The pathophysiological mechanisms underlying mucus hypersecretion induced by cold temperatures in cigarette smoke-exposed rats

MIN-CHAO LI[1], GANG YANG[2], XIANG-DONG ZHOU[1], SERGEY TSELLUYKO[3] and JULIY M. PERELMAN[4]

[1]Department of Respiratory Medicine, The Second Affiliated Hospital, [2]Department of Neurosurgery, The First Affiliated Hospital, Chongqing Medical University, Chongqing 400010, P.R. China; [3]Department of Histology, Amur State Medical Academy; [4]Far Eastern Scientific Center of Physiology and Pathology of Respiration, Blagoveshchensk 675000, Russia

Received July 24, 2013; Accepted October 11, 2013

DOI: 10.3892/ijmm.2013.1535

Abstract. In a recent study, we demonstrated that transient receptor potential melastatin 8 (TRPM8), a calcium-permeable cation channel that is activated by cold temperatures, is localized in the bronchial epithelium and is upregulated in subjects with chronic obstructive pulmonary disease, which causes them to be more sensitive to cold air. In the present study, we found that exposure to cold temperatures induced ciliary ultrastructural anomalies and mucus accumulation on the epithelial surface. Male Sprague-Dawley rats were exposed to cold temperatures to determine the effects of cold air on ultrastructural changes in cilia and the airway epithelial surface. The rats were also exposed to cigarette smoke and/or cold temperatures to determine the effects of smoke and cold air on TRPM8 expression and the role of cold air in cigarette smoke-induced mucus hypersecretion. Following real-time RT-PCR and western blot analysis, we observed a high expression of TRPM8 mRNA and protein in the bronchial tissue following cigarette smoke inhalation. As shown by ELISA, concurrent cold air enhanced the levels of mucin 5AC (MUC5AC) protein, as well as those of inflammatory factors [tumor necrosis factor (TNF)-α and interleukin (IL)-8] that were induced by cigarette smoke inhalation to a greater extent than stimulation with separate stimuli (cold air and cigarette smoke separately). The results suggest that cold air stimuli are responsible for the ultrastructural abnormalities of bronchial cilia, which contribute to abnormal mucus clearance. In addition, cold air synergistically amplifies cigarette smoke-induced mucus hypersecretion and the production of inflammatory factors through the elevated expression of the TRPM8 channel that is initiated by cigarette smoke inhalation.

Correspondence to: Professor Xiang-Dong Zhou, Department of Respiratory Medicine, The Second Affiliated Hospital, Chongqing Medical University, 74 Linjiang Road, Yuzhong, Chongqing 400010, P.R. China
E-mail: zxd999@263.net

Key words: chronic obstructive pulmonary disease, mucin 5AC, transient receptor potential melastatin 8

Introduction

Previous studies on animals and humans have demonstrated that cold air elicits a series of respiratory pathological and physiological changes. Prolonged exposure to cold air can induce the activation of macrophages, an increase in inflammatory factors and granulocytes, as well as the recruitment of alveolar macrophages in the airway (1-3). Long-term and continuous cold stimulation may cause a series of morphological changes in the airways, such as an increase in the number of goblet cells and mucus glands, hypertrophy of the airway muscular fascicles, and a thickening of the muscle layers of the terminal arteries and arterioles. Gradually, these changes may play a role in the symptoms of chronic obstructive pulmonary disease and bronchitis (4). Moreover, low ambient temperatures are associated with a reduction in lung function and an increased frequency of exacerbation in patients with chronic obstructive pulmonary disease (COPD) (5). Therefore, exposure to cold air is a major environmental factor that exacerbates chronic inflammatory airway diseases, such as COPD and asthma (5).

Studies on the expression of the functional cold-sensing transient receptor potential melastatin 8 (TRPM8) variant in human lung epithelial cells have demonstrated that the TRPM8 channel is involved in an underlying molecular mechanism of respiratory cold temperature detection (6). The transient receptor potential (TRP) channels are a family of cation channels that are involved in diverse cellular functions, such as vision, taste, olfaction, hearing, touch, pain and thermo- and osmosensation (7,8). TRPM8 and TRPA1 have been reported to be the molecular transducers of innocuous cooling perception and painfully cold temperatures, respectively (9-13). TRPM8 is a non-selective calcium-permeable cation channel that is expressed in a subset of sensory neurons, including the dorsal root and trigeminal ganglia (9-14), as well as in non-neuronal areas (15-17). TRPM8 is activated by cold temperatures below 25˚C and cooling agents, such as menthol, eucalyptol and icilin agents (9,18,19).

In our previous study, we demonstrated that upregulated TRPM8 expression in bronchial epithelial cells of subjects with COPD can provoke mucus hypersecretion through the cold-mediated activation of the TRPM8 channel (20). These findings provide a molecular mechanism by which cold air increases the susceptibility of the airways to exacerbation in subjects with COPD. However, the direct effects of cold air on the ultrastructure of cilia, which is involved in the function of mucociliary clearance, and the mechanisms that underlie the upregulated expression of TRPM8 in COPD are currently unknown.

In this study, we identified the direct effects of cold air on the ultrastructure of cilia. We present the hypothesis that cigarette smoke, which is a well-established risk factor for COPD (21,22), is responsible for the enhanced basal expression of the TRPM8 receptor. Mucin 5AC (MUC5AC), which is one of the predominant mucins found in airway secretions, has been implicated in pulmonary diseases that are associated with mucus hypersecretion (23), interleukin (IL)-8 and the tumor necrosis factor (TNF)-α protein. These are pro-inflammatory mediators that are associated with the pathophysiology of mucus hypersecretion in COPD (24-26). In this study, we analyzed these factors to elucidate the effects of cold air and cigarette smoke on mucus hypersecretion and the production of inflammatory factors. Since the co-presence of cold air and cigarette smoke is common in the lungs of patients with COPD, the synergistic effects of cold air on mucus hypersecretion and the upregulation of inflammatory factors that are induced by cigarette smoke inhalation were also analyzed.

