齒醫科學博士學位論文

Modulatory mechanisms on the inflammation and muscarinic receptor function in salivary gland epithelial cells

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2014 년 8 월

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Modulatory mechanisms on the inflammation and muscarinic receptor function in salivary gland epithelial cells

by

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August, 2014

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Abstract

Salivary gland epithelial cells (SGEC) release several cytokines that play important roles in the inflammatory process. Muscarinic receptors, particularly the type 3 subtype (M3R), play an important role in exocrine secretion. However, the regulatory mechanism of the inflammation and the functional expression of M3R in SGEC remains to be elucidated.

In chapter Ⅰ, I examined whether capsaicin can modulate the cytokine release in SGEC. These findings demonstrated that the increases in TNFα and IL-6 mRNA transcripts were highest at 3h and 1h after incubation with poly(I:C) and LPS, respectively. Pretreatment of the cells with 10 μM capsaicin, however, significantly inhibited mRNA transcripts and its protein levels. The simultaneous application of 10 μM capsazepine with capsaicin did not block the inhibitory effect of capsaicin. Furthermore, the inhibitory effect of capsaicin was also shown in primary cultured cells from TRPV1$^{-/-}$ mice. I found that both poly(I:C) and LPS induce IκB-α degradation and phosphorylation, which results in NF-κB activation and capsaicin inhibits this NF-κB pathway. These results demonstrate that SGEC release pro-inflammatory cytokines by TLR stimulation, and capsaicin inhibits this process by inhibiting the NF-κB pathway.

In chapter Ⅱ, I examined A253 cells derived from human salivary gland tumor tissue, in which muscarinic receptor function is suppressed. In this study, I examined whether M3R function is suppressed by epigenetic modulation of the receptor. I found that A253 cells expressed all subtypes of muscarinic receptors, except subtype 3, at the mRNA and protein level. However, treatment of cells with 5-aza-2'-deoxycytidine (5-Aza-CdR) restored the

functional expression of the M3R. Treatment of cells with 5-Aza-CdR completely restored the carbachol-induced calcium response, which was not observed in untreated A253 cells. Global methylation levels in A253 cells were also reduced by 5-Aza-CdR-treatment. I also examined whether 5-Aza-CdR treatment induced demethylation of the M3R CpG island, and found that one of the methylated CGs was demethylated by bisulfite sequencing. Thus, I conclude that suppression of M3R function in A253 cells results from hypermethylation of the CpG island; moreover, M3R function can be restored by DNA demethylation.

This study suggests that capsaicin could potentially alleviate the inflammation and 5-Aza-CdR could potentially be used to restore function to the M3R, which is suppressed in salivary gland epithelial cells.

Key words: salivary gland, capsaicin, TNFα, IL-6, capsazepine, NF-κB, epigenetic, muscarinic receptor, A253, hypermethylation, 5-Aza-CdR

Student number: 2010-30650

Contents

Chapter Ⅱ. Epigenetic modulation of the muscarinic type 3 receptor in salivary epithelial cells

List of Figures

Chapter Ⅰ

Chapter Ⅱ

Abbreviations

HSG	Human submandibular gland
TLRs	Toll-like receptors
Poly(I:C)	polyinosinic-polycytidylic acid
LPS	lipopolysaccharide
TNFα	Tumor necrosis factor alpha
IL-6	Interleukin 6
TRPV1	transient receptor potential vanilloid1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
M3R	Muscarinic acetylcholine receptor M_3
GPCR	G protein-coupled receptor
MSP	Methylation-specific PCR
CCh	Carbachol
ATP	Adenosine triphosphate

General Introduction

Pattern recognition receptors that detect specific molecular motifs are decisive factors for activation of immune system (Roach et al., 2005). Toll-like receptors (TLRs) are a type of pattern recognition receptors, and play a major role in activation of innate immune responses against microbial pathogen. So far, ten TLRs have been identified in human (Chuang & Ulevitch, 2001; Tabeta et al., 2004). They bind to distinct components of various pathogens such as bacteria, fungi or viruses known as pathogen-associated molecular patterns (PAMPs) (Xu et al., 2000). For instance, double-stranded RNA (dsRNA) which is generated during viral replication are recognized by the TLR3 that binds to poly(I:C) (Alexopoulou, Holt, Medzhitov, & Flavell, 2001). Gram-negative bacteria-derived LPS in the cell walls are also recognized by the TLR4 (Takeda, Kaisho, & Akira, 2003).

TLR signaling is an essential mechanism that causes initiation of inflammatory responses. After TLR recognizes microbial pathogens, they trigger intracellular signaling pathways through the induction of pro-inflammatory cytokines, chemokines, and type I interferon (IFN) (Han et al., 2003; Kawai et al., 2001). Moreover, signaling from TLRs allows upregulation of costimulatory molecules on specialized antigen-presenting cells. Furthermore, TLR triggering actively participates in the pathogenesis of autoimmune disorders. It promotes organ-specific autoimmune lesions such as Sjögren syndrome (SS), which is a chronic autoimmune inflammatory disease that primarily affects salivary glands, resulting in their functional impairment (Spachidou et al., 2007).

Deteriorated inflammation can promote cancer development and progression. For instance, persistent infection of *Helicobacter pylori* is closely related to gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma (Karin, 2006). In general, as a part of normal host defense system, the main function of inflammatory response triggered by infection is to prevent tumor development. However, tumorigenic pathogens debase host immunity and lead to persistent infections implicated in inflammation (Rakoff-Nahoum & Medzhitov, 2009).

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is known as a colorless, pungent, and irritant compound extracted from hot and red chili peppers. Capsaicin is used to help relieve neuropathic pain, itching, and exhibits anti-cancer or anti-inflammatory effects (Morre et al., 1996). Furthermore, it has been reported to selectively suppress the growth of a number of human tumor cells (Morre, Chueh, & Morre, 1995). Its immunosuppressive effects have been linked to its ability to suppress NF-κB activation. Nuclear transcription factor κB (NF-κB) is a heterodimeric factor that regulates genes involved in immunity, inflammation and malignant diseases (Liu & Chen, 2011). Links between NF-κB activation and cancer have been found, and it has officially been revealed that the stimulation of muscarinic receptors can trigger NF-κB activation (Kim, Hawke, & Baldwin, 2006; Lee, Jeon, Kim, & Song, 2007).

Salivary gland tumors can be developed in a wide range of histological locations. The submandibular gland-derived tumor cell line A253 presents characteristics of differentiated epithelial cells, and it is considered a useful tool to study the mechanisms of saliva modification, chloride transport, and intracellular signal transduction pathways, which could be activated by different stimuli or agonists (Trzaskawka et al., 2000).

The components of the cholinergic system were detected in non-neuronal cells and tissues, which comprise of the non-neuronal cholinergic system. It is composed by acethylcholine, the neurotransmitter at neuromuscular junctions, synapses, and other biological regions within the central nervous system. It is the organic molecule that is synthesized and degraded by nicotinic and muscarinic receptors (Wessler, Kirkpatrick, & Racke, 1999).

According to several types of experiments and observation in molecular levels, five subtypes of muscarinic receptors have been discovered to date. Muscarinic M1, M3 and M5 receptor subtypes couple to $G_{q/11}$ and can activate phospholipases (Eglen, 2006). Muscarinic M2 and M4 receptor subtypes favor interactions with the G_i protein family and decrease adenylate cyclase activity, reducing intracellular levels of cAMP (Caulfield, 1993). Changes in the expression and/or function of muscarinic receptors have been shown to be implicated in many pathophysiological processes, like degenerative nervous diseases, chronic inflammation and cancer (Koch, Haas, & Jurgens, 2005). Activation of the muscarinic receptors has been associated with proliferation, angiogenesis and tumor growth (Espanol, de la Torre, Fiszman, & Sales, 2007). In epigenetic perspectives, factors that affect salivary gland carcinoma uniquely sensitive to inflammation are currently unknown. Since the field of epigenetics continues to shed light on the importance of clinical treatments and further researches, it is significant to inquire the questions about how we can efficiently cope with inflammatory defects and diseases in epigenetic cancer studies.

Epigenetic modifications may play a role in the development of tumors. Changes in DNA methylation including both hypomethylation and hypermethylation may affect transcriptional inactivation of tumor suppressor genes in human cancer. In actively transcribed genes, the CpG sites in CpG

islands of promoter regions are unmethylated, whereas increased cytosine methylation in the CG sites within CpG island is associated with reduced gene expression and possible gene silencing (Ehrlich, 2009).

DNA methylation has been reported to play a fundamental role in a large spectrum of biological processes, including aging, infectious diseases, and human cancers including salivary gland cancer (Herman & Baylin, 2003). CpG hypermethylation is critical for silencing the expression of certain tumor suppressor genes, and influences comprehensive regulations resulting in differentiated programs in numerous types of tumor. For that reason, the levels of CpG methylation is effectively used to subclassify tumors, predict response to chemotherapeutic agents that are metabolized or antagonized by cellular enzymes regulated by promoter methylation, and to assess the effects of methylating and demethylating therapies (Herman & Baylin, 2003).

There are two major purposes in this thesis. First, it is essential to reveal that capsaicin is involved in anti-inflammatory effect. Second, the function of the M3R is suppressed by epigenetic modulation in salivary gland epithelial cells.

CHAPTER Ⅰ

Capsaicin regulates the NF-κB pathway in salivary gland inflammation

1. Introduction

Toll-like receptors (TLRs), known as pathogen-associated molecular patterns (PAMPs), are a conserved family of type I transmembrane receptors. The TLRs comprised by 11 mammalian proteins play an essential role in activating both innate and adaptive immune responses, which recognize the molecular patterns associated with microbial pathogens. Toll-like receptor 3 (TLR3) recognizes double-stranded RNA (dsRNA) associated with viral infection by binding viral polyinosine-polycytidylic acid (poly[I:C]). Toll-like receptor 4 (TLR4) recognizes bacterial infection by binding lipopolysaccharides (LPS) derived from gram-negative bacteria (Takeda et al., 2003).

