

Salidroside reduces cold-induced mucin production by inhibiting TRPM8 activation

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Received April 18, 2013; Accepted June 28, 2013

DOI: 10.3892/ijmm.2013.1434

Abstract. Salidroside is an effective component of the traditional Chinese herb, *Rhodiola rosea*, that is known to have the ability to protect individuals from cold attacks. In the present study, we investigated the effects of salidroside on respiratory epithelial cells exposed to cold temperatures. We wished to determine whether salidroside exerts any effect on cold-induced mucin (MUC) production and the possible mechanisms involved in this process. We incubated HBE16 cells with salidroside, exposed them to a cold stimulus (18°C), and assayed the following endpoints: MUC production (the expression of MUC5AC), concentration intracellular of free calcium ($[Ca^{2+}]_i$), the activation of the transient receptor potential melastatin 8 (TRPM8) channel and the cAMP response element-binding protein (CREB). Our results revealed a significant increase in the $[Ca^{2+}]_i$ concentration, as well as in TRPM8 and CREB expression in the cold-stimulated cells. MUC5AC expression was also increased. Treatment of the cells with salidroside at concentrations of 50 and 100 μ M decreased the $[Ca^{2+}]_i$ concentration, with a maximal effect detected in the cells treated with 100 μ M salidroside. The expression of TRPM8 and TRPM8 channel conductivity were also repressed by salidroside; salidroside decreased the high levels of CREB activity and phosphorylation observed in the cold-stimulated cells. Furthermore, we transfected the cold-stimulated cells with CREB small interfering RNA (siRNA) to analyze TRPM8

gene expression in the absence of CREB activity. The results revealed that the cells treated with either CREB siRNA or salidroside expressed low levels of TRPM8 mRNA and protein. These results indicate that salidroside reduces MUC overproduction induced by cold stimuli and that salidroside exerts its protective effects by inhibiting TRPM8 activation, mainly by decreasing CREB activity.

Introduction

Cold stimuli are known to exacerbate chronic obstructive pulmonary disease (COPD) and have been shown to promote mucin (MUC) hypersecretion (1). Excessive MUC production leads to airway obstruction and enhances inflammation. Previous studies have mainly focused on secondary inflammatory processes (2) and bacterial (3) or viral infections (4,5) that occur in the airways following exposure to cold temperatures; however, the molecular mechanisms behind cold-induced mucus secretion have not yet been fully elucidated. Human organs express ion channels of the transient receptor potential (TRP) family, including some ion channels that respond at distinct temperature thresholds (6-9). TRP melastatin 8 (TRPM8) is a member of this family that is activated by temperatures ranging from 8 to 25°C. TRPM8 is a ligand-gated cation channel with moderate to high selectivity for calcium ions (10,11).

TRPM8 can be activated by cold temperatures and by cooling agents, such as menthol, eucalyptol and icilin. In a previous study, we found that TRPM8 plays a critical role in cold-induced MUC secretion in the human respiratory system (12). Human airway epithelial cells exposed to cold temperatures exhibited activated TRPM8 channels, and MUC5AC expression was upregulated through the TRPM8- Ca^{2+} -phospholipase C (PLC)-phosphatidylinositol biphosphate (PIP2) pathway. Thus, we hypothesized that TRPM8 plays a role in cold-induced MUC secretion. In this study, we aimed to discover methods of inhibiting cold-induced MUC hypersecretion.

Salidroside (rhodioside) is a glycoside compound primarily found in the plant, *Rhodiola rosea*, which grows in cold, high-latitude areas. It is well known that salidroside has pharmacological effects on many pathological conditions, including radiation poisoning (13), inflammation (14), fatigue,

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Abbreviations: TRPM8, transient receptor potential melastatin 8; CRE, cAMP response element; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; MUC, mucin; siRNA, small interfering RNA

Key words: salidroside, mucin, airway, cold stimuli, transient receptor potential melastatin 8

aging and hypoxia (15,16). Other studies have found that salidroside exerts its protective effects mainly by repressing the inflow of intracellular free calcium (Ca^{2+}) ($[\text{Ca}^{2+}]_i$) into cells. Considering what is already known about salidroside and TRPM8, we hypothesized that salidroside has the ability to protect cells from cold nociceptive stimuli and that it may play a positive role in the airway system. In the present study, we explored the regulation of TRPM8 gene transcription in cold-assaulted cells and examined the effects of salidroside on TRPM8 channels and MUC production. We used the HBE16 human airway epithelial cell line for *in vitro* studies. The cells were pre-treated with salidroside and exposed to cold temperatures; the protein and mRNA levels of MUC5AC were then determined. The expression levels of TRPM8 and the transcription factor, cAMP response element-binding protein (CREB), were assayed to investigate whether there were any differences in the levels of these factors following treatment with salidroside. Furthermore, we transfected the cells with CREB small interfering RNA (siRNA) to determine whether the transcription factor, CREB, is involved in TRPM8 gene transcription.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium, HEPES, fetal bovine serum (FBS), anti- β -actin monoclonal antibody, salidroside (>98% purity; National Institute for Food and Drug Control), CREB siRNA and negative control siRNA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine[®] 2000 and OPTI-MEM Reduced Serum Medium were purchased from Invitrogen (San Diego, CA, USA). The rabbit anti-TRPM8 polyclonal antibody was purchased from Abcam (Cambridge, MA, USA) and the horseradish peroxidase (HRP)-goat anti-rabbit IgG antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). CREB-Luc reporter constructs were purchased from Biocat (Catalonia, Spain). The Dual Luciferase Reporter Gene Assay kit was purchased from BioTime Technology (Beijing, China). The pRL-TK Renilla Luciferase Reporter Vector was purchased from Promega (Madison, WI, USA). The high-purity total RNA extraction kit was purchased from Biotake Biotechnology (Beijing, China). The PrimeScript RT Reagent kit was purchased from Takara (Dalian, China).

Cell culture. HBE16 human airway epithelial cells were plated in 6-well plates at a concentration of $5\text{--}6 \times 10^5$ cells/well in 2 ml DMEM/Ham's F12 medium that contained 10% FBS. The cells were then incubated at 37°C and 5% CO_2 . The culture medium was changed to growth factor-free medium prior to the start of each experiment. Cell supernatants and lysates were collected and assays were performed, as described below.