Materials and methods

Chemicals. Mouse monoclonal MUC5AC antibody and non-immune mouse IgG were obtained from Chemicon (Temecula, CA, USA); horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China); bovine serum albumin and mouse anti-β-actin monoclonal antibody were from Sigma (St. Louis, MO, USA); TNF-α and the IL-8 enzyme-linked immunosorbent assay (ELISA) kit were obtained from Wuhan Boster Biological Technology (Wuhan, China); the First-strand cDNA Synthesis kit was from Fermentas (Burlington, ON, Canada); SYBR Premix EX Taq™ was purchased from Takara Biotechnology (Dalian, China); rabbit anti-TRPM8(C-term) Polyclonal Antibody was from Abgent (San Diego, CA, USA); ECL-Plus chemiluminescence was obtained from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK); and the rTRPM8 and 18S rRNA primers were purchased from Shanghai Bioengineering Co., Ltd. (Shanghai, China).

Treatment of animals. Pathogen-free male Sprague-Dawley (SD) rats (8 weeks old, 260-280 g body weight) were purchased from the Laboratory Animal Center of Chongqing Medical University (Chongqing, China) [certificate: SCXK (YU) 2007-0001]. The SD rats were maintained under optimal conditions for hygiene, temperature (20±2˚C) and photoperiods (12 h light:12 h dark) and were provided food and water *ad libitum* according to the institutional guidelines for the care and use of laboratory animals. All animal procedures were approved by the Ethics Committee of Chongqing Medical University.

The first experiment was designed to determine the effects of cold air on the ultrastructural changes in cilia and the airway epithelial cell surface in SD rats. The rats were divided into 2 groups: the control group and the group exposed to cold air. There were 8 rats in each group. The control group was maintained at a room temperature of 20±2˚C, and the group exposed to cold air was maintained in a cold air therapeutic apparatus (Zimmer Elektromedizin GmbH, Bayern, Germany), which provided cold air stimulus to the rats through a breathing mask for 3 h daily at -18˚C for 40 days.

The second experiment was designed to determine the effects of cigarette smoke on TRPM8 expression and the role of cold air in cigarette smoke-induced mucus hypersecretion. In this experiment, the SD rats were divided into a control group, a cigarette inhalation group, a group exposed to cold air and a group exposed to cigarette inhalation plus cold air. Each group contained 6 rats. The rats in the cigarette inhalation group were exposed to filtered mainstream smoke (Chongqing Cigarette Factory, Chongqing, China) with 10 cigarettes/h for 6 h/day (morning, noon and evening) over a period of 40 days using a smoking machine that was assembled in our laboratory. The air flow rate was 1.5 l/min. The animals were placed in a restraining box, and the smoke was delivered cyclically. The rats in the group exposed to cigarette inhalation plus cold air were co-treated with cigarette inhalation and cold air. The procedure of cold air inhalation was the same as in the first experiment. All the rats were sacrificed on day 40 using 2 ml of 10% chloral hydrate anesthesia and samples were obtained for analysis.

ELISA for the detection of MUC5AC protein levels in bronchoalveolar lavage fluid (BALF). At the end of the experiment, a thoracotomy was performed and the left main bronchus was ligated proximally. BALF was prepared by carefully instilling 2 ml of normal saline into the right lung. The fluid was withdrawn and the process was repeated 3 times until 80% of the instilled fluid was collected. The BALF was centrifuged at 3,000 rpm for 15 min, and the supernatant was stored in a freezer at -70˚C. The total protein levels in BALF were estimated by Bradford assay. Next, 100 mg of total protein were incubated with bicarbonate-carbonate buffer at 40˚C in a 96-well plate until dry. The plates were blocked with 2% bovine serum albumin (Sigma) for 1 h at room temperature and were incubated with a mouse monoclonal MUC5AC antibody (1:100) for 1 h. Subsequently, HRP-conjugated goat anti-mouse IgG (1:10,000) was dispensed into each well and incubated for 1 h. A color reaction was developed with 3,3'-5,5'-tetramethylbenzidine peroxidase solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) and the reaction was terminated with 1 M H_2SO_4. The absorbance was read at 450 nm.

ELISA for the detection of IL-8 and TNF-α protein levels in BALF. The TNF-α and IL-8 protein levels in BALF were measured using an ELISA kit (Wuhan Boster Biological Technology) according to the manufacturer's instructions. Briefly, 100 µl of assay diluent and 50 µl of sample were added to each well for a 2-h incubation at room temperature. Subsequently, 200 µl of conjugate were added to each well for another 2 h at room temperature, and 200 µl of substrate reaction solution were added to each well for a 30-min incuba-

Table I. Primers used for real-time RT-PCR.

Gene	Sense (5'→3')	Antisense (5'→3')	Product size (bp)	GenBank accession no.
rTRPM8	GCAGTGGTACATGAACGGAGT	TGAAGAGTGAAGCCGGAATAC	109	NM_134371
18S rRNA	CTTAGAGGGACAAGTGGCG	GGACATCTAAGGGCATCACA	71	X01117

PCR, polymerase chain reaction.

tion. Finally, 50 µl of stop solution were added to terminate the reaction. The absorbance was read at 450 nm.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Small fragments (1x1x2 mm) of bronchial tissue in the right main bronchus were washed twice in phosphate-buffered saline (PBS) and immediately fixed by immersion in 2.5% glutaraldehyde in 0.15 M phosphate buffer (pH 7.2) at 4˚C for 24 h. After fixation, the tissues were dehydrated through a graded series of ethanol. After being air-dried at room temperature, the samples were coated with platinum/palladium and analyzed under a scanning electron microscope (JSM-6340F; Jeol, Ltd., Tokyo, Japan).

The tissue fragments were fixed in 2.5% glutaraldehyde in 0.15 M phosphate buffer (pH 7.2) at room temperature for 30 min and post-fixed with 1% osmium tetroxide in the same buffer for 30 min at room temperature, following 2 5-min washings with phosphate buffer. The samples were dehydrated in a series of graded ethanol and embedded in Epon 812 (Nissin EM Co., Ltd., Tokyo, Japan). The samples were cured at 60˚C for 48 h and sectioned on a Reichert ultramicrotome (70 nm; Leica, Wetzlar, Germany). Following staining with uranyl acetate-lead citrate, the samples were visualized in a Philips EM 400 (Philips, Eindhoven, The Netherlands) TEM system.