Epithelial cells expressing various TLRs may initiate an immune response (Greene and McElvaney, 2005). Salivary gland epithelial cells (SGEC) from patients with Sjögren's syndrome (SS) express much higher levels of mRNA for pro-inflammatory cytokines, such as TNFα and IL-6 (Fox et al., 1994). Both IL-6 and TNFα seem to be highly associated with salivary gland inflammation, since these cytokines are overexpressed in saliva of patients with SS but not patients with drug-induced xerostomia (Vucicevic Boras et al., 2006).

Transient receptor potential vanilloid subtype 1 (TRPV1) is a ligand-gated, selective cation channel expressed in nociceptors (Caterina et al., 1997). Capsaicin is a colorless irritant phenolic amide $C_{18}H_{27}NO_3$ found in various capsicums that gives hot peppers their hotness. Capsaicin is used in topical creams for its analgesic properties (Karnka et al., 2002). Vanilloid receptor signaling induced by either capsaicin (Su et al., 1999) or heat (Kirschstein et al., 1999) was completely blocked by capsazepine, one of the most competitive TRPV1 antagonists, against capsaicin (Szallasi and Blumberg, 1999). TRPV1

also induces pain, desensitization, neurotoxicity, and neurogenic inflammation responses (Oxholm et al., 1992). It was originally identified in nociceptive neurons, but recent evidence has identified functional TRPV1 in various cell types, including bladder epithelial cells (Birder et al., 2001), bronchial epithelial cells (Veronesi et al., 1999), and synovial fibroblasts (Engler et al., 2007).

Capsaicin has been associated with anti-inflammatory properties (Joe et al., 1997). Capsaicin induce an intracellular signaling in neuronal cells via vanilloid receptors (Su et al., 1999), but the mechanism of the anti-inflammatory action in non-neuronal cells by capsaicin is not fully understood. Salivary epithelial cells can also initiate inflammation, but whether capsaicin has an anti-inflammatory activity in salivary glands remains unknown. This prompted us to evaluate possible mechanisms of anti-inflammatory activities of capsaicin in SGEC.

2. Materials and Methods

Reagents

Poly(I:C) and LPS (*Escherichia coli 0111:B4*) (Sigma Aldrich, St. Louis, MO, USA); Capsaicin and capsazepine (Sigma Aldrich); Antibodies to TRPV1, IκB-α, and phospho-IκB-α (p-IκB-α) (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cell Signaling Technology Danvers, MA, USA), respectively, were used in this study.

Cell culture

HSG cells, originating from human submandibular ducts, were cultured in Dulbecco's modified Eagle's medium (Welgene, Daegu, South Korea), supplemented with 10% (v/v) fetal bovine serum (Welgene,) and 1% penicillin/streptomycin (Life Technologies, Seoul, Korea) at 37 ℃ in a humidified atmosphere of 5% CO_2 and 95% air. HSG cells and acinar cells were pre-incubated for 3 h or 1 h with poly(I:C) and LPS at the concentrations indicated for each experiment.

RT-PCR

HSG cells were treated with 10 μg/ml of poly(I:C) or 1 μg/ml of LPS, and harvested for RNA extraction. Total RNA was purified using Trizol (Invitrogen, Carlsbad, CA, USA). Reverse transcriptase with an oligo-dT primer (Invitrogen) was used to prepare cDNA from 1 μg of total RNA. PCR with specific primers was performed using 1 μl of cDNA. The primer sets were as follows: TNF-α: forward 5'-CCAGGCAGTCAGATCATCTTC-3' and reverse 5'-TTGATGGCAGAGAGGAGGTT-3'; IL-6: forward 5'-

CACACAGACAGCCACTCACC-3' and reverse 5'-AGCTCTGGCTTGTTCCTCAC-3'; and TRPV1: forward 5'-TTTCAGGCAGACACTGGAAGA-3' and reverse 5'-TTGAAGACCTCAGCGTCCTCT-3'; GAPDH: forward 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse 5'-GAAGATGGTGATGGGATTTC-3'. PCR conditions were as follows: 35 cycles of denaturation at 95℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 30 s; and a final step at 72℃ for 10 min. (PTC-1148C; Bio-Rad Laboratories Inc., Hercules, CA, USA). PCR products were separated by using a Mupid® -2 plus electrophoresis system (OPTIMA, Tokyo, Japan) on 1.5% agarose gels (Sigma Aldrich) containing 0.1 μg/ml ethidium bromide (Sigma Aldrich) and were visualized under UV light with a bioimaging system (TS-312R; Spectroline, Westbury, NY, USA).

Quantitative real-time PCR

Total RNA was extracted from HSG cells and mouse SMGs using Trizol (Invitrogen) according to the manufacturer's protocol. One microgram of total RNA was converted to cDNA using Superscript II reverse transcriptase (Invitrogen) and oligo-(dT) primers according to the manufacturer's protocol. The primers were based on the mouse TNFα and IL-6 cDNA sequences. The primers were TNFα: forward 5'-TCCCAGGTTCTCTTCAAGGGA-3' and reverse 5' GGTGAGGAGCACGTAGTCGG-3'; IL-6: forward 5'-AACGATGATGCACTTGCAGA-3'; and reverse 5'-GGAAATTGGGGTAGGAAGGA-3'; GAPDH: forward 5'-TTCACCACCATGGAGAAGGC-3'; and reverse 5'-

TCATGACCACAGTCCATGCC-3'. Quantitative real-time PCR was performed in a reaction containing cDNA and SYBR PCR master mix (Applied BioSystems, Foster City, CA, USA). Samples were analyzed with the ABI PRISM 7500 sequence detection system (Applied BioSystems). All PCRs were performed in triplicate, and the specificity of the reaction was determined by melting curve analysis at the dissociation stage.

MTT assay

Cells were subcultured into coated multiwell cell culture plates (SPL Lifescience, Pocheon, Korea) and allowed to reach ~80% confluence over 24 h. The cells were washed once with sterile phosphate-buffered saline and treated for 3 h with capsaicin. Cell viability was assessed using the C-Chip DHC-NO1 (iNCYTO) (Digital Bio Technology, Suwon, Korea), according to the supplier recommendations. Cell viability was determined by spectrophotometrically assaying the production of water-soluble formazan dye (Sigma Aldrich) by active mitochondrial dehydrogenase enzymes in viable cells. Data are expressed as the percentage of viable cells relative to untreated control cells.

Enzyme-linked immunosorbent assay for cytokines

Cell culture media were collected at various time points in each experiment. The cytokine levels secreted into the medium were measured using TNFα and IL-6 ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Serum was used immediately after thawing from -70℃.

Experimental animals and cell preparation

All procedures were conducted in accordance with the Institutional Animal Care and Use Committee at the School of Dentistry, Seoul National University. TRPV1$^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The salivary phenotype of this mice looks normal; the average weights for SMG and flow rate were not significantly different to those of littermate (data not shown). Mouse SMG samples were finely minced into small pieces in KRH (Krebs-Ringer Hepes) solution (120 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgCl_2$, 11.1 mM glucose, 20 mM HEPES, pH 7.4) (Sigma Aldrich), aerated with 95% O_2, and then digested in KRH solution containing 100 units/ml of collagenase (Worthington, Lakewood, UK) and 1% BSA (Sigma Aldrich) for 60 min with continuous agitation (MSH-20A; Daihan Scientific, Seoul, Korea). After the incubation by shaking water bath (SWB-10; Jeio Tech, Seoul, Korea), dissociated cells were harvested by filtering the suspension through a nylon mesh (200 μm) (Lockertex, Warrington, Cheshire, UK) followed by centrifugation (Micro 17R centrifuge; Hanil Science industrial Co., Ltd., Incheon, Korea).

Western blotting

Cultured cells were collected and protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL, USA) with BSA as standard. Protein samples were separated by 8% SDS-PAGE (Bio-Rad, Hercules, CA, USA). After electrophoresis using a Power-PacTM Basic system (Bio-Rad), the proteins were transferred to nitrocellulose membranes (Whatman, Dassel, Germany). The membranes were blocked with 5% non-fat milk (Seoul-milk, Seoul, Korea) and probed with the following antibodies: anti-TRPV1 antibody

(1:200 dilution) (Santa Cruz Biotechnology), anti-IκB-α/p-IκB-α antibodies (1:1000 dilution) (Cell Signaling Technology Danvers), then incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnology). The immunoreactive protein was visualized by ECL reagent (iNtRON Biotechnology, Sungnam, Korea).

Statistical Analysis

All experiments were conducted in triplicate. Statistical analysis was performed by t-tests using Graph Pad Prism5 software (GraphPad Software, Inc., La Jolla, CA, USA). Asterisks indicate that P values of less than 0.05 are considered statistically significant ($^{*}P < 0.05$).

3. Results

Expression of pro-inflammatory cytokines induced by poly(I:C) and LPS in HSG cells

I examined whether TLR stimulation induces expression of pro-inflammatory cytokines, TNFα and IL-6, using RT-PCR and real-time PCR (Fig. 1). HSG cells were treated with 10 μg/ml of poly(I:C) or 1 μg/ml of LPS for up to 48 h. TNFα and IL-6 mRNA levels are significantly increased, particularly at 3 h or 1 h incubated with poly(I:C) or LPS, respectively (upper panels in Fig. 1A and B). The increased mRNA levels of both cytokines were further confirmed by real-time PCR (lower panels in Fig. 1A and B). The maximal increase of TNFα and IL-6 mRNA expression was also observed at 3 h or 1 h after incubation in real time PCR, which corresponded well to the RT-PCR time course. Thus, 3 h poly(I:C) treatments and 1 h LPS treatments were carried out throughout the experiments. My results demonstrate that poly(I:C) and LPS up-regulate TNFα and IL-6 mRNA expression in HSG cells.