Cell viability measurements. Confluent HBE16 cells were incubated with 10, 50, 100, or 200 μM salidroside for 24 h. We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method to detect cell viability and to select the appropriate dose of salidroside for the subsequent experiments.

siRNA transfection. Cells were plated at a density of $1\text{--}2 \times 10^6$ cells/ml and incubated at 37°C until the cells were 60–80% confluent. CREB siRNA (0.5 μg) were diluted in 100 μl of siRNA transfection medium (solution A); 6 μl siRNA transfection reagent was diluted in 100 μl of siRNA transfection medium (solution B). The solutions (A + B) were subsequently mixed gently and incubated at room temperature for 45 min. The cells were washed once with 2 ml of siRNA transfection medium followed by the addition of 0.8 ml of the siRNA transfection reagent mixture (solutions A + B). The cells were then incubated for 7 h at 37°C in a CO_2 incubator and were recovered in 2 ml of DMEM/Ham's F12 medium containing 10% FBS for an additional 24 h. The medium was then aspirated and replaced with fresh growth medium for the following assays (see below for details).

RNA isolation and real-time PCR for MUC5AC and TRPM8 mRNA. After the HBE16 cells were harvested, total RNA was extracted from the cells using the Biotake high-purity total RNA extraction kit, and RNA integrity was verified using 1.5% agarose gel electrophoresis. The absorbance at 260/280 nm ($A_{260/280}$) was in the range of 1.8–2.0. Total RNA was primed with an oligo(dT) primer and reverse transcribed using the PrimeScript RT Reagent kit. Real-time PCR was performed using the SYBR Premix EX Taq[™] II real-time PCR kit. The sequences of the primers used for real-time PCR were as follows: MUC5AC (U06711) forward, 5'-CAGCCACGTCCCC TTCAATA-3' and reverse, 5'-ACCGCATTTGGGCATCC-3'; TRPM8 (NM024080) forward, 5'-ACTCAGAAGGCTGAGG TACA-3' and reverse, 5'-TTCAGTCGGAGTCTCACTCT-3'; and GAPDH (BC026907) forward, 5'-GAAGGTGAAGGT CGGAGT-3' and reverse, 5'-GAAGATGGTGATGGGA TTTC-3'. GAPDH was used as the loading control. Relative mRNA expression was determined by comparing it to a standard curve.

Enzyme-linked immunosorbent assay (ELISA) for MUC5AC protein. MUC5AC protein levels in the cell lysates and supernatants were measured using ELISA. Cell lysates at multiple dilutions were prepared with phosphate-buffered saline (PBS). Cell culture supernatants were collected for this assay. In addition, 50 μl of each sample were incubated with bicarbonate-carbonate buffer (50 μl) at 40°C in a 96-well plate until dry. The wells were washed 3 times with PBS and blocked with 2% bovine serum albumin (BSA) for 1 h at room temperature. The wells were then incubated with mouse anti-human MUC5AC monoclonal antibody (45M1) (10 $\mu\text{g}/\text{ml}$; Neomarkers Inc., Fremont, CA, USA) diluted in PBS containing 0.05% Tween-20 for 1 h at room temperature. After washing 3 times, the wells were incubated with HRP-conjugated goat anti-mouse IgG (1 $\mu\text{g}/\text{ml}$) for 1 h. HRP was developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase solution, quenched with 1 M H_2SO_4 and the absorbance at 450 nm was measured.

Western blot analysis for TRPM8 and phosphorylated CREB (p-CREB) protein levels. Total protein was determined by BCA analysis. The cells were lysed in lysate buffer, disrupted on ice for 20 min, and then centrifuged at 12,000 rpm for 15 min at 4°C to remove the nuclei and unbroken cells. The

total protein concentration in the cell lysates was determined by BCA assay. Equal amounts of protein were diluted in SDS sample buffer and boiled for 5 min. Proteins were resolved on 6% SDS gels. After running, the gels were equilibrated in transfer buffer containing 25 mM Tris-HCl, 192 mM glycine and 20% methanol (pH 8.3). The proteins were then transferred by electrophoresis onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated in 5% milk dissolved in PBS and 0.05% Tween-20 for 1 h at room temperature and subsequently incubated with rabbit anti-TRPM8 (diluted 1:200) or rabbit anti-p-CREB (Ser-129) polyclonal antibodies (diluted 1:500) overnight at 4°C. After washing 3 times, the membranes were incubated with an HRP-labeled goat anti-rabbit IgG antibody (diluted 1:1,000) for 2 h at room temperature. Enhanced chemiluminescence (ECL) and autoradiography were performed to visualize TRPM8 and p-CREB protein. The membranes were also blotted for β -actin and α -tubulin to ensure equal protein loading.

Immunofluorescence for TRPM8. Cells were fixed with 4% paraformaldehyde and subsequently permeabilized with 0.1% Triton X-100 in PBS for 30 min. The cells were rinsed, blocked in 1% BSA plus 1% normal goat serum and incubated with rabbit anti-TRPM8 (diluted 1:500) overnight at 4°C. After 3 washes of 10 min each in 0.1% Triton X-100/PBS, the cells were incubated with FITC-conjugated fluorescent goat anti-rabbit IgG (1:500) for 2 h at room temperature followed by cellular nuclear staining with 4',6-diamidino-2-phenylindole (DAPI). The samples were examined using a Leica inverted TCS-SP2 confocal microscope that was equipped with appropriate fluorescence filters.

Determination of TRPM8 channel conductivity. Whole-cell patch-clamp experiments were performed using an EPC-10 amplifier and IGOR Pro 4.0 software for data acquisition and analysis. Frequencies of 10 KHz were selected for sampling and 2 KHz for filtration. The extracellular solution used for electrophysiological recordings consisted of 140 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 0.3 mM Na_2HPO_4 , 0.4 mM KH_2PO_4 , 4 mM NaHCO_3 , 5 mM glucose and 10 mM HEPES (the pH was adjusted to 7.4 using NaOH). The intracellular solution filled into patch-clamp pipettes contained 140 mM KCl, 1 mM MgCl_2 , 2.5 mM CaCl_2 , 4 mM EGTA and 10 mM HEPES. The calculated free Ca^{2+} concentration in this solution was 150 nM, and the pH was adjusted to 7.2 using KOH as previously described (17). The borosilicate glass patch pipettes used for the whole-cell recordings had resistances of 2–4 M Ω when filled with intracellular solutions.