In total, 50 cross-sections of cilia from each specimen were observed. The ciliary ultrastructural anomalies were counted under a magnification of x50,000 by an observer who was blinded to the experimental design, and the anomalies were expressed as a percentage of the total number of cilia in the fields of vision.

Real-time reverse transcription polymerase chain reaction (qRT-PCR) for the detection of TRPM8 mRNA in the bronchial tissues. The test specimens were obtained from the right main bronchus. TRPM8 mRNA transcripts were measured by qRT-PCR. 18S rRNA was selected as the endogenous control gene. Total RNA was isolated from the tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In total, 5 µg of total RNA were reverse transcribed into cDNA using the first-strand cDNA synthesis kit according to the manufacturer's instructions. The PCR primers for rat TRPM8 and 18S rRNA were designed according to the published cDNA sequences (Table I). The specificity of the PCR primers was tested under normal PCR conditions, and the products of the reaction were electrophoresed onto 2% agarose gels. Real-time PCR was performed using SYBR Premix EX Taq™ in a Bio-Rad IQ5 PCR System (Bio-Rad Laboratories, Hercules, CA, USA). PCR was performed under the following conditions: denaturation at 94˚C for 15 min, 40 cycles of denaturation at 94˚C for 15 sec, annealing at 58˚C for 45 sec and extension at 72˚C for 1 min, followed by a final extension at 72˚C for 5 min. Finally, the melting curve analysis was performed to confirm that a single product was amplified and that no primer dimers had interfered with the reaction. The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used for the relative mRNA quantification.

Western blot analysis for the detection of TRPM8 protein expression. TRPM8 protein expression was measured by western blot analysis. Briefly, the right main bronchus was washed 3 times with ice-cold PBS. The tissues were resuspended in lysate buffer, lysed on ice for 20 min and centrifuged at 12,000 rpm for 15 min at 4˚C. The protein content was determined by Bradford assay. Equivalent amounts of protein (30 µg) from each sample were separated on 10% SDS-PAGE gels and transferred onto a polyvinylidene fluoride membrane (Sigma). The membrane was blocked with 5% non-fat milk in Tris-buffered saline, incubated with a rabbit anti-TRPM8 (C-term) polyclonal antibody (1:100) and a mouse anti-β-actin monoclonal antibody (1:1,000) overnight at 4˚C, followed by incubation with corresponding HRP-conjugated secondary antibodies (1:2,000) overnight at 4˚C. Specific blots were developed using ECL-Plus Chemiluminescence. The densitometric quantification of the bands was performed using Quantity One software (Bio-Rad Laboratories). The results were expressed as the ratio of the expression of TRPM8 to β-actin.

Immunofluorescence for the detection of MUC5AC expression in the bronchial tissue sections. The frozen sections (10-12 µm) from the right main bronchus were placed at room temperature for 30 min and immersed in cold acetone for 10 min. The sections were rinsed with PBS, and 3% hydrogen peroxide was added for 5 min. After washing, the sections were blocked using 1% BSA plus 1% normal goat serum and incubated with a mouse monoclonal MUC5AC antibody (1:200) overnight at 4˚C. Non-immune mouse IgG was used as a negative control. Following 3 10-min washes in PBS, the slides were incubated with an FITC-conjugated secondary antibody (1:200) for 2 h at room temperature. The samples were examined under a Leica inverted TCS-SP2 confocal microscope (Leica, Heidelberg, Germany) that was fitted with the appropriate fluorescence filters. The quantification of the MUC5AC immunostaining in the bronchial epithelium was performed using software equipped in the TCS-SP2 confocal microscope. The entire bronchial epithelium was selected as a region of interest (ROI). The mean value was

