Procaine stimulates aquaporin-5 expression in human salivary gland ductal cells via the suppression of DNA methyltransferase-1

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Received July 11, 2017; Accepted December 11, 2017

DOI: 10.3892/mmr.2018.8821

Abstract. The present study aimed to investigate whether procaine may upregulate the expression of aquaporin-5 (AQP5) in human salivary gland ductal cells and the underlying mechanisms of this upregulation. Immortalized normal human salivary gland ductal cells (NS-SV-DC), lacking AQP5 protein expression, were used to measure the glandular secretion rate following treatment with procaine, and the protein expression levels of AQP5 in NS-SV-DC cells were measured by western blotting. In order to investigate the mechanism of procaine action on AQP5 protein expression, the protein expression and activity of DNA methyltransferase (DNMT)1, and the CpG methylation of AQP5, were investigated further. In NS-SV-DC cells treated with procaine, the mRNA and protein levels of AQP5, and the secretion rate of cells, were significantly increased. Although no significant alterations were observed in the protein expression of DNMT1 following procaine treatment, its enzymatic activity was reduced, resulting in CpG island demethylation at Sp1-2 and Sp1-3 sites of the AQP5 gene, which may contribute to the significantly upregulated AQP5 gene expression. The results of the present study indicate that procaine may upregulate the protein expression of AQP5 in human submandibular glands by inhibiting the activity of DNMT1 and promoting liquid secretion. The procaine-mediated expression of AQP5 may provide a novel regimen for the treatment of SS syndrome.

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Key words: Sjogren's syndrome, procaine, aquaporin-5, salivary gland ductal cells, DNA methyltransferase-1, suppression

Introduction

Sjogren's syndrome (SS) is a chronic inflammatory autoimmune disease that usually results from a functional decline caused by lymphocyte invasion into salivary and lacrimal glands, and is associated with a dry mouth, dry eyes and other symptoms (1,2). In the United States, SS has been listed as the second most common human autoimmune disease (3). Worldwide, >80% of patients with SS suffer from oral dryness, diminished taste, angular cheilitis and oral bacterial infections (4). Although SS syndrome has been previously investigated, an understanding of its pathogenesis remains incomplete (5,6). At present, the treatment of SS syndrome primarily depends on traditional Chinese medicinal therapy, in which the effects occur slowly, and its effectiveness and safety require further investigation (7).

Aquaporins (AQPs) are a family of transmembrane proteins that selectively allow the passage of water molecules. In 1988, the first member of the AQP family was identified and termed 'AQP1' by Denker *et al* (8). A total of 10 AQPs with tissue-specific distributions were identified later, among which AQP5 was the only protein that was confirmed to be expressed in the salivary gland acinar cells with a high density in the lacrimal and submandibular glands (9-11). Previous studies demonstrated that the distribution of AQP5 was consistent with the sites of SS syndrome, and AQP5 expression in salivary gland cells increased with stimulation by interferon-α and increased the level of glandular secretion (12-14).

Motegi *et al* (15) have demonstrated that hypomethylation of the CpG island in the AQP5 promoter region increases AQP5 mRNA expression, thereby increasing AQP5 protein expression in the human submandibular gland and subsequent glandular secretion. Reports have indicated that procaine was investigated at the clinical stage as a candidate for anticancer treatment due to its specific inhibition of DNA methyltransferase (DNMT) activity (16,17). This mechanism indicates that procaine may induce AQP5 expression by inhibiting the activity of DNMT1. In the present study, human immortalized submandibular gland ductal cells (NS-SV-DC), lacking AQP5 protein expression, were used as cell models to investigate the effect of procaine on SS syndrome.

Materials and methods

Materials and reagents. PcDNA3.1 (+) expression vector and simian virus 40 T antigen (SV40T) template plasmid (Promega Corporation, Madison, WI, USA); DH5α competent cells (Takara Bio, Inc., Otsu, Japan); Takar Ex Taq® enzyme and T4 DNA ligase (Takara Bio, Inc.); reverse transcription-quantitative polymerase chain reaction (RT-qPCR) primers for GAPDH and AQP5 (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA); mouse monoclonal immunoglobulin (Ig)G1 antibodies against AQP5, β-actin and DNMT1 (cat. nos. sc-514022, sc-8432 and sc-271729, respectively; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); TOP10F' competent cells (Invitrogen; Thermo Fisher Scientific, Inc.).

Plasmid Extraction kit and Agarose Gel Electrophoresis kit (Tiangen Biotech Co., Ltd., Beijing, China); Serum-free Keratinocyte Medium (SFKM; Gibco; Thermo Fisher Scientific, Inc.); MTT solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); TRIzol™ reagent kit and TA cloning system (Invitrogen; Thermo Fisher Scientific, Inc.); Advantage cDNA PCR kit (Clontech Laboratories, Inc., Mountainview, CA, USA). Mem-PER Eukaryotic Membrane Protein Extraction reagent kit (Pierce; Thermo Fisher Scientific, Inc.); Transwell-COL culture chamber (Corning Incorporated, Corning, NY, USA); EpiQuik™ DNA Methyltransferase Activity assay kit (Epigentek Group, Inc., Farmingdale, NY, USA); Wizard® DNA Purification kit and Luciferase Assay System (Promega Corporation); EZ DNA Methylation kit (Zymo Research Corp., Irvine, CA, USA).

