



Cold-inducible RNA-binding protein migrates from the nucleus to the cytoplasm under cold stress in normal human bronchial epithelial cells via TRPM8-mediated mechanism

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Background: Cold-inducible RNA-binding protein (CIRP or hnRNP A18) is a multifunctional stress-responsive protein. Our previous study demonstrated that cold stress increased CIRP expression and migrated from the nucleus to the cytoplasm in airway epithelial cells. However, the mechanism through which CIRP migrates from the nucleus to the cytoplasm upon cold stress remains unknown.

Methods: The expression of CIRP in the bronchial epithelium was examined using immunofluorescence, real-time polymerase chain reaction (RT-PCR), and Western blotting. The expression of inflammatory factors interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor- α (TNF- α) were detected by ELISA and RT-PCR. Transient receptor potential melastatin 8 (TRPM8) receptor function was characterized by Ca²⁺ imaging.

Results: Cold stress upregulated the expression of CIRP, inflammatory factors and promoted the translocation of CIRP from the nucleus to the cytoplasm in normal human bronchial epithelial (NHBE) cells. Cold stress activated the TRPM8/(Ca²⁺)/PKC α /glycogen synthase kinase 3 β (GSK3 β) signaling cascade, and that inhibition of this signaling pathway attenuated the migration of CIRP from the nucleus to cytoplasm but did not decrease its overexpression induced by cold stress. Knocked down CIRP expression or blocked CIRP migration between the nucleus and cytoplasm significantly decreased inflammatory factor expression.

Conclusions: These results indicate that cold stress leads to the migration of CIRP from the nucleus to the cytoplasm with alteration of expression, which are involved in the expression of inflammatory factors (IL-1 β , IL-6, IL-8 and TNF- α) induced by cold air, through TRPM8/Ca²⁺/PKC α /GSK3 β signaling cascade.

Keywords: Cold-inducible RNA-binding protein (CIRP); cold stress; migration; transient receptor potential melastatin 8 (TRPM8); glycogen synthase kinase 3 β (GSK3 β)

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Introduction

The cold-inducible RNA-binding protein (CIRP or hnRNP A18) belongs to a group of cold-shock proteins (1) and contains 172 amino acid residues (2). CIRP, the first cold-shock protein identified in mammals (3), has various biological functions. CIRP can be enhanced by stressors, including mild cold temperature (4,5), UV irradiation (6), hypoxia (7), and osmotic pressure (8). Additionally, CIRP protects cells from death induced by genotoxic stress and inhibits UV- and hydrogen peroxide (H₂O₂)-induced cell apoptosis (9). However, several studies have revealed that CIRP released from cells into circulation promotes inflammation, contributing to poor prognosis in brain inflammation (10), hemorrhagic shock, and sepsis (11). In addition, the spatial distribution of CIRP is not fixed. Originally, CIRP was detected in the nucleus of fibroblastic cells and induced by hypothermic shock (12). Nishiyama *et al.* and Matsumoto *et al.* then observed CIRP in the cytoplasm of human spermatid cells (13) and *Xenopus* oocytes (14), respectively. Further, De Leeuw *et al.* revealed that CIRP translocated from the nucleus to the cytoplasm in response to osmotic stress (15). These data imply that the localization of CIRP is subjected to cell type and the cell type-specific response to diverse stressors.

Our previous study demonstrated that cold stress increased CIRP expression and mediated airway mucin production through toll-like receptor 4 (TLR4)/nuclear factor- κ B (NF- κ B) signaling in normal human bronchial epithelial (NHBE) cells (16). While CIRP is mainly localized in the nucleus, TLR4 is on the plasma membrane and NF- κ B is a cytoplasmic signaling molecule. Therefore, we speculated that under cold stress, CIRP migrated from the nucleus to the cytoplasm in human bronchial epithelial (16HBE) cells.

CIRP consists of a highly conserved RNA-recognition motif (RRM) in the N-terminal and a less conserved arginine-glycine-rich (RGG) domain in the C-terminal, which is a nuclear localization signal and associated with nucleocytoplasmic shuttling (17). Previous studies have reported that CIRP migrates from the nucleus to cytoplasmic stress granules through a methylation-dependent mechanism (14). Other data have confirmed that glycogen synthase kinase 3 β (GSK3 β), a serine/threonine kinase, upregulates CIRP transcription, increases CIRP protein phosphorylation level, and facilitates its translocation to the cytoplasm (18). However, the mechanism through which CIRP migrates from the nucleus

to the cytoplasm upon cold stress in airway epithelial cells remains unknown.

The discovery of the transient receptor potential (TRP) family of thermosensitive ion channels revealed a new biological mechanism for temperature sensing (19,20). TRP melastatin 8 (TRPM8), which is a nonselective calcium (Ca²⁺)-permeable cation channel, is mainly expressed on the sensory neurons of the trigeminal ganglia and dorsal root ganglion (19-21). TRPM8 is also expressed on 16HBE cells (22). Several studies have revealed that TRPM8 can be activated by diverse stimulators, including cold temperature, Icilin, eucalyptol, and menthol (19,20,23,24). Electrophysiological studies have found that TRPM8 activation leads to a large release of intracellular Ca²⁺ (20,25,26). TRPM8-induced Ca²⁺ influx activates phospholipase C (PLC), thereby hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP₂), resulting in the generation of inositol trisphosphate (IP₃) and diacylglycerol (27,28). Daniels *et al.* revealed that IP₃ promotes Ca²⁺ release from the endoplasmic reticulum (ER), which is consistent with research findings by Puzianowska-Kuznicka and Kuznicki (29,30). Rebecchi and Pentylala demonstrated that diacylglycerol is an activator of protein kinase C (PKC) (31). Phosphorylated PKC further activates GSK3 β (32,33), which has been identified as a contributor to the cytosolic translocation of CIRP protein (18,34). Accordingly, we supposed that cold stress-mediated activation of TRPM8 phosphorylated GSK3 β and led to the migration of CIRP protein from the nucleus to the cytoplasm in 16HBE cells under cold stress. We present the following article in accordance with the MDAR reporting checklist (available at <https://dx.doi.org/10.21037/atm-21-4447>).

Methods

Reagents

16HBE cells were purchased from the Experimental Medical Research Center of Guangzhou Medical College (SCC150, Guangzhou, Guangdong, China). Roswell Park Memorial Institute (RPMI) 1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Rabbit anti-CIRP polyclonal antibody was purchased from Proteintech Group, Inc. (10209-2-AP, Chicago, IL, USA). Rabbit anti-TRPM8 anti-body and anti-phospho-PKC (p-PKC) were purchased from Abcam (ab109308 and ab109539, Cambridge, MA, USA). Anti-phospho-

GSK3 β ser 9 (p-GSK3 β ser 9) was bought from Bioworld Technology, Inc. (BS4084P, Shanghai, China). Anti-t-PKC (ab107166), anti-t-GSK3 β (ab32391), monoclonal anti- β -actin (ab5694), anti-Lamin B1 (ab133741), and secondary immunofluorescence DyLight 488 antibody (ab96899) were purchased from Abcam. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) was purchased from Jinqiao Biotech (ZB-2306, Beijing, China). TRPM8 inhibitor BCTC (ab141517), (Ca²⁺)_i chelating agent BAPTA-AM (ab120503), and PKC α specific inhibitor safinol (ab144070) were purchased from Abcam. TRPM8-, GSK3 β - and scrambled-small interfering RNA (siRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Real-time polymerase chain reaction (RT-PCR) primers were synthesized by Sangon Biotechnology (Shanghai, China).

Cell culture and hypothermia incubation

The 16HBE cells were grown on a 12 mm transwell membrane insert (0.45 μ m pore size; Costar, Corning Inc., NY, USA) and maintained at the air-liquid interface. The cells were cultured in RPMI 1640 medium containing 10% FBS, penicillin (100 IU/mL), and streptomycin (100 μ g/mL) at 37 °C in a 5% CO₂ humidified incubator. The culture media were refreshed every 2–3 days. When the cells were 80–90% confluent, they were passaged with trypsin or ethylenediaminetetraacetic acid (EDTA) and reseeded in the air-liquid interface culture at 1 \times 10⁵ cells/cm² of density. When the cells reached 80–90% confluence again after being cultured for another 24–48 hours, they were used for the experiments. For temperature-shift experiments, 16HBE cells starved by RPMI 1640 without FBS for an additional 24 hours were first incubated at 15, 18, 22, 25, or 32 °C in a 5% CO₂ humidified incubator for 12 hours. The cells were then harvested for further experiments. For the inhibition experiments, cells were pretreated with BCTC (15 μ M), BAPTA-AM (10 μ M), or safinol (30 μ M) 30 minutes before cold air treatment.

siRNA transfection

The 16HBE cells were plated at a density of approximately 2 \times 10⁶ cells/mL in each well of a 24-well plate at 60–80% confluence. The cells were then transfected with 20 nM of siRNA (siCIRP, siTRPM8, siGSK3 β , or siNT) using Lipofectamine 2000, according to the manufacturer's

instructions. Twenty-four hours later, the cells were washed 3 times with PBS and incubated in serum-free RPMI 1640 medium for another 24 hours before cold stimulation.