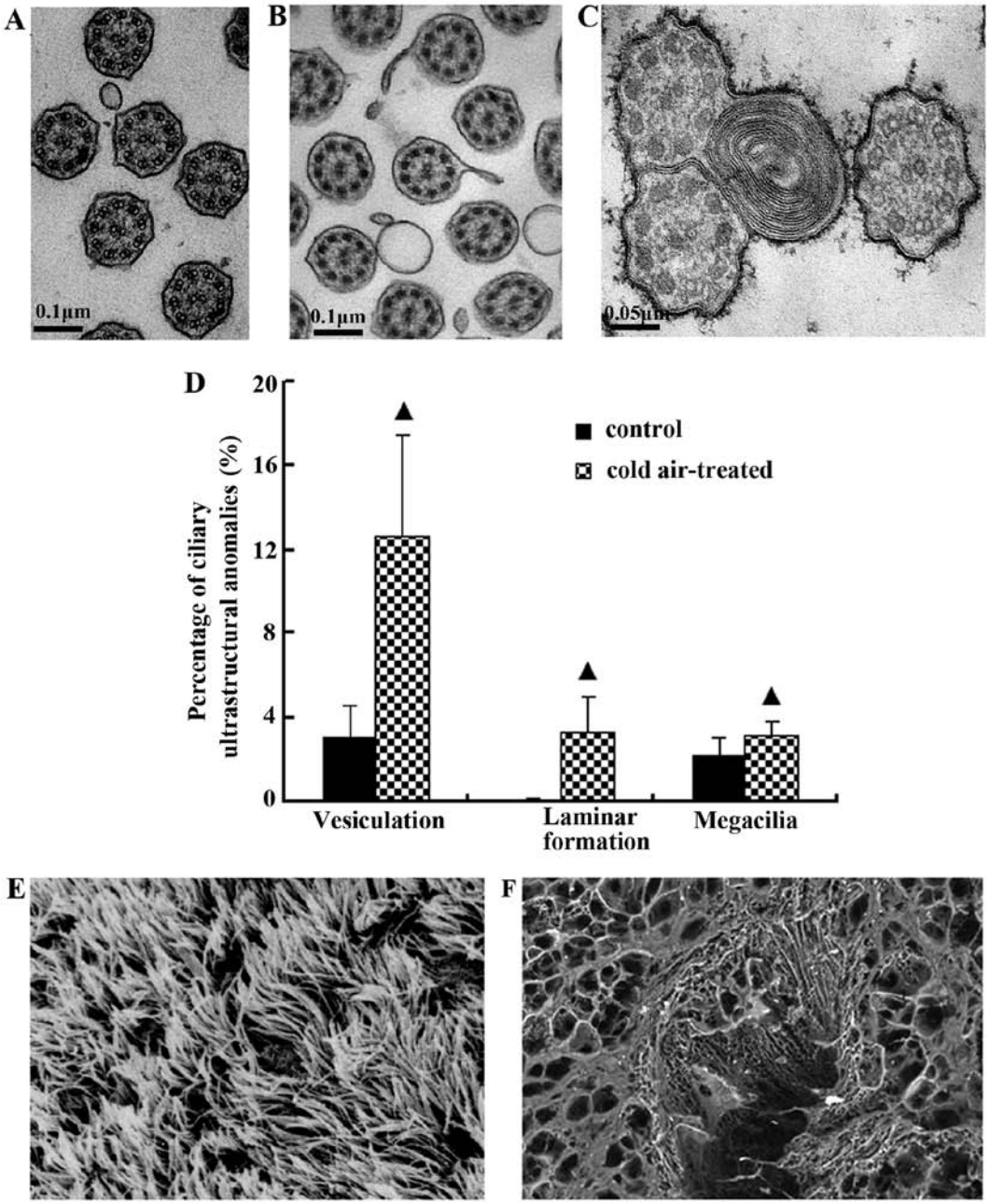

Figure 1. Transmission electron microscopy of ciliary transections, scanning electron microscopy of the epithelial surface and comparison of the percentages of ciliary ultrastructural anomalies in the control and cold air-treated rats. (A) Cross-section of normal cilia in the control group. (B) Formation of diverticula in the lateral membrane walls of cilia in the group exposed to cold air. (C) Laminar formations in the cilia and megacilia anomalies in the group exposed to cold air. (D) Comparison of the percentages of ciliary ultrastructural anomalies in the control and cold air-treated rats. The values are the means ± SD; N=8. ▲P<0.05 compared with the control group. (E) Ultrastructural organization of the epithelial surface in the control group (x2,500). (F) Mucus accumulation on the epithelial surface in the cold air-treated group (x5,000).

recorded and analyzed. The measurement of TRPM8 expression was performed by an observer who was unaware of which group the biopsy specimens were obtained from.

Statistical analysis. The data are presented as the means ± SD, and data analyses were performed using SPSS version 10.0 for Windows software (SPSS Inc., Chicago, IL, USA). Differences were examined for statistical significance using one-way ANOVA to compare TRPM8 expression between the different groups. The main effects of cold air and smoking on the expression of MUC5AC and inflammatory factors as well as the coordinated interaction between cold air and smoking were analyzed by ANOVA with a factorial design. P-values <0.05 were considered to indicate statistically significant differences.

Results

Effects of cold air stimuli on the ultrastructure organization of cilia and the airway epithelial cell surface. Normal ciliary

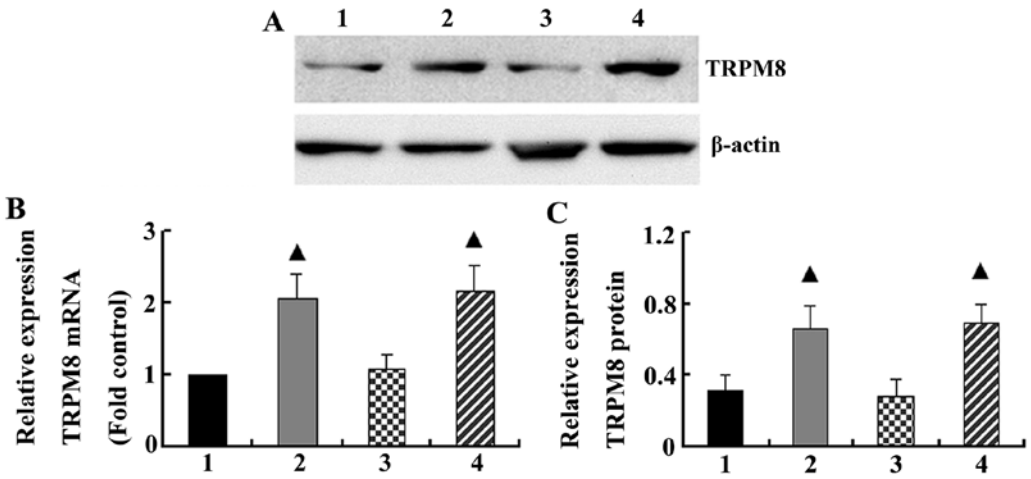

Figure 2. Relative quantification of transient receptor potential melastatin 8 (TRPM8) mRNA and protein in the bronchial tissue in lane 1, control group; lane 2, group exposed to cigarette smoke; lane 3, cold air-treated group; and lane 4, group exposed to cigarette smoke plus cold air. (A) The levels of the TRPM8 protein were determined by western blot analysis. (B) The levels of TRPM8 mRNA were determined by real-time RT-PCR, and a comparative Ct method ($2^{-\Delta\Delta Ct}$) was used for the relative mRNA quantification. (C) Densitometry quantification of the bands in (A) was performed using Quantity One software, and the results are expressed as the ratio of the expression of TRPM8 to β-actin. The values in (B) and (C) are shown as the means ± SD; N=6. ▲P <0.05 vs. control.

axonema and ciliary membranes were observed in the control group (Fig. 1A). The radial spokes from 2 central microtubules radiated to 9 peripheral pairs of microtubules were joined by a nexin link, and the whole structure formed an axonema. Each pair comprised microtubules A and B with 2 side arms that were arranged clockwise. Following repeated cold air stimulation, the fromation of diverticula and vesiculation (Fig. 1B), laminar formations in cilia and megacilia anomalies (3 axonemas constituted a compound cilium) (Fig. 1C) were significantly increased when compared with the control group (P<0.05) (Fig. 1D).

The normal orientation of cilia was observed on the epithelial surface in the control group (Fig. 1E). However, the bronchial ciliated epithelium in the rats repeatedly exposed to cold air was covered by varying degrees of accumulated mucus (Fig. 1F).