Establishment of immortalized NS-SV-DC or acinar (NS-SV-AC) phenotype. Primers were designed according to the SV40T sequence as follows: SV40T-forward, 5'-GGAATTCATGGA TAAAGTTTTAAACAGAGGAATCTTTGCA-3' (*Eco*R I, underlined sequence); and SV40T-reverse, 5'-CCTCGAGTTATG TTTCAGGTTCAGGGGGAGGTGTGGGAGGTT-3' (*Xho* I, underlined sequence).

The reaction conditions of the PCR amplification were as follows: 5 min of pre-denaturation at 94˚C; 30 sec of denaturation at 94˚C, 30 sec of annealing at 58˚C and 2.5 min of extension at 72˚C for 30 cycles; and 10 min of extension at 72˚C. The PCR product was analyzed using an agarose gel electrophoresis kit (cat. nos. DP209-03, Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions.

For the construction and identification of vectors, the pcDNA3.1 (+) expression vector and the PCR product were digested at 37˚C for 2 h with *EcoR* I and *Xho* I restriction endonucleases. Following ligation with T4 DNA ligase overnight at 4˚C, the ligated product was added to DH5α competent cells for transformation (Takara Bio, Inc.) according to the manufacturer's protocols and incubated at 37˚C for 3 days. Then, the plasmid was extracted from DH5α competent cells and subsequently digested using *EcoR* I and *Xho* I restriction endonucleases. The linearized product obtained by enzyme digestion was stored at -20˚C. Simultaneously, the plasmid was performed by sequencing identification by Sangon Biotech Co., Ltd. (Shanghai, China).

Establishment of cell lines. The characteristics of immortalized NS-SV-DC and NS-SV-AC cell clones have already been described previously (18,19). The cryopreserved human submandibular gland cells from a submandibular gland with no histopathological disorders were resuscitated and cultured in an incubator with SFKM medium and 0.5% CO_2 at 37˚C. When the cell confluence reached 90%, cells were transfected with the pcDNA3.1-SV40T plasmid using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols, then the cells were cultured for 24 h and diluted. Following culture for about 12 passages, the cells with unaltered phenotype observed by inverted-phase contrast microscope (Olympus Corporation, Tokyo, Japan, magnification, x100) were collected for the subculture of human NS-SV-AC and NS-SV-DC monoclonal cells, as previously described (18,19). The submandibular gland tissue was obtained from a 32-year old healthy female who had given written informed consent at October 2013 in The Affiliated Hospital of Hebei University (Baoding, China). The present study was approved by the Medical Ethics Committee of The Affiliated Hospital of Hebei University (Baoding, China).

Detection of cell growth. Following 3-4 passages, the NS-SV-DC cells were seeded into 96-well plates ($1x10^4$/well) and incubated for 12 h at 37˚C until adhered to the bottom, and the supernatant was discarded. A volume of 200 µl SFKM medium containing different concentrations of procaine (0, 500 nM, and 1 and 2 µM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added into the 96-well plates. A total of 20 µl MTT solution (5 mg/ml) was added to the cells on the 1st, 2rd, 4th and 7th day of incubation. The cells incubated with MTT solution was continued for 4 h at 37˚C and then formazan crystals were dissolved with 200 µl dimethyl sulfoxide at 37˚C for 10 min. The cell number was calculated by measuring optical density at 450 nm (Synergy 4; BioTek Instruments, Inc., Winooski, VT, USA).

Primer design and RT-qPCR. According to the whole gene sequence of AQP5 and GAPDH (Seq ID nos. K09867 and K00134, respectively) described by the Kyoto encyclopedia of Genes and Genomes (http://www.kegg.jp/), primers were designed respectively as follows: AQP5 upstream primer 5'-CAAGGCCGTGTTCGCAGAGTTCT-3', AQP5 downstream primer 5'-TCTTCCGCTCTTCCCGCTGCTCC-3', amplified product 739 bp; GAPDH upstream primer 5'-ACG CATTTGGCTGTATTGGG-3', GAPDH downstream primer 5'-TGATTTTGGAGGGATCTCGC-3', amplified product 280 bp.