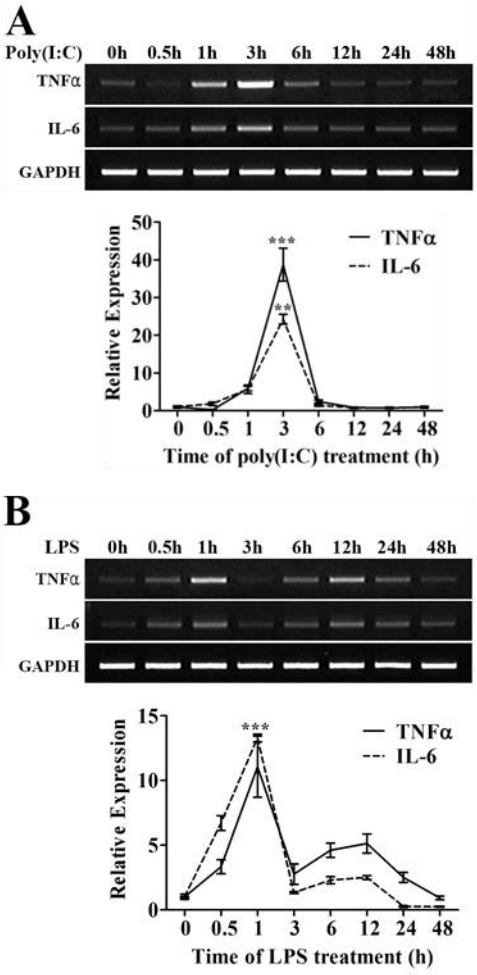

A
Poly(I:C)
0h
0.5h
1h
3h
6h
12h
24h
48h
TNFα
IL-6
GAPDH
Relative Expression
50
40
30
20
10
0
***
**
TNFα
IL-6
0
0.5
1
3
6
12
24
48
Time of poly(I:C) treatment (h)
B
LPS
0h
0.5h
1h
3h
6h
12h
24h
48h
TNFα
IL-6
GAPDH
Relative Expression
15
10
5
0
***
TNFα
IL-6
0
0.5
1
3
6
12
24
48
Time of LPS treatment (h)

Figure 1. TNFα and IL-6 mRNA levels in HSG cells stimulated with poly(I:C) or LPS.

The expression levels of TNFα and IL-6 mRNA in HSG cells using RT-PCR **(upper panels in Fig. 1A and B)** and real-time PCR **(lower panels in Fig. 1A and B)**. Total RNA was extracted from HSG cells stimulated with poly(I:C) or LPS. Vertical bars in the graphs indicate mean ± standard deviation (SD) mRNA levels calibrated to the amount of GAPDH mRNA as determined using real-time PCR (n=3). Experiments were repeated three times with essentially identical results. I compared the maximum value with the control at prestimulus time indicated by "0" in horizontal axis. *P*-values of less than 0.05 were considered statistically significant ($^{**}P < 0.01$, $^{***}P < 0.001$).

Expression of transient receptor potential vanilloid 1 (TRPV1)

I next examined whether TRPV1 is expressed in HSG cells and human submandibular gland (SMG) tissues using RT-PCR and Western blot analysis. TRPV1 mRNA and protein in both cells were detected with primers or antibodies specific for human TRPV1 (Fig. 2A). I then performed MTT assay to check the viability of cells after incubation with capsaicin. In MTT assay, cells treated with 10 μM of capsaicin for 3 h were >95% viable (Fig. 2B). I also examined whether 10 μM of capsaicin affects cytokines level in HSG cells by real-time PCR (Fig. 2C). Although there were transient increases of TNFα and IL-6 mRNA transcripts after 0.5 and 1 h incubation, expression levels of TNFα and IL-6 mRNA after 3 h incubation with capsaicin were not significantly different from the prestimulus group ($P > 0.1$, indicated by "0"). The result demonstrates that 10 μM capsaicin alone does not affect cell viability and levels of cytokines at least in 3 h incubation time.

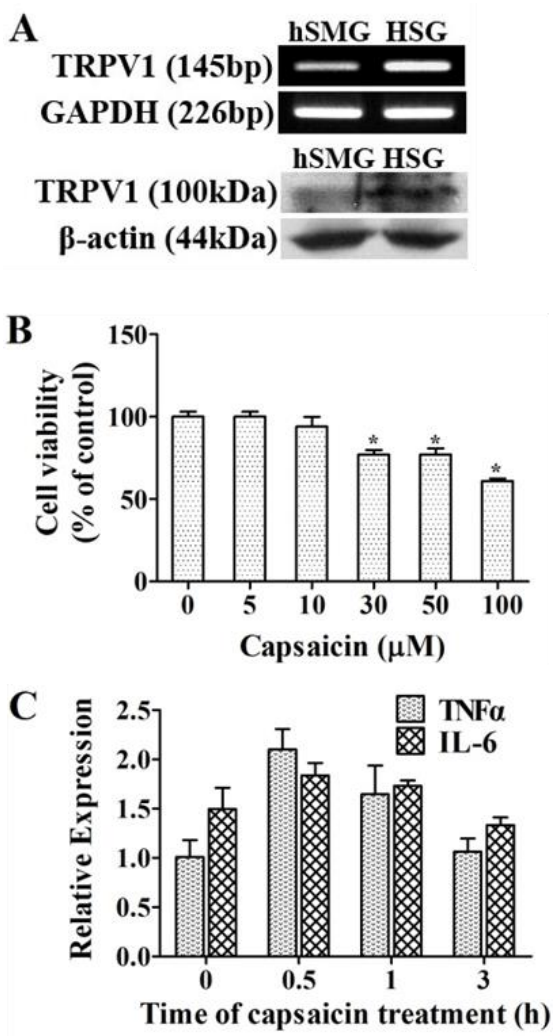

A
hSMG HSG
TRPV1 (145bp)
GAPDH (226bp)
hSMG HSG
TRPV1 (100kDa)
β-actin (44kDa)
B
Cell viability (% of control)
150
100
50
0
0 5 10 30 50 100
Capsaicin (μM)
C
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
TNFα
IL-6
0 0.5 1 3
Time of capsaicin treatment (h)

Figure 2. Expression of TRPV1 and application of capsaicin.

(A) Expression of TRPV1 mRNA in HSG cells and human submandibular gland (SMG) tissue using RT-PCR (the upper panel) and Western blot (the lower panel). **(B)** Effects of capsaicin on the cell viability, which was estimated by MTT assay. The HSG cells (7.7×10^3/well) were grown in 96-well plates in media supplemented with capsaicin (5 μM, 10 μM, 30 μM, 50 μM, 100 μM) for 24 h. No significant suppression of cell growth was detected in 5 μM or in 10 μM capsaicin. **(C)** Expression of TNFα and IL-6 in 10 μM of capsaicin-treated HSG cells. Capsaicin-induced expression levels of TNFα and IL-6 mRNA were compared with the expression levels of those in the pre-stimulus group.

Effect of capsaicin on TNFα and IL-6 expression in HSG cells

The ability of capsaicin to inhibit TNFα and IL-6 mRNA expression induced by poly(I:C) or LPS was evaluated by real-time PCR (Fig. 3A and B, $P < 0.05$). TNFα and IL-6 mRNA expression levels were increased by poly(I:C) or LPS. However, simultaneous application of capsaicin with poly(I:C)- or LPS significantly decreased TNFα and IL-6 mRNA levels. Interestingly enough, the inhibitory effect of capsaicin was not blocked by capsazepine. Capsazepine was ineffective to reverse recover the decreased TNFα and IL-6 mRNA level induced by capsaicin. To confirm the effect of capsaicin, we measured levels of TNFα and IL-6 protein released into the medium induced by poly(I:C)- or LPS using ELISA (Fig. 3C and D, $P < 0.05$). The amount of TNFα and IL-6 protein in poly(I:C)- or LPS-treated cells also significantly reduced by capsaicin. Simultaneous application of capsazepine with capsaicin does not reverse the decreased TNFα and IL-6 protein level induced by capsaicin, either, suggesting that capsaicin may not act through TRPV1.

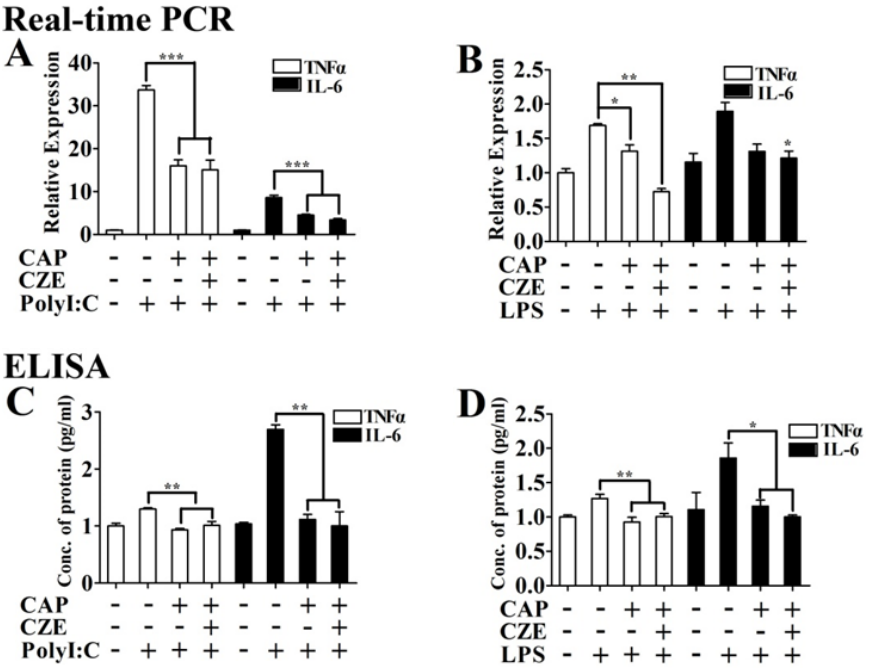

Real-time PCR
A
Relative Expression
TNFα
IL-6
CAP
CZE
PolyI:C
B
Relative Expression
TNFα
IL-6
CAP
CZE
LPS
ELISA
C
Conc. of protein (pg/ml)
TNFα
IL-6
CAP
CZE
PolyI:C
D
Conc. of protein (pg/ml)
TNFα
IL-6
CAP
CZE
LPS

Figure 3. Effect of capsaicin on TNFα and IL-6 mRNA expression and protein level in poly(I:C) and LPS-stimulated HSG cells.