Effects of cigarette smoke on TRPM8 expression. Real-time PCR and western blot analysis demonstrated that cigarette smoke increased the basal mRNA and protein levels of TRPM8 in the bronchial tissue (2.07±0.35 and 0.66±0.12, respectively), whereas the mRNA and protein levels of TRPM8 were 1.0±0.00 and 0.31±0.09 in the control group, respectively (P=0.006 and P=0.00). However, cold air stimuli had no effect on TRPM8 mRNA and protein expression in the bronchial tissue compared with the control group (P>0.05). When the rats were exposed to cigarette inhalation and cold air, the levels of TRPM8 mRNA and protein (2.16±0.36 and 0.70±0.10, respectively) were similar to those from the group exposed to cigarette smoke only (P=0.772 and P=0.640, respectively) (Fig. 2).

Effects of cold air stimuli on the cigarette smoke-induced intracellular synthesis and secretion of MUC5AC protein. An immunofluorescence assay and ELISA revealed that the relative levels of intracellular MUC5AC protein expressed in goblet cells and MUC5AC protein secreted in BALF were increased in the group exposed to cigarette smoke (17.74±2.92 and 66.08±3.86 μg/mg, respectively) compared with the control group, in which the intracellular MUC5AC and secreted MUC5AC protein relative levels were 3.40±1.00 and 56.74±7.83 μg/mg, respectively (P<0.001). Significant increases in intracellular and secreted MUC5AC protein (21.65±3.90 and 71.40±4.38 μg/mg, respectively) were observed in the rats that were exposed to cold air compared with those in the control group (P<0.01). The intracellular and secreted MUC5AC protein levels were markedly increased (47.84±6.61 and 182.39±56.90 μg/mg, respectively; P<0.01) in the rats that were exposed to cigarette smoke and cold air, and the coordinated interaction between cold air and cigarette smoke was significant (F=12.33, P=0.002; F=18.60, P=0.00) with 1.21- and 1.32-fold increases in the total amounts of intracellular and secreted MUC5AC that were induced by separate stimuli, respectively. Collectively, the increased levels of MUC5AC in the rats that were exposed to cigarette smoke and cold air suggested that cold air synergistically increased MUC5AC mucin synthesis and the secretion induced by cigarette smoke (Fig. 3).

Effects of cold air stimuli on the cigarette smoke-induced production of inflammatory cytokines. The release of the TNF-α and IL-8 proteins in BALF was increased following exposure to cigarette smoke (138.37±36.69 and 271.24±82.03 ng/l, respectively) compared with the control group (58.81±17.48 and 156.48±35.56 ng/l, respectively; P<0.001). Similarly, cold air induced a significant increase in TNF-α and IL-8 protein levels in BALF (142.62±49.40 and 203.65±107.73 ng/l, respectively) compared with the control group (P<0.001). Co-stimulation with cold air and cigarette smoke resulted in a stronger synergistic increase in TNF-α and IL-8 levels (379.46±133.76 and 596.75±148.74 ng/l, respectively) compared with stimulation by separate stimuli (1.35- and 1.25-fold increases in the total amounts of TNF-α and IL-8 following stimulation with cold air and cigarette smoke, respectively) and the coordinated interaction between cold air and cigarette smoke was significant (F=6.75, P=0.017; F=11.21, P=0.003). Overall, these data indicate that co-exposure to cigarette smoke and cold air induced the production of TNF-α and IL-8 in a synergistic manner (Fig. 3).

