Abstract. Airway remodeling is a central event in the pathology of chronic obstructive pulmonary disease (COPD) that leads to airway narrowing and subsequently, to increased mechanical pressure. High mechanical pressure can exacerbate airway remodeling. Thus, a treatment regimen aimed at disrupting this high-pressure airway remodeling vicious cycle may improve the prognosis of patients with COPD. Recent studies have demonstrated that mechanical stress induces lung epithelial-mesenchymal transition (EMT), which is commonly present in airway epithelial cells of patients with COPD. As TRPC1 functions as a mechanosensitive channel that mediates non-selective cation entry in response to increased membrane stretch, the present study investigated the role of TRPC1 in the occurrence of EMT induced by mechanical stress. In the present study, the expression of TRPC1 in the bronchial epithelium was examined in vivo by immunohistochemistry. In vitro, human bronchial epithelial (16HBE) cells were subjected to mechanical stretching for up to 48 h, and TRPC1 expression was then examined by RT-qPCR and western blot analysis. In addition, TRPC1 receptor function was assessed by Ca2+ imaging and siRNA transfection. EMT was identified using immunofluorescence, western blot analysis and RT-qPCR. It was found that TRPC1 expression was upregulated in patients with COPD and in 16HBE cells subjected to mechanical stretch. The mechanical stress-induced activation of TRPC1 in 16HBE cells increased the intracellular calcium concentration and subsequently decreased the expression of cytokeratin 8 and E-cadherin, and increased the expression of α-smooth muscle actin, indicating the occurrence of EMT. On the whole, the findings of the present study demonstrate that TRPC1 plays a key role in the occurrence of EMT in human lung epithelial cells in response to mechanical stretch; thus, this protein may serve as a novel therapeutic target for progressive airway remodeling in COPD.

Introduction

Cells and the extracellular matrix (ECM) in the airways are subjected to various mechanical stimuli, such as the continuous cyclic stretch of breathing and the contraction of the airway smooth muscle, due to the dynamic nature of lung function (1). Pathologically high levels of stretch exerted on lung tissues play a key role in many pathological situations. This process can induce the expression of various inflammatory mediators, including interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-13 and matrix metalloproteinase (MMP-9) (2,3), and an increase in goblet cell number and mucin5AC (MUC5AC).
protein secretion (4,5). Furthermore, excessive stretching is a
major cause of airway remodeling (2,6-8). Over the past few
years, extensive research has been conducted to investigate
the role of abnormal mechanical stress in airway remodeling
in asthma; however, very little attention has been devoted
to stress-induced airway remodeling in chronic obstructive
pulmonary disease (COPD), which is characterized by chronic
coughing, sputum production and recurrent episodes of
wheezing that result in the pathological upregulation of airway
pressure.

Airway remodeling is a critical feature of COPD, char-
acterized by the aberrant repair of the epithelium and the
accumulation of fibroblasts, which contribute to ECM deposi-
tion, leading to irreversible airway obstruction and progressive
high pressure in the airways (9,10). Thus, airway remodeling
and increased airway mechanical stress function in a vicious
cycle that may contribute to the ongoing decline in lung func-
tion and quality of life and, ultimately, the poor prognosis of
patients with COPD. Thus, further elucidation of the mecha-
nisms of mechanical stress-induced airway remodeling in
COPD is important for the treatment of this disease.

Recently, epithelial-mesenchymal transition (EMT)
has been identified as a new source of fibroblasts that can
contribute to the remodeling of the airways (11). EMT is a
biological process that allows a polarized epithelial cell with
cell-cell contacts that is attached to the basal membrane to
acquire the characteristics of mesenchymal cells through
multiple biochemical alterations (12,13). Significant EMT
has been demonstrated in the airway epithelial cells of
patients with COPD (14-16), and EMT has been suggested as
an important factor for airway remodeling in COPD (17,18).
Previous studies have demonstrated that primary airway
epithelial cells subjected to mechanical stretch acquire EMT
phenotypes (19-21). However, the specific molecular mecha-
nisms of EMT in response to mechanical stress remain poorly
understood.