Cell viability assay

Cell viability was assessed using a tetrazolium-based (MTT) assay. Cells (5 \times 10⁴ cells/well) were seeded in 96-well plates and cultured in 200 μ L of RPMI 1640 medium under different treatment conditions for the indicated time periods. Next, 20 μ L of MTT was added, and the plates were incubated at 37 °C for an additional 4 hours. After incubation, 150 μ L of dimethyl sulfoxide (DMSO) was added to each well and incubated at room temperature for 10 minutes. Subsequently, the absorbance was measured at 570 nm with a Sunrise Remote microplate reader (Tecan, Männedorf, Switzerland).

Protein extraction

Cells in each group were collected and washed 3 times with PBS; the supernatant was removed, and the cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing a protease inhibitor and phosphatase inhibitor. Samples were then centrifuged at 14,000 \times g for 15 minutes at 4 °C. The supernatants were transferred to new tubes for subsequent analysis. The samples were then tested using a bicinchoninic acid (BCA) protein assay reagent. The quantitative changes in CIRP, TRPM8, PKC α , p-PKC α , GSK3 β , and p-GSK3 β were tested by western blot, while interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), interleukin-8 (IL-8), and interleukin-6 (IL-6) were detected by ELISA assay.

Subcellular fractionation

Cytoplasmic and nuclear extracts were prepared according to the nuclear and cytoplasmic extraction kit guide (BestBio, Shanghai, China). Additionally, all samples were tested using a BCA protein assay reagent. Quantitative changes of CIRP in the nucleus and cytoplasm were measured by western blot.

Western blotting

The extracted protein samples were separated with SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the proteins were transferred through

electrophoresis onto polyvinylidene difluoride (PVDF) membranes and blocked with 5% skimmed milk or 5% bovine serum albumin (BSA) at room temperature for 1 hour. The PVDF membranes were then probed with specific primary antibodies against CIRP (1:500), TRPM8 (1:200), p-PKC α (1:500), and p-GSK3 β (ser 9) (1:500) overnight at 4 °C. After being washed 3 times with phosphate-buffered saline with Tween-20 (PBST) containing 500 mL of PBS and 250 μ L of Tween-20, the membranes were incubated with the HRP-conjugated goat anti-rabbit and anti-mouse secondary antibodies (1:1,000) for 1 hour at room temperature. The blots were developed according to the instructions of the enhanced chemiluminescence (ECL) reagent kit (KeyGen Biotech Co., Ltd., Nanjing, China). Lamin B1 was used as a nuclear envelope marker and β -actin was used as cytosolic protein or total protein marker. The amount of protein present was normalized with either β -actin or Lamin B1.

ELISA assay

After various treatments, the amount of IL-6, IL-8, TNF- α , and IL-1 β protein was detected according to the guidelines of the ELISA kit (KeyGen Biotech Co., Ltd.) Cell lysates were prepared with PBS at multiple dilutions. A 50 μ L aliquot of each sample was then incubated with an equal volume of bicarbonate-carbonate buffer at 40 °C in a 96-well plate until dry. The plates were washed 3 times with PBS and blocked with 2% BSA for 1 hour at room temperature followed by incubation with 50 μ L of the primary antibody (1:200 for mouse IL-1 β , IL-6, IL-8, and TNF- α antibodies) at 37 °C for 1 hour. Following a wash step, the plates were incubated with the HRP-conjugated goat anti-mouse IgG (1:5,000) for 1 hour at room temperature. The color reaction was performed with a 3,3',5,5'-tetramethylbenzidine peroxidase solution (TMB) and quenched with sulfuric acid (H₂SO₄). The absorbance was read at 450 nm using a microplate reader. The results were calculated based on a comparison with standard samples.

RT-PCR

Total RNA in each group was extracted using TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China). Samples with OD260/OD280 ratios between 1.6 and 1.8 were stored at -20 °C. Subsequently, 2 μ g of total RNA was used to synthesize complementary DNA (cDNA) using the iScript complementary DNA synthesis kit,

and 20 ng cDNA was used for the RT-PCR reaction with iQ SYBR Green supermix on an iCycler (Takara Biotechnology Co., Ltd.). The following primers were prepared: CIRP, forward 5'-ACAGATCATGGCATCAGATGAAGGCAAAC-3' and reverse 5'-TAAGCTTACTCGTTGTGTGTAGCGTAACTG-3'; IL-1 β , forward 5'-CAGGATGAGGA CATGAGCACC-3' and reverse 5'-CTCTGCAGACTCAAACCTCCAC-3'; IL-6, forward 5'-AGTGAGG AACAAGCCAGAGC-3' and reverse 5'-GAGGTGCCCATGCTACATTT-3'; IL-8, forward 5'-CCTGATTTCTGCAGCTCTGT-3' and reverse 5'-AACTTCTCCACAACCCTCTG-3'; TNF- α , forward 5'-AGGACTCAGCTTCGACACCA and reverse 5'-CGTCCACAGACTTCCCA TTC-3', and; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5'-GAAGGTGAAGGTCCGAGT-3' and reverse 5'-GAAG ATGGTGATGGGATTTTC-3'. PCR reactions were performed as follows: CIRP, 94 °C for 1 minute, 30 cycles of 53 °C for 1 minute, and 72 °C for 1.5 minutes, followed by 5 minutes of incubation at 72 °C; IL-1 β , 94 °C for 2 minutes, 28 cycles of 94 °C for 30 seconds, and 58 °C for 30 seconds, followed by 10 minutes of incubation at 72 °C; IL-6 and IL-8, 94 °C for 2 minutes, 28 cycles of 94 °C for 30 seconds, and 72 °C for 30 seconds, followed by 10 minutes of incubation at 72 °C, and; TNF- α , 95 °C for 30 seconds, 30 cycles of 95 °C for 5 seconds, and 64 °C for 30 seconds, followed by 10 minutes of incubation at 72 °C. The comparative Ct method (2^{- $\Delta\Delta$ Ct}) was used for relative mRNA quantification. GAPDH mRNA was also amplified as an internal control.

Immunofluorescence

The 16HBE cells from each group were plated at a density of 1 \times 10⁶/mL in 24-well plates on a poly-L-lysine coated glass coverslip in each well. The culture media was renewed each day. After reaching 60–80% confluence, the cells were washed 3 times for 5 minutes with ice-cold PBS. Cells were then fixed with 4% paraformaldehyde for 20 minutes at room temperature and permeabilized with 0.1% Triton X-100 for 30 minutes. After a wash step, cells were blocked with 1% BSA plus 1% normal goat serum and followed by incubation with anti-CIRP or a mouse anti-MUC5AC antibody (1:200) overnight at 4 °C. Cells were then rinsed again with PBS (3 times for 10 minutes) and incubated with a FITC-conjugated fluorescent secondary antibody (1:200) in the dark for 1 hour at 37 °C. Finally, the cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The

samples were visualized using a TCS-SP2 laser scanning confocal microscope (Leica, Wetzlar, Germany) equipped with ZEN 2009 software for image acquisition and analyses.

Calcium imaging assay

The concentration of $(Ca^{2+})_i$ was measured by calcium imaging. First, the cell suspensions from each treatment group were collected. Next, 200 μ L suspensions were loaded with 200 μ L of 5 μ M Fluo-3/AM in Hank's balanced salt solution for 30 minutes in the dark at 37 °C. Cell suspensions were then washed twice with D-Hank's solution to remove the extracellular fluo-3/AM. Laser scanning confocal microscope (Leica) analysis was performed to determine intracellular Ca^{2+} concentration which was indicated by fluo-3-fluorescence and expressed as the fluorescence intensity.

Statistical analysis

Data are presented as the mean \pm SD of five independent experiments. All experiments were independently repeated 5 times. SPSS statistics version 22.0 software was used to analyze the data and one-way analysis of variance (ANOVA) followed by Bonferroni analysis were performed to evaluate the levels of difference between groups. P values <0.05 were considered statistically significant.