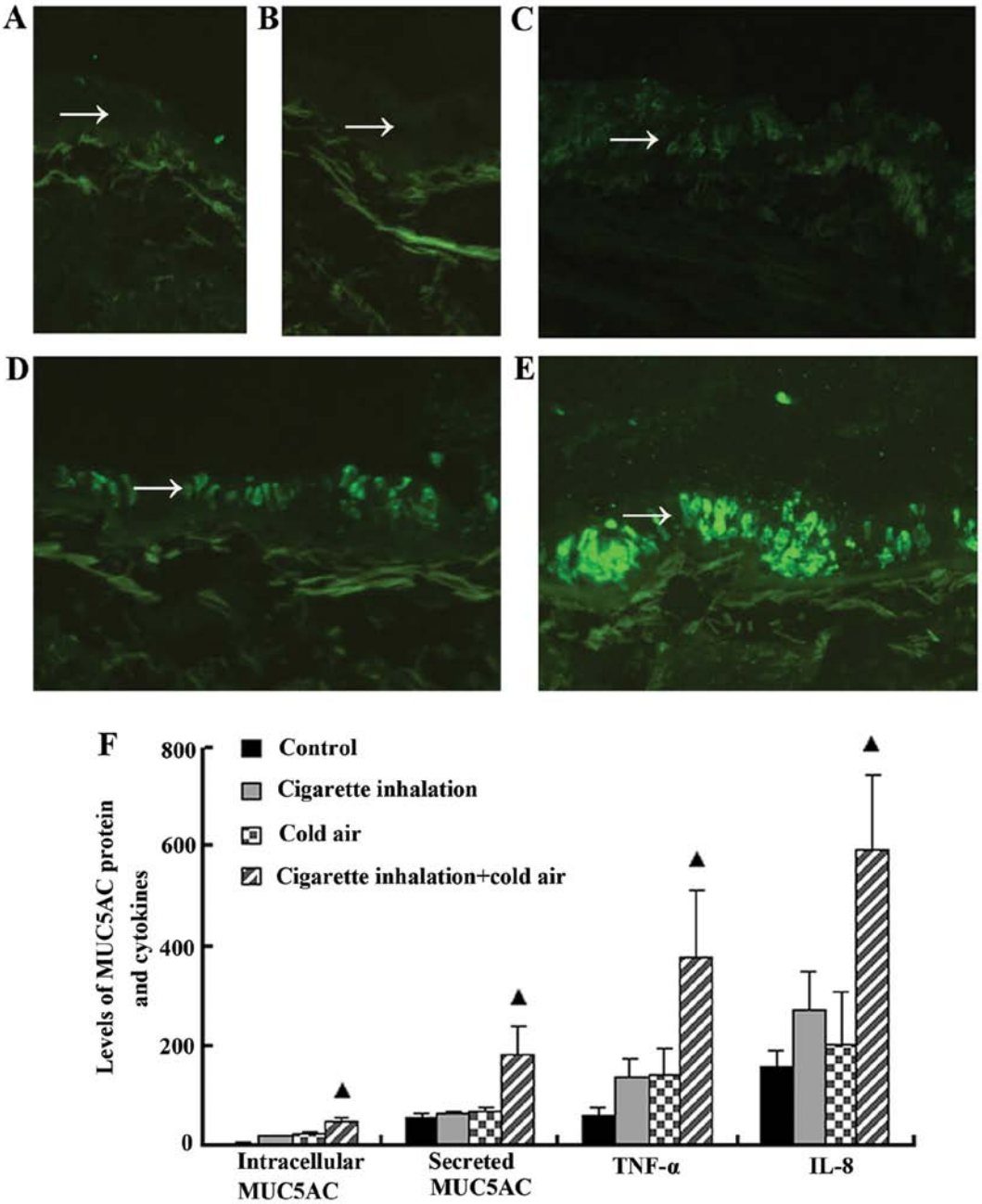

Figure 3. Effects of cold air stimuli on the cigarette smoke-induced production of intracellular and secreted mucin 5AC (MUC5AC) protein and inflammatory cytokines. (A-E) Photomicrographs of representative histological sections of the bronchial epithelium (x400) in the (A) control group, (B) negative control group, (C) group exposed to cigarette smoke, (D) group exposed to cold air and (E) group exposed to cigarette smoke plus cold air. Arrows indicate the bronchial epithelium. The sections were incubated with a mouse monoclonal antibody against MUC5AC, followed by anti-mouse IgG coupled to FITC. In the negative control group, non-immune IgG was used instead of the primary antibody. (F) Quantification of intracellular MUC5AC in the bronchial epithelium and MUC5AC, TNF-α, IL-8 in BALF. Values are the means ± SD (μg/mg for secreted MUC5AC protein, ng/l for TNF-α and IL-8); N=6. ▲P<0.05 the coordinated interaction between cold air and cigarette smoke was significant by ANOVA with a factorial design.

Discussion

Cold air-induced COPD exacerbation is a well known phenomenon that may elicit a series of respiratory pathological and physiological changes, such as a reduction in lung function, an increased frequency of exacerbation and morphological changes in the airways (4,5,27). In a recent study, we demonstrated that cold air that is temporarily inhaled provokes robust excessive secretions of airway mucus through the cold-mediated activation of the TRPM8 channel and contributes to cold-induced COPD exacerbation (20). Airway secretions are cleared by mucociliary clearance and other mechanisms, such as cough, peristalsis, and two-phase gas-liquid flow. Mucociliary clearance is a very complex process that involves several variables, such as the structure, number, movement and co-ordination of cilia that are present in the airways (28,29). Therefore, we in this study, investigated whether the penetration of cold air into the lower airway elicits

ciliary ultrastructural anomalies and contributes to mucus accumulation. We analyzed the ultrastructure organization of the bronchial ciliary system in rats that were exposed to cold air stimuli. Two types of ultrastructural anomalies were observed: anomalies of the ciliary membrane and architectural ciliary anomalies. Following repeated cold air stimulation, the formation of diverticula and vesiculation were observed in the cilia. Striking architectural ciliary anomalies were observed in the cilia, such as 3 axonemas that constituted a compound cilium, which are described as megacilia or compound cilia and laminar formations.

In addition, we investigated the association between repeated cold air stimulation and the amount of mucus on the epithelial surface. We found that cilia on the epithelial surface were covered by accumulated mucus following repeated cold air stimulation. Previous studies have demonstrated that the ciliary beat frequency decreased at low temperatures (30). Therefore, we speculated that the ciliary ultrastructural anomalies that were induced by cold air partly resulted in a lower ciliary beat frequency and defective mucociliary clearance, which led to mucus accumulation on the epithelial surface (28,31). Collectively, these results indicate that ciliary anomalies and excessive MUC5AC secretion that are induced by cold air stimuli may be the reason why the bronchial ciliated epithelium was covered by accumulated mucus after the rats were treated with repeated cold air stimulation.

Owing to the excessive accumulation of mucus in the airways due to cold air, obstructive lung diseases may be more common in cold areas; however, these results do not support this hypothesis. The first-line defense against an inhaled insult that impinges on and damages the epithelium is the production of mucus. In a healthy subject, the production of mucus is an important homeostatic defense mechanism to combat the onslaught of cold. Due to the adaptation of TRPM8 to cold stimuli (32,33), cold air cannot evoke a continuous cascade of MUC5AC secretion (20). Moreover, the cilia can undergo morphological regeneration and functional restoration following a mechanical injury (34,35). Thus, cold air can trigger symptoms; however, it is unlikely to be a causal factor that initiates respiratory diseases (1).

Previous studies have indicated that the TRPM8 receptor, which is expressed in human lung epithelial cells, plays an essential and predominant role in mediating the respiratory detection of cold stimuli (6,20). Previous studies in our laboratory have demonstrated that the upregulated expression of the TRPM8 channel in the bronchial epithelial cells of subjects with COPD provokes an excessive production of airway mucus in response to cold air and further contributes to cold-induced COPD exacerbation (20). An intriguing issue is why COPD patients present the state of upregulated expression of the TRPM8 channel in the bronchial epithelium. This issue was addressed by determining the effects of cigarette smoke, the principal risk factor for the development of COPD, on the basal expression of the TRPM8 receptor *in vivo*. In this study, we demonstrate, using animal models, that cigarette smoke upregulated the basal levels of the TRPM8 channel in bronchial tissue and that cold air stimuli had no effect on TRPM8 expression. This finding indicates that cigarette smoke is a potential etiological factor for the elevated expression of the TRPM8 channel. However, the detailed mechanisms of cigarette smoke that are involved in this process require further clarification. Cold air stimuli do not play a role in the regulation of TRPM8 expression, but may exert their effects by activating the TRPM8 channel (6).