The NS-SV-DC cells were seeded into 96-well plates ($1x10^4$/well) for 12 h and cultured with SFKM medium containing procaine (2 µM). Following 0, 48, 72, 96 and 120 h culture, the cells were collected. The total RNA was extracted by TRIzol reagent kit, with normal human submandibular gland cells used as a positive control and untreated NS-SV-AC cells served as a negative control. cDNA synthesis was performed by Advantage cDNA PCR kit according to the manufacturer's instructions. The reaction of RT-qPCR primers (AQP5: Hs00387048 m1; Applied Biosystems; Thermo Fisher Scientific, Inc.) was performed using the synthesized AQP5 cDNA as template with GAPDH as the internal reference. RT-qPCR measurements were performed on CFX96 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with a SYBR® Premix

Ex Taq kit (Takara Bio, Inc.). The temperature process was 95˚C for 30 sec followed by 40 cycles of amplification (95˚C for 5 sec, 60˚C for 30 sec, and 72˚C for 30 sec). The results were analyzed using $2^{-\Delta\Delta Cq}$ method (20).

Detection of protein expression by western blotting. The NS-SV-DC cells treated with procaine (2 μM) at different times (0, 48, 72, 96 and 120 h) were collected and rough extraction of cell membranes was performed using Eukaryotic Membrane Protein Extraction Reagent kit. Following protein quantification with BCA protein assay reagent kit (Invitrogen; Thermo Fisher Scientific, Inc.), SDS-PAGE electrophoresis was performed using a total of 30 μg proteins separated on 12% SDS-PAGE gels. The protein aggregations were collected, transferred to a polyvinylidene fluoride membrane (END Millipore, Billerica, MA, USA), blocked with 3% bovine serum albumin (Sigma-Aldrich; Merck KGaA) and kept overnight at 4˚C. Following blocking, the membranes were incubated with mouse monoclonal IgG1 AQP5 antibody (dilution: 1:100) for 1 h at 37˚C, washed three times with PBS-Tween-20 (PBST, 0.1% Tween) and subsequently incubated with horseradish peroxidase (HRP)-conjugated antibodies (dilution: 1:1,000; cat. no. sc-516102, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, Inc.) for 1 h at 37˚C, washed with PBST and detected by an Enhanced Chemiluminescence assay kit (Thermo Fisher Scientific, Inc.). The protein expression of DNMTs was analyzed by the same method with mouse monoclonal IgG1 DNMT1 (1:100), DNMT3A (dilution: 1:100; cat. no. sc-373905, Santa Cruz Biotechnology, Inc.) and DNMT3B (dilution: 1:100; cat. no. sc-70984, Santa Cruz Biotechnology, Inc.) antibodies and corresponding HRP-conjugated antibodies (dilution: 1:1,000; cat. no. sc-516102). β-actin protein was used as internal reference and detected as aforementioned with mouse monoclonal β-actin antibody (dilution: 1:100; cat. no. sc-8432, Santa Cruz Biotechnology, Inc.) and corresponding HRP-conjugated antibodies (dilution: 1:1,000; cat. no. sc-516102). Images were captured using a ChemiDoc™ XRS+ image analyzer (Bio-Rad Laboratories, Inc.) and quantification of the protein results was performed using ImageJ 1.47i software (National Institutes of Health, Bethesda, MD, USA).

Determination of glandular secretion rate. The glandular secretion rate was determined as described previously (21). NS-SV-DC cells were seeded into the upper chamber of the Transwell-Col culture chamber, which had a diameter of 24.5 mm, and cells were cultured with 500 or 2 μM procaine. The non-treated cells were used as controls. Following 48 h, the medium in the upper chamber was replaced with 0.4 ml hypertonic medium (400 mOsm, i.e. 100 mM sucrose), while in the lower chamber fresh isotonic medium (300 mOsm) was added. Following 4 h, the liquid in the upper chamber was collected and measured by determining the alteration in volume in the upper chamber to determine the secretion rate with a calibrated pipette.

Analysis of DNMT activity. DNA was extracted from the NS-SV-DC cells treated with procaine (500 nM and 2 μM) at different times (0, 6, 12, 24 and 48 h) using a Wizard DNA Isolation kit (cat. no. A1120, Promega Corporation), and DNA methyltransferase activity was determined with an EpiQuik™ DNA Methyltransferase Activity Assay kit.

Analysis of CpG methylation in AQP5 gene. The genomic DNA was extracted from NS-SV-DC cells treated with procaine for 48 h using Promega's Wizard® DNA Purification kit. The non-treated cells were used as controls. The extracted DNA was modified by bisulfite using the EZ DNA Methylation kit for PCR amplification (upstream primer, 5'-GGGAATTTCGGT TTGGGAGA-3'; downstream primer, 5'-CCCGTCCGAACC ACGTAAC-3'). The amplification conditions were as follows: 1 min pre-denaturation at 94˚C; 30 sec denaturation at 94˚C and 2 min annealing at 68˚C, repeated 35 cycles; and 3 min of extension at 72˚C. The amplified fragment was inserted into the pCR2.1-TOPO plasmid vector (Invitrogen; Thermo Fisher Scientific, Inc.) by TA cloning system and transformed into TOP10 F' competent cells (One Shot™ TOP10F' Chemically Competent *E. coli*, Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols, followed by plasmid extraction. A total of six monoclonal strains were selected from the experimental group and the control group, respectively, for plasmid sequencing by Sangon Biotech Co. Ltd.