Transient receptor potential canonical 1 (TRPC1), a
member of the vertebrate mechanosensitive cation (MscCa)
channels, plays a critical role in converting the sensed mechan-
ical stimuli into biological signals by transducing stretch into
Ca2+ flux across the cell membrane (22,23). TRPC1 has been
reported to be abundantly expressed in airway epithelial
cells and its expression is markedly increased in patients
with COPD (18). Furthermore, the recent study by Xu et al
indicated that the increase in TRPC1 expression was closely
related to the occurrence of EMT in patients with COPD
where they exposed human bronchial epithelial (HBE; 16HBE
cells) to 5% cigarette smoking extract (CSE) (18). Therefore,
it was hypothesized that TRPC1 plays a vital role in the
process of mechanical stress-induced airway remodeling in
COPD via the occurrence of EMT. To test this hypothesis,
in vivo, the present study examined the expression of TRPC1
in patients with COPD. In vitro, 16HBE cells we exposed to
mechanical stretch for up to 48 h to mimic the effects of high
airway pressure, and TRPC1 expression was then measured by
RT-qPCR and western blot analysis. The function of TRPC1
was assessed by Ca2+ imaging and siRNA transfection, and the
occurrence of EMT was identified by immunofluorescence,
western blot analysis and RT-qPCR. It was that TRPC1 expres-
sion was upregulated in patients with COPD and in 16HBE
cells subjected to mechanical stretch. The TRPC1-mediated
increase in intracellular Ca2+ played a key role in the occurre-
cence of EMT in human lung epithelial cells in response to
mechanical stretch. Thus, this molecule may serve as a novel
therapeutic target for progressive airway remodeling in COPD.

Materials and methods

Reagents. 16HBE cells were purchased from the American
Type Culture Collection (ATCC). Lipofectamine RNAiMAX
reagent was obtained from Invitrogen; Thermo Fisher
Scientific, Inc. Rabbit polyclonal anti-TRPC1 antibody
(ab75322) was purchased from Abcam. Mouse monoclonal
anti-cytokeratin 8 (sc-58736) and E-cadherin (sc-8426)
antibodies were purchased from Santa Cruz Biotechnology,
Inc. Rabbit anti-α-smooth muscle actin (α-SMA) mono-
clonal antibodies (ab32575) were purchased from Abcam.
Rabbit monoclonal anti-GAPDH antibody (AF1186) was
from Beyotime Institute of Biotechnology. Cy3-labeled
goat anti-mouse antibodies (A0521) and FITC-labeled goat
anti-rabbit antibodies (A0562) were purchased from Beyotime
Institute of Biotechnology. TRPC1 and NC siRNAs were
purchased from Ribobio Biotechnology Co, Ltd. SYBR Premix
EX Taq was purchased from Takara Biotechnology. The
bicinchoninic acid (BCA) assay kit and Fluoro-3 AM were
purchased from Beyotime Institute of Biotechnology, and
TRIzol reagent was purchased from Invitrogen; Thermo
Fisher Scientific, Inc. The PCR primers for TRPC1, GAPDH,
cytokeratin 8, E-cadherin and α-SMA were from Sangon
Biotech. The Revert Aid First Strand cDNA synthesis kits
were purchased from Fermentas; Thermo Fisher Scientific,
Inc. 3,3'-Diaminobenzidine tetrahydrochloride (DAB) was
from Sigma-Aldrich; Merck KGaA; 1,2-bis(2-aminophenoxy)
ethane-N,N,N’,N’-tetraacetic acid (BAPTA-AM) was
purchased from Santa Cruz Biotechnology, Inc. ECL reagent
was from Beyotime Institute of Biotechnology.