Results

Cell viability

The cell viability of each group was detected by MTT assay. We found that none of the conditions used in our study showed significant influence on cell viability.

Cold stress induced CIRP upregulation in 16HBE cells

CIRP is induced by multiple environmental stresses, including cold temperature, hypoxia, UV irradiation, and osmotic pressure. The present study further verified that cold stress enhances the expression of CIRP protein and mRNA. Our data showed that the expression of CIRP protein and mRNA were both increased markedly by mild cold stress (32–18 °C), peaking at 18 °C, but the increase was reduced under severe cold treatment of 15 °C (Figure 1A,1B). Further, when 16HBE cells were exposed to cold air (a temperature of 18 °C), we found that cold stress-

induced CIRP protein expression in a time-dependent manner, with the increase beginning at 4 hours and peaking at 12 hours (Figure 1C). Meanwhile, CIRP mRNA expression also began to increase at 4 hours, but the plateau expression was observed at 8 hours and persistent for 12 hours (Figure 1D). based on these results, a temperature of 18 °C for 12 hours was selected as the best reaction condition for subsequent experiments because according to the previous observation, we assume that this is the best condition to observe CIRP protein expression increase.

Cold-air triggered TNF- α , IL-1 β , IL-6, and IL-8 expression and CIRP may participate in inflammatory responses induced by cold air in 16HBE cells

Previous studies have confirmed that exposure to cold air can aggravate chronic airway inflammation diseases, such as asthma and chronic obstructive pulmonary disease (COPD), by triggering cold airway inflammation (35,36). In this paper, again we confirmed that cold air triggered airway inflammatory responses and explored the underlying mechanism. Our data showed that the expression of TNF- α , IL-1 β , IL-6, and IL-8 mRNA and protein were significantly increased by cold stress (18 °C) compared to the control group (37 °C), which indicated that cold stress could trigger airway inflammation. In addition, we found that compared with the control nontargeting siRNA (siNT) and no transfection groups, increased expression of TNF- α , IL-1 β , IL-6, and IL-8 mRNA and protein were significantly decreased by CIRP siRNA, which attenuated CIRP protein expression effectively (Figure 2). These findings demonstrated that cold air could trigger TNF- α , IL-1 β , IL-8, and IL-6 expression, and that CIRP was involved in the expression of these inflammatory factors.

Cold stress promoted CIRP migration from the nucleus to the cytoplasm in 16HBE cells

Previous studies have reported that the spatial distribution of CIRP is based on different environmental stressors and is subjected to the environment in which the cells are located. We further observed the spatial distribution of the CIRP before and after cold stimulation using confocal laser-scanning microscopy (CLSM). The CLSM showed that cold stress enhanced the expression of CIRP, which was mainly observed in the cytoplasm (Figure 3A). We further determined the level of CIRP expression at the cellular and subcellular levels, respectively. At the cellular

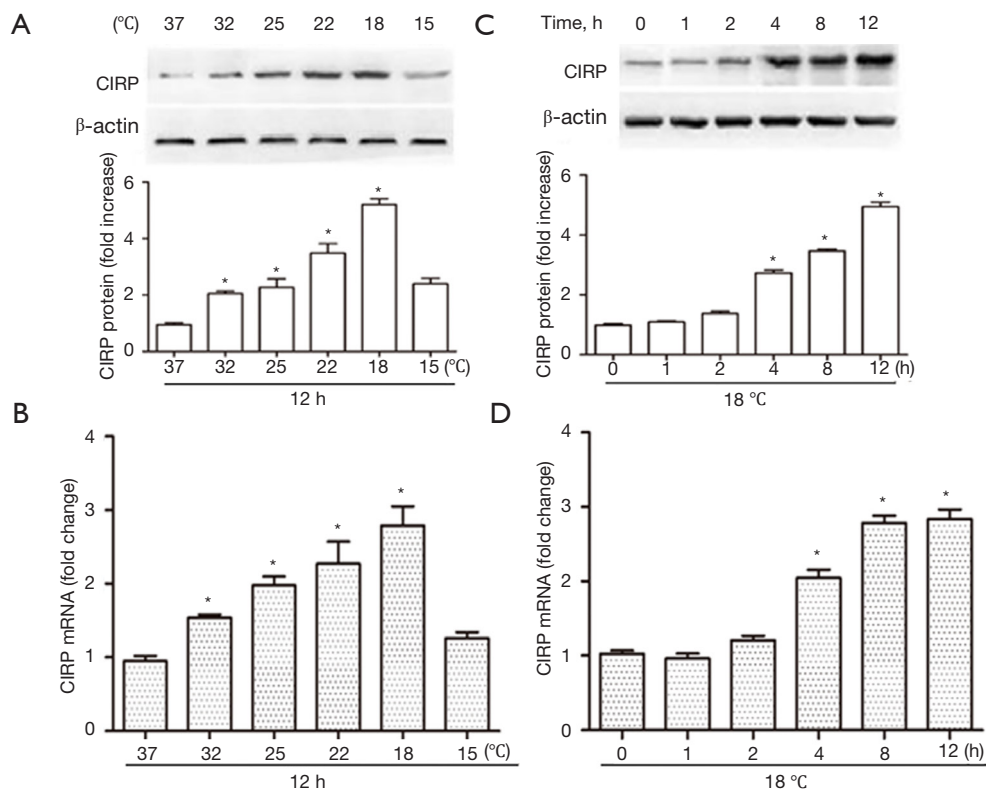


Figure 1 Effect of cold air on CIRP expression in 16HBE cells. Cells were cultured at various temperatures (37, 32, 25, 22, 18, or 15 °C) and for various times (0, 1, 2, 4, 8, or 12 hours). Total RNA and protein of CIRP were collected. The levels of CIRP mRNA and protein were then measured by RT-PCR and western blotting, respectively. (A) Effect of decreasing temperatures on CIRP protein production over 12 hours. The expression of CIRP protein increased markedly in response to mild cold treatment (32–18 °C), peaking at 18 °C. Compared with 18 °C, the increase induced by severe cold treatment (15 °C) was reduced. (B) Effect of decreasing temperatures on CIRP mRNA expression. The expression of CIRP mRNA increased markedly in response to mild cold treatment (32–18 °C) and peaked at 18 °C, but severe cold treatment (15 °C) did not affect the levels of CIRP mRNA. (C) Effect of cold air (18 °C) treatment for various time intervals on CIRP protein production. The expression of CIRP was increased in a time-dependent manner. (D) Effect of cold air (18 °C) treatment for various time intervals on CIRP mRNA expression. CIRP mRNA increased markedly starting at 4 hours, and this increase peaked at 8 hours and persisted for 12 hours. * $P < 0.01$ compared with control (temperature 37 °C or time 0). These data are presented as the means \pm SD ($n=5$). CIRP, cold-inducible RNA-binding protein; 16HBE, human bronchial epithelial; RT-PCR, real-time polymerase chain reaction.

level, western blot assay demonstrated that cold treatment markedly increased CIRP expression (Figure 3B). At the subcellular level, as shown in Figure 3C,3D, CIRP was mainly detected in the nucleus rather than the cytoplasm in the control group. Conversely, in the cold group, CIRP expression in the cytoplasm was higher than in the nucleus, which was consistent with the CLSM results. These results suggested that cold stress increased CIRP expression and also promoted the migration of CIRP from the nucleus to the cytoplasm in 16HBE cells.