Detection of luciferase activity. The AQP5 promoter fragment (575 bp) containing the transcription initiation site was amplified according to the genomic DNA sequence (GenBank no. AH006636, https://www.ncbi.nlm.nih.gov/nuccore/). The amplification primers were as follows: Upstream primer 5'-CTC GAGAAGGGGAACCCCGGCCGGGAGAG-3', underlined *Xho*l site; downstream primer 5'-AAGCTTGTCCGGGCC ACGTGACCCAGG-3', underlined *Hind*III site. The amplified fragment was inserted into the PGL3 Basic vector (Promega Corporation) containing luciferase reporter gene to perform transformation, coating, sequencing and double-enzyme digestion. Subsequently, the human luciferase reporter plasmid of AQP5 promoter pC3-Luc was successfully constructed. PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to analyze the AQP5 promoter sequence. The AQP5 promoter fragment contains three Sp1 binding sites: Sp1-1, Sp1-2 and Sp1-3 sites. In order to further investigate the effect of methylation on the expression of AQP5 protein, methylation of the above three sites was performed with different methylation modification. All CGs (Sp1-1: 1st CG; Sp1-2: 23rd and 24th CGs; 31st CG and Sp1-3: 33rd CG) in the pD1-Luc plasmid were methylated; all CGs in pD2-Luc plasmid were methylated except for the 24th CG; the 1st, 23rd and 31st CGs in pD3-Luc plasmids were methylated; and the 1st, 23rd, 24th and 31st CGs in pD4-Luc plasmids were methylated. The four constructed plasmids were transfected into untreated NS-SV-DC cells using Lipofectamine® 2000 (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocols. Following incubation for 24 h at 37˚C after transfection, the luciferase activity was detected by Luciferase Assay System. The luciferase activity of each sample was normalized to the amount of protein in the cell lysate which was measured with BCA protein assay reagent kit (Pierce; Thermo Fisher Scientific, Inc.).

Statistical analysis. GraphPad Prism 7.0 (GraphPad software, Inc., La Jolla, CA, USA) was used for data analysis and the

data were presented as mean ± standard deviation. Quantitative data between groups were compared and analyzed using one- and two-way analysis of variance and Dunnett's multiple comparisons test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of procaine on cell growth. Cell numbers were measured by MTT reagent at 1st, 2rd, 4th and 7th day of incubation to test the effects of different concentrations of procaine (0, 500 nM, and 1 and 2 μM) on the growth of NS-SV-DC cells (Fig. 1). The results demonstrated that 2 μM procaine had no significant impact on cell growth, which indicated that this concentration of procaine is nontoxic or of low toxicity to cells (Fig. 1). Therefore, 2 μM procaine was used for subsequent experiments.

Effect of procaine on fluid secretion of NS-SV-DC cells. Although procaine is widely used as a clinical drug, to the best of our knowledge, there have been no previous reports of procaine use for SS syndrome treatment. Therefore, it was important to confirm whether procaine may promote fluid secretion by NS-SV-DC cells. AQP is considered to mediate the passive transmembrane transport of free water. In the present study, Transwell culture plates were used to simulate the osmotic pressure gradient model with the osmotic pressure of the cell culture higher compared with the lower chamber. Following incubation for 4 h, the volume of liquid was quantified and the cell capacity of fluid secretion was calculated by the alteration in volume in the upper chamber (Fig. 2). The liquid transport capacity of the untreated NS-SV-DC cells was ~3.8 μl/cm^2/h, while the cell transport capacity of NS-SV-DC cells treated with 500 or 2 μM procaine was 5.87 and 7.0 μl/cm^2/h, respectively, which was significantly increased compared with the control group (Fig. 2), indicating that procaine promoted fluid secretion by NS-SV-DC cells.

Effect of procaine on AQP5. The mRNA expression of AQP5 in NS-SV-DC cells was measured by RT-qPCR at 0, 48, 72, 96 and 120 h (Fig. 3). The AQP5 mRNA content of the untreated NS-SV-DC cells was ~10% of that in normal submandibular gland cells. The AQP5 mRNA content of NS-SV-DC cells treated with procaine was significantly increased with the extension of processing time and the mRNA content was close to that in the normal submandibular gland cells following 120 h incubation (Fig. 3).

The protein expression of AQP5 was determined by western blotting (Fig. 4), with untreated cells at the different time points serving as controls. Consistent with the alteration of AQP5 mRNA expression, the AQP5 protein expression increased as the processing time increased. The protein content ratios of AQP5/β-actin at the different time points (0, 48, 72, 96 and 120 h) were 0, 0.25, 0.25, 0.27 and 0.78, respectively, which revealed that procaine significantly upregulated the expression of AQP5 at 120 h compared with other durations (Fig. 4).