Tissue samples. Normal human lung tissues were obtained
from surgical specimens that were resected in the Department
of Thoracic Surgery, the 2nd Clinical Hospital of Chongqing
Medical University from 2014 to 2015. The test population
consisted of 20 patients who were pathologically diagnosed
with stage I non-small cell lung cancer (NSCLC) according
to the 8th edition of the TNM classification for lung cancer.
All patients were informed of the research content of the
project and signed an agreement to participate in the present
study. Ethics committee approval was obtained for the Second
Clinical Hospital of Chongqing Medical University. None of
the patients had a history of any disease other than COPD
and lung cancer. All the normal lung tissues that were obtained
from the organization were separated from the tumor by at
least 5 cm and were examined by a pathologist to confirm
that they were non-cancerous. The subjects were divided
into the COPD group (8 males, 5 females) with a mean age
of 58.7 years (SD, 3.9 years) and the control group (4 males,
3 females) with a mean age of 60.4 years (SD, 5.4 years).
The diagnosis of COPD was based on the Global Initiative
for Chronic Obstructive Lung Disease (GOLD) criteria (24)
namely a post bronchodilator FEV1/FVC <0.7 [forced expira-
tory volume in the first second (FEV1)]; forced vital capacity
Transient transfection with TRPC1 siRNA. The 16HBE cells were plated into 6-well plates at 200,000 cells per well in medium containing 10% fetal bovine serum and incubated overnight. The cells were then cultured in serum-free medium before being divided into 3 groups as follows: The control group, the TRPC1 siRNA group and the NC siRNA group. A total of 100 pmol TRPC1 siRNA or NC siRNA were transfected into the 16HBE cells using Lipofectamine RNAiMAX reagent (10:1) according to the manufacturer's instructions. The detailed sequence of TRPC1 siRNA and NC siRNA are shown as follows: TRPC1 siRNA sense, 5’-GGA UUGUGCGGGAGGUAGAA-3’ and antisense, 5’-CUUCAC CUCCCCGAACAUC-3’; NC siRNA sense, 5’-UUUCC AGAGCGUGACUGTT-3’ and antisense, 5’-ACUGAGAC GGGAGGAATTT-3’. At 6 h following transfection, the cells were fed with medium containing 10% fetal bovine serum and incubated at 37°C for a further 48 h. RT-qPCR and western blot analysis were then performed to determine the efficiency of TRPC1 knockdown.

Mechanical stretch system. In the in vitro experiments, a FX-4000 Flexcell tension system (Flexcell International Corp.) was used to apply sinusoidal strain to the 16HBE cells as previously described (2,8) to imitate the pathological conditions that are associated with airway mechanical stress. Briefly, the 16HBE cells were first separately seeded at 4x10^5 cells/well in Flexcell biaxial 6-well plates coated with type I collagen (Flexcell International Corp.) in medium containing 10% fetal bovine serum for 24 h before it was replaced with serum-free medium. The cells were then divided into a control group and a group exposed to mechanical stretch. Cells in the control group were cultured on similar plates in the same incubator without any additional intervention, including mechanical loading, whereas cells in the mechanical stretch group were subjected to sinusoidal stretching for 48 h at a frequency of 60 cycles per min at a 15% elongation.

Ca^{2+} imaging. The levels of intracellular Ca^{2+} were measured using the membrane- and cell-permeable Ca^{2+}-indicator, Fluo-3-AM. Following mechanical stretching, 16HBE cells were seeded into confocal dishes at a density of 1,000,000 cells per dish. After the cells had been stretched for 48 h at a 15% elongation, western blot analysis, immunofluorescence and RT-qPCR were performed to determine the protein and mRNA expression profiles of EMT-related genes. Inhibition experiments were performed with 16HBE cells transfected with TRPC1 siRNA/NC siRNA.

Cell culture and grouping. The 16HBE cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum and incubated at 37°C in a humidified water-jacketed incubator containing 5% CO2 during the subsequent experiments. Cultured cells were divided into a control group and a group exposed to mechanical stretch. Cells in the control group were cultured on similar plates in the same incubator without any additional intervention, whereas cells in the mechanical stretch group were subjected to sinusoidal stretching for 48 h at a frequency of 60 cycles per min at a 15% elongation.

RT-qPCR. The mRNA levels of TRPC1, cytokeratin 8, E-cadherin and α-SMA were measured by RT-qPCR. TRIzol reagent was used to extract total RNA from the cultured 16HBE cells according to the manufacturer’s instructions. A total of 5 μg total RNA was reverse transcribed into complementary DNA (cDNA) with a first-strand cDNA synthesis kit. The specific primer sequences for RT-qPCR were as follows:
TRPC1 sense, 5'-TCGGTGTTGTATGTTGCTT-3' and antisense, 5'-TGAGGAGGGAATGATGTTGA-3'; GAPDH sense, 5'-AGAAAGGGTGGCTATTG-3' and antisense, 5'-AGGGGACATCCAGTCTTT-3'; cyclerokin 8 sense, 5'-GAGGCATCCACAGTTTAC-3' and antisense, 5'-TTGCTTCCAGGGCTTTCTT-3'; E-cadherin sense, 5'-GCCAAACAGAGCCGACTAT-3' and antisense, 5'-ATGTTGTCAGCTCAGCCGC-3'; and c-SMA sense, 5'-GCCAGCGAATGAGCAGGA-3' and antisense, 5'-ACAGAGTTATTTGCGCTCCGA-3'. The PCR reaction was performed for 35 cycles under the following conditions: Pre-denaturation at 94°C for 2 min; denaturation at 94°C for 30 sec, annealing at 60°C (TRPC1 and c-SMA), 65°C (cyclerokin 8), or 58°C (E-cadherin) for 30 sec and extension at 72°C for 10 min. The comparative Cq method (2-ΔΔCq) (25) was used to determine the relative mRNA quantification, and GAPDH was used as the endogenous control gene.