(A, B) Effect of 10 μM capsaicin on the poly(I:C)- and LPS-induced expression levels of TNFα and IL-6 mRNA transcripts using real-time PCR. After stimulation with 10 μg/ml poly(I:C) for 3 h or 1 μg/ml LPS for 1 h, the cells are further incubated with capsaicin for 3 h with or without 10 μM capsazepine. **(C, D)** Effect of 10 μM capsaicin on the poly(I:C)-and LPS-induced expression levels of TNFα and IL-6 proteins. The amount of proteins were measured from the incubation medium using ELISA kits. CAP, capsaicin; CZE, capsazepine ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

The inhibitory mechanism of capsaicin through IκB-α degradation

To ensure whether the inhibitory effect of capsaicin is mediated by TRPV1 or not, I repeated the same experiment using primary cultured SMG acinar cells, which was immediately used after harvest from $TRPV1^{-/-}$ mice. I obtained the similar results from $TRPV1^{-/-}$ mice compared to the $TRPV1^{+/+}$ mice. Capsaicin also significantly inhibited TNFα and IL-6 mRNA expression in SMG acinar cells not only from $TRPV1^{+/+}$ (Fig. 4A, $^{*}P < 0.05$) but also from $TRPV1^{-/-}$ mice (Fig. 4B, $^{**}P < 0.01$, $^{***}P < 0.001$). The results strongly suggest that the anti-inflammatory effect of capsaicin in salivary epithelial cells is not mediated by TRPV1. Thus, I further investigated the mechanism of capsaicin inhibition of cytokine release. NF-κB is a major transcription factor that regulates expression of cytokines such as TNFα and IL-6 (Beinke and Ley, 2004; Novotny et al., 2008). Thus, I examined whether NF-κB signaling is responsible for capsaicin inhibition of cytokine release. An immunoblot analysis shows that poly(I:C) and LPS induce IκB-α phosphorylation and degradation (the second lanes both in Fig. 4C and D), which lead to NF-κB activation. Interestingly, simultaneous incubation of cells with capsaicin almost completely inhibited IκB-α phosphorylation and degradation induced by poly(I:C) or LPS (the third lanes both in Fig. 4C and D). Furthermore, the reduced IκB-α phosphorylation induced by capsaicin was not recovered by capsazepine. Consecutive three different experiments showed similar results. These results strongly suggest that anti-inflammatory effect of capsaicin was associated with IκB-α/NF-κB signaling pathway in salivary gland epithelial cells.

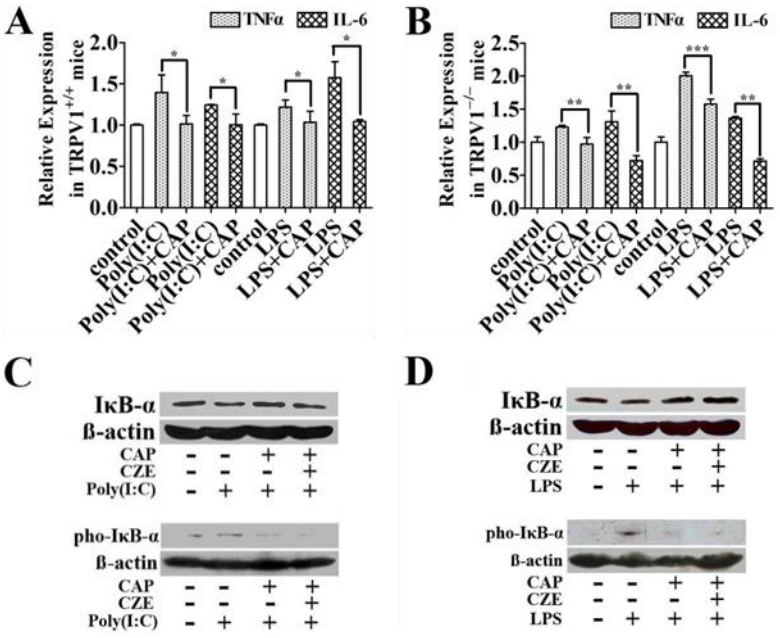

A
TNFα
IL-6
Relative Expression in TRPV1+/+ mice
2.0
1.5
1.0
0.5
0.0
control
Poly(I:C)
Poly(I:C)+CAP
Poly(I:C)
Poly(I:C)+CAP
control
LPS
LPS+CAP
LPS
LPS+CAP
B
TNFα
IL-6
Relative Expression in TRPV1−/− mice
2.5
2.0
1.5
1.0
0.5
0.0
control
Poly(I:C)
Poly(I:C)+CAP
Poly(I:C)
Poly(I:C)+CAP
control
LPS
LPS+CAP
LPS
LPS+CAP
C
IκB-α
ß-actin
CAP
CZE
Poly(I:C)
pho-IκB-α
ß-actin
CAP
CZE
Poly(I:C)
D
IκB-α
ß-actin
CAP
CZE
LPS
pho-IκB-α
ß-actin
CAP
CZE
LPS

Figure 4. Inhibitory effect of capsaicin in TRPV1$^{-/-}$ mice and IκB-α/NF-κB signaling pathway.

The expression levels of TNFα and IL-6 mRNA in primary cultured acinar cells from the TRPV1$^{+/+}$ **(A)** or TRPV1$^{-/-}$ mice **(B)** using real-time PCR. After stimulation with 10 μg/ml poly(I:C) for 3 h or 1 μg/ml LPS for 1 h, the cells further treated with 10 μM of capsaicin for 3 h. The results are mean ± SD of three independent experiments. CAP, capsaicin; CZE, capsazepine ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). **(C, D)** Western blot analysis for IκB-α and p-IκB-α (phosphorylated form) in HSG cells. The cells were stimulated with poly(I:C) for 3 h **(C)** or LPS for 1 h **(D)** followed by capsaicin treatment for 3 h with or without capsazepine.

4. Discussion

The TLR family plays an essential role in innate immune responses as pathogen-recognition transmembrane receptors expressed on various cells types, including macrophages and dendritic cells. So far, ten TLRs have been reported in humans, all of which recognizes distinct molecular patterns of bacteria, viruses, and fungi (Takeda et al., 2003). One of mechanisms that activate salivary gland epithelial cells is mediated by TLRs (Alexopoulou et al., 2001; Zeuke et al., 2002). Activation of TLR-mediated immune response in Sjögren's syndrome also has been reported (Kawakami et al., 2007). Thus, viral and bacterial infections of the salivary glands can trigger localized inflammatory chemokine and/or cytokine production. Activation of these TLRs give rise to multiple signaling pathways that produce pro-inflammatory chemokines (Li et al., 2010) and/or cytokines, such as TNFα, IL-6, IFN-α, and IFN-β (Vercammen et al., 2008). Localized and swift pro-inflammatory cytokine production forms the first line of defense against invading viruses and bacteria. However, chronic or repeated infection's results lead to persistent inflammatory response that might cause organ dysfunction or autoimmune disease (Roescher et al., 2009).

My study demonstrated that treating cells with poly(I:C) or LPS, increase inflammatory cytokine production in SGEC. Poly(I:C) and LPS can activate innate immune responses by binding TLR3 (Alexopoulou et al., 2001) and TLR4 (Zeuke et al., 2002). Respectively, In my experiments showed that the stimulation of the cells with poly(I:C) or LPS increase TNFα and IL-6 mRNA levels, and peaked maximum at 3 h or 1 h after incubation with poly(I:C) or LPS, respectively. Apoptosis and glandular destruction, due to localized

autoimmune responses, are considered to be major factors in salivary gland hypofunction (Jonsson et al., 2007). However, non-apoptotic mechanisms, such as an inflammatory cytokine inhibition of the salivary secretion, also play an equally important role for salivary gland hypofunction (Dawson et al., 2006). These cytokines are not only generated by infiltrating lymphocytes but also activated by SGEC (Manoussakis and Kapsogeorgou, 2007).

Capsaicin has analgesic and anti-inflammatory properties in primary sensory neurons (Gonzalez et al., 1993; Joe et al., 1997). Capsaicin receptors, called vanilloid receptors, plays a fundamental role in the signal transduction of peripheral tissue injury and inflammation responses (Planells-Cases et al., 2000), but repeated application results in anti-inflammatory properties (Lee et al., 2007). It was found that capsaicin also has anti-inflammatory effect in SGEC. Capsaicin not only reduced TNFα and IL-6 mRNA expression but also inhibited their release at the protein levels. Interestingly enough, however, our data showed that capsazepine does not block capsaicin inhibition of TNFα and IL-6 release by capsaicin.

NF-κB, a family of inducible transcription factors, regulates the expression of specific genes involved in various pathological conditions, including inflammation. Extracellular signals, such as inflammatory cytokine or oxidative stress, stimulate the IκB-α/NF-κB signaling pathway that produce pro-inflammatory mediators. These signals activate IκB-α degradation leading to NF-κB activation (Yamamoto and Gaynor, 2001). Thus, the inhibitory action of capsaicin in SGEC may be mediated by inhibition of IκB-α/NF-κB signaling pathway rather than TRPV1 and my results also showed that capsaicin effect on SGEC was not mediated by activation of TRPV1, conventional signaling pathway for capsaicin. First, capsazepine did not antagonize the inhibitory

action of capsaicin on the cytokine release induced by poly(I:C) or LPS. Second, such an inhibitory action of capsaicin was also shown even in the TRPV1$^{-/-}$ mice. Finally, capsaicin inhibited the poly(I:C) and LPS-induced IκB-α phosphorylation and degradation process, a different signaling pathway from TRPV1 activation. Capsazepine, again, has little effect on this IκB-α phosphorylation process.