Content and activity of DNMT1 in the cells treated with procaine. Procaine, which specifically inhibits the activity of DNMT1, has been investigated up to clinical stage II as a potential anticancer drug. A study has confirmed that hypomethylation of the CpG island in the AQP5 promoter region promoted AQP5 protein expression in NS-SV-DC cells (15). In order to study the effect of procaine on DNMTs in the present study, the expression of DNMTs (DNMT1/3A/3B) in NS-SV-DC cells was detected by western blotting, presented in Fig. 5A. The results demonstrated that there were no significant alterations in the expression of DNMTs with increasing time of procaine treatment, which indicated that procaine did not influence the expression of DNMTs protein in NS-SV-DC cells.

The effect of procaine on the activity of DNMT1 was further investigated using an EpiQuik™ DNA Methyltransferase Activity assay kit, which demonstrated that procaine significantly reduced the activity of DNMT1 methyltransferase (Fig. 5B).

Effect of procaine on CpG island demethylation in AQP5 of NS-SV-DC cells. In this experiment, the CpG island methylation in AQP5 was analyzed by methylation-specific PCR. The extracted DNA was initially treated with bisulfite to convert unmethylated C to U, followed by primer design with specific

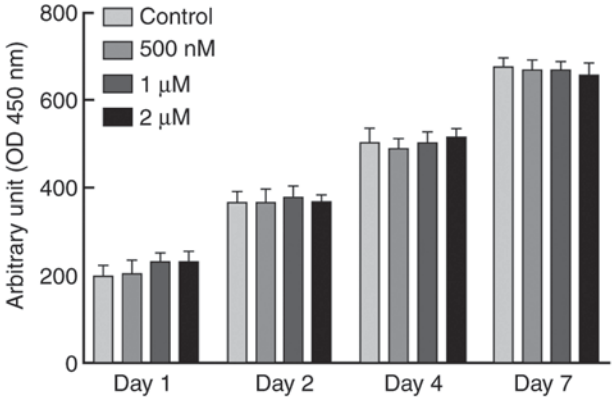

Figure 1. Growth properties of NS-SV-DC cells cultured in the presence or absence of procaine. Cells ($1x10^4$/well) were cultured in 96-well plates in medium supplemented with procaine (0, 500 nM, and 1 and 2 μM) for up to 7 days. Viable cells were estimated by an MTT assay. No significant suppression of cell growth was detected at any concentrations of procaine. NS-SV-DC, normal human salivary gland ductal cells; OD, optical density.

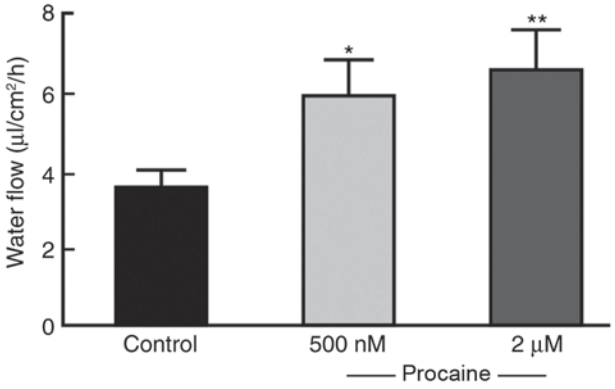

Figure 2. Net fluid secretion rates across NS-SV-DC monolayers. Net fluid secretion rates of control (untreated NS-SV-DC cells) and procaine (500 or 2 μM)-treated NS-SV-DC cells were measured using a hypertonic medium (400 mOsm) at the apical side and isotonic medium at the basolateral side. The results are expressed as fluid flow in μl secreted/cm^2/h and are the mean + standard deviation of three separate experiments performed in triplicate. Untreated cells were used as the control. $^*P<0.05$, $^{**}P<0.01$ vs. control. NS-SV-DC, normal human salivary gland ductal cells.

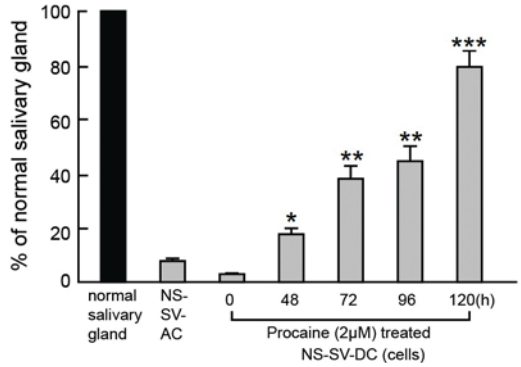

Figure 3. Steady-state levels of AQP5 mRNA was measured using reverse transcription-quantitative polymerase chain reaction. GAPDH mRNA was used as an internal standard. Induced expression levels of AQP5 mRNA in NS-SV-DC cells were compared with the expression level (100%) in normal salivary gland tissues. Each bar represents at least three separate mRNA isolations performed in duplicate. NS-SV-AC cells expressed a relatively small amount of AQP5 mRNA compared with normal salivary gland tissues. Procaine (2 μM)-treated NS-SV-DC ductal cells exhibited a marked increase in the expression of AQP5 mRNA at 120 h following treatment. *P<0.05, **P<0.01, ***P<0.001 vs. NS-SV-DC at 0 h, without procaine. AQP5, aquaporin-5; NS-SV-DC, normal human salivary gland ductal cells; NS-SV-AC, normal human salivary gland acinar cells; normal salivary gland, normal salivary gland cells.