Functional analysis of TRPM8 under cold stress in 16HBE cells

To measure the characteristic function of the TRPM8 channel, TRPM8 defective 16HBE cells were generated by transfection with siTRPM8. 16HBE cells were pretreated with TRPM8 inhibitor BCTC and the $(Ca^{2+})_i$ chelating agent BAPTA-AM for 30 minutes. The cells were then given 18 °C cold air stress for 12 hours. The $(Ca^{2+})_i$ was measured by calcium imaging. As shown in Figure 4, cold stress significantly increased intracellular Ca^{2+} , and increases

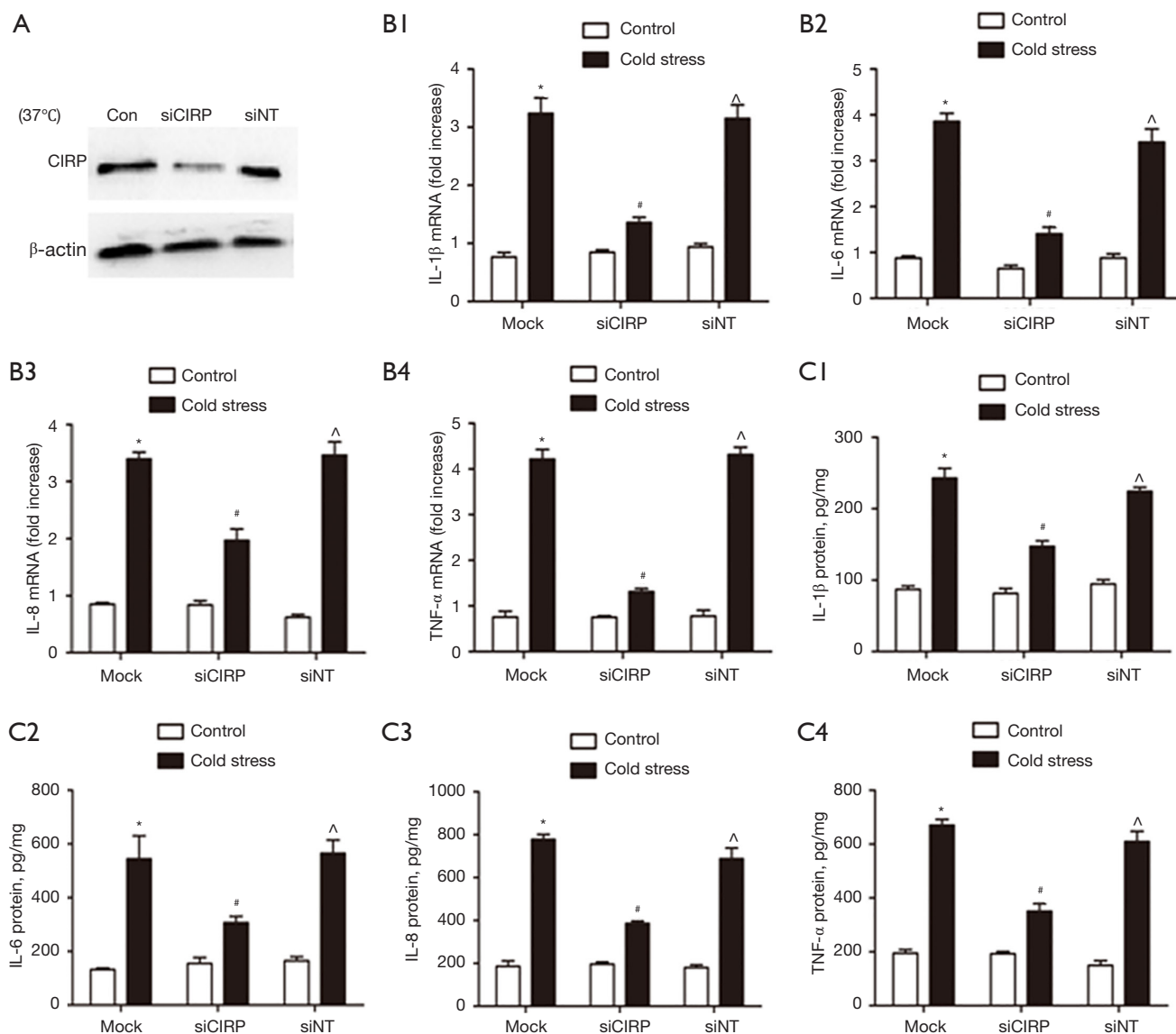


Figure 2 The effect of CIRP downregulation on inflammatory factors (IL-1 β , IL-6, IL-8, and TNF- α) mRNA and protein expression levels. The 16HBE cells were transfected with the specific siCIRP or siNT, or left untransfected as the negative control. After transfection, the cells were incubated at 18 °C (cold stress) or 37 °C (control) for 12 hours to measure IL-1 β , IL-6, IL-8, and TNF- α mRNA and protein expression, respectively. (A) Western blotting demonstrated that CIRP expression was notably blocked by siCIRP. (B) The RT-PCR suggested that the transfection of siCIRP significantly attenuated cold stress-induced expression of IL-1 β , IL-6, IL-8, and TNF- α mRNA. (C) The ELISA indicated that the expression of IL-1 β , IL-6, IL-8, and TNF- α protein enhanced by cold air was decreased markedly by siCIRP. * $P < 0.05$ vs. control group cells; # $P < 0.05$ vs. untransfected cells with cold stress stimulation; and ^ $P > 0.05$ vs. untransfected cells with cold stress stimulation. These data are presented as the means \pm SD ($n = 5$). CIRP, cold-inducible RNA-binding protein; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; IL-8, interleukin-8; TNF- α , tumor necrosis factor- α ; siRNA, small interfering RNA; siCIRP, siRNA targeting CIRP; siNT, non-targeting siRNA; RT-PCR, real-time polymerase chain reaction.

