Original Article

Silencing of poly(ADP-ribose) polymerase-1 suppresses hyperstretch-induced expression of inflammatory cytokines in vitro

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In addition to biochemical stimuli, physical forces also play a critical role in regulating the structure, function, and metabolism of the lung. Hyperstretch can induce the inflammatory responses in asthma, but the mechanism remains unclear. Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme that can regulate a variety of inflammatory cytokines expression. In the present study, we aimed to investigate the role and mechanism of PARP-1 in mechanical stretch-induced inflammation in human bronchial epithelial cells (HBEPiCs). HBEPiCs were simulated by mechanical stretch and cells under static were used as the control. PARP-1 expression was interfered by small interfering RNA. Oxidative stress was evaluated by DHE staining. DNA damage was assessed by comet assay. The results showed that interleukin-8 (IL-8) and vascular cell adhesion molecule-1 (VCAM-1) expression were regulated by hyperstretch in a time-dependent manner. Hyperstretch could increase PARP-1 expression and activity by inducing superoxide production and DNA damage. Silencing of PARP-1 attenuated hyperstretch-induced IL-8 and VCAM-1 up-regulation as well as monocytes adhesion, which were related to the inhibition of nuclear factor-kappa B (NF-κB) translocation. Our study showed that hyperstretch could induce inflammatory response and superoxide production as well as DNA damage in HBEPiCs. PARP-1 silencing decreased IL-8 and VCAM-1 expression, partly through inhibition of NF-κB translocation. PARP-1 played a fundamental role in hyperstretch-induced inflammation. PARP-1 silencing could be used as a potential therapeutic approach to reverse bronchial epithelial inflammation in asthma.

Keywords asthma; mechanical stretch; poly(ADP-ribose) polymerase-1; inflammatory factor; NF-κB

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Introduction

Asthma is a disease of airway dysfunction that involves epithelial damages, bronchoalveolar lavage fluid accumulation, cytokines up-regulation, and epithelial hyperplasia [1]. Current research in pathology of asthma has focused on inflammatory reactions initiated by immunological responses to allergens and irritants [2]. In addition to biochemical stimuli, physical forces also play an important role in regulating the structure, function, and metabolism of the lung [3–5]. Cells in the respiratory airway are constantly exposed to physical forces induced by cyclic expansions and deflations of the lung. Physical forces, such as shear stress and stretching force, can regulate airway remodeling and gene expression [6]. It has been shown that mechanical stretch can regulate epithelial signaling, gene expression, and pulmonary functions in vitro [7,8]. Asthma can trigger deep inspirations with increases of amplitude and frequency, which can induce hyperstretch and result in deterioration of the airway obstruction [9,10].

The bronchial epithelial cells (BEpiCs) contacts first with aeroallergens and have recently become an interesting target for many studies to investigate their involvement in the modulation of inflammatory responses [11]. BEpiCs are not only considered to be a physical barrier, but also to actively contribute to airway inflammation. They can synthesize inflammatory cytokines, which may affect the recruitment and activation of immune cells to the local tissue and also create a microenvironment where these activated immune cells can take part in the inflammatory processes [12].

Poly(ADP-ribose) polymerase-1 (PARP-1), an abundant nuclear enzyme present in eukaryotes, accounts for nearly 90% of total cellular PARP activity [13]. In the nucleus, PARP-1 can be activated by DNA breaks and participates in a variety of cellular processes by catalyzing the cleavage of nicotinamide adenine dinucleotide (NAD⁺) into nicotinamide and ADP ribose to form long branches of ADP-ribose polymers on target proteins, including transcription factors and itself [14]. However, excessive activation of PARP-1 results in the intracellular depletion of NAD⁺ and ATP, thereby causing a cellular energy crisis and cell death [15]. Moreover, PARP-1 has been suggested to regulate the expression of a variety of key inflammatory genes, such as...
vascular cell adhesion molecule-1 (VCAM-1), which is regulated by nuclear factor-κB (NF-κB) [16]. PARP-1 has been proved to be closely associated with the pathogenesis of asthma. It has been shown that PARP-1 deficiency can block interleukin-5 (IL-5) expression through calpain-dependent degradation of STAT-6 in a murine model of asthma [17]. Moreover, PARP-1 inhibition could suppress the inflammatory cell migration [18]. Inhibition of PARP-1 protected against both reactive oxygen species-induced airway epithelial cell injury in vitro and airway inflammation in vivo [19]. Pretreatment with the PARP-1 inhibitors before ovalbumin challenge significantly reduced the severity of cough and the occurrence of dyspnea and delayed the onset of respiratory abnormalities. PARP-1 inhibitors were able to prevent the morphological and biochemical changes of lung tissue or bronchoalveolar lavage fluid induced by ovalbumin challenge [20].

In the present study, we stimulated the human BEpiCs (HBEpiCs) with mechanical stretch and aimed to investigate the role and mechanism of PARP-1 in hyperstretch-induced inflammation response.