Western blot analysis. First, the 16HBE cells were homogenized on ice in RIPA lysis buffer containing protease inhibitors. After the mixture was centrifuged at 11,000 rpm for 10 min at 4°C, the supernatant was used for western blot analysis. The total protein was assessed using a BCA protein assay. Equivalent amounts of protein (30 μg) were obtained from each sample and fractionated using 10% SDS-PAGE. The blots were then transferred onto polyvinylidene fluoride membranes. The membranes were blocked in skim milk for 1 h at room temperature and then incubated overnight at 4°C with rabbit anti-TRPC1 polyclonal antibodies (1:1,000), mouse anti-cytokeratin 8 monoclonal antibodies (1:1,000), mouse anti-E-cadherin monoclonal antibodies (1:1,000), rabbit anti-c-SMA monoclonal antibodies (1:1,000) and rabbit anti-GAPDH monoclonal antibodies (1:1,000). The membranes were then incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:2,000) for 1 h at 37°C. Finally, the membranes were incubated with western blot ECL reagent in the dark for 5 min. Quantitative results were acquired by measuring the optical density of the labeled protein bands using Quantity One software (Bio-Rad Laboratories, Inc.). The values were normalized to the intensity level of GAPDH.

Immunofluorescence. First, the 16HBE cells were seeded into 24-well plates at a density of 100,000 cells per well and cultured in medium containing 10% fetal bovine serum overnight. The cells were then washed 3 times with PBS, fixed with 4% paraformaldehyde for 20 min and permeabilized using 0.3% Triton X-100 for 5 min. After the cells were blocked with goat serum for 1 h at room temperature, they were incubated with rabbit anti-TRPC1 polyclonal antibodies (1:200), mouse anti-cytokeratin 8 monoclonal antibodies (1:100), mouse anti-E-cadherin monoclonal antibodies (1:100), or rabbit anti-c-SMA monoclonal antibodies (1:500) at 4°C overnight. Finally, all the culture dishes were incubated with Cy3-labeled goat anti-mouse antibodies (1:500) and FITC-labeled goat anti-rabbit antibodies (1:500) for 1 h at 37°C. The cells were then stained with DAPI (10 mg/ml) for 15 min. Fluorescent labeling was analyzed using a fluorescence upright microscope (BX51, Olympus Corp.). Fluorescence intensities were evaluated using Image-Pro Plus software (Media Cybernetics).

The results are presented as the fold control of fluorescence intensity.

Statistical analysis. All data are expressed as the means ± SD. Data analyses were performed using SPSS 22.0 software (IBM Corp.). Statistical analysis was performed using a Student's t-test for comparisons between 2 groups and one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) for comparisons involving >2 groups. The level of statistical significance was established at P<0.05.

Results

TRPC1 protein and mRNA levels in bronchial epithelial cells are increased in patients with COPD. The results of immunohistochemistry revealed that the majority of TRPC1 immunoreactivity was localized at the basal surface in epithelial cells (Fig. 1A and B). The value of the integrated optical density/area of the TRPC1 protein in the bronchial epithelium was higher in patients with COPD (0.246±0.027) than in patients in the control group who had no underlying chronic inflammatory airway disease (0.161±0.021, P=0.012, Fig. 1C). The results of RT-qPCR and western blot analysis revealed that the TRPC1 mRNA (COPD vs. control, 2.42±0.702 vs. 1±0.00, P<0.001, Fig. 1D) and protein (COPD vs. control, 1.32±0.337 vs. 0.710±0.164, P=0.002, Fig. 1E and F) levels in the lungs of patients with COPD were significantly higher than those of the control group.