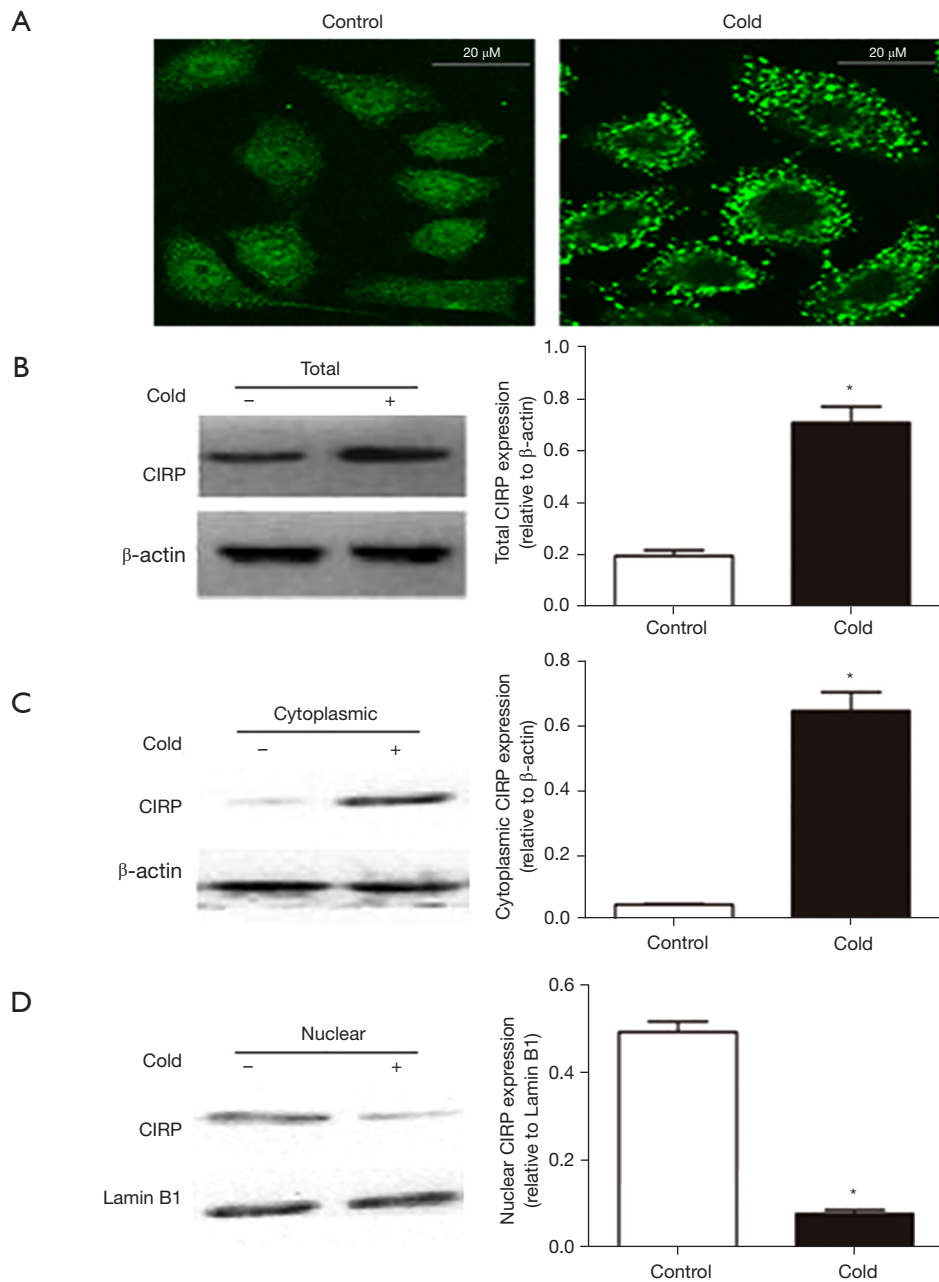


Figure 3 The expression of CIRP in 16HBE cells treated with or without cold stress (18 °C) for 12 hours. The expression and localization of CIRP were detected by CLSM and western blot. (A) CLSM showed that the expression of CIRP was notably increased when the cells were exposed to cold stress and was mainly located in the cytoplasm. The cellular CIRP protein expression in each group was detected using the CIRP antibody and fluorescein isothiocyanate (green) goat anti-mouse IgG, magnification $\times 800$. (B) Total protein was prepared, and CIRP expression was measured by western blot analysis. Anti- β -actin was used as the marker. Higher CIRP levels were observed in the cold stress group. (C,D) Cytosolic and nuclear fractions were prepared, and the expression levels of CIRP in each fraction were analyzed by western blot. Anti-Lamin B1 and anti- β -actin were used as markers of nuclear and cytoplasmic proteins, respectively. In subcellular fractionation, the expression of cytoplasmic CIRP was markedly increased, while nuclear CIRP was decreased after cold stress treatment. * $P < 0.05$ vs. control group cells. These data are presented as the means \pm SD (n=5). CIRP, cold-inducible RNA-binding protein; 16HBE, human bronchial epithelial; CLSM, confocal laser-scanning microscopy.

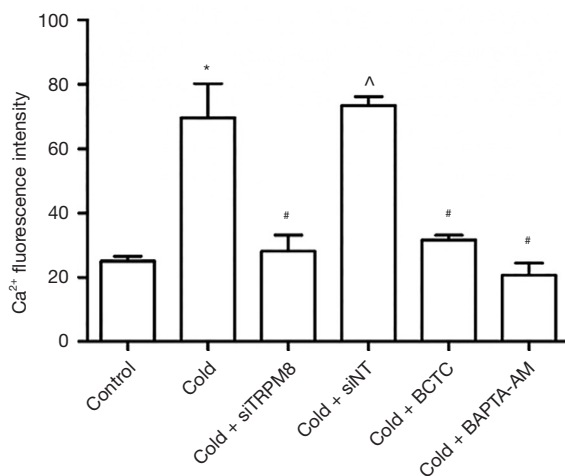


Figure 4 The $(Ca^{2+})_i$ in different experimental groups was measured by calcium imaging. The Ca^{2+} concentration indicated by fluo-3-fluorescence is shown as fluorescence intensity. The Ca^{2+} concentration was increased by cold air and decreased by pretreatment with siTRPM8, TRPM8 inhibitor BCTC, and $(Ca^{2+})_i$ chelating agent BAPTA-AM. * $P < 0.01$ compared with the control group; # $P < 0.05$ compared with the cold stress group; and ^ $P > 0.05$ compared with the cold stress group. These data are presented as the means \pm SD ($n = 5$). TRPM8, transient receptor potential melastatin 8.

in Ca^{2+} induced by cold stress were significantly decreased by siTRPM8, BCTC, and BAPTA-AM but not by siNT. These results indicated that cold stress, at least partially, activated TRPM8, which led to an increase in $(Ca^{2+})_i$.

The effect of cold stress on the TRPM8/PKC α /GSK3 β signaling pathway in 16HBE cells

As shown in *Figure 5*, western blot clearly demonstrated that TRPM8- and GSK3 β -siRNA effectively attenuated the expression of TRPM8 and GSK3 β protein compared to the control group, while siNT did not have the same effect (*Figure 5A, 5B*). Moreover, cold stress significantly increased the phosphorylated levels of PKC α and GSK3 β (*Figure 5C, 5D*, * $P < 0.05$ vs. control). In contrast, the TRPM8-siRNA markedly attenuated the increased phosphorylation of PKC α and GSK3 β by cold stress (*Figure 5C, 5D*, # $P < 0.05$ vs. cold group). Further, we found that pretreatment with TRPM8 inhibitor BCTC, $(Ca^{2+})_i$ chelating agent BAPTA-AM, or PKC α specific inhibitor safinolol, significantly attenuated

the phosphorylation level of PKC α as well as the phosphorylation level of GSK3 β . The above findings illustrated that cold stress, at least partially, facilitated the activation of PKC α and GSK3 β in a TRPM8-dependent manner.

The TRPM8/PKC α /GSK3 β signaling pathway may be involved in cold-mediated migration of CIRC from the nucleus to the cytoplasm in 16HBE cells

At the cellular level, the TRPM8-siRNA, BCTC, BAPTA-AM, safinolol, and GSK3 β -siRNA groups did not exhibit decreased expression of the CIRC increased by cold stress compared to the cold group (*Figure 6A*, # $P > 0.05$, there was no statistical significance). At the subcellular level, after pretreatment with siTRPM8, BCTC, BAPTA-AM, safinolol, and siGSK3 β , the expression of CIRC in the cytoplasm was decreased, while the expression of CIRC in the nucleus was markedly increased (*Figure 6B, 6C*, # $P < 0.05$). These data indicated that the blocking of the TRPM8/PKC α /GSK3 β signaling pathway suppressed migration of CIRC from the nucleus to the cytoplasm but did not inhibit the overexpression of CIRC induced by cold stress.

In addition, the alternation in CIRC expression before and after cold stress was directly observed by CLSM. Compared with the control, CLSM (*Figure 7*) showed that cold stress significantly increased CIRC protein expression, which was enriched in the cytoplasm. However, compared with the cells treated with cold stress and those treated with non-targeting siRNA, the cells treated with TRPM8-siRNA, BCTC, BAPTA-AM, safinolol, and GSK3 β -siRNA displayed a significant reduction of cytosolic CIRC expression and higher nuclear CIRC expression. Collectively, these findings confirmed that cold stress induced CIRC overexpression and promoted CIRC migration from the nucleus to the cytoplasm at least partly via the TRPM8/PKC α /GSK3 β signaling pathway.

Effect of CIRC location on the expression of IL-1 β , IL-6, IL-8, and TNF- α induced by cold stress in 16HBE cells

Figure 2 shows that CIRC contributed to cold stress-inducible inflammatory cytokine expression. To determine the effect of CIRC location on the expression of inflammatory factors induced by cold stress, we transfected cells with TRPM8-siRNA, GSK3 β -siRNA, or non-targeting siRNA, or pretreated cells with BCTC, BAPTA-