Ca^{2+} influx detection. The cells were exposed to cold temperatures (18°C) or pre-treated with salidroside and collected at different time intervals. We resuspended the cells in D-Hanks buffer containing 0.2% FBS (10^6 cells/ml), removed 1 ml of the cell suspension to an Eppendorf tube and then added 2 μl Fura-2/AM stock solution to the cells followed by incubation with shaking at 37°C for 60 min. The cells were centrifuged at 1,300 rpm for 6 min, washed twice with pre-heated D-Hanks buffer containing 0.2% BSA and resuspended in D-Hanks buffer. The fluorescence intensity was then observed using a dual wavelength spectrophotometer (Hitachi F-3000).

The 340/380 nm fluorescence intensity ratio of each sample is abbreviated as R. The 380 nm fluorescence intensity of Fura-2 under Ca^{2+} -free and Ca^{2+} saturation conditions are abbreviated as Fmin and Fmax, respectively. As the controls, 40 μl 2.5% Triton X-100 were added to the cells to detect the maximum fluorescence intensity ratio (Rmax) and 40 μl 250 mM Ca^{2+} chelator EGTA were added to the cells to detect the minimum fluorescence intensity ratio (Rmin). The $[\text{Ca}^{2+}]_i$ concentrations were calculated using the formula $[\text{Ca}^{2+}]_i = \text{Kd}[(\text{R} - \text{Rmin})/(\text{Rmax} - \text{R})](\text{Fmin}/\text{Fmax})$.

CREB activity assay. CREB activity was determined using the CREB-Luciferase Reporter Assay System (Biocat). The CREB-Luc and pRL-TK Renilla Luciferase Reporter plasmids were transfected into the HBE16 cells using Lipofectamine® 2000. Eighteen hours after transfection, the cells were pre-incubated with 100 μM salidroside for 30 min and exposed to cold temperatures (18°C). CREB activity was assayed in accordance with the manufacturer's instructions provided with the Luciferase Reporter Gene Assay kit. The results were expressed as detected luciferase relative light units (RLU)/Renilla luciferase RLU.

Statistical analysis. Data analysis was performed using the SPSS 17.0 statistical software package (SPSS, Inc., Chicago, IL, USA). All *in vitro* experiments using cell lines included samples from at least 6 separate wells and 2–3 independent experiments. Comparisons of >2 groups were made using the Student's t-test or a one-way analysis of variance (ANOVA) followed by Bonferroni's analysis. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Viability of HBE16 cells following exposure to salidroside. The cells were incubated with 10, 50, 100, or 200 μM salidroside for various periods of time to determine cellular proliferation rates following exposure to salidroside. Salidroside at a concentration of 200 μM inhibited cell proliferation after 16 h. Following incubation with 10, 50, or 100 μM salidroside for 24 h, the viability of these cells did not differ significantly from that of the non-treated cells (Fig. 1). These results indicate that lower doses of salidroside do not affect cell viability. Therefore, we selected to use the concentrations of 50 and 100 μM for the following experiments.

Salidroside decreases cold-induced MUC5AC expression. To investigate the effects of cold temperatures on MUC5AC expression in airway epithelial cells, we exposed the HBE16 cells to cold temperatures (18°C) for various periods of time. MUC5AC mRNA levels were upregulated after 1 h of exposure to cold temperatures, with the concentration of MUC5AC protein in the cell lysates and culture supernatants increasing as well. The mRNA and protein levels of MUC5AC continued to increase, reaching a plateau at 8 h. These results indicate that exposure to cold temperatures actively induces MUC5AC expression. The cells were then incubated with 50 μM salidroside prior to exposure cold temperatures. The MUC5AC protein and mRNA levels in the salidroside-treated cells were lower than those observed in the cold-stimulated group at 6 h.

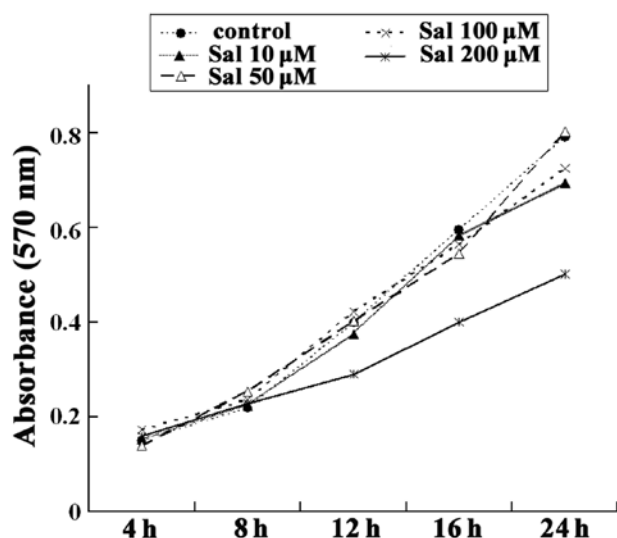


Figure 1. The viability of the HBE16 cells was not affected by 100 μ M salidroside. HBE16 cells were incubated with various concentrations of salidroside and the growth rates of the cells in each group were measured by MTT assay at 4, 6, 8, 12, 16 and 24 h of incubation. Sal, salidroside.

Treatment of the cells with 100 μ M salidroside for 8 h significantly attenuated the cold-induced intracellular synthesis and secretion of the MUC5AC protein (Fig. 2), indicating that salidroside time- and dose-dependently reduced MUC5AC expression induced by cold stimulation.

Salidroside decreases I_{TRPM8} membrane currents. In a previous study, we found that cold temperatures induce TRPM8 activation (12). We further assayed the activation of TRPM8 in cold-stimulated HBE16 cells by patch-clamping the cells at various time points following exposure to cold temperatures. The current density of cold-stimulated TRPM8 channels increased at 15 sec and reached a plateau at 6 min. In the salidroside-treated cells, TRPM8 activation began to decrease after 40 sec. Salidroside significantly decreased the current through cold-stimulated TRPM8 channels at 10 min of recording in comparison with the untreated cold-stimulated channels ($P < 0.05$). The 100 μ M concentration of salidroside was more effective at reducing currents than the 50 μ M concentration, showing maximal effects at 20 min (Fig. 3). These results indicate that TRPM8 activation is involved in cold-stimulated MUC expression and that salidroside inhibits currents through TRPM8, suggesting a previously unknown property of salidroside.