Transfection with TRPC1 siRNA suppresses TRPC1 protein expression in 16HBE cells. The knockdown efficiency was determined by RT-qPCR and western blot analysis. A significantly lower level of TRPC1 mRNA (control vs. TRPC1 siRNA, 1±0.00 vs. 0.378±0.086, P<0.001, Fig. 2A) and protein (control vs. TRPC1 siRNA, 0.930±0.085 vs. 0.476±0.051, P=0.0014, Fig. 2B and C) was observed in the 16HBE cells transiently transfected with TRPC1 siRNA than in the control cells. However, there was no significant difference in the TRPC1 mRNA and protein levels between the NC siRNA transfection group and the control group (P>0.6726, Fig. 2).

Mechanical stretch upregulates the mRNA and protein expression of TRPC1 levels in 16HBE cells. RT-qPCR and western blot analysis were used to quantify changes in the mRNA and protein levels of TRPC1 following exposure of the 16HBE cells to cyclic stretch for 48 h. As shown in Fig. 3, significant increases in the mRNA (control vs. stretch, 1±0.00 vs. 1.95±0.343, P=0.004, Fig. 3A) and protein (control vs. stretch, 0.762±0.111 vs. 1.13±0.173, P=0.035, Fig. 3B and C) of TRPC1 were observed in the 16HBE cells following exposure to cyclic stretch.

Mechanical stretch activated the TRPC1 channels in 16HBE cells. Ca2+ imaging was used to assess TRPC1 function in the 16HBE cells stimulated with cyclic stretch. As shown in Fig. 4A and B, cyclic stretch produced a robust increase in the intracellular Ca2+ of the 16HBE cells. The intracellular Ca2+ levels reached a maximum at approximately 0.5 h after the cells were exposed to stretch and then declined slowly to a plateau level that was still higher than the baseline level.
at approximately 4 h following stretch initiation. As shown in Fig. 4C and D, the stretch-induced increase in intracellular Ca\textsuperscript{2+} levels at 48 h after stretching was substantially attenuated in the 16HBE cells that were transfected with TRPC1 siRNA (stretch vs. stretch + TRPC1 siRNA, 1.63±0.205 vs. 1.08±0.096, P=0.03), but not in the 16HBE cells transfected with NC siRNA (stretch vs. stretch + NC siRNA, 1.63±0.205 vs. 1.56±0.211, P=0.572). These results indicated that TRPC1 plays a vital role in mechanical stress-induced increase in intracellular Ca\textsuperscript{2+} in 16HBE cells.

Mechanical stretch alters the expression of EMT-related markers in 16HBE cells. After the 16HBE cells were exposed to cyclic stretch, changes in the protein expression of lung epithelial cell markers (cytokeratin 8 and E-cadherin) and mesenchymal marker (\(\alpha\)-SMA) were observed using immunofluorescence staining, RT-qPCR and western blot analysis. The results of immunofluorescence staining revealed that the protein levels of cytokeratin 8 (control vs. stretch, 1±0.00 vs. 0.335±0.111, P<0.001) and E-cadherin (control vs. stretch, 1±0.00 vs. 0.345±0.033, P<0.001) were significantly decreased after the cells were stretched to 15% elongation for 48 h at a frequency of 60 cycles per min, and the protein expression of \(\alpha\)-SMA (control vs. stretch, 1±0.00 vs. 4.71±0.530, P<0.001) was increased (Fig. 5A and B). Moreover, the altered fluorescence intensities of cytokeratin 8, E-cadherin and \(\alpha\)-SMA induced by mechanical stretch were significantly attenuated by transfection with TRPC1 siRNA (stretch vs. stretch + TRPC1 siRNA, cytokeratin 8, 0.335±0.111 vs. 0.345±0.033 vs. 0.909±0.008, \(\alpha\)-SMA, 4.71±0.53 vs. 1.19±0.235, P<0.001). RT-qPCR and western blot analysis were used to quantify the changes in the mRNA and protein levels of cytokeratin 8, E-cadherin and \(\alpha\)-SMA in the 16HBE cells following mechanical stretch. As shown in Fig. 5C-E,
mechanical stretch substantially enhanced both the mRNA and protein levels of α-SMA and attenuated the mRNA and protein levels of cytokeratin 8 and E-cadherin (P≤0.001). Additionally, these changes were significantly attenuated by transfection with TRPC1 siRNA (P≤0.001). However, transfection with NC siRNA did not alter the immunofluorescence intensity, mRNA or protein levels of cytokeratin 8, E-cadherin or α-SMA (P≥0.069).