In the present study, we demonstrate that cold air induces the production of TNF-α and IL-8 in the airways, which is in agreement with previous *in vitro* studies that demonstrated that the activation of the TRPM8 variant in human lung epithelial cells by cold exposure leads to increased expression levels of several cytokine and chemokine genes, including IL-8 and TNF-α (36). Moreover, these results are consistent with those of previous studies (37-40), and suggest that cigarette smoke has the potential to induce the production of IL-8 and TNF-α in the airways. In this study, the concomitant presence of cigarette smoke and cold air resulted in a synergistic enhancement of the production of IL-8 and TNF-α. The underlying mechanisms of this synergistic modulation may depend on the upregulated basal levels of the TRPM8 channel in the bronchial epithelia that is induced by cigarette smoke; this upregulated basal level of the TRPM8 channel may be activated by cold stimuli and lead to signal amplification, which causes further production of IL-8 and TNF-α. We demonstrated that cigarette smoke and cold air promoted mucin synthesis and mucus secretion. Cigarette smoke is a common agent that promotes mucin synthesis and mucus secretion through a variety of mechanisms, such as the epidermal growth factor receptor signaling pathway (41), oxidant-dependent mechanisms (42), goblet cell hypertrophy and hyperplasia (43,44). However, the mechanisms responsible for cold air-induced mucin synthesis and mucin secretion are largely unknown. In our previous study, we demonstrated that cold air provoked airway mucus hypersecretion through the TRPM8-mediated influx of the Ca^{2+} signaling pathway (20). In addition, potential cold-related products, including IL-8 and TNF-α, which are the inducers of mucin gene expression, mucin synthesis and mucus secretion (24-26), act as secondary stimuli and may be responsible for the upregulation of MUC5AC synthesis and secretion. Therefore, the synergistic amplified production of MUC5AC that was induced by the combination of cold air and cigarette smoke in our study was due to the influx of Ca^{2+} through the upregulated expression of the TRPM8 channel caused by cigarette smoke and the synergistically enhanced expression of IL-8 and TNF-α, which was provoked by the co-exposure of cold air and cigarette smoke. The synergistic effect of cold air on cigarette smoke-driven cytokine and MUC5AC upregulation may constitute an amplification step that contributes to the severity and persistence of mucus hyperproduction observed in COPD exacerbations that are induced by cold air. Simultaneously, the ciliary ultrastructural anomalies caused by cold air may further intensify the accumulation of mucus.

Taken together, these results suggest that cigarette smoke is a potential etiological factor for the elevated expression of the TRPM8 channel, thus causing subjects with COPD to have greater sensitivity to cold than healthy subjects; this greater sensitivity is coupled with mucus hyperproduction and hypersecretion in the airways when exposed to cold air. The present study may provide a new rationale for therapies that target an upregulated TRPM8 channel level in the treatment of cold air-associated pathological mucus hyperproduction.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (nos. 81370111 and 81270102), the Chongqing Nature Science Foundation (no. KJ120301) and the Scientific and Technological Research Program of Chongqing Municipal Education Commission (no. cstc2012jjA10050). The authors would also like to thank the editors of the American Journal Experts, for professional English language editing of this article.

