Cold temperature induces mucin hypersecretion from normal human bronchial epithelial cells *in vitro* through a transient receptor potential melastatin 8 (TRPM8)–mediated mechanism

MinChao Li, PhD,[a] Qi Li, PhD,[a] Gang Yang, PhD,[b] Victor P. Kolosov, MD, PhD,[c] Juliy M. Perelman, MD, PhD,[c] and Xiang Dong Zhou, MD, PhD[a] *Chongqing, China, and Blagoveschensk, Russia*

Background: Cold air stimulus is a major environmental factor that exacerbates chronic inflammatory airway diseases, such as chronic obstructive pulmonary disease (COPD) and asthma. At the molecular level, cold is detected by transient receptor potential melastatin 8 (TRPM8). To date, TRPM8 expression has not been characterized in the airway epithelium of patients with COPD. The role of TRPM8 channels in a series of airway responses induced by cold stimuli and the molecular and biochemical pathways of TRPM8 in regulating cold-induced responses are largely unknown.

Objective: We sought to explore the role of TRPM8 in cold air–provoked mucus hypersecretion and the potential signaling pathway involved in this process.

Methods: The expression of TRPM8 in the bronchial epithelium was examined by means of immunohistochemistry, RT-PCR, and Western blotting. TRPM8 receptor function and hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) were characterized by means of Ca^{2+} imaging and spatiotemporal dynamics of phospholipase C (PLC) δ1–pleckstrin homology–green fluorescent protein, respectively. The expression of MUC5AC mRNA and MUC5AC mucin protein was measured by using real-time PCR and ELISA, respectively. Four serine residues in the myristoylated alanine-rich C kinase substrate (MARCKS)–phosphorylation site domain were mutated to identify the function of MARCKS in TRPM8-mediated airway mucus hypersecretion.

Results: TRPM8 protein and mRNA expression were significantly increased in patients with COPD compared with expression seen in healthy subjects. Cold produced robust increases in intracellular Ca^{2+} levels and promoted translocation of PLCδ1–pleckstrin homology–green fluorescent protein. Cold increased expression of MUC5AC mRNA and intracellular and secreted MUC5AC protein in a nonsustained way. Phosphorylation site domain–mutant MARCKS cDNA hindered MUC5AC secretion induced by cold.

Conclusions: These results indicate that the TRPM8 receptor is involved in cold-induced mucus hypersecretion through the Ca^{2+}-PLC-PIP2-MARCKS signaling pathway. (J Allergy Clin Immunol 2011;128:626-34.)

Key words: *Transient receptor potential melastatin 8, mucus hypersecretion, MUC5AC, cold air, myristoylated alanine-rich C kinase substrate, Ca^{2+}, phosphatidylinositol 4,5-bisphosphate*

Exposure to cold air is a major environmental factor that exacerbates chronic inflammatory airway diseases, such as chronic obstructive pulmonary disease (COPD) and asthma.[1,2] Cold temperature–related exacerbation of these diseases is often followed by a subsequent increase in bacterial and viral infections of the airway, infiltration of inflammatory factors, and mucus secretion.[2,3] Cold stimulus for such diseases, to a certain extent, plays a "trigger point" effect, leading to the exacerbation. An in-depth exploration of the regulatory mechanisms of cold stimulation in patients with COPD might have important clinical implications for the prevention of such exacerbations in these diseases.

The discovery of thermosensitive ion channels of the transient receptor potential (TRP) family has demonstrated an underlying molecular mechanism for temperature detection.[4,5] Transient receptor potential melastatin 8 (TRPM8), a nonselective calcium (Ca^{2+})–permeable cation channel, is expressed on a subset of sensory neurons from the dorsal root ganglion and trigeminal ganglia,[4-6] as well as in a number of nonneuronal areas.[7-10] TRPM8 is activated by cold temperatures of less than 25°C and cooling agents, such as menthol, eucalyptol, or icilin agents.[4,5,11,12] Antagonists of TRPM8 include the compounds N-(4-tert-butylphenyl)-4-(3-chloropyridin-2-yl) piperazine-1-carboxamide (BCTC), thio-BCTC, 2-aminoethoxydiphenyl borate, and capsazepine.[11,12] Electrophysiological studies have indicated that activation of TRPM8 results in a large increase in intracellular Ca^{2+} levels.[5,13,14] TRPM8-mediated Ca^{2+} influx activates phospholipase C (PLC), thereby hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP2),[15-17] and leads to the generation of inositol trisphosphate (IP3) and diacylglycerol. Diacylglycerol is an activator of protein kinase C (PKC),[16,18] and IP3 can induce Ca^{2+} release from endoplasmic reticulum (ER).[19] Activated PKC phosphorylates myristoylated alanine-rich C kinase substrate (MARCKS), which is a central regulatory

From [a]the Department of Respiratory Medicine, Second Affiliated Hospital of Chongqing Medical University; [b]the Department of Neurosurgery, First Affiliated Hospital of Chongqing Medical University; and [c]Far Eastern Scientific Center of Physiology and Pathology of Respiration, Siberian Branch, Russian Academy of Medical Sciences, Blagoveschensk.

Supported by the National Nature Science Foundation of China (no. NSF81070031) and the China-Russia Cooperation Research Foundation (no. NSF81011120108; RFBR10-04-91160).

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication September 27, 2010; revised March 10, 2011; accepted for publication April 14, 2011.

Available online July 18, 2011.

Reprint requests: Xiang Dong Zhou, MD, PhD, Department of Respiratory Medicine, Second Affiliated Hospital of Chongqing Medical University, No. 74, Linjiang Road, Yuzhong District, Chongqing 400010, China. E-mail: longman185@163.com.