Salidroside reduces cold-induced TRPM8 expression in HBE16 cells. To further confirm the inhibitory effects of salidroside on TRPM8, we investigated the mRNA and protein expression of TRPM8 following incubation with salidroside. The cells were pre-treated with salidroside and then exposed to cold temperatures (18°C). The TRPM8 mRNA and protein levels were then detected by real-time PCR, western blot analysis and immunofluorescence assay, respectively. The cells were exposed to cold temperatures (18°C) for 6 h; exposure to cold temperatures significantly increased TRPM8 protein and mRNA levels compared with the normal control group ($P < 0.05$), and the cellular TRPM8 levels continued to increase

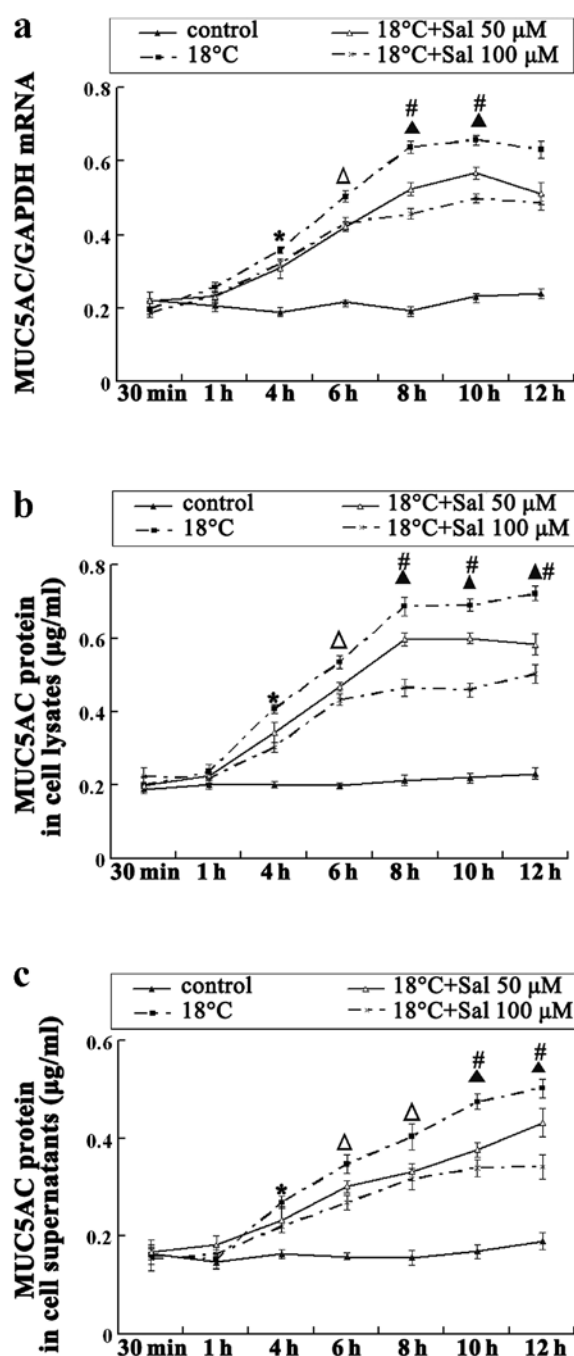


Figure 2. Salidroside downregulates cold-induced MUC5AC expression. (a) Cells were treated with various concentrations of salidroside (50 or 100 μ M) prior to exposure to cold temperatures. The MUC5AC mRNA levels in each group were detected by real-time PCR at various time points (1, 4, 6, 8, 10, 12 h) as described in Materials and methods. The data shown are the means \pm SD from 6 different samples. (b) MUC5AC protein levels in cell lysates. Cells were treated with salidroside or exposed to cold temperatures (18°C) as described in (a) and subsequently harvested, lysed and analyzed by ELISA. (c) MUC5AC protein levels in cell supernatants. Cells were treated as described in (a) and cell supernatants were collected and tested for MUC5AC protein levels by ELISA. * $P < 0.05$ compared with the control group; $^{\Delta}P < 0.01$ compared with the control group; $^{\Delta}P < 0.05$ compared with the 50 μ M salidroside-treated group; $^{\#}P < 0.01$ compared with the 100 μ M salidroside-treated group. P-values were calculated using the paired Student's t-test. Sal, salidroside.

in a time-dependent manner. The cells pre-treated with salidroside prior to exposure to cold temperatures had lower

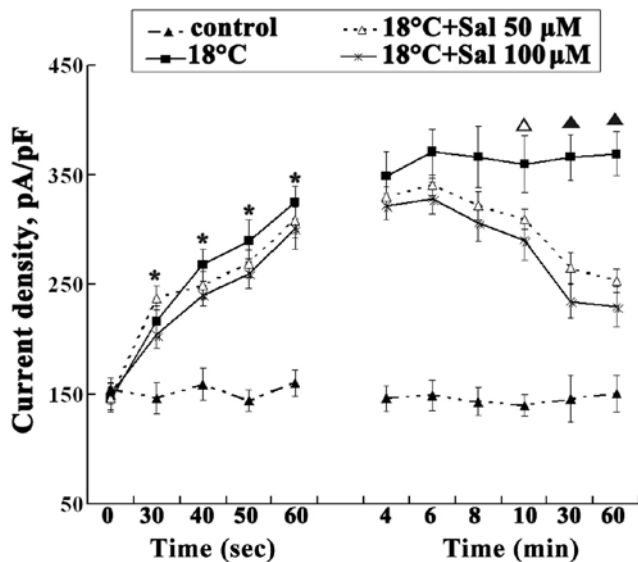


Figure 3. Salidroside inhibits cold-induced membrane currents through transient receptor potential melastatin 8 (TRPM8) channels in HBE16 cells. Cells were pre-incubated with salidroside (50 and 100 μ M) and then exposed to cold temperatures (18°C). The TRPM8 currents at various time points following exposure to cold temperatures were determined by a patch clamp. * $P < 0.05$ compared with the control group; $^{\Delta}P < 0.05$ compared with the 100 μ M salidroside-treated group; $^{\bullet}P < 0.01$ compared with the 100 μ M salidroside-treated group. P-values were calculated using the paired Student's t-test. Sal, salidroside.

TRPM8 expression levels than the non-treated cells ($P < 0.05$). The high dose of salidroside (100 μ M) showed more potent inhibitory effects on increased TRPM8 expression than the low dose (50 μ M) (Figs. 4 and 5). These results indicate that salidroside represses TRPM8 not only by reducing TRPM8 activation but also by inhibiting TRPM8 mRNA and protein expression.