Materials and Methods

Cell culture, stretch system, and gene silencing
HBEpiCs (ScienCell, Carlsbad, USA) were grown in high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin, seeded into 6-well Flexcell plates (Flexcell, Hillsborough, USA) coated with collagen I, and assembled in a stretch chamber placed in a cell-culture incubator at 37°C with 5% CO₂. The hyperstretch experiments were carried out with 10% area increase at a frequency of 40 cpm, which was applied using a computer-controlled Flexcell 5000-Tension apparatus [3]. Cells up to passage 4 were used in the experiment. To inhibit PARP-1 expression, cells were transiently transfected with siRNA (GenePharma, Shanghai, China) in OptiMEM Medium (Invitrogen) by using Lipofectamine™ 2000 (Invitrogen). Experiments were carried out 24 h after transfection.

Reverse transcription-polymerase chain reaction analysis
RNA was extracted from HBEpiCs using Trizol (Invitrogen). The cDNA was reversely transcribed from ~1 μg of RNA using the First strand cDNA synthesis kit (Fermentas, Burlington, Canada). The real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using iQ™SYBR green supermix (Bio-Rad, Hercules, USA). Each sample was analyzed in triplicate, and the expression was normalized to that of β-actin. The primers for VCAM-1 were as follows: forward, 5'-ATGACATGCTTTGACCCA-GG-3'; reverse, 5'-GTGTCTCCTCCTTGGACACT-3'; and β-actin: forward, 5'-TGGACATCCGGCAAGAC-3'; reverse, 5'-GAAAGGGTGTAACGCAACTA-3'. Quantitative values were obtained by the threshold cycle (CT) values. The relative mean fold change of expression was calculated by the 2^-ΔΔCT method.

Western blot analysis
Protein was extracted from HBEpiCs. Equal amount of protein was separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membrane (Millipore, Billerica, USA). After being blocked with 5% non-fat milk for 2 h at room temperature, membranes were washed three times (10 min for each time) with Tris-buffered saline containing Tween-20 (TBST) and incubated with primary antibodies at 4°C overnight. The primary antibodies were as follows: rabbit monoclonal anti-β-actin (1:2000; Cell Signaling Technology, Beverly, USA), mouse monoclonal anti-VCAM-1 (1:500; Santa Cruz Biotechnology, Santa Cruz, USA), and rabbit polyclonal anti-PARP-1 (1:500; Sigma-Aldrich, St Louis, USA). After being washed with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature. Signals were detected with enhanced chemiluminescence kit (Millipore) and analyzed by Image-Pro Plus 6.0.

Enzyme-linked immunosorbent assay
The IL-8 concentration in the media was measured with ELISA kit (R&D, Minneapolis, USA) according to the manufacturer’s instruction. Briefly, cell culture collected from stretch experiments or static controls was centrifuged to remove cell debris, and then 100 ml of the media were applied in the experiment. The absorbance levels of proteins were measured at 450 nm by a Varioskan Flash microplate reader (Thermo Scientific, Waltham, USA). Sampling was performed in triplicates.

Measurement of O₂⁻ production
To measure O₂⁻ production in cells, we used dihydroethidium (DHE; Beyotime, Beijing, China) as described previously [21]. Stretch-preconditioned and static cells were incubated in 5 μM DHE for 30 min at 37°C in a light-protected environment, then washed with DMEM without FBS for three times. Fluorescence was acquired by use of fluorescence microscope.

Comet assay
The cells were examined for DNA damage by comet assay as described previously [22]. Briefly, the cells were collected in phosphate saline buffer (PBS, pH 7.4) under cold condition. The cell suspension (50 μl) was mixed with 500 μl of low melting point agarose (Trevigen, Gaithersburg, USA), and then the mixture (50 μl) was dropped and spread over...
sample area, and allowed to gel at 4°C. The slide was immersed in a cold electrophoresis buffer (200 mM NaOH and 1 mM EDTA, pH ≥ 12) and run for 30 min at 25 V, 300 mA. The slides were then put in a solution to be neutralized with 0.4 M Tris-EDTA (pH 8.0), followed by methanol treatment for 5 min. Finally, the slides were stained by immersion in SYBR Green I and observed under a fluorescence microscope. The slides were scored using CometScore software v1.5 (TriTek Corporation, Sumerduck, USA).

PARP-1 activity
PARP-1 activity was determined in cells with a colorimetric PARP assay kit (Trevigen) [23]. Briefly, cultured cells were collected and centrifuged at 400 g for 10 min at 4°C and then resuspended in 100 μl of PARP lysis buffer. The cell lysates were incubated on ice for 30 min and centrifuged at 10,000 g for 10 min at 4°C. The supernatants were collected and quantified by BCA protein assay kit (Beyotime). PARP cocktail was added to each well and incubated for 60 min. Then, diluted streptavidin-HRP was added, incubated for 20 min at room temperature, followed by TACS-Sapphire incubation for additional 30 min in the dark. The reaction was stopped by addition of 0.2 M HCl and then the absorbance