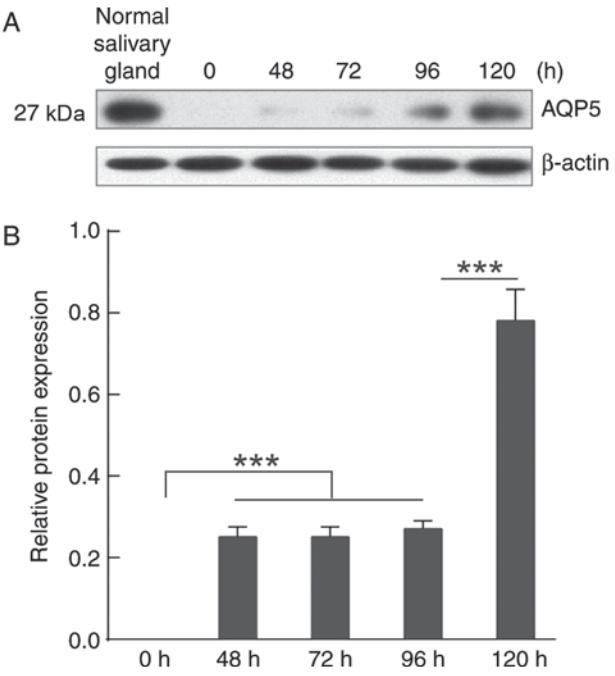

Figure 4. Western blot analysis of AQP5 and β-actin proteins in crude plasma membranes from procaine-treated NS-SV-DC ductal cells. (A) Although the basal level of AQP5 expression (0 h) was not detected, increased expression of AQP5 protein with a molecular weight of 27 kDa was observed in NS-SV-DC cells following treatment with 2 μM procaine between 48 and 120 h of treatment. (B) Densitometric analysis (AQP5/β-actin ratio) revealed the expression level of AQP5 protein in procaine-treated NS-SV-DC cells relative to that of normal salivary gland. These results were similar to those observed for mRNA levels using reverse transcription-quantitative polymerase chain reaction analysis. ***P<0.001. AQP5, aquaporin-5; NS-SV-DC, normal human salivary gland ductal cells; normal salivary gland, normal salivary gland cells.

PCR. The target fragment was obtained and sequenced. Through analysis of the AQP5 promoter sequence by PLANTCARE, three Sp1 binding sites in CpG island of AQP5 were identified, Sp1-1, Sp1-2 and Sp1-3, respectively (Fig. 6A).

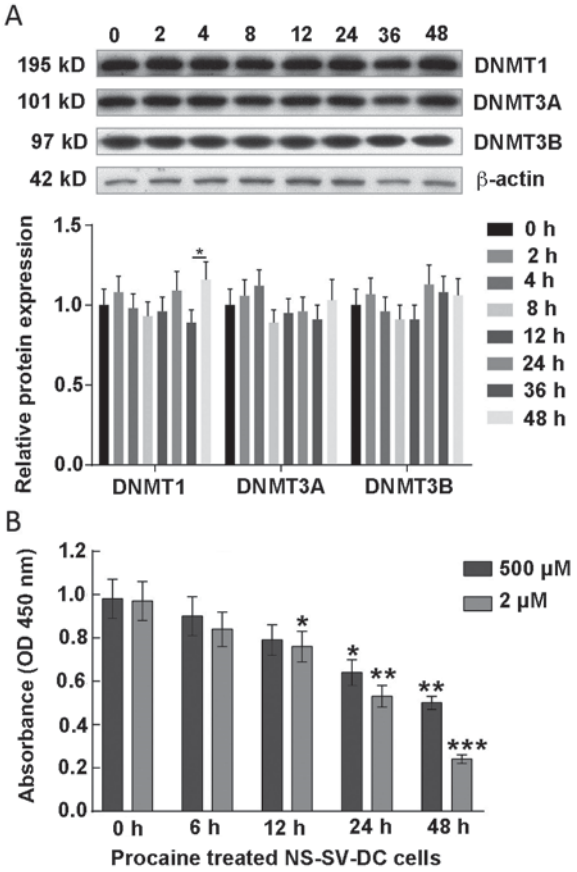

Figure 5. Expression and activation levels of DNMTs in procaine-treated NS-SV-DC cells. (A) Western blot analysis of DNMT1, DNMT3A and DNMT3B proteins in procaine (2 μM)-treated NS-SV-DC cells. (B) Methylation activity assay of procaine-treated NS-SV-DC cells. The methylation of DNMT1 procaine-treated NS-SV-AC cells was measured by using the EpiQuik™ DNA Methyltransferase Activity assay kit. The results are expressed as OD at a wavelength of 450 nm and are the mean ± standard deviation of three separate experiments. *P<0.05, **P<0.01, ***P<0.001 vs 0 h within procaine-treated (500 or 2 μM) group. DNMT, DNA methyltransferase; NS-SV-DC, normal human salivary gland ductal cells; OD, optical density.