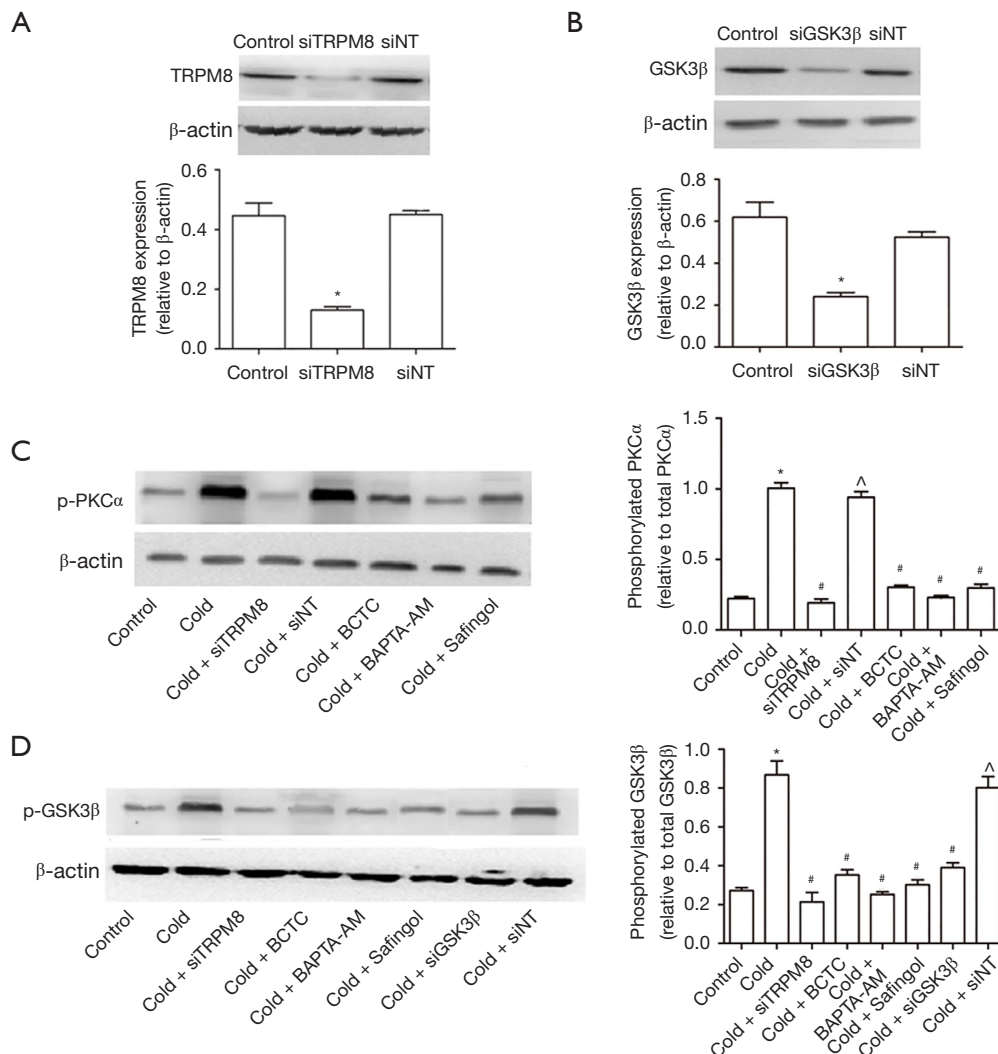


Figure 5 Cold stress-activated PKC α -related signaling pathway in 16HBE cells. Cells were transfected with TRPM8-siRNA, GSK3 β -siRNA, or siNT, or pretreated with TRPM8 inhibitor BCTC (15 μ M), (Ca^{2+}) chelating agent BAPTA-AM (10 μ M), or PKC α specific inhibitor safinol (30 μ M) for 30 minutes before cold stress (18 $^{\circ}$ C, 12 hours). The expression levels of TRPM8, PKC α , and GSK3 β were measured by western blot. Anti- β -actin was used as the marker. (A,B) TRPM8 and GSK3 β were decreased by TRPM8-siRNA and GSK3 β -siRNA, respectively, but not by siNT. (C) TRPM8-siRNA, BCTC, and BAPTA-AM inhibited the phosphorylation of PKC α induced by cold stress. (D) TRPM8-siRNA, BCTC, BAPTA-AM, and GSK3 β -siRNA inhibited the phosphorylation of GSK3 β induced by cold stress. * $P < 0.01$ compared with the control group; # $P < 0.05$ compared with the cold stress group; ^ $P > 0.05$ compared with the cold stress group. These data are presented as the means \pm SD ($n=5$). 16HBE, human bronchial epithelial; TRPM8, transient receptor potential melastatin 8; siRNA, small interfering RNA; GSK3 β , glycogen synthase kinase 3 β .

AM, or safinol before cold stimulation. As shown in Figures 6, 7, CIRP localized within the cytoplasm after cold stress but resided in the nucleus after treatment with TRPM8-siRNA, GSK3 β -siRNA, BCTC, BAPTA-AM, and safinol. Figure 8 shows that TRPM8-siRNA, BCTC,

BAPTA-AM, safinol, and GSK3 β -siRNA also reduced TNF- α , IL-1 β , IL-8, IL-6 mRNA, and protein expression induced by cold stress. These results suggested that CIRP migration from the nucleus to the cytoplasm was required for inflammatory responses induced by cold stress.

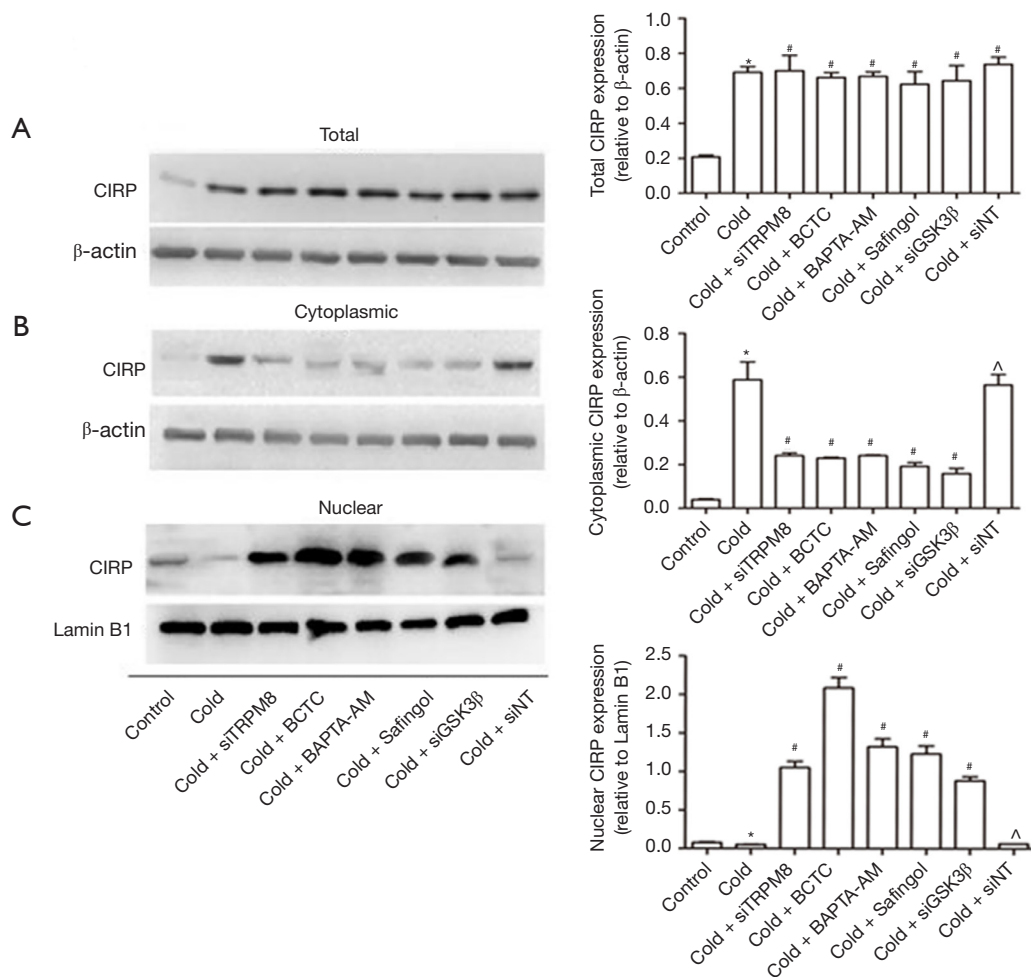


Figure 6 Western blot analysis for the expression of CIRP protein in 16HBE cells. Cells were transfected with TRPM8-siRNA, GSK3 β -siRNA, or siNT, or pretreated with TRPM8 inhibitor BCTC (15 μ M), (Ca²⁺)_i chelating agent BAPTA-AM (10 μ M), or PKC α specific inhibitor safingol (30 μ M) for 30 minutes before cold stress (18 °C, 12 hours). (A) At the cellular level, compared with the cold group, the expression of CIRP increased by cold stress were not decreased by TRPM8-siRNA, BCTC, BAPTA-AM, safingol, or GSK3 β -siRNA ([#]P>0.05, there was no statistical significance). (B,C) After subcellular fractionation, the expression of nuclear CIRP was markedly increased, while cytoplasmic CIRP was decreased in the siTRPM8, BCTC, BAPTA-AM, safingol, and siGSK3 β groups compared with the cold group. *P<0.01 compared with the control group; [#]P<0.05 compared with the cold stress group; Δ P>0.05 compared with the cold stress group. These data are presented as the means \pm SD (n=5). CIRP, cold-inducible RNA-binding protein; 16HBE, human bronchial epithelial; TRPM8, transient receptor potential melastatin 8; siRNA, small interfering RNA; GSK3 β , glycogen synthase kinase 3 β .

Discussion

The results of this study showed that apart from increased CIRP synthesis and its migration from the nucleus to the cytoplasm in NHBE cells, cold stress also induced inflammation responses through CIRP migration from the nucleus to the cytoplasm via the TRPM8/(Ca²⁺)/PKC α /GSK3 β pathway. These data support the discovery that

increased CIRP and inflammatory factor (IL-1 β , IL-6, IL-8, and TNF- α) production promoted by cold stress are blocked by siCIRP. Inhibition of activation of the TRPM8/(Ca²⁺)/PKC α /GSK3 β signaling pathway did not decrease the upregulation of CIRP induced by cold stress, but it attenuated CIRP expression in the cytoplasm and reduced expression of the above inflammatory factors.