The Ca^{2+} imaging results revealed that mechanical stretch induced an increase in intracellular Ca^{2+} levels. To further explore the role of intracellular Ca^{2+} in the expression of EMT markers under the condition of mechanical stretch, an
inhibitory experiment was also performed by the addition of the intracellular Ca\textsuperscript{2+} chelator, BAPTA-AM. As shown in Fig. 5, changes in immunofluorescence intensity, and in the mRNA and protein levels of cytokeratin 8, E-cadherin and α-SMA induced by mechanical stretch were markedly attenuated by BAPTA-AM (P<0.001).

Figure 4. Fluorescence intensity of intracellular Ca\textsuperscript{2+} levels in 16HBE cells was determined using laser confocal microscopy. (A) Representative images of fluorescence-positive cells stretched for 0, 0.5, 1, 2, 4, 8, 12, 24 and 48 h at 15% elongation respectively. (B) The fluorescence intensities for intracellular Ca\textsuperscript{2+} levels in (A) were analyzed with the quantification tools and were shown by fold of control. (C) Representative images of fluorescence-positive cells stretched for 48 h at 15% elongation with or without transfection of TRPC1 siRNA or NC siRNA. (D) The fluorescence intensities for intracellular Ca\textsuperscript{2+} in (C) were analyzed with the quantification tools and are shown as the fold of the control. Values in (B and D) are shown as means ± SD (n=3). *P<0.05 vs. the control group.
Figure 5. Epithelial-mesenchymal transition (EMT) markers in 16HBE cells after stretching with 15% elongation for 48 h were determined using immunofluorescence, RT-qPCR and western blot analysis. (A) Representative images of fluorescence-positive cells stretched for 48 h at 15% elongation. (B) The fluorescence intensities for EMT markers were analyzed with the quantification tools and were shown by fold of control. (C) The levels of the EMT markers mRNA were determined using real-time RT-qPCR. (D and E) The protein levels of the EMT-related markers were determined by western blot analysis. Values in (B, C and E) are shown as the means ± SD (n=3). *P<0.05 vs. the control group. #P<0.05 vs. the stretch group.
Discussion

In the present study, it was observed that the expression of TRPC1 was increased in airway epithelial cells in patients with COPD. In vitro, a Flexcell FX-4000 Tension System was used to stretch the 16HBE cells at a 15% elongation to imitate the pathological increased airway mechanical pressure in COPD. The decreased expression of cytotokeratin 8 and E-cadherin and the simultaneous increase in the expression of α-SMA supported the occurrence of EMT. Furthermore, the mechanisms underlying these changes was mediated, at least in part, by a TRPC1-mediated intracellular Ca\(^{2+}\) increase since transfection with TRPC1 siRNA or treatment with intracellular Ca\(^{2+}\) chelator BAPTA-AM abolished these alterations in EMT marker expression induced by mechanical stress. These results may thus provide a novel mechanistic insight into the treatment or alleviation of airway remodeling in COPD.

Cells and the ECM in the lung exist in a mechanically dynamic environment. Mechanical stretch is essential for the regulation of respiratory physiology and pathophysiology. Pathologically increased mechanical stretch exerted on lung tissues is one of the most common characteristics of chronic inflammatory airway diseases, such as COPD and asthma due to bronchoconstriction, mucus hypersecretion and airway remodeling. In turn, increased mechanical stretch has been reported to increase goblet cell number and MUC5AC protein expression (5), upregulate the expression levels of IL-13, transforming growth factor (TGF-β1) and MMP-9 in bronchial epithelial cells (2), finally further aggravating mucus hypersecretion as well as airway remodeling in these conditions. Thus, a better understanding of the mechanisms through which how lung cells respond to mechanical stretch is of key importance for identifying targets for the treatment and prevention of chronic inflammatory airway diseases.

Mechanotransduction is a fundamental process of the conversion of mechanical stimuli into biochemical or electrical signals (26,27). This phenomenon is widely studied in several areas of medical science, including vascular biology (6,28,29) and skeletal biology (7,30). It has been proposed that a number of TRP isoforms exhibit mechanosensitivity (TRPA1; TRPV1, 2, 4; TRPC1, 5, 6; TRPM3, 7; TRPP2) (31,32). Among these, TRPC1 was the first cloned TRP channel (33) and exhibited high mechanosensitivity in vertebrates (22). It is widely present in the heart, arteries, skeletal muscle and was reported to be plentifully expressed in airway epithelial cells and show a significant increase in patients with COPD (18). Previous studies have demonstrated that mechanical stretch can induce an influx in Ca\(^{2+}\) levels and can upregulate the expression of airway remodeling-associated factors, IL-13, MMP-9 and TGF-β1 in the 16HBE cells via the activation of TRPC1, indicating its critical role in mechanical stretch induced airway remodeling (2,8).

