed to control and pSS patients’ serum and found that IFN-γ expression was significantly decreased in the Jurkat cells exposed to pSS patient’s serum (Data not shown). These data also showed that the internalization and decreased expression levels of M3R and MHC I in Jurkat cells were both
prevented by treatment with IFN-γ. IFN-γ is known to upregulate MHC I expression and membrane localization by inducing the expression of several genes related to antigen processing and membrane presentation via the JAK/STAT1 pathway (Zhou, 2009). Interestingly, my data indicate that IFN-γ upregulates both MHC I and M3R expression in pSS IgG-treated Jurkat cells (Fig. 12B and 12D). Mita et al. also reported that “IFN-γ increases the expression levels of muscarinic receptor subtypes that activate phosphatidylinositol turnover in EoL-1 cells”, and suggested that this process helped EoL-1 cells to differentiate (Mita et al., 1996).

Based on these data, I hypothesized that pSS autoantibody-mediated internalization of MHC I in leukocytes activates NK cells to attack “missing-self” leukocytes, thus leading to the observed leukopenia. MHC I is known to inhibit activation of NK cells - NK cells are generally maintained in a suppressed state by the continuous recognition of MHC I molecules expressed on the host cell surface, preventing them from attacking host tissues. However, when host cells are infected, damaged, or stressed with decreased MHC I expression, NK cells are released from their suppressed state to attack the “missing-self” host cells (Fogel et al., 2013). Previous studies have also reported the migration and accumulation of NK cells in host tissues exposed by autoimmune diseases such as RA (Ahern and Brennan, 2011), type 1 diabetes mellitus (Dotta et al., 2007), and juvenile dermatomyositis (Li et al., 2004).

Since there was no significant cell viability difference in the groups treated with pSS IgG and control IgG, I consider it unlikely that the internalization of MHC I or M3R itself can elicit Jurkat cell death (Fig. 10H). However, when I exposed pSS IgG-treated Jurkat cells to the NK cells, the ratio of lysed Jurkat cells was
significantly higher than the Jurkat cells treated with control IgG. This is critical
evidence that the T cells exposed to the pSS IgG become more vulnerable to the
NK cell-mediated cytotoxicity. Nevertheless, this result seems contradictory to the
previous clinical studies on pSS. NK cells collected from patients with pSS showed
significantly decreased activity compared to NK cells collected from control
patients (Pedersen and Oxholm, 1988). However, they also reported that there was
no impairment in effector/target cell conjugation of pSS patient-derived NK cells,
thus my hypothesis is still plausible since the pSS patient-derived T cells with
decreased surface MHC I expression are still recognizable to the NK cells, even if
they become less potent over the course of disease progression. In addition, I found
that NK cells exposed to the pSS IgG showed significantly increased cell death
compared to those exposed to control IgG. This may indicate that even NK cells
can lose MHC I and kill each other. Therefore it is highly possible that the effect of
pSS IgG is not only specific to T lymphocytes but also generally applied to various
kinds of leukocytes such as neutrophil, eosinophil, basophil, and monocyte.
Disruption of physiological functions of M3R on T lymphocytes is another
possible cause for pSS-induced leukopenia. T lymphocytes are known to express
type 1-5 muscarinic receptors and other cholinergic receptors (Sato et al., 1999).
Activation of M3 receptors on T lymphocytes via paracrine and autocrine
acetylcholine stimulation plays a key role in delivering calcium signals to the
nucleus and in increasing the expression of prosurvival genes such as c-fos (Fujii
and Kawashima, 2000). Thus, a long-term decrease in para/autocrine Ach
stimulation may slowly inhibit leukocyte proliferation and/or survival.
In conclusion, this study suggests that pSS autoantibody-induced co-internalization of M3R and MHC I in T lymphocytes followed by NK cell attack could explain the high prevalence of leukopenia in pSS patients. Further studies using primary human T lymphocytes or pSS animal disease model should be conducted to fully prove my hypothesis.
Conclusions

In chapter I, I identified the cellular location of specific isoforms of sodium bicarbonate cotransporters (NBCs) in human submandibular gland (hSMG). NBCe1-B is located on the apical membrane of hSMG duct cells, while NBCn1 is expressed at the basolateral membrane of hSMG acinar cells and luminal side of hSMG duct cells. Using intracellular pH measurement and co-immunoprecipitation studies, I also confirmed that NBCe1-B and NBCn1 have different activation mechanisms. Only NBCe1-B is phosphorylated and regulated by Src tyrosine kinase; NBCn1 is not. These results revealed a new mechanism for NBCe1-B, which could be used as molecular target to overcome decreased HCO₃⁻ secretion in Sjögren’s syndrome. Therapeutic artificial regulation of NBCs using gene delivery therapy would improve the oral health and quality of life of Sjögren’s syndrome patients. Further examination focusing on the molecules that regulate NBCn1 activity and their respective mechanisms of action may provide a clue to the reports linking hyposalivation with Sjögren’s syndrome.