CIRP was the first cold shock protein to be identified.

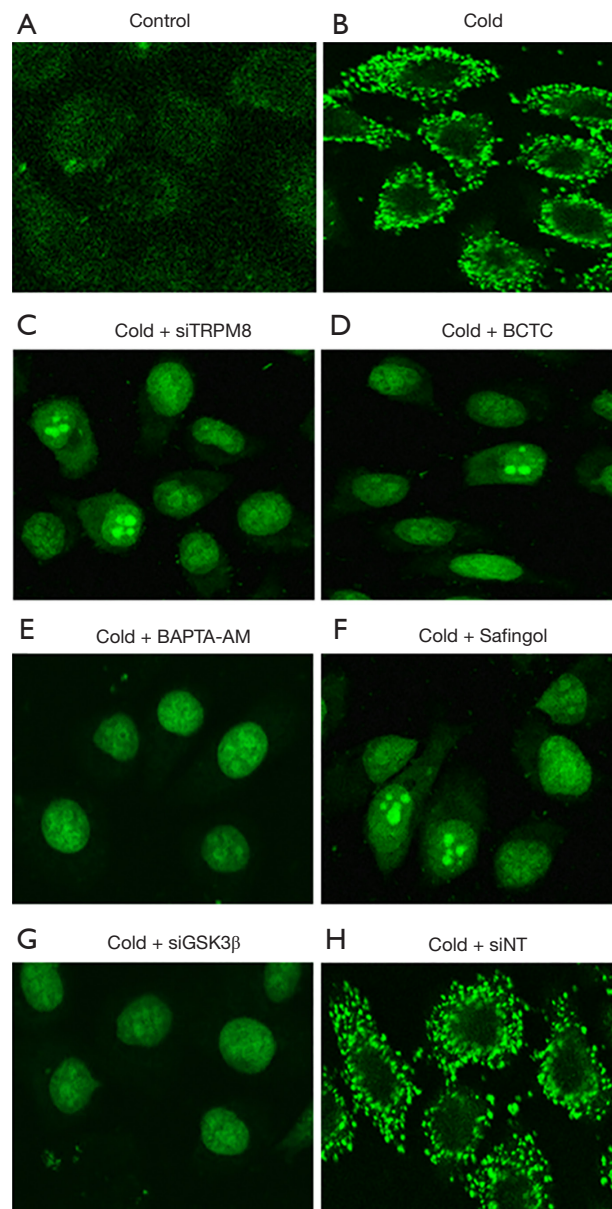


Figure 7 CLSM for the expression of CIRP protein in 16HBE cells. Cells were pretreated with or without siRNA, or pretreated with the TRPM8 inhibitors BCTC (15 μM), $(\text{Ca}^{2+})_i$ chelating agent BAPTA-AM (10 μM), or PKC α specific inhibitor safingol for 30 minutes and then exposed to cold air (18 $^{\circ}\text{C}$, 12 hours). Intracellular CIRP protein expression in 16HBE cells was detected by CLSM. The cellular CIRP protein expression in each group was detected using the CIRP antibody and fluorescein isothiocyanate (green) goat anti-mouse IgG, magnification $\times 800$. The results showed that cold stress (18 $^{\circ}\text{C}$) significantly increased CIRP protein expression, which was enriched in the cytoplasm compared with the control group (37 $^{\circ}\text{C}$). However, the cells treated with TRPM8-siRNA, TRPM8 inhibitor BCTC, $(\text{Ca}^{2+})_i$ chelating agent BAPTA-AM, PKC α specific inhibitor safingol, or GSK3 β -siRNA exhibited remarkably decreased cytosolic CIRP expression with higher nuclear CIRP expression compared with the cold stress group and the non-target siRNA group. (A) The control group (37 $^{\circ}\text{C}$); (B) the cold stress group (18 $^{\circ}\text{C}$); (C) the cold stress plus TRPM8-siRNA group; (D) the cold stress plus BCTC group; (E) the cold stress plus BAPTA-AM group; (F) the cold stress plus safingol group; (G) the cold stress plus GSK3 β -siRNA group; (H) the cold stress plus non-target siRNA group. CLSM, confocal laser-scanning microscopy; CIRP, cold-inducible RNA-binding protein; 16HBE, human bronchial epithelial; siRNA, small interfering RNA; TRPM8, transient receptor potential melastatin 8; IgG, immunoglobulin G; GSK3 β , glycogen synthase kinase 3 β .

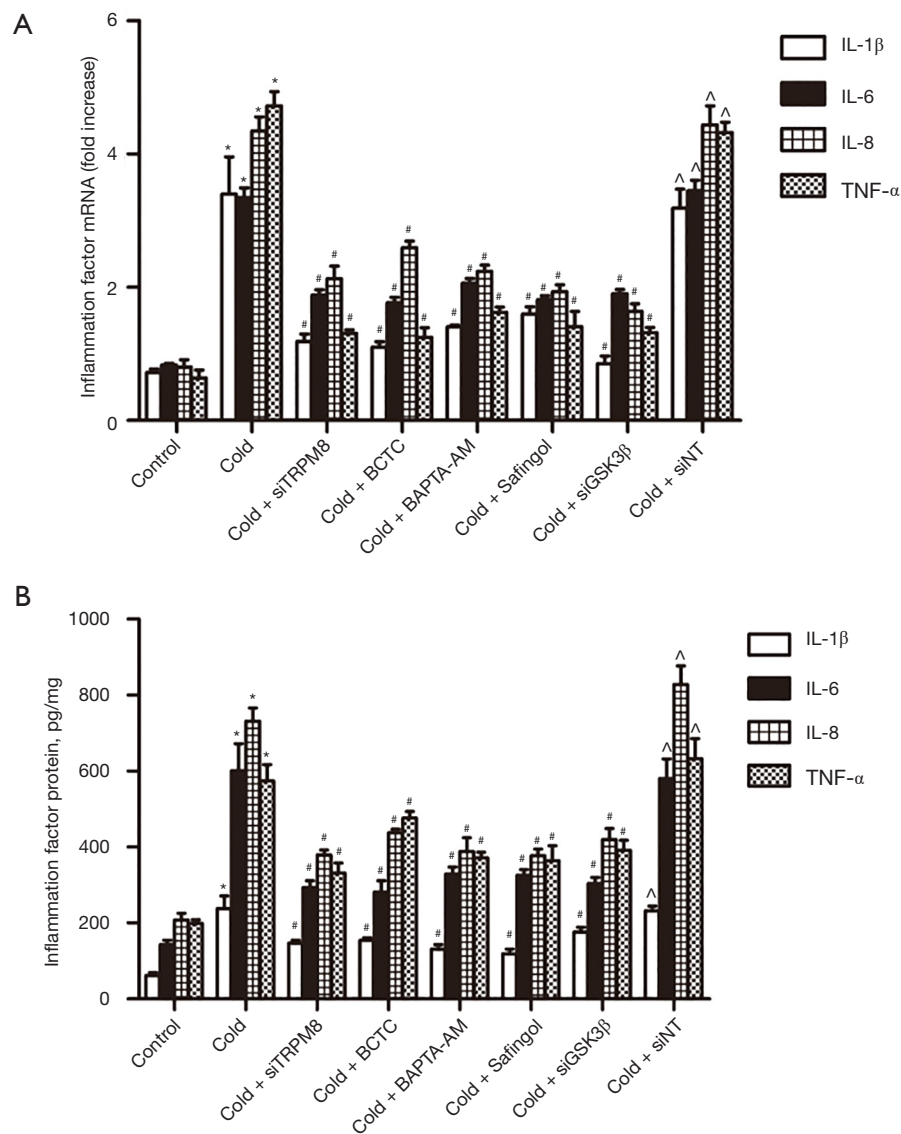


Figure 8 TRPM8/PKC α /GSK3 β activation contributes to cold stress promoting effects on inflammatory response. Cells were pretreated with or without siRNA, or pretreated with the TRPM8 inhibitors BCTC (15 μ M), [Ca²⁺]_i chelating agent BAPTA-AM (10 μ M), or PKC α specific inhibitor safingol for 30 minutes and then exposed to cold air (18 $^{\circ}$ C, 12 hours). RT-PCR and ELISA were used to test the expression of inflammatory factor (IL-1 β , IL-6, IL-8, and TNF- α) mRNA and protein, respectively. (A) Cold stress enhanced IL-1 β , IL-6, IL-8, and TNF- α mRNA expression, while siTRPM8, BCTC, BAPTA-AM, safingol, and siGSK3 β significantly decreased these cold-induced responses. (B) Cold stress enhanced IL-1 β , IL-6, IL-8, and TNF- α protein expression, while siTRPM8, BCTC, BAPTA-AM, safingol, and siGSK3 β significantly decreased these cold-induced responses. *P<0.01 compared with the control group; #P<0.05 compared with the cold stress group; ^P>0.05 compared with the cold stress group (there was no significance). The data in the column graph are the means \pm SD (n=5). TRPM8, transient receptor potential melastatin 8; GSK3 β , glycogen synthase kinase 3 β ; siRNA, small interfering RNA; RT-PCR, real-time polymerase chain reaction; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; IL-8, interleukin-8; TNF- α , tumor necrosis factor- α .