The analysis of CpG islands in the experiment and control groups is presented in Fig. 6B. The three Sp1 binding sites in the NS-SV-DC cells not treated with procaine were highly methylated, while the treated cells demonstrated marked demethylation at the Sp1-2 and Sp1-3 sites, which demonstrated that hypermethylation of these two sites may inhibit the expression of the AQP5 gene.

Effect of demethylation at different sites on the expression of AQP5. In order to determine whether procaine may promote the expression of AQP5 via CpG island demethylation at Sp1-2 and Sp1-3 sites, four fluorescent protein reporter plasmids (pD1-Luc, pD2-Luc, pD3-Luc and pD4-Luc) were constructed (Fig. 7A), and transfected into NS-SV-DC cells. Following incubation for 24 h, the luciferase content was measured (Fig. 7B). The results demonstrated that the luciferase activity in the NS-SV-DC cells transfected with the pD2-Luc, pD3-Luc and pD4-Luc plasmids was significantly elevated compared with the empty vector group (P<0.01, P<0.001; Fig. 7B), which indicated that procaine may lead to CpG demethylation of the 23rd and 24th and 33rd CG sites in the AQP5 promoter by inhibiting the activity of DNMT1, subsequently resulting in the upregulation of AQP5 expression.

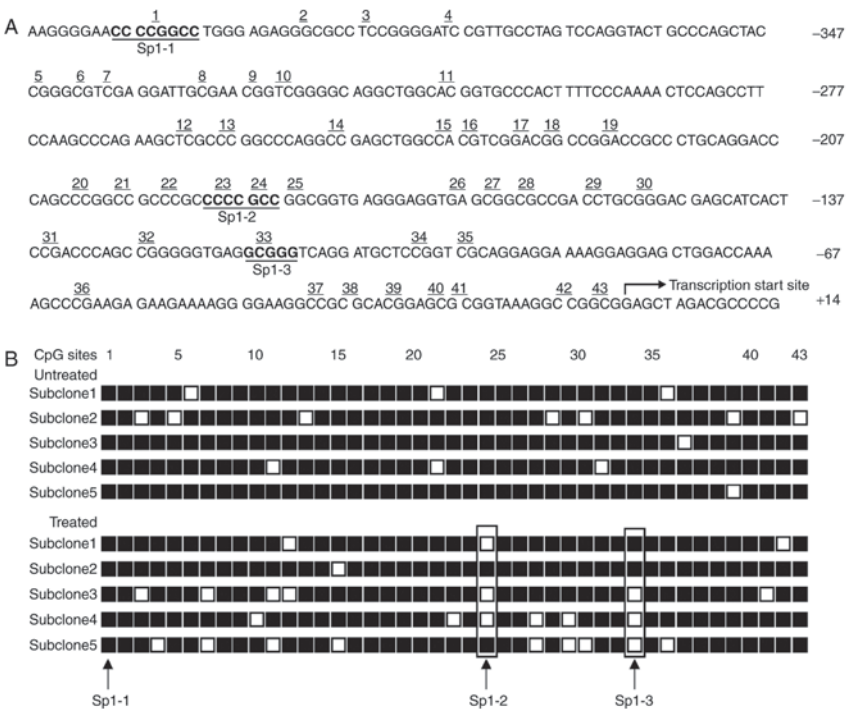

Figure 6. Bisulfite sequencing of the CpG islands in the AQP5 promoter. (A) The CpG island of the AQP5 promoter (GenBank/EMBL Data Bank no. U46566) was analyzed. This sequence spans 578 bp between positions -406 to +172 relative to the transcription start site, including 43 CGs upstream of the transcriptional start site. Three CG-containing Sp1-binding sites within this sequence are indicated as underlined and in bold, corresponding to the 1st, 23rd, 24th and 33rd CGs within this island. (B) DNA from the control NS-SV-DC and procaine (2 μM)-treated NS-SV-DC cells was treated with bisulfite, and the AQP5 promoter was PCR amplified. The PCR product was ligated into pCR2.1-TOPO using the TA cloning system. Five subclones from the control cells and procaine-treated cells were selected and sequenced. Demethylation was observed at the CGs in the second Sp1 (Sp1-2) and the third Sp1 (Sp1-3) sites, as indicated by the boxes. AQP5, aquaporin-5; NS-SV-DC, normal human salivary gland ductal cells; PCR, polymerase chain reaction; □, unmethylated cytosines; ■, methylated cytosines.