References

1. Davis MS, Malayer JR, Vandeventer L, Royer CM, McKenzie EC and Williamson KK: Cold weather exercise and airway cytokine expression. J Appl Physiol 98: 2132-2136, 2005.
2. Koskela HO: Cold air-provoked respiratory symptoms: the mechanisms and management. Int J Circumpolar Health 66: 91-100, 2007.
3. Larsson K, Tornling G, Gavhed D, Muller-Suur C and Palmberg L: Inhalation of cold air increases the number of inflammatory cells in the lungs in healthy subjects. Eur Respir J 12: 825-830, 1998.
4. Giesbrecht GG: The respiratory system in a cold environment. Aviat Space Environ Med 66: 890-902, 1995.
5. Donaldson GC, Seemungal T, Jeffries DJ and Wedzicha JA: Effect of temperature on lung function and symptoms in chronic obstructive pulmonary disease. Eur Respir J 13: 844-849, 1999.
6. Sabnis AS, Shadid M, Yost GS and Reilly CA: Human lung epithelial cells express a functional cold-sensing TRPM8 variant. Am J Respir Cell Mol Biol 39: 466-474, 2008.
7. Montell C: The TRP superfamily of cation channels. Sci STKE 2005: re3, 2005.
8. Song MY and Yuan JX: Introduction to TRP channels: structure, function, and regulation. Adv Exp Med Biol 661: 99-108, 2010.
9. Peier AM, Moqrich A and Hergarden AC, *et al*: A TRP channel that senses cold stimuli and menthol. Cell 108: 705-715, 2002.
10. Kwan KY, Allchorne AJ, Vollrath MA, Christensen AP, Zhang DS, Woolf CJ and Corey DP: TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. Neuron 50: 277-289, 2006.
11. Karashima Y, Talavera K and Everaerts W, *et al*: TRPA1 acts as a cold sensor in vitro and in vivo. Proc Natl Acad Sci USA 106: 1273-1278, 2009.
12. Colburn RW, Lubin ML and Stone DJ Jr, *et al*: Attenuated cold sensitivity in TRPM8 null mice. Neuron 54: 379-386, 2007.
13. Huang J, Zhang X and McNaughton PA: Modulation of temperature-sensitive TRP channels. Semin Cell Dev Biol 17: 638-645, 2006.
14. Nealen ML, Gold MS, Thut PD and Caterina MJ: TRPM8 mRNA is expressed in a subset of cold-responsive trigeminal neurons from rat. J Neurophysiol 90: 515-520, 2003.
15. Van Haute C, De Ridder D and Nilius B: TRP channels in human prostate. ScientificWorldJournal 10: 1597-1611, 2010.
16. Li Q, Wang X, Yang Z, Wang B and Li S: Menthol induces cell death via the TRPM8 channel in the human bladder cancer cell line T24. Oncology 77: 335-341, 2009.
17. Yang XR, Lin MJ, McIntosh LS and Sham JS: Functional expression of transient receptor potential melastatin- (TRPM) and vanilloid-related (TRPV) channels in pulmonary arterial and aortic smooth muscle. Am J Physiol Lung Cell Mol Physiol 290: L1267-L1276, 2006.
18. Voets T, Owsianik G and Nilius B: TRPM8. Handb Exp Pharmacol 179: 329-344, 2007.
19. Mälkiä A, Madrid R, Meseguer V, de la Peña E, Valero M, Belmonte C and Viana F: Bidirectional shifts of TRPM8 channel gating by temperature and chemical agents modulate the cold sensitivity of mammalian thermoreceptors. J Physiol 581: 155-174, 2007.
20. Li M, Li Q, Yang G, Kolosov VP, Perelman JM and Zhou XD: Cold temperature induces mucin hypersecretion from normal human bronchial epithelial cells in vitro through a transient receptor potential melastatin 8 (TRPM8)-mediated mechanism. J Allergy Clin Immunol 128: 626-634, 2011.
21. Laniado-Laborín R: Smoking and chronic obstructive pulmonary disease (COPD). Parallel epidemics of the 21 century. Int J Environ Res Public Health 6: 209-224, 2009.
22. Taylor JD: COPD and the response of the lung to tobacco smoke exposure. Pulm Pharmacol Ther 23: 376-383, 2010.
23. Voynow JA, Gendler SJ and Rose MC: Regulation of mucin genes in chronic inflammatory airway diseases. Am J Respir Cell Mol Biol 34: 661-665, 2006.
24. Rogers DF: Physiology of airway mucus secretion and pathophysiology of hypersecretion. Respir Care 52: 1134-1146, 2007.
25. Wang IJ, Wu CY and Hu FR: Effect of proinflammatory cytokines on the human MUC5AC promoter activity in vitro and in vivo. Clin Ophthalmol 1: 71-77, 2007.
26. Bautista MV, Chen Y, Ivanova VS, Rahimi MK, Watson AM and Rose MC: IL-8 regulates mucin gene expression at the posttranscriptional level in lung epithelial cells. J Immunol 183: 2159-2166, 2009.
27. Davis MS, Lockard AJ, Marlin DJ and Freed AN: Airway cooling and mucosal injury during cold weather exercise. Equine Vet J Suppl 34: 413-416, 2002.
28. Houtmeyers E, Gosselink R, Gayan-Ramirez G and Decramer M: Regulation of mucociliary clearance in health and disease. Eur Respir J 13: 1177-1188, 1999.
29. Armengot M, Milara J, Mata M, Carda C and Cortijo J: Cilia motility and structure in primary and secondary ciliary dyskinesia. Am J Rhinol Allergy 24: 175-180, 2010.
30. Smith CM, Hirst RA, Bankart MJ, Jones DW, Easton AJ, Andrew PW and O'Callaghan C: Cooling of cilia allows functional analysis of the beat pattern for diagnostic testing. Chest 140: 186-190, 2011.
31. Calderón-Garcidueñas L, Valencia-Salazar G and Rodríguez-Alcaraz A, *et al*: Ultrastructural nasal pathology in children chronically and sequentially exposed to air pollutants. Am J Respir Cell Mol Biol 24: 132-138, 2001.
32. Abe J, Hosokawa H, Sawada Y, Matsumura K and Kobayashi S: Ca^{2+}-dependent PKC activation mediates menthol-induced desensitization of transient receptor potential M8. Neurosci Lett 397: 140-144, 2006.
33. Daniels RL, Takashima Y and McKemy DD: Activity of the neuronal cold sensor TRPM8 is regulated by phospholipase C via the phospholipid phosphoinositol 4,5-bisphosphate. J Biol Chem 284: 1570-1582, 2009.
34. Baroody FM: Mucociliary transport in chronic rhinosinusitis. Clin Allergy Immunol 20: 103-119, 2007.
35. Kim YM, Lee CH, Won TB, Kim SW, Kim JW, Rhee CS and Min YG: Functional recovery of rabbit maxillary sinus mucosa in two different experimental injury models. Laryngoscope 118: 541-545, 2008.
36. Sabnis AS, Reilly CA, Veranth JM and Yost GS: Increased transcription of cytokine genes in human lung epithelial cells through activation of a TRPM8 variant by cold temperatures. Am J Physiol Lung Cell Mol Physiol 295: L194-L200, 2008.
37. Arnson Y, Shoenfeld Y and Amital H: Effects of tobacco smoke on immunity, inflammation and autoimmunity. J Autoimmun 34: J258-J265, 2010.
38. Adcock IM, Caramori G and Barnes PJ: Chronic obstructive pulmonary disease and lung cancer: new molecular insights. Respiration 81: 265-284, 2011.
39. Mulligan RM, Atkinson C, Vertegel AA, Reukov V and Schlosser RJ: Cigarette smoke extract stimulates interleukin-8 production in human airway epithelium and is attenuated by superoxide dismutase in vitro. Am J Rhinol Allergy 23: e1-e4, 2009.
40. Li YT, He B and Wang YZ: Exposure to cigarette smoke upregulates AP-1 activity and induces TNF-alpha overexpression in mouse lungs. Inhal Toxicol 21: 641-647, 2009.
41. Takeyama K, Jung B and Shim JJ, *et al*: Activation of epidermal growth factor receptors is responsible for mucin synthesis induced by cigarette smoke. Am J Physiol Lung Cell Mol Physiol 280: L165-L172, 2001.
42. Baginski TK, Dabbagh K, Satjawatcharaphong C and Swinney DC: Cigarette smoke synergistically enhances respiratory mucin induction by proinflammatory stimuli. Am J Respir Cell Mol Biol 35: 165-174, 2006.
43. Innes AL, Woodruff PG, Ferrando RE, Donnelly S, Dolganov GM, Lazarus SC and Fahy JV: Epithelial mucin stores are increased in the large airways of smokers with airflow obstruction. Chest 130: 1102-1108, 2006.
44. Haswell LE, Hewitt K, Thorne D, Richter A and Gaça MD: Cigarette smoke total particulate matter increases mucous secreting cell numbers in vitro: a potential model of goblet cell hyperplasia. Toxicol In Vitro 24: 981-987, 2010.