Previous studies have found that CIRP inhibited cell death from UV, moderate hypothermia, and genotoxic stress (6,7,37) and also significantly decreased DNA damage and H₂O₂-inducible neural cell apoptosis (9,38). In contrast to these protective actions, more and more studies have reported that CIRP is secreted into circulation, activating detrimental endogenous proinflammatory responses (39). Rajayer *et al.* reported that CIRP secreted by BV2 cells exposed to alcohol induced proinflammatory (TNF- α and IL-1 β) expression (10). Zhou *et al.* also found that the release of CIRP from microglia promoted TNF- α production and modulated brain inflammation, subsequently leading to neural damage (40). In addition, Davis *et al.* reported that CIRP promoted an inflammatory reaction under hemorrhagic shock and sepsis (41). Sakurai *et al.* showed that CIRP modulated apoptosis and the expression of IL-23 and TNF- α to enhance intestinal inflammation and colorectal tumors (42), and also that CIRP accelerated tumorigenesis of liver cancer by regulating reactive oxygen species (ROS) accumulation under oxidative stress (43). These findings indicate that CIRP has numerous biological functions. We found that the expression of CIRP mRNA and protein began to increase at 32 °C, peaked at 18 °C, and began to decrease at 15 °C. CIRP protein expression changed over time, and the plateau expression was observed at 12 hours. CIRP mRNA expression peaked at 8 hours and did not further increase with the extension of time. The maximum time interval was earlier for CIRP mRNA expression (8 hours) induced by cold air than for CIRP protein expression (12 hours), which indicated that cold air has prolonged effect on CIRP protein expression than mRNA expression (Figure 1). In addition, our experiments showed that cold stress increased inflammatory factor (TNF- α , IL-1 β , IL-8, and IL-6) synthesis, which was reduced by siCIRP (Figure 2). These results revealed that the cold air increased CIRP production in a temperature- and time-dependent manner, and that CIRP was partly necessary for cold stress-inducible inflammatory responses to cold stress. CIRP, a well-known stress protein, migrates from the nucleus to the cytoplasm under physiological and environmental stress. Hence, we determined the subcellular localization of CIRP in 16HBE cells. CIRP was observed in the nucleus under normal conditions but detected in cytoplasm after cold stimulation (Figure 3), which demonstrated that CIRP could migrate from the nucleus to the cytoplasm upon cold stress. This result is consistent with the findings of previous reports (7,8,14,44). Further, we found that CIRP expression was increased significantly

after cold treatment. These observations indicate that cold stress might play a dual role in CIRP regulation.

De Leeuw *et al.* (15) found that phosphorylation of GSK3 β kinase promoted the movement of CIRP from the nucleus to the cytoplasm under ER stress and cytoplasmic stress. Other studies have also indicated that CIRP migrated from the nucleus to the cytoplasm either through its arginine methylation or phosphorylation by GSK3 β kinase (7). GSK3 β is a multifunctional serine/threonine protein kinase which is involved in a number of cell signaling pathways, playing important roles in glycogen metabolism, cell differentiation, cell proliferation, and gene expression (45,46). Hartigan *et al.* reported that transient increases in intracellular calcium increased Tyr216 phosphorylation of GSK3 β (47). Research by both Gao *et al.* and Park *et al.* found that activated PKC could phosphorylate GSK3 β as well (32,33). Sabnis *et al.* reported that a functional truncated TRPM8 variant was detected in NHBE cells (26). Additionally, it has been reported that truncated TRPM8 contains a voltage sensor region, the transmembrane segment 4 and the segment 4/segment 5 linker, which is involved in cold sensation. These findings illustrate that TRPM8 expressed in NHBE cells has an ability to perceive cold stimulation (48,49) and can be activated, resulting in an obvious increase in (Ca²⁺)_i concentration and phosphorylation of PKC α . Based on this, our study aimed to determine whether TRPM8, PKC α , or GSK3 β were related to CIRP movement under cold conditions.

Figure 4 shows that cold temperature induced a significant increase in (Ca²⁺)_i concentration, and increased (Ca²⁺)_i concentration was inhibited by TRPM8 siRNA, specific TRPM8 antagonist BCTC, as well as by (Ca²⁺)_i chelating agent BAPTA-AM. These results are consistent with those of a previous study (23) showing that the TRPM8 channel can be activated by cold stimulation and that NHBE cells sense cold stimulus primarily due to the TRPM8 channel. Western blotting (Figure 5) showed that cold stimulation augmented the phosphorylation of PKC α and GSK3 β , and the above phenomena were blocked by TRPM8 siRNA, BCTC, BAPTA-AM, and safinol (a specific PKC inhibitor). These results suggested that cold stimulation-induced activation of PKC α /GSK3 β is highly dependent on the TRPM8 channel. Next, we tested whether TRPM8/(Ca²⁺)_i/PKC α /GSK3 β involved CIRP migration from the nucleus to the cytoplasm. At the cellular level (Figure 6A), cold stimulation significantly induced CIRP expression in 16HBE cells, and the overproduction

of CIRP was not reduced by TRPM8 siRNA, BCTC, BAPTA-AM, safinol, or GSK3 β siRNA. At the subcellular level (Figure 6B,6C), 16HBE cells pretreated with TRPM8 siRNA, BCTC, BAPTA-AM, safinol, or GSK3 β siRNA significantly attenuated CIRP expression in the cytoplasm, with overproduction of CIRP in the nucleus. These results demonstrated that cold stimulus increased CIRP synthesis and promoted CIRP movement from the nucleus to the cytoplasm via the TRPM8/(Ca²⁺)_i/PKC α /GSK3 β signaling pathway. In addition, the changes in CIRP expression were observed directly by CLSM, strengthening the credibility of our results. As shown in Figure 7, CIRP expression was remarkably increased under cold conditions and was mainly located in the cytoplasm. However, transfection of the 16HBE cells with TRPM8 siRNA or GSK3 β siRNA remarkably decreased cytoplasmic CIRP expression. Similar to previous experimental results, incubation with the TRPM8 specific inhibitor BCTC, (Ca²⁺)_i chelating agent BAPTA-AM, or PKC α specific inhibitor safinol prior to cold stimulation notably reduced cytoplasmic CIRP expression with nuclear CIRP upregulation, confirming previous conclusions. Finally, we found that limiting CIRP movement from the nucleus to the cytoplasm by inhibiting the TRPM8/(Ca²⁺)_i/PKC α /GSK3 β signaling pathway attenuated the expression levels of IL-1 β , IL-6, IL-8, and TNF- α (Figure 8). These data suggest that the inflammatory factor expression modulated by CIRP was dependent on CIRP migration from the nucleus to the cytoplasm. While it is widely accepted that CIRP can bind RNAs and to modulate them at the post-transcriptional level via increasing mRNA stabilization, our previous research showed that CIRP mediated airway mucin by interaction with TLR4 directly. Thus, it would be interesting to study whether CIRP regulating inflammatory factor expression is a direct or indirect effect of CIRP.

In conclusion, the present study showed that cold stress upregulated CIRP mRNA and protein expression in 16HBE cells and promoted CIRP movement from the nucleus to the cytoplasm via TRPM8/(Ca²⁺)_i/PKC α /GSK3 β . CIRP mediated inflammatory factor (IL-1 β , IL-6, IL-8, and TNF- α) expression induced by cold stress through CIRP migration from the nucleus to the cytoplasm. To our knowledge, this study is the first to demonstrate the mechanism of CIRP migration from the nucleus to the cytoplasm upon cold stress in bronchial airway epithelial cells. Although we have clarified how CIRP migrates from the nucleus to the cytoplasm, the exact molecular mechanisms by which cytosolic CIRP mediates

inflammatory factor expression are unknown. Thus, more studies are needed.

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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