In chapter II, my data confirm that the autoantibody against muscarinic 3 receptor (M3R) is present in immunoglobulin G (IgG) of primary Sjögren’s syndrome (pSS) patients. It was also confirmed that the binding of pSS IgG autoantibodies elicits co-internalization of M3R and MHC I in Jurkat cells. This internalization of M3R and MHC I does not affect cell viability directly, however; it activates NK cells to attack “missing-self” leukocytes, leading to the observed leukopenia. In conclusion, this study suggests that pSS autoantibody-induced co-internalization of M3R and MHC I in T lymphocytes followed by NK cell attack could explain the
high prevalence of leukopenia in pSS patients. Further studies with primary human T lymphocytes or pSS animal disease model should be conducted to fully prove my hypothesis.

These findings may indicate potential therapeutic targets for the treatment of Sjögren syndrome patients with hyposalivation and leukopenia.
References


국문초록

타액선 중탄산이온 수송체 및 자가면역항체에 관한 연구

남궁은

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쇼그렌 증후군 (Sjögren’s syndrome, SS)은 만성 자가면역질환의 일종으로 1차 증후군에서는 침샘과 눈물샘에 림프구가 침윤되면서 구강건조증 및 안구건조증을 일으킨다. 뿐만 아니라 이후 2차 증후군으로 진행되며, 관절염, 근육통, 폐 질환, 혈관염, 림프종, 백혈구 감소증 등 전신적인 증상이 나타난다. 또한 이 시기 구강에서는 타액 완충능 감소로 인한 다발성 치아우식증 등이 흔히 관찰된다. 소그렌 증후군에서 보이는 타액 완충능 감소는 부분적으로 구강 내 산도를 중화시키고 병원성 침식을 막아준다고 알려져 있는 타액 내 중탄산이온 ($HCO_3^-$)의 감소에 기인한다. 하지만 타액선에서의 이런 중탄산이온의 형성 및 분비 기전과 그로 인한 타액 세포 내 pH 조절 기전에 대해서는 많은 연구가 되어있지 않다. 한편, 소그렌 증후군 환자의 혈청 내에는 침 또는 눈물 분비에 있어서 주된 작용을 하는 수용체인 muscarinic acetylcholine type 3 receptor (M3R)에 대한 자가면역항체가 존재함이 최근 밝혀졌다. 이 M3R 자가면역
항체를 가지고 있는 쇼그렌 증후군 환자에게서 백혈구 감소증 발생 빈도가 높다는 보고가 있지만 쇼그렌 증후군 환자의 혈청 내에 존재하는 M3R 자가면역항체가 백혈구 감소증에서 어떠한 역할을 하는지는 잘 알려지지 않았다. 따라서 본 연구는 타액 완충능에 크게 기여하는 중탄산이온의 형성 및 분비와 관련된 세포막 수송체 sodium bicarbonate cotransporter (NBC)의 작용 기전 및 쇼그렌 증후군에서 보이는 자가면역항체의 병리학적 기전에 대해 연구하였다. 이 연구를 통해 사람의 악하선에서 NBC 아형(isoform)의 종류 또는 그 위치에 따른 발현 양상 및 세포 내 pH 조절 기전에 관한 기여도를 밝혀내었다. NBCe1-B 는 악하선 도관에 위치하고, NBCn1 은 악하선의 선포(acinar) 세포와 도관세포에 모두 위치하는 것을 확인하였다. 또한, tyrosine kinase inhibitor 에 의한 각 NBC의 활성 변화를 조사하고, NBC의 활성에 따른 tyrosine residue의 인산화 여부를 조사한 바, NBC가 활성화되었을 때, NBCn1의 tyrosine residue는 인산화되지 않았고, NBCe1-B의 tyrosine residue는 직접적으로 인산화되었다. 즉, NBCe1-B는 tyrosine이 인산화 될 때 그 활성이 조절되지만, NBCn1은 조절되지 않음을 밝혀내었다. NBCe1-B와 NBCn1은 모두 세포 내 pH 조절에 있어 중요한 역할을 하지만, 그 작용 기전은 다음을 확인함으로써 기존에 밝혀지지 않은 NBCe1-B의 작용 기전을 제시하였다. 다음으로 쇼그렌 증후군 환자와 건강인의 혈청에서 항체를 분리 분리 정제하여 쇼그렌 증후군 환자의 혈청에서 M3R 자가면역항체의 존재 유무를 확인하였다. 이후, 사람 T 세포주(jurkat cells)에 자가면역항체와 함께 전배양한 후, 이 자가면역항체가 T 세포의 M3R과 MHC class I에 미치는 영향 및 직접적으로 T 세포의 기능에 미치는 영향을 살펴보았다. SS 자가면역항체의 전 처리는 T 세포의 세포막에 존재하는 M3R과 MHC class I를 감소시켰으나 세포 수명에 직접적으로 영향을 미치지 않았다. 하지만 홍미롭게도 NK 세포와 T 세포를 함께 배양할 때 SS 항체를 처리 시, T 세포의 수명이 감소하였다. 이상의 연구결과는
쇼그렌 증후군 환자에게서 높은 빈도로 발생하는 백혈구 감소 기전을 부분적으로 설명해 줄 수 있을 것으로 사료된다.

주요어: NBC, 악하선, 세포 내 pH, 쇼그렌증후군, 자가면역항체, 무스카리닉 수용체, 백혈구 감소증

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