At the moment, it is not certain why the capsaicin did not act on the TRPV1. It is speculated that although TRPV1 is barely expressed in HSG cells at the protein level, TRPV1 is found to have no function. In fact, we could not see the $[Ca^{2+}]_i$ response to capsaicin, which was observed in most of cells expressing TRPV1 including sensory neurons (Ding et al., 2010). My study demonstrates that capsaicin inhibition is associated with NF-κB inactivation in SGEC, which differs from primary sensory neurons. Capsaicin might be one of potential drugs to alleviate the inflammation in salivary glands, although it is uncertain at the moment whether the capsaicin also effect on the chronic inflammatory condition, such as Sjögren's syndrome.

Acknowledgments

This work was supported by a National Research Foundation of Korea Grant, through the Oromaxillofacial Dysfunction Research Center for the Elderly (No. 2012-000912) at Seoul National University in Korea.

CHAPTER Ⅱ

Epigenetic modulation of the muscarinic type 3 receptor in salivary epithelial cells

1. Introduction

Epigenetic regulation by CpG methylation plays an important role in tumorigenesis, and is also crucial to mounting a successful response to cancer therapy. Recent studies have indicated that hypermethylation of CpG islands within the promoter and 5' regions of genes is an important epigenetic mechanism for suppressing gene expression (Baylin et al., 1998; Jones and Laird, 1999; Laird and Jaenisch, 1996). DNA hypermethylation may directly affect the basal transcriptional machinery by altering the secondary structure of DNA, thereby leading to transcriptional repression (Di Croce et al., 2002). 5-Aza-2'-deoxycytidine (5-Aza-CdR, decitabine) is a prodrug that is phosphorylated by deoxytidine kinase activation. 5-Aza-CdR is a nucleotide analog that can irreversibly inactivates DNA methyltransferase after incorporated into DNA, thus results in the demethylation of DNA (Momparler, 2005). Therefore, 5-Aza-CdR can reactivate many genes that have been inactivated by hypermethylation (Cameron et al., 1999; Chen et al., 2003; Daskalakis et al., 2002; Kaneda et al., 2002; Zhu et al., 2002).

Muscarinic cholinergic receptors are members of the heterotrimeric G protein-coupled receptor (GPCR) superfamily. Muscarinic cholinergic receptors are abundant in the central nervous system, as well as in non-neural tissues that are innervated by the parasympathetic nervous system. There are 5 subtypes of muscarinic receptors, M1–M5, which encode muscarinic receptor proteins that exhibit a rhodopsin-like architecture with seven transmembrane domains (Hulme et al., 2003; Wess et al., 2007). Changes in the expression and/or function of muscarinic receptors were involved in many pathophysiological processes such as degenerative nervous diseases, chronic inflammation, and

cancer (Koch et al., 2005; Paleari et al., 2008; Sales, 2010). In addition, muscarinic receptor activation were also involved in proliferation, angiogenesis, and tumor growth (Espanol and Sales, 2004; Espanol et al., 2007). However, the functional expression of the muscarinic type 3 receptor (M3R), a subtype which plays a key role in secretory epithelia, has not yet been rigorously studied in pathological condition. In particular, the role of this subtype in cancer cells remains to be elucidated.

In this study, I investigated the extent to which epigenetic modulation determines the expression of the M3R in A253 cells. These cells were originally derived from a submandibular gland carcinoma. In A253 cells, functional expression of the M3R has not yet been described, even though these cells originate from secretory epithelia and the M3R is known to play an important role in secretion in this tissue. In contrast to HSG cells, a $[Ca^{2+}]_i$ response to the typical muscarinic agonist is not observed in A253 cells. Thus, we hypothesized that M3R function may be suppressed on the epigenetic level, which may also contribute to the development of submandibular gland tumors. I identified a causal relationship between hypermethylation of the CpG island and the expression level of the M3R in A253 cells. I also found that demethylation at CG sites may directly activate transcription of the M3R in A253 cells, indicating that the M3R CpG island is hypermethylated.

2. Materials and Methods

Reagents

5-aza-2'-deoxycytidine (5-Aza-CdR) was obtained from Sigma Aldrich (St. Louis, MO, USA). Antibodies against M3R were purchased from Abcam (Cambridge, UK).

Cell culture

HSG and A253 cells, originating from human submandibular ducts, were cultured in Dulbecco's modified Eagle's medium (Welgene, Daegu, South Korea) supplemented with 10% (v/v) fetal bovine serum (Welgene) and 1% penicillin/streptomycin (Life Technologies, Seoul, Korea). Cells were propagated at 37℃ in a humidified atmosphere of 5% CO_2.

RT-PCR

HSG and A253 cells were harvested for RNA extraction. Total RNA was purified using Trizol (Invitrogen, Carlsbad, CA, USA). Reverse transcriptase with an oligo-dT primer (Invitrogen) was used to prepare cDNA from 1 μg of total RNA. PCR with specific primers was performed using 1 μl of cDNA. The primer sets used were as follows: M1R: forward 5'-ACGGAGCTCCCCAAATACAG-3', reverse 5'-TAGCACATGGGGTTGATGGT-3'; M2R: forward 5'-AGCCTTCTATTTGCCAGTGA-3', reverse 5'-GCAACAGCACTGACTGAGGT-3'; M3R: forward 5'-GTTACCCTCATCGGACAACCT-3', reverse 5'-TTACCCACTGAGGAGTTGACG-3'; M4R: 5'-

CAGTTTGTGGTGGGTAAGCG-3', reverse 5'-GTACAGCACCGTCATGATGA-3'; M5R: 5'-GACCCAAGAGTCAGAAATGTG-3', reverse 5'-AGAAGGTAGAAACCAGGACCA-3'; GAPDH: forward 5'-GAAGGTGAAGGTCGGAGTC-3', reverse 5'-GAAGATGGTGATGGGATTTC-3'. PCR conditions were as follows: 35 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 30 s, followed by a final extension at 72℃ for 7 min on a PCR thermocycler (PTC-1148C; Bio-Rad Laboratories Inc., Hercules, CA, USA). PCR products were resolved using a Mupid®-2 plus electrophoresis system (OPTIMA, Tokyo, Japan) on 1.5% agarose gels containing 0.1 μg/ml ethidium bromide. PCR products were visualized under UV light with a bioimaging system (TS-312R; Spectroline, Westbury, NY, USA).

Membrane preparation and western blotting

Cultured cells were collected and lysed. Cell lysates were spun by centrifugation at 600 x g at 4℃, and the resultant supernatants were spun again at 20,000 xg at 4℃ (Popova and Rasenick, 2004). The pellets were solubilized and then subjected to SDS/PAGE and immunoblotting. After electrophoretic separation using a Power-PacTM Basic system (Bio-Rad, Hercules, CA, USA), the proteins were transferred to nitrocellulose membranes (Whatman, Dassel, Germany). The membranes were then blocked with 5% non-fat milk (Seoul-milk, Seoul, Korea) and probed with anti-M3R antibodies (1:1500 dilution) (Abcam, Cambridge, UK). After washing, membranes were then incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz,

CA, USA). Immunoreactive bands were ultimately visualized using ECL reagents (iNtRON Biotechnology, Sungnam, Korea).

Immunofluorescence confocal laser microscopy

To visualize the M3R, HSG and A253 cells (treated with or without 5-Aza-CdR) were grown on cell culture slides. Cells were washed three times with PBS, fixed in 4% paraformaldehyde at room temperature for 15 min, and incubated overnight at 4℃ with anti-human M3R antibodies (1:200; Abcam, Cambridge, UK). Cells were then washed three times with PBS containing 1% bovine serum albumin, and then incubated for 1 h with Alexa Fluor® 488 goat anti-rabbit IgG antibodies (1:200). All slides were mounted using VECTASHIELD H-1200 with DAPI (Vector Laboratories, Burlingame, CA, USA), and images were captured on a laser scanning confocal microscope (Fluoview300; Olympus, Tokyo, Japan).

Measurement of $[Ca^{2+}]_i$

HSG and A253 cells, treated with or without 5-Aza-CdR, were loaded with the Ca^{2+}-sensitive fluorescent probe, fura-2/AM (Molecular Probes, Eugene, OR, USA) for 45 min at room temperature. A MetaFluor® imaging system (version 6.1; Universal Imaging, West Chester, PA, USA) was used for recording and analysis. Approximately 15-20 cells were recorded at 37℃ and analyzed in each experiment. Fura-2 fluorescence was recorded at excitation wavelengths of 340 and 380 nm, with an emission wavelength of 510 nm. Results are presented as 340 nm/380 nm ratios (Ca^{2+} fluorescence ratio, F340/F380).

Determination of 5-methylcytosine DNA content

Genomic DNA was extracted from HSG and A253 cells, treated with or without 5-Aza-CdR, using a Qiagen Blood and Cell Culture DNA Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. The global DNA methylation levels were then examined in the DNA samples isolated from these two salivary gland epithelial cell lines. The relative degrees of methylation in the DNA samples were quantified using a MethylFlashTM Methylated DNA Quantification Kit (EPIGENTEK, Farmingdale, NY, USA) according to the manufacturer's instructions.

Methylation-specific PCR and 5-aza-2'-deoxycytidine treatment

Bisulfite modification of genomic DNA was carried out using an EpiTect Bisulfite Kit (Qiagen). Methylation-specific PCR was performed using bisulfite-treated DNA as template, using specific primer sequences for either the methylated or unmethylated form of the gene. HSG and A253 cells were seeded in 100-mm culture dishes one day prior to treatment. Cells were then treated with 5-aza-2'-deoxycytidine (Sigma Aldrich) at a final concentration of 10 μM for 1, 2, 3, and 4 days. Culture medium was renewed daily, and treated cells were harvested at the end of the fourth day. Genomic DNA and total RNA were then isolated using a QIAamp DNA Blood Mini Kit and an RNeasy Mini Kit (Qiagen), respectively. The following methylation-specific primers were used: M forward, 5'-GGTTTGTGTCGATTTGATTATC-3'; M reverse, 5'-ACTCGATACGTAAACGACCTC-3'; U forward, 5'-TTTGGTTTGTGTTGATTTGATTATT-3'; U reverse, 5'-TTAACTCAATACATAAACAACCTC-3'.