0091-6749/$36.00

doi:10.1016/j.jaci.2011.04.032

Abbreviations used

BCTC: N-(4-tert-butylphenyl)-4-(3-chloropyridin-2-yl) piperazine-1-carboxamide
COPD: Chronic obstructive pulmonary disease
ER: Endoplasmic reticulum
ERK: Extracellular signal-regulated kinase
FVC: Forced vital capacity
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GFP: Green fluorescent protein
IP3: Inositol trisphosphate
MARCKS: Myristoylated alanine-rich C kinase substrate
NHBE: Normal human bronchial epithelial
PH: Pleckstrin homology
PIP2: Phosphatidylinositol 4,5-bisphosphate
PKC: Protein kinase C
PLC: Phospholipase C
PSD: Phosphorylation site domain
shRNA: Short hairpin RNA
TRP: Transient receptor potential
TRPM8: Transient receptor potential melastatin 8

molecule linking secretagogue stimulation at the cell surface to mucin granule release by differentiated normal human bronchial epithelial (NHBE) cells.[20-22] Accordingly, we speculated that the cold stimulation–induced activation of the TRPM8 channel could lead to the activation of MARCKS and participate in the exocytosis of mucus secretion.

Recent studies have identified and characterized a novel cold- and menthol-activated TRPM8 variant in lung epithelial cells.[14] Activation of the TRPM8 variant in lung epithelial cells by cold air leads to increased expression of several cytokine and chemokine genes.[23] Airway vagal afferent nerves express TRPM8 receptors, and activation of TRPM8 receptors by cold excites these airway autonomic nerves.[24] These results show that TRPM8 receptors are potentially involved with the airway inflammatory response and resistance induced by cold air. However, there is no direct evidence that TRPM8 channels are present in lung tissue from patients with COPD. The role of TRPM8 channels in cold-induced airway responses has not been explored in detail, and the molecular and biochemical pathways for TRPM8-induced regulation of cold-induced responses are largely unknown.

The goals of the studies described here were as follows: (1) Clarify TRPM8 expression in lung tissue from patients with COPD and healthy subjects; (2) explore the hypothesis that cold-mediated activation of the TRPM8 channel is responsible for the enhanced expression of MUC5AC, which is one of the predominant airway mucins implicated in pulmonary diseases with mucus hypersecretion[25]; and (3) determine the role of MARCKS in the molecular mechanisms of cold-initiated airway hypersecretion.

METHODS

Reagents

PLCδ1–pleckstrin homology (PH)–green fluorescent protein (GFP) plasmids were kind gifts from Tamas Balla (Public Health Service, National Institute of Health, Bethesda, Md). BCTC, exon 18–specific TRPM8 small hairpin RNA (shRNA), and scramble shRNA were kind gifts from Christopher A. Reilly (University of Utah, Salt Lake City, Utah). L-menthol (abbreviated as menthol) was purchased from Crystal Pure Reagent Co Ltd (Shanghai, China) and dissolved in 75% ethanol to the final concentration indicated. Fluro-3 AM was purchased from Beyotime Biotechnology (Jiangsu, China). Rabbit anti-TRPM8 (C-term) polyclonal antibody was purchased from Abgent (San Diego, Calif). SYBR Premix EX Taq was purchased from TaKaRa Biotechnology (Dalian, China).

Tissues

Experiments were approved by the institutional review board, and informed consent was obtained before sampling. For more information, see the Methods section in this article's Online Repository at www.jacionline.org.

For more information on immunohistochemical localization of TRPM8 in human lung tissue quantitative analysis, see the Methods section in this article's Online Repository.

Cell culture

Proliferating NHBE cells were purchased from the American Type Culture Collection (ATCC, Rockville, Md). For more information, see the Methods section in this article's Online Repository.

PLCδ1-PH-GFP plasmid transient transfection and TRPM8 shRNA plasmid stable transfection

PLCδ1-PH-GFP, exon 18–specific TRPM8 shRNA, and scramble shRNA were identified and transfected into the NHBE cells according to the manufacturer's instruction. For more information, see the Methods section in this article's Online Repository. Attenuated TRPM8 protein expression was observed in NHBE cells stably transfected with TRPM8 shRNA, and the extent of gene knockdown did not change over time. The basal activation of critical pathways was not affected by G418 selection in our experiments (see Figs E1 and E2 and the Results section in this article's Online Repository at www.jacionline.org for more information).

For more information on cytotoxicity assessment, see the Methods section in this article's Online Repository.

For more information on Ca^{2+} imaging and spatiotemporal dynamics of PLCδ1-PH-GFP, see the Methods section in this article's Online Repository.

Construction of MARCKS–phosphorylation site domain–targeted mutations and stable transfection

The effector domain of MARCKS has the sequence KKKKKRFS FKKSFKLSGFSFKKSKK, in which 4 serine residues are the PKC-dependent phosphorylation site domain (PSD).[22] Four serine residues (Ser^{159}, Ser^{163}, Ser^{167}, and Ser^{170}) in the wild-type PSD were mutated to aspartic acids to construct MARCKS-PSD–targeted mutations. The tetra-Asp mutant should not be phosphorylated by PKC.[26] For more information, see the Methods section in this article's Online Repository.

RNA isolation, reverse transcription, and real-time PCR analysis

The TRPM8 mRNA and MUC5AC mRNA transcripts were measured by using real-time quantitative PCR. The PCR primers for human *TRPM8*, human *MUC5AC*, and human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were designed according to the published cDNA sequences (see Table E1 and the Methods section in this article's Online Repository at www.jacionline.org).

For more information on Western blotting, see the Methods section in this article's Online Repository.

Determination of MUC5AC protein level

Production of MUC5AC mucin protein in cell lysates and culture supernatants was measured by means of ELISA, as previously reported.[27] For more information, see the Methods section in this article's Online Repository.