Salidroside inhibits $[Ca^{2+}]_i$ in HBE16 cells. To investigate the mechanisms underlying the salidroside-mediated TRPM8 inhibition, we assayed the free calcium influx in the cells, which is crucial to TRPM8 activation and is known to play a role in many other salidroside-mediated protective events. The HBE16 cells were loaded with Fura-2/AM to examine the intracellular calcium concentration. When the HBE16 cells were exposed to cold temperatures (18°C), we observed that the $[Ca^{2+}]_i$ concentration began to increase after 4 min and continued to increase, reaching a plateau at 10 min, compared with the normal group ($P < 0.01$). In the HBE16 cells incubated with salidroside prior to exposure to cold temperatures, the increase in the $[Ca^{2+}]_i$ concentration was significantly attenuated at 8 min. Furthermore, pre-treatment of the cells with both concentrations of salidroside (50 and 100 μ M) decreased the $[Ca^{2+}]_i$ concentration in a dose-dependent manner (Fig. 6).

Salidroside decreases TRPM8 expression by inhibiting CREB activity in HBE16 cells. Previous studies have reported that the TRPM8 promoter may have a CREB binding site (18). To determine whether CREB is involved in TRPM8 activation, we first assayed the expression and activity of CREB in the cells under cold assault, and then transfected the cells with CREB siRNA to investigate the effects of CREB on TRPM8

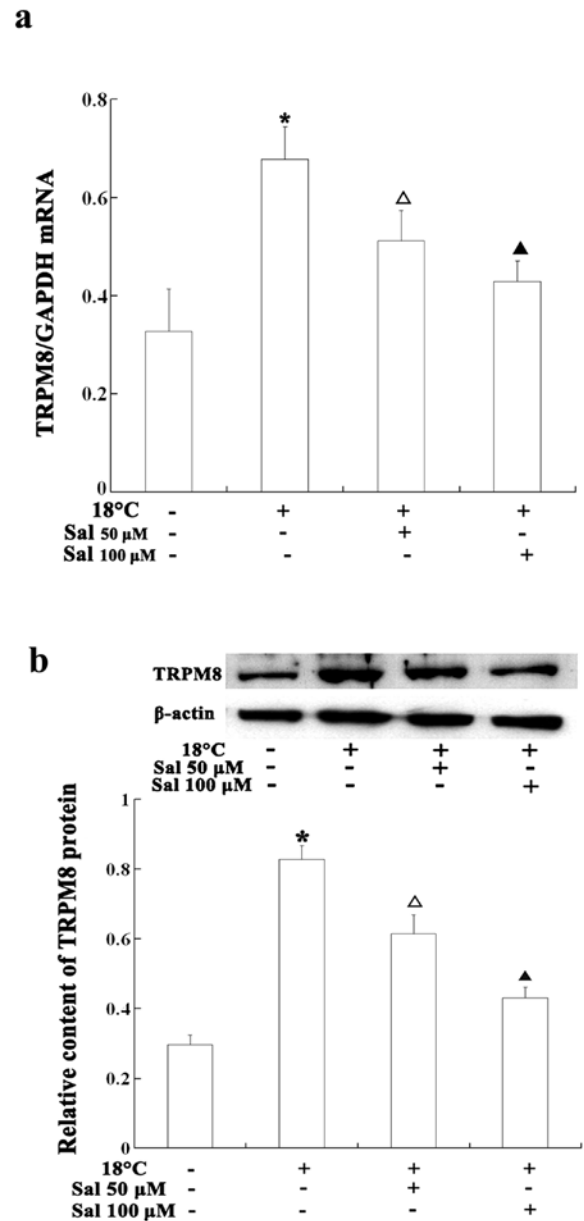


Figure 4. Salidroside decreases transient receptor potential melastatin 8 (TRPM8) expression in cold-stimulated cells. HBE16 cells were treated with 2 different concentrations of salidroside (50 and 100 μ M) for 6 h. (a) The level of TRPM8 mRNA in the HBE16 cells following treatment with salidroside was detected by real-time PCR. (b) The level of TRPM8 protein in the HBE16 cells following treatment with salidroside was determined by western blot analysis. TRPM8 expression levels were determined using densitometry analysis. Protein levels were calculated as the ratio of TRPM8 to β -actin and normalized to the control. Data shown are the means \pm SD from 6 different samples. * $P < 0.05$ compared with the control group; $^{\Delta}P < 0.05$ compared with the cold-stimulated (18°C) group; $^{\bullet}P < 0.01$ compared with the cold-stimulated (18°C) group. P-values were calculated using the paired Student's t-test. Sal, salidroside.

expression. Following the incubation of the HBE16 cells under cold conditions for 6 h, the mRNA and protein levels of TRPM8 increased when compared with the control cells kept at warm temperatures. When the cells were transfected with CREB siRNA prior to exposure to cold temperatures (18°C), the TRPM8 mRNA and protein levels were considerably lower than the levels observed in the non-transfected cells