Bisulfite sequencing

In total, 2 μg of genomic DNA isolated from A253 cells, treated either with or without 5-Aza-CdR, was modified with sodium bisulfite. The modified genomic DNA was then amplified with the following primers: forward, 5'-GGTATTTTGGTTTTGGTGATTA-3'; reverse, 5'-TCTTTCCAACAAAATATTACCAA-3'. PCR reactions were performed as follows: 94℃ for 5 min, followed by 35 cycles of 94℃ for 40 s, 55℃ for 40 s, and 72℃ for 1 min. The resultant PCR products were purified with a PCR Purification Kit (Qiagen) and ligated into pCR2.1-TOPO using the TA cloning system (Invitrogen). Five separate clones from treated cells and five separate clones from untreated cells were chosen for sequence analysis.

Statistical analysis

All experiments were conducted in triplicate. Statistically significant differences in the data were determined by Student's t-test, using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). *P* values less than 0.05 were considered statistically significant (designated with an asterisk; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

3. Results

Muscarinic receptor subtypes in HSG and A253 cells

I first examined the expression levels of various muscarinic receptor subtypes in two different salivary gland epithelial cell lines, HSG and A253. RT-PCR was used to determine the mRNA expression levels of various muscarinic receptors. HSG cells, employed as a positive control, expressed all subtypes (1 to 5) of muscarinic receptors. A253 cells also expressed all subtypes of muscarinic receptors, with the exception of type 3. The type 3 muscarinic receptor (M3R) was expressed only at extremely low levels in A253 cells (Fig. 1A), as summarized in Fig. 1B (n=3, $^{***}P < 0.001$). These results indicated that expression of the M3R is suppressed in A253 cells. I next examined whether the low level of M3R mRNA expression is related to DNA methylation of the M3R CpG island.

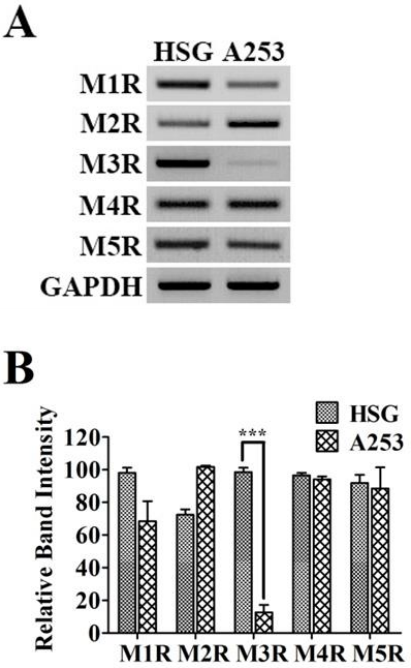

A
HSG
A253
M1R
M2R
M3R
M4R
M5R
GAPDH
B
HSG
A253
Relative Band Intensity
120
100
80
60
40
20
0
***
M1R
M2R
M3R
M4R
M5R

Figure 1. mRNA expression levels of various muscarinic receptor subtypes in HSG and A253 cells.

(A) Total RNA was extracted from HSG and A253 cells, and RT-PCR was used to examine the mRNA expression levels of various muscarinic receptors. HSG cells were used as a positive control. A253 cells expressed all muscarinic receptor subtypes except type 3 (M3R). The muscarinic receptor subtype 3 (M3R) was expressed only at an extremely low level in A253 cells. **(B)** Quantitative analysis of band densities. The intensity of each band was measured by densitometry, and showed that the mRNA expression level for the M3R was greatly reduced in A253 cells. The vertical bars in the graphs indicate mean ± standard deviations (SDs) of mRNA levels, normalized to the amount of GAPDH mRNA, as determined using RT-PCR. Experiments were repeated three times with essentially identical results; a summarized result is shown (n=3; $^{***}P < 0.001$).

Induction of the M3R in A253 cells by treatment with 5-Aza-CdR

I next examined whether the low expression level of the M3R is increased by 5-Aza-CdR treatment. A time course analysis of RT-PCR data is shown in Fig. 2A. Cells were incubated with 10 μM of 5-Aza-CdR, a well-characterized demethylating agent. At 24 h after 5-Aza-CdR treatment, the mRNA expression level of the M3R was significantly increased. Furthermore, the expression level of M3R mRNA gradually increased in proportion to the length of the incubation period tested for 4 days. After 3 days of treatment with 5-Aza-CdR, the expression level of M3R mRNA in A253 cells was nearly the same as in HSG cells, as summarized in Fig. 2B (n=3, $^{*}P < 0.05$; $^{**}P < 0.01$). The increased expression level of the M3R was further confirmed at the protein level using Western blot analysis (Fig. 2C). Before 5-Aza-CdR treatment, only a relatively small amount of M3R protein was expressed in A253 cells. However, the amount of M3R protein increased significantly, in proportion to the length of the incubation period with 5-Aza-CdR, as summarized in Fig. 2D (n=3, $^{*}P < 0.05$). These results demonstrate that expression of the M3R is markedly suppressed in A253 cells, and indicate that this suppression is due to hypermethylation of the M3R gene.

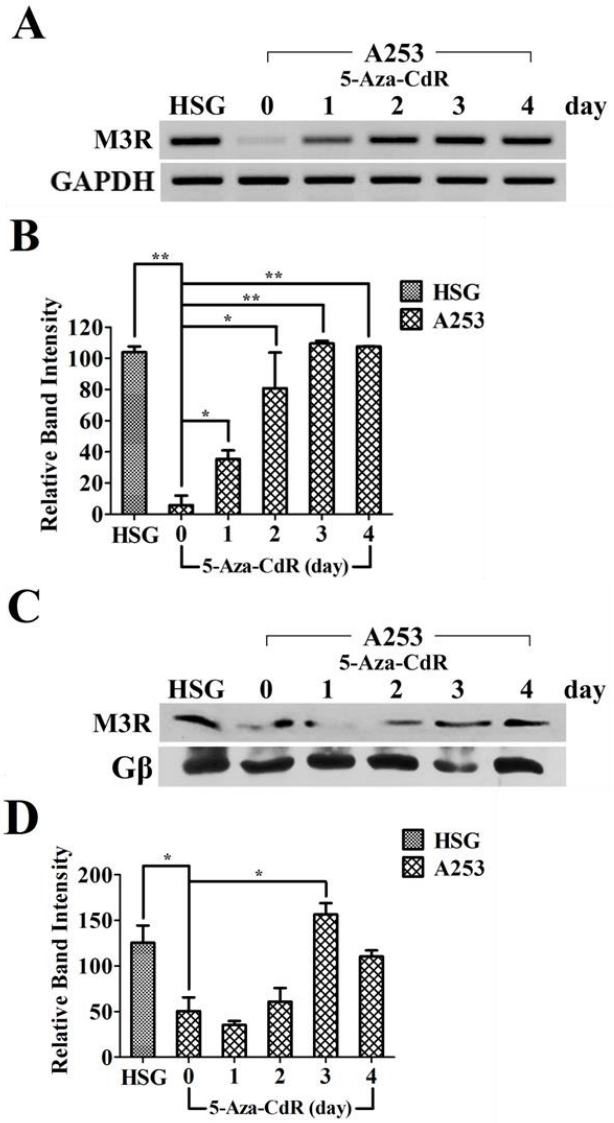

A
A253
5-Aza-CdR
HSG 0 1 2 3 4 day
M3R
GAPDH
B
Relative Band Intensity
120
100
80
60
40
20
0
HSG 0 1 2 3 4
5-Aza-CdR (day)
HSG
A253
C
A253
5-Aza-CdR
HSG 0 1 2 3 4 day
M3R
Gβ
D
Relative Band Intensity
200
150
100
50
0
HSG 0 1 2 3 4
5-Aza-CdR (day)
HSG
A253

Figure 2. Induction of M3R mRNA expression and protein production by 5-Aza-CdR.

A253 cells were treated with 5-Aza-CdR (10 μM) for 24, 48, 72, or 96 h. The expression of M3R mRNA in HSG and A253 cells was then determined by RT-PCR **(A, B)** and Western blot analysis **(C, D)**. Before 5-Aza-CdR treatment, only a small amount of M3R protein was present. However, the amount of M3R protein increased proportionally with the length of the incubation period with 5-Aza-CdR. A protein with an apparent molecular weight of 66 kDa, corresponding to the M3R, was clearly observed in lysates from A253 cells after treatment with 5-Aza-CdR for 24 to 96 h. All experiments were performed in triplicate ($^{*}P < 0.05$; $^{**}P < 0.01$).

Rescue of M3R function by 5-Aza-CdR-treatment

The increased level of M3R protein upon 5-Aza-CdR treatment was further confirmed by confocal microscopy imaging. Although the M3R was highly expressed in both membrane and cytosolic preparations of HSG cells (Fig. 3A), the M3R was barely little expressed in A253 cells (Fig. 3B). However, after 3 days of treatment of A253 cells with 10 μM 5-Aza-CdR, the expression level of the M3R was markedly increased in both membrane and cytosolic preparations (Fig. 3C). I next measured the magnitude of the intracellular free Ca^{2+} ($[Ca^{2+}]_i$) response in fura-2 loaded cells to verify that the M3R regained its function, even after demethylation by 5-Aza-CdR treatment. HSG cells exhibited a robust response to 10 μM carbachol (CCh), a known M3R agonist, as evidenced by the increased $[Ca^{2+}]_i$ response (Fig. 4A). In contrast, CCh exerted no effect on the calcium response in A253 cells (Fig. 4B). Importantly, A253 cells responded well to the control stimulus (100 μM ATP, a known purinergic agonist), as shown by the increased $[Ca^{2+}]_i$ response. After 3 days of treatment with 10 μM 5-Aza-CdR, CCh consistently evoked a calcium response in A253 cells; the amplitude of this response was not much different to that in HSG cells (Fig. 4C), as summarized in Fig. 4D (n=3, $^{***}P < 0.001$). These results demonstrate that 5-Aza-CdR treatment rescues the expression of the M3R, as well as its function in A253 cells.