Currently, the EMT mechanism for human airway remodeling in COPD has attracted the attention of various researchers. Emerging evidence suggests that primary human lung epithelial cells subjected to mechanical stretch develop EMT phenotypes (19,20,34); Xu et al recently reported that a high expression of TRPC1 in the airway epithelia of patients with COPD was accompanied by an increased level of vimentin and a simultaneously decreased level of E-cadherin, as well as morphological changes from a typical epithelial cobblestone appearance with close connection to a more loosely connected elongated fusiform appearance, demonstrating its role in occurrence of EMT in COPD (18). Given the mechanosensitive properties of TRPC1 and the fact that TRPC1 overexpression in COPD promoted EMT process, the present study investigated the role of TRPC1 in the occurrence of COPD-associated EMT in response to mechanical stress. The results demonstrated that TRPC1-mediated intracellular Ca\(^{2+}\) increase plays an important role in this process.

As a well-established mechanosensitive, Ca\(^{2+}\)-permeable TRP channels (35), the activation of TRPC1 depends on both phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)) and protein kinase C (PKC) (36). The intracellular Ca\(^{2+}\) increase following TRPC1 activation can activate phospholipase C (PLC), which subsequently hydrolyze PIP\(_2\), generating inositoltrisphosphate (IP\(_3\)) and diacylglycerol (DAG). DAG can activate PKC, and IP\(_3\) may bind to its calcium channel-coupled receptor on the ER membrane and induce Ca\(^{2+}\) release from endoplasmic reticulum (37). Thus, it was hypothesized that the intracellular Ca\(^{2+}\) increase observed in the present study may result from both an influx of extracellular Ca\(^{2+}\) and Ca\(^{2+}\) release from the ER, and the reduced level of PIP\(_2\), and the simultaneous increase of PKC may explain the plateau of the intracellular calcium concentration when exposed to chronic mechanical stress. However, further studies are required to address these issues.

Studies on in vitro alveolar epithelial cell cultures using mechanical stretch suggest that a 5 to 12% elongation is physiological, while mechanical stretch at 37 to 50% elongation is associated with pathophysiological conditions produced by mechanical ventilation (34,38,39). Therefore, in the present study, a 15% elongation for 48 h was used as a stimulus for 16HBE cells. This magnitude of stimulation and timing likely reflects the pathologic conditions of chronic airway diseases such as COPD.

A higher TRPC1 expression was observed level in the lung tissues of patients with COPD compared with those of the control group, which was consistent with the findings of a previous study (18). It has previously been reported that 5% CSE for 48 h significantly increased TRPC1 protein expression in 16HBE cells (18), and nicotine treatment obviously upregulated TRPC1 expression in cultured rat distal pulmonary arterial smooth muscle cells (PASMcs) (40), indicating a role of cigarette smoke in upregulation of TRPC1 expression. However, Jiang et al found no significant difference in TRPC1 expression between patients with NSCLC who were smokers and non-smokers (41). Thus, it is currently not clear whether the upregulation of TRPC1 in COPD is due to cigarette smoke or other factors associated with COPD.

In conclusion, the present study demonstrated that the TRPC1-mediated increase in intracellular Ca\(^{2+}\) levels play a key role in mechanical stress-induced occurrence of EMT. There were some limitations to the present study. First, the possible involved signaling pathway downstream TRPC1-mediated intracellular Ca\(^{2+}\) in EMT occurrence induced by mechanical stretch was not investigated. Second, in the present study, 16HBE cells subjected to a Flexcell FX-4000 Tension System used to mimic the effects of high airway pressure. This model may not fully reflect the in vivo condition, where other cell types are present as well. In addition, primary normal human
bronchial epithelial (NHBE) cells may be more suitable since they were established without any genetic background changes (42). Therefore, further studies are required to address these questions thoroughly, which will aid in the elucidation of the mechanisms of mechanical stretch-induced airway remodeling in COPD and may provide a novel therapeutic target for patients with COPD.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

ML and MZ designed the research; JW wrote the manuscript and analyzed data, YH, NL and GY performed the research and analyzed data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethics committee approval was obtained for the Second Clinical Hospital of Chongqing Medical University [reference no. 2014(65)], and all subjects provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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