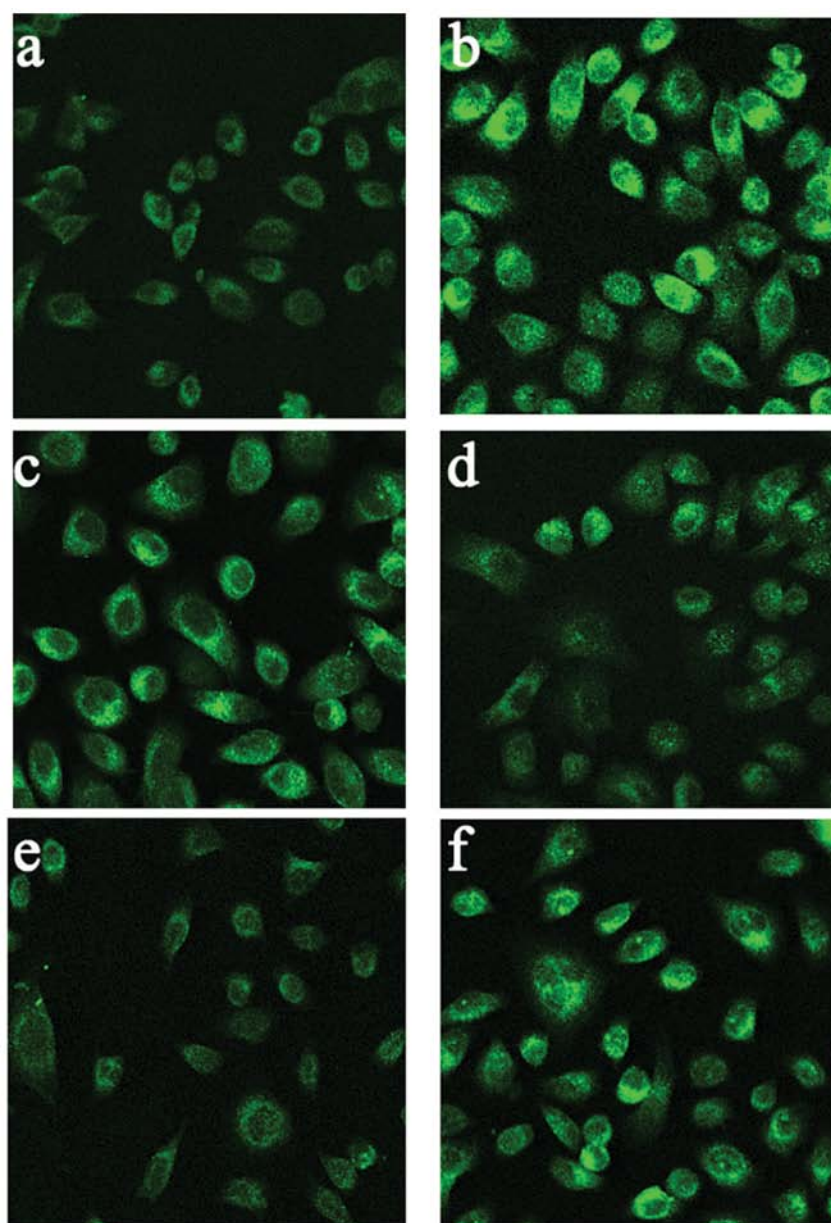


Figure 5. Salidroside and cAMP response element-binding protein (CREB) small interfering RNA (siRNA) decrease the expression of transient receptor potential melastatin 8 (TRPM8) protein in HBE16 cells. HBE16 cells were treated with 2 different concentrations of salidroside (50 and 100 μ M) or transfected with CREB siRNA. The protein expression was assayed by immunofluorescence. (a) Untreated HBE16 cells; (b) cold-stimulated (18°C) group; (c) cold-stimulated (18°C) + 50 μ M salidroside-treated group; (d) cold-stimulated (18°C) + 100 μ M salidroside-treated group; (e) cold-stimulated (18°C) + CREB siRNA-transfected group; (f) cold-stimulated (18°C) + control siRNA-transfected group. The samples were run in triplicate and in 3 separate experiments. Data are expressed as the means \pm SE, n=6.

(Figs. 5 and 7). Exposure to cold temperatures also increased the levels of p-CREB. The cells transfected with CREB siRNA showed lower levels of p-CREB, an effect similar to the one observed in the salidroside-treated cells ($P<0.05$ compared with the cold-stimulated (18°C) cells (Fig. 8A). We additionally transfected the cells with a CREB-Luc Reporter plasmid to measure the activity of CREB in the cold-stimulated cells. The cells exposed to cold temperatures (18°C) showed higher RLU levels than the normal control cells, while the cells incubated with salidroside prior to exposure to cold temperatures exhibited lower RLU levels than the cold-stimulated cells not treated with salidroside ($P<0.01$) (Fig. 8B). These results suggest that CREB plays an important role in TRPM8 gene

transcription and that salidroside represses TRPM8 expression by inhibiting CREB activity.

Discussion

Mucus overproduction is a clinical hallmark of acute exacerbation of COPD. One of the important aggravating factors is exposure to cold temperatures, which can cause mucociliary dysfunction and the accumulation of inflammatory factors, which may ultimately result in a bacterial infection in the respiratory system. The effective management of COPD exacerbation requires a thorough understanding of the underlying pathophysiological mechanisms that shape its clinical manifes-

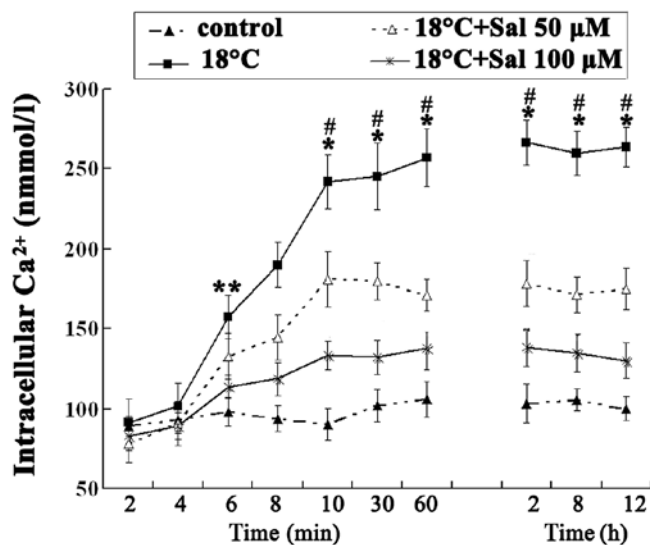
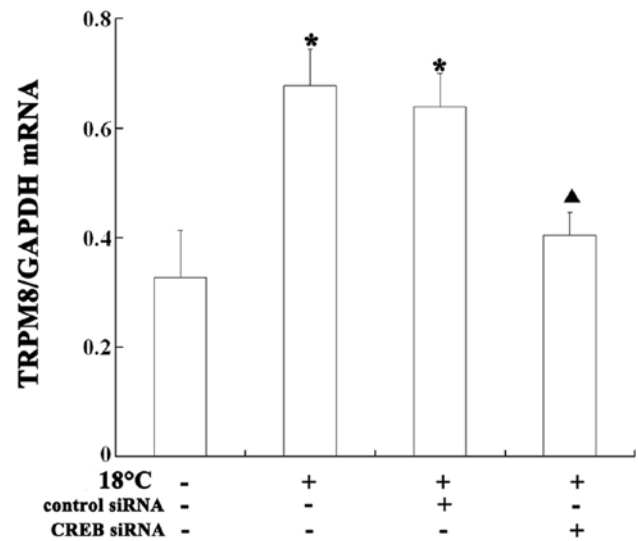