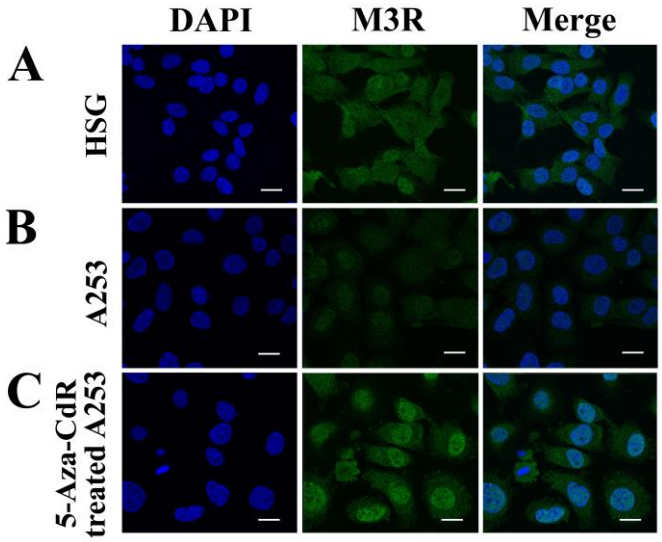

DAPI
M3R
Merge
A
HSG
B
A253
C
5-Aza-CdR
treated A253

Figure 3. Immunocytochemical staining of the M3R before and after 5-Aza-CdR treatment.

The subcellular localization of the M3R was determined by immunostaining in HSG and A253 cells, both before and after 5-Aza-CdR treatment. **(A)** Ubiquitous expression of the M3R, both at the plasma membrane and in the cytosol, in HSG cells which were used as a positive control. **(B)** Expression of the M3R in A253 cells. **(C)** Expression of the M3R in A253 cells after 5-Aza-CdR-treatment. (Original magnification, ×400; scale bar, 50 μm)

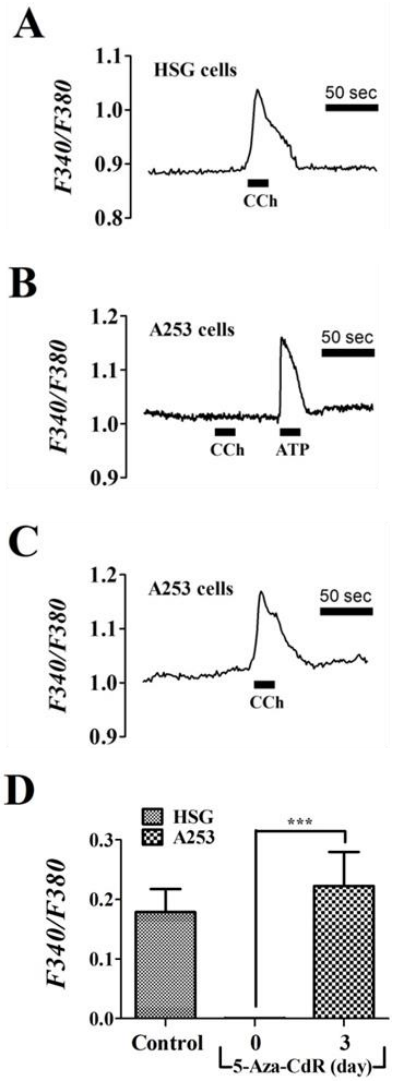
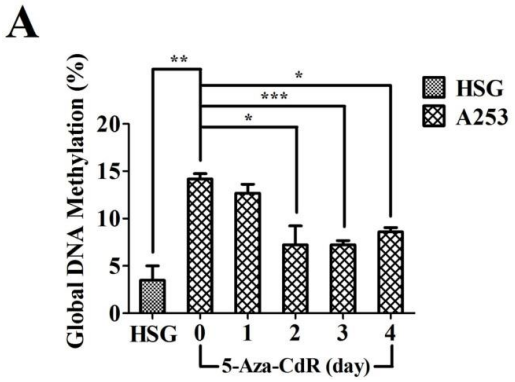

A
HSG cells
50 sec
F340/F380
1.1
1.0
0.9
0.8
CCh
B
A253 cells
50 sec
F340/F380
1.2
1.1
1.0
0.9
CCh
ATP
C
A253 cells
50 sec
F340/F380
1.2
1.1
1.0
0.9
CCh
D
HSG
A253
***
F340/F380
0.3
0.2
0.1
0.0
Control
0
3
5-Aza-CdR (day)

Figure 4. Rescue of M3R function by 5-Aza-CdR treatment.

Measurements of intracellular free calcium concentrations ($[Ca^{2+}]_i$) in fura-2 loaded cells. The magnitude of the $[Ca^{2+}]_i$ response induced by carbachol (CCh), a typical M3R agonist, was used to assess the extent of M3R function in HSG cells, untreated A253 cells, and treated A253 cells. **(A)** A typical $[Ca^{2+}]_i$ response to 10 μM CCh in HSG cells. **(B)** A representative $[Ca^{2+}]_i$ response to 10 μM CCh and 100 μM ATP in A253 cells. **(C)** A representative $[Ca^{2+}]_i$ response to 10 μM CCh in A253 cells treated with 5-Aza-CdR. **(D)** Summary of all experiments, each of which was performed in triplicate ($^{***}P < 0.001$).

Hypermethylation of the CpG island in A253 cells

My data demonstrate that hypermethylation, possibly in CpG islands, may have occurred in the M3R gene in A253 cells. Thus, I next examined the global DNA methylation levels in A253 cells, both before and after treatment with 5-Aza-CdR. We extracted genomic DNA from 5-Aza-CdR-treated and untreated A253 cells, and then quantitatively determined the 5-methylcytosine content in the isolated genomic DNA. Untreated A253 cells exhibited significantly higher 5-methylcytosine content compared with HSG cells (Fig. 5A). However, treatment of A253 cells with 5-Aza-CdR reduced their 5-methylcytosine content; moreover, this reduction was proportional to the length of the incubation period. After 2 days of treatment with 5-Aza-CdR, the 5-methylcytosine content of A253 cells was significantly reduced to a level similar to that detected in HSG cells (n=3, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). This result indicates that A253 cells (a salivary gland tumor cell line) are hypermethylated compared with HSG cells. The methylation status of the M3R CpG island was further investigated by methylation-specific PCR using DNA isolated from HSG, A253, and 5-Aza-CdR-treated A253 cells. 5-Aza-CdR treatment induced a strong unmethylation-specific band, which was not observed in untreated A253 cells (Fig. 5B). This finding suggests that the expression level of the M3R is at least partially regulated by the methylation status of the M3R CpG island region.


A
Global DNA Methylation (%)
20
15
10
5
0
**
*
***
*
HSG
A253
HSG
0
1
2
3
4
5-Aza-CdR (day)

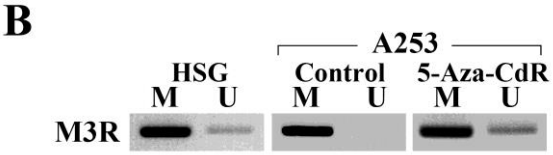

B
A253
HSG
Control
5-Aza-CdR
M
U
M
U
M
U
M3R

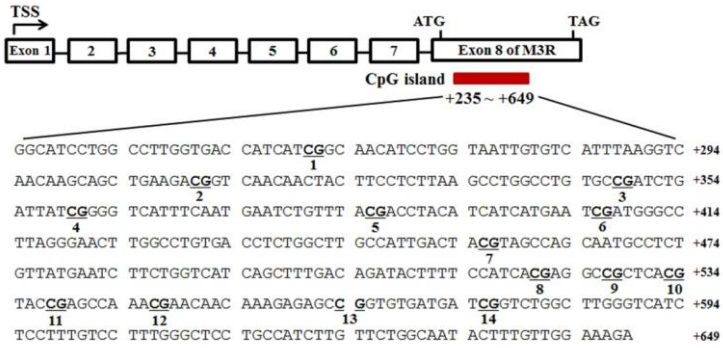
Figure 5. Methylation levels in total DNA and the M3R CpG island upon treatment with 5-Aza-CdR.

(A) Global DNA hypomethylation in A253 cells upon 5-Aza-CdR treatment. Total DNA was extracted from HSG cells (as a control) and A253 cells, both before and after 5-Aza-CdR treatment. The 5-methylcytosine content of each preparation of DNA was then determined quantitatively. The 5-methylcytosine content decreased proportionally to the length of the incubation period with 5-Aza-CdR. **(B)** Induction of hypomethylation by 5-Aza-CdR in hypermethylated A253 cells. Methylation-specific PCR was performed with sodium bisulfite-modified DNA samples, obtained from either 5-Aza-CdR-treated or untreated A253 cells. M, methylated M3R CpG island PCR products. UM, unmethylated M3R CpG island PCR products. 5-Aza-CdR treatment induced an unmethylation-specific band in A253 cells. All experiments were performed in triplicate ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

Bisulfite sequencing of the M3R CpG island, both before and after 5-Aza-CdR-treatment

I next studied the methylation pattern within the M3R CpG island in A253 cells, both before and after treatment with 5-Aza-CdR. PCR products were cloned into pCR2.1-TOPO, and five separate clones were sequenced. The DNA methylation status of each clone was determined by analyzing the sequence of the CpG island in the human M3R. I observed 14 CG pairs, located +235 to +649 base pairs away from the start codon of exon 8, a coding region in the M3R gene (Fig. 6A). In untreated A253 cells, these CG pairs were heavily methylated, from the 1st to 14th CG pair, as expected. However, 5-Aza-CdR-treatment demethylated one of the methylated CG pairs in untreated A253 cells (Fig. 6B). In my study, the 4th, 7th, 8th and 11th methylated CG pairs were demethylated by 5-Aza-CdR-treatment in A253 cells; moreover, M3R function was completely restored in these cells, as evidenced by their calcium responses to CCh.