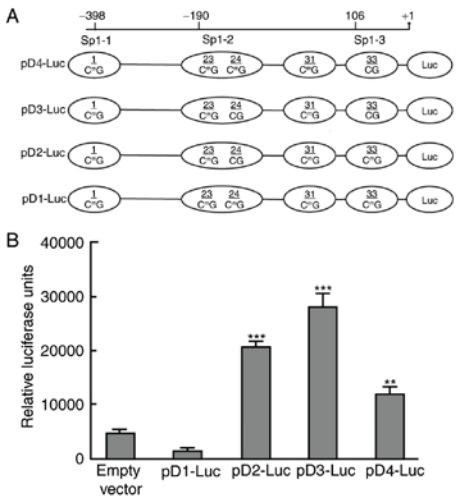

Figure 7. Analysis of relative luciferase activity in NS-SV-DC cells. (A) AQP5-promoter constructs used for the luciferase transfection assay. The human wild-type AQP5 promoter luciferase fusion plasmid, pD1-Luc, contained all methylated CGs, including the 1st, 23rd, 24th, 31st and 33rd, as well as the transcription start site. pD2-Luc contains an unmethylated CG at the 24th position and methylated CGs at the 1st, 23rd, 31st and 33rd positions. pD3-Luc contains unmethylated CGs at the 24th and 33st positions, and methylated CGs at the 1st, 23rd and 31rd positions. pD4-Luc contains an unmethylated CG at the 33rd position and methylated CGs at the 1st, 23rd, 24th and 31st positions. (B) At 24 h following transfection, NS-SV-DC cells were harvested for analysis of luciferase activity. The luciferase activity of each sample was normalized to the amount of protein in the cell lysate. The experiments were performed at least two times in triplicate. The luciferase activity of NS-SV-DC cells transfected with an empty vector served as a control. *P<0.05, **P<0.01 and ***P<0.001 vs. empty vector. NS-SV-DC, normal human salivary gland ductal cells; AQP5, aquaporin-5; Luc, luciferase.

Discussion

Previous studies have investigated whether AQP5 is specifically expressed in the salivary gland. It has been demonstrated that AQP5 was closely associated with salivary secretion through regulation of AQP5 protein expression, which is an area of interest in SS syndrome treatment research (22,23).

In the present study, human NS-SV-DC cells lacking AQP5 gene expression were successfully constructed and it was confirmed that 500 nM or 2 μM procaine promoted fluid secretion by these cells. The results of the present study further demonstrated that procaine upregulated the protein expression of AQP5 in NS-SV-DC cells, which may promote gland secretion. In human salivary glands, AQP5 was previously demonstrated to localize to the apical membranes in acinar cells instead of those in ductal cells (24). AQP5 stimulates water to flow into the acinar lumen. Reduced salivary gland secretion was observed in mice harboring a mutant form of the AQP5 channel (25). In addition, it has been reported that AQP3 is localized to the basolateral surface in acinar cells, where it regulates water movement into those cells (26). Therefore, it may be hypothesized that AQP5 and AQP3 may be responsible for the control of normal fluid outflow in acinar cells. A previous study has demonstrated that in alveolar epithelial cells (AECs), CpG islands exist within the promoter region of AQP5. In MLE-12 cells and AECs, high AQP5 expression and hypomethylation was demonstrated in the AQP5 promoter. Furthermore, endogenous SP1 was reported to bind to the hypomethylated Sp1 binding sites in the AQP5 promoter region, instead of the hypermethylated Sp1 binding

sites (27). In the present study, protein expression of DNMT1 in NS-SV-DC cells was measured, and the results demonstrated no significant alterations in DNMT1 content between the experimental and control groups. However, the activity of DNMT1 methyltransferase was reduced following procaine treatment, indicating that procaine may induce the expression of AQP5 protein through the demethylation of CpG islands in the AQP5 promoter region. Previous research has demonstrated that procainamide was a partial competitive inhibitor of DNMT1 and reduced the affinity of the enzyme for its two substrates, S-adenosyl-l-methionine and hemi-methylated DNA (17). In animal experiments, it was demonstrated that procaine inhibited the levels of DNMT1 and 5-methylcytosine in the lungs of endotoxemic animals and simultaneously ameliorated neutrophil infiltration and the production of hyperoxide (16). In the present study, CpG bisulfite-sequencing PCR demonstrated that the CpG island demethylation in AQP5 in the cells treated with procaine was marked compared with the control group, and that the demethylation sites were two Sp1 binding sites: Sp1-2 and Sp1-3 sites, which upregulated the expression of the AQP5 gene when analyzed by a luciferase reporter assay. Collectively, the results of the current study demonstrated that procaine may suppress the methylation of CpG islands in the AQP5 promoter region by inhibiting the activity of DNMT1 methyltransferase. Hypomethylation of Sp1-2 and Sp1-3 sites in the AQP5 promoter region caused an upregulation of AQP5 expression, which may subsequently lead to the observed increase in fluid secretion by NS-SV-DC cells following procaine treatment.

Procaine, as an anticancer treatment candidate, has been widely used in the clinic to inhibit DNMT1 activity. The present study demonstrated that procaine may promote the secretion of NS-SV-DC cells by promoting the expression of AQP5, providing further information concerning its upregulation mechanism and a potential novel regimen for the treatment of SS syndrome.

Acknowledgements

The authors would like to give their sincere appreciation to the reviewers for their helpful comments on this article.

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