Figure 6. Salidroside attenuates cold-induced increase in intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) concentration in HBE16 cells. Cells were exposed to cold temperatures (18°C) for 4 min, at which point the $[\text{Ca}^{2+}]_i$ concentration began to increase. This effect became more significant at 6 min. ** $P < 0.01$ compared with the control group. Salidroside attenuated the increase in the $[\text{Ca}^{2+}]_i$ concentration at 10 min. * $P < 0.05$ compared with the $50 \mu\text{M}$ salidroside-treated group; * $P < 0.01$ compared with the $100 \mu\text{M}$ salidroside-treated group. Paired Student's t-tests were used to calculate the P-values.

tation. In a previous study, we reported that cold temperatures induce MUC hypersecretion in human airway epithelial cells through a TRPM8-mediated mechanism (12). TRPM8 is a major cold receptor in the body with variants that are expressed by the human respiratory system (19). TRPM8 is activated by temperatures between 8 and 25°C (20) and is a member of the TRP family of Ca^{2+} -permeable, non-selective cation channels and the TRPM subfamily of TRP proteins (21-23). We found that TRPM8 is highly expressed in human airway epithelial cells following exposure to cold stimuli. Functional assays revealed that TRPM8 activation significantly reduced MUC secretion induced by cold temperatures; however, the intervention mechanisms for TRPM8-related events have not yet been fully elucidated.

Rhodiola rosea has long been used in traditional Chinese medicine and it primarily grows at high altitude in thin air and cold weather conditions. Salidroside is the major active ingredient isolated from the plant, *Rhodiola rosea*, and possesses the ability to enhance the resistance of the body to fatigue and cold (24). Salidroside has also been reported to have potent antioxidant and anti-aging properties (25). Salidroside has been shown to reduce H_2O_2 -induced cell apoptosis in SH-SY5Y human neuroblastoma cells (26,27). However, the potential effects of salidroside on cold-induced airway inflammation have not yet been fully elucidated. The respiratory epithelium communicates directly with the external environment. When cold air is inhaled, the respiratory tract loses a considerable amount of heat. This heat loss may be followed by a series of stress reactions. Cold air-induced respiratory symptoms include MUC oversecretion and inflammation, and these phenomena are common in countries or areas with a cold climate (28). It has also been reported that people living at high latitudes exhibit

a



b

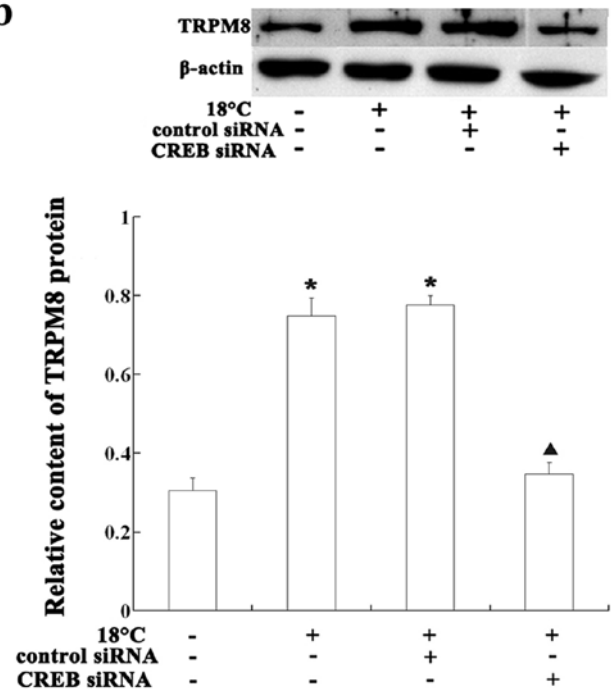


Figure 7. Transfection of cAMP response element-binding protein (CREB) small interfering RNA (siRNA) represses cold-induced transient receptor potential melastatin 8 (TRPM8) expression. HBE16 cells were transfected with CREB siRNA prior to exposure to cold temperatures (18°C). Cells were then cultured for an additional 6 h, at which time TRPM8 mRNA levels were assayed by real-time PCR and protein levels were measured by western blot analysis. (a) TRPM8 mRNA expression following transfection with CREB siRNA. (b) TRPM8 protein expression following transfection with CREB siRNA. * $P < 0.05$ compared with the control group. * $P < 0.01$ compared with the cold-stimulated (18°C) group. P-values were calculated using the paired Student's t-test. Sal, salidroside.

perturbed mucociliary function, placing them at a higher risk of developing nasal obstruction and cough (29). Considering the known properties of salidroside, we hypothesized that it may play a role in regulating homeostasis in the airway system.