A


TSS
ATG
TAG
Exon 1
2
3
4
5
6
7
Exon 8 of M3R
CpG island
+235 ~ +649
GGCATCCTGG CCTTGGTGAC CATCATCGGC AACATCCTGG TAATTGTGTC ATTTAAGGTC +294
AACAAGCAGC TGAAGACGGT CAACAACTAC TTCCTCTTAA GCCTGGCCTG TGCCGATCTG +354
ATTATCGGGG TCATTTCAAT GAATCTGTTT ACGACCTACA TCATCATGAA TCGATGGGCC +414
TTAGGGAACT TGGCCTGTGA CCTCTGGCTT GCCATTGACT ACGTAGCCAG CAATGCCTCT +474
GTTATGAATC TTCTGGTCAT CAGCTTTGAC AGATACTTTT CCATCACGAG GCCGCTCACG +534
TACCGAGCCA AACGAACAAC AAAGAGAGCC GGTGTGATGA TCGGTCTGGC TTGGGTCATC +594
TCCTTTGTCC TTTGGGCTCC TGCCATCTTG TTCTGGCAAT ACTTTGTTGG AAAGA +649

B

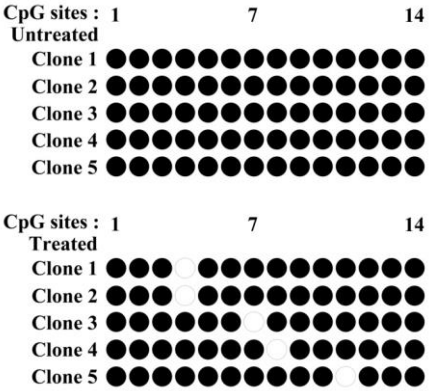

CpG sites : 1 7 14
Untreated
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
CpG sites : 1 7 14
Treated
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5

Figure 6. Bisulfite sequencing of the M3R CpG island before and after 5-Aza-CdR-treatment.

(A) The M3R CpG island (GenBank no. NM_000740) was analyzed. This sequence spans from base pair +235 to +649 (relative to the start codon), and includes 14 CG pairs inside exon 8, a coding region. The start (ATG) and stop (TAG) codons in exon 8 are shown. The CG sites within this sequence are underlined and shown in bold. TSS indicates transcription start site. **(B)** DNA isolated from either 5-Aza-CdR-treated or untreated A253 cells was treated with bisulfite, and the M3R CpG island was PCR amplified. The resultant PCR product was ligated into pCR2.1-TOPO using the TA cloning system. Five clones from control cells and five clones from 5-Aza-CdR-treated cells were picked and sequenced. Symbols: ○, unmethylated cytosines; ●, methylated cytosines. Demethylation was observed at one of the 14 CG pairs.

4. Discussion

Epigenomic studies are well-established, and have been successfully applied to screen for novel candidate genes in many types of human cancer (Glazer et al., 2009; Hoque et al., 2008; Smith et al., 2009; Yamashita et al., 2002). Abnormal gene expression in tumor cells may result from alterations in copy number, sequence mutations, and/or epigenetic dysregulation (McCabe et al., 2009).

Here, I employed an epigenetic strategy to compare the A253 and HSG cell lines. The A253 cell line was originally derived from a human submandibular gland tumor (Giard et al., 1973), and has been used to study secretory mechanisms such as chloride transport (Roomans, 1998) and intracellular signal transduction pathways (Sugita et al., 1999). Although A253 cells possess β-adrenergic and P2-purinergic receptors, they lack the functional expression of α-adrenergic or muscarinic cholinergic receptors (Marmary et al., 1989; Zhang et al., 1997). Thus, in contrast to HSG cells, A253 cells do not respond to carbachol, which normally increases $[Ca^{2+}]_i$. The M3R, a G protein-coupled receptor (GPCR), belongs to the largest transmembrane receptor superfamily of muscarinic receptors, and is expressed in both human and mice. The M3R is characterized by a seven-transmembrane α-helix structure, and is comprised of 8 exons with the receptor-coding sequence located within exon 8 (Forsythe et al., 2002; Pierce et al., 2002). In general, the binding of a ligand to its cognate GPCR elicits Ca^{2+} and/or PKC signaling cascades that induce the expression of genes required for multiple fundamental functions, including exocrine and endocrine secretion, smooth muscle and cardiac muscle contraction, pain

transmission, fluid homeostasis, blood pressure regulation, and immune responses (Pierce et al., 2002).

The M3R is well established to play a key role in fluid secretion by increasing $[Ca^{2+}]_i$. However, the expression pattern and function(s) of the M3R are still poorly understood, especially in salivary gland tumor cells such as A253 cells. I hypothesized that the mechanism of suppression of M3R function might involve hypo and/or hypermethylation of the M3R gene in salivary gland tumor cells. In our study, one methylated CG pair at the 4th, 7th, 8th, and 11th sites was demethylated by 5-Aza-CdR-treatment. This treatment also rescued M3R function, as demonstrated by the CCh-induced calcium response observed in A253 cells. My results are also consistent with previous studies. The CpG island of the 14-3-3 σ gene in salivary gland adenoid cystic carcinoma cells was found to be methylated inside the exon, whereas HSG cells showed very limited pattern of hemimethylation in the 14-3-3 σ gene. Methylation occurring at CpG sites inside the exon, a coding region, has been shown to significantly reduce the expression of 14-3-3 σ (Uchida et al., 2004). Therefore, it is highly likely that internal methylation within an exon, a coding region, can affect the DNA-binding activity of transcription factors; moreover, inhibition of this methylation is likely to correlate with increased expression of the relevant gene. Of particular note in our study, I found that any demethylation occurring at even only one site among a total of 14 methylated CG sites was sufficient to increase the transcriptional activity of the M3R gene, resulting in increased M3R protein expression and increased M3R function. However, it is not yet clear whether these four CG sites among the 14 CG sites are functionally specific, or if any site of CG unmethylation is sufficient to restore M3R function.

To the best of in my knowledge, this is the first study reporting that 5-Aza-CdR can epigenetically regulate the expression and function of the M3R found in salivary gland tumor cells. 5-Aza-CdR is a pyrimidine nucleoside analog that strongly inhibits DNA methyltransferase activity, and is one of the strongest known inhibitors of DNA methylation. Several lines of evidence have indicated that demethylation of gene-specific sequences is associated with cell differentiation (Lubbert et al., 1996). At low concentrations of 5-Aza-CdR, an immortalized normal human salivary gland duct cell line lacking the expression of aquaporin 5 was shown to acquire aquaporin 5 expression (Motegi et al., 2005).

In conclusion, the results of this study strongly suggest that 5-Aza-CdR-mediated demethylation in the M3R CpG island induces M3R gene expression in A253 cells, a salivary gland tumor cell line, thereby increasing the level of the M3R protein. This increased level of M3R protein restores M3R function, as evidenced by the increased $[Ca^{2+}]_i$ response to CCh stimulation. Since low doses of 5-Aza-CdR have been used to treat patients with myelodysplastic syndrome, and have been shown to be well-tolerated by this group of patients (Wijermans et al., 2000), this drug might be an effective therapeutic option for patients with salivary gland tumors. Thus, My results may provide a clue for developing new therapeutic drugs for cancer.

5. References

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국문 초록

타액선 상피세포 내 염증 및 무스카리닉 수용체 조절기전

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정상적인 타액분비는 구강건강을 유지하는데 필수 조건이다. 타액선에서 염증이 있을 경우 이는 타액분비에 영향을 미쳐 구강건강을 해치게 된다. 하지만, 타액선에서 일어나는 염증기전에 대해서는 잘 알려져 있지 않다. 타액분비 시 가장 큰 역할을 하는 것은 콜린 동작성 무스카리닉 수용체이다. 따라서, 본 연구에서는 TRPV1 수용체와 그 효현제인 캡사이신 (capsaicin) 을 중심으로 한 타액선 염증기전 및 무스카리닉 수용체 조절기전을 후성유전학적인 연구 방법을 통해 규명해 보았다.

본 연구 결과, 타액서 상피세포는 poly(I:C)나 LPS와 같은 Toll-like

receptors (TLRs) 의 효현제에 의해 자극을 받으면, pro-inflammatory 사이토카인인 TNFα와 IL-6가 유리됨을 관찰하였고, 캡사이신은 이들 사이토카인의 유리를 유의하게 억제시켰다. 이러한 캡사이신의 항염증 효과는 길항제인 캡사제핀 (capsazepine) 에 의해 억제되지 않았으며, 이는 TRPV1 Knock Out을 이용한 생쥐 실험에서도 확인되었다. 결론적으로 캡사이신에 의한 항 염증효과는 NF-κB를 통한 신호전달 경로를 조절함으로써 일어남을 밝혀내었다.

무스카리닉 수용체 조절기전에 관한 연구에서는, A253세포의 경우 무스카리닉 수용체 타입 3 (M3R) 유전자가 과메틸화 (hypermethylation) 되어 있음을 밝혀내었다. A253 세포에서 과메틸화된 M3R은 DNA demethylating agent인 5-aza-2-deoxycytidine (5-Aza-CdR) 처리에 의해 M3R 단백질의 발현량이 증가하고, M3R의 기능도 회복되는 것을 관찰할 수 있었다. 끝으로 5-Aza-CdR처리에 의해 실제로 M3R이 demethylation 된 것을 bisulfite sequencing 분석실험을 통해 확인하였다. 이러한 연구를 통해 타액선 상피세포내 캡사이신이 염증 조절기전에 관여함과 무스카리닉 수용체의 조절기전에 있어 후성유전학의 한 분야인 DNA 메틸화가 영향을 미친다는 것을 알 수 있다.

주요어: 타액선, 캡사이신, TNFα, IL-6, 캡사제핀, NF-κB, 후성유전학, 무스카리닉 수용체, A253, 과메틸화, 5-Aza-CdR

학 번: 2010-30650