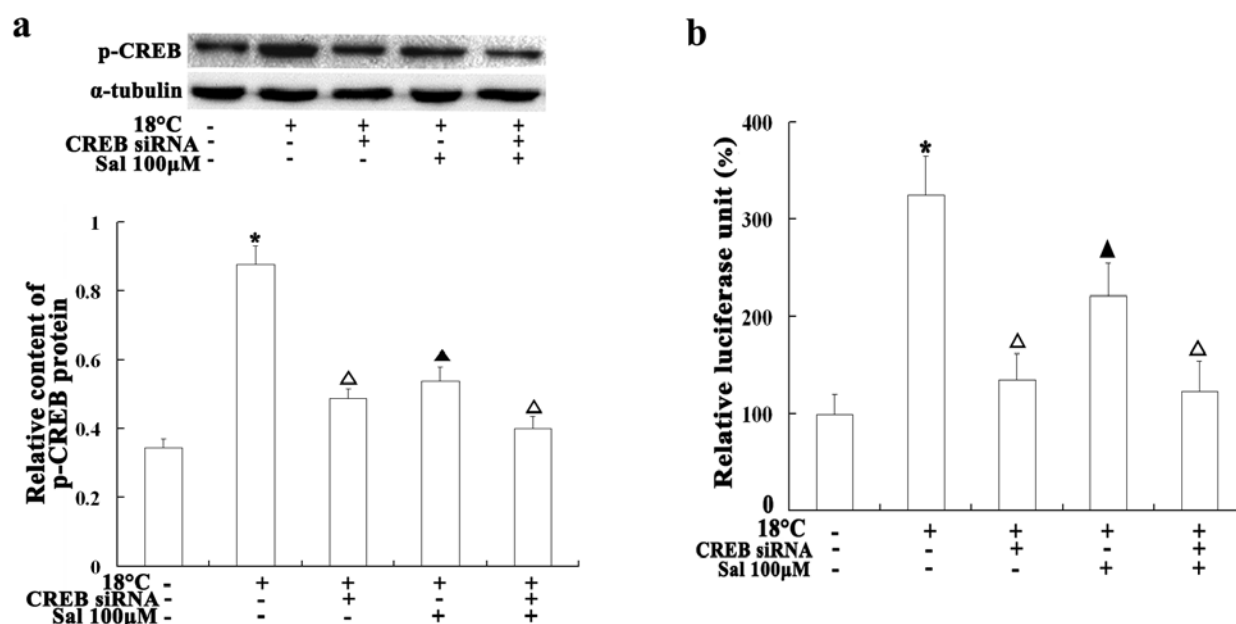


Figure 8. Salidroside reduces cold-induced cAMP response element-binding protein (CREB) phosphorylation and activity. (a) HBE16 cells were cultured in the presence of 100 μ M salidroside or transfected with CREB small interfering RNA (siRNA) and then exposed to cold temperatures (18°C). p-CREB and α -tubulin protein levels were measured by western blot analysis. To quantitatively determine p-CREB levels, we performed densitometry analysis for each sample. Protein levels are calculated as the ratio of p-CREB to α -tubulin and normalized to the control. Data shown are the means \pm SD from 6 different samples. (b) Cells were transfected with a CREB-Luc Reporter plasmid to measure CREB activity. * P <0.05 compared with the control group; $^{\Delta}$ P <0.01 compared with the cold-stimulated (18°C) group; $^{\Delta}$ P <0.05 compared with the cold-stimulated (18°C) group. P -values were calculated using the paired Student's t -test. Sal, salidroside.

In this study, we found that HBE16 cells exposed to cold temperatures exhibited a marked increase in MUC5AC levels and TRPM8 activation. By contrast, cells pre-treated with various concentrations of salidroside (10, 50 and 100 μ M) prior to the cold assault exhibited a dose- and time-dependent decrease in MUC5AC expression, indicating that salidroside is capable of protecting HBE16 cells from cold-induced MUC oversecretion. Furthermore, we found that the $[Ca^{2+}]_i$ concentration rapidly and robustly increased in the cold-stimulated cells, reaching a plateau at 6 to 10 min following exposure to cold temperatures. The cells pre-treated with salidroside showed decreased levels of $[Ca^{2+}]_i$ and TRPM8 activation, with the $[Ca^{2+}]_i$ concentration decreasing as early as 1 min following incubation with salidroside and reaching a minimal level at 2 h post-treatment. High doses of salidroside inhibited the inflow of Ca^{2+} almost completely.

$[Ca^{2+}]_i$ is an important second messenger in various types of cells, regulating functional and physiological cellular activities, such as contraction, secretion, signal transmission and transcriptional regulation. Under normal circumstances, the extracellular concentration of calcium is usually 0.1–10 mM and the intracellular calcium concentration is 10^{-7} – 10^{-8} M, much lower than the calcium concentrations in the extracellular environment. Ca^{2+} -dependent cellular signaling pathways are activated by alterations in the levels of $[Ca^{2+}]_i$. When cells are exposed to stimuli such as inflammation (30), hypoxia (31) and cold temperatures (32), there is an influx of extracellular calcium or a release of calcium from the cellular endoplasmic reticulum, either of which overloads the $[Ca^{2+}]_i$ level. The $[Ca^{2+}]_i$ concentration plays an important role in the activation of TRPM8. Studies have also shown that TRPM8 mediates transmembrane Ca^{2+} flux (33). However, the excessive inflow

of Ca^{2+} leads to desensitization of TRPM8, a process that is mediated through the Ca^{2+} -dependent activation of protein kinase C (PKC) (34,35). TRPM8 is also regulated by a number of kinases and second messengers, such as PLC, PIP2 (36,37), phospholipase A2 (PLA2), lysophospholipids (LPLs) (38,39), arachidonic acid (AA) (40) and cAMP (41).

CREB is a 43-kDa leucine zipper transcription factor that belongs to the activating transcription factor (ATF) subfamily of the basic-region leucine zipper (bZIP) family. CREB primarily responds to the cAMP signaling pathway, binding to CREs to increase or decrease the transcription of certain genes (42). CREB-mediated transcription regulates various cellular responses, including intermediary metabolism, neuronal signaling, cell proliferation, apoptosis and other molecular events. The CREB protein contains an N-terminal transactivation domain (kinase-induced domain), a basic region and a leucine zipper motif at the C-terminus. The transactivation domain includes a phosphorylation site at Ser133 that is recognized by certain protein kinases (43). CREB can be activated by a variety of stimuli, including peptide hormones, growth factors, forskolin, phorbol esters and a number of protein kinases, including protein kinase A (PKA), pp90 ribosomal S6 kinase (pp90RSK), and Ca^{2+} /calmodulin-dependent protein kinases (CaMKs) (44,45).

It has been reported that the TRPM8 core promoter region contains a CRE, which is possibly located between base pairs -1,417 and -1,406. It may play an important role in promoting the transcription of TRPM8 (18,46). Therefore, we further examine whether the transcription factor, CREB, is involved in TRPM8 expression. We found that the expression of p-CREB protein and CREB activity were significantly elevated in the cells exposed to cold temperatures compared

with the control cells. CREB phosphorylation and activity levels were lower in the cells pre-treated with salidroside than the untreated cells. While TRPM8 expression decreased in the cells transfected with CREB siRNA, the expression of TRPM8 in the cells transfected with CREB siRNA and incubated with salidroside prior to exposure to cold temperatures decreased even more significantly. These results indicate not only that CREB activation directly contributes to TRPM8 expression, but also that salidroside inhibits TRPM8 expression by decreasing the activation and activity of CREB.

The known chemical antagonists of TRPM8 are capsaiz-epine, N-(4-tert-butylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carboxamide (BCTC) and thiol BCTC. While these are all synthesized chemicals, salidroside is a component of the Chinese herb, *Rhodiola rosea*, which may be a natural candidate for the treatment of diseases involving TRPM8 overexpression. The regulation of TRPM8 expression by salidroside is important for the identification of alternative treatment modalities for airway mucus hypersecretory diseases induced by cold stimuli.

Acknowledgements

The present study was supported by the National Nature Science Foundation of China (grant no. 81100003 and 31171346), and the China-Russia Cooperation Research Foundation (grant no. 31211120168), and the New Teacher Fund for Doctor Station, the Ministry of Education, China (no. 20115503120006), and Chongqing Nature Science Foundation (grant no. cstc2011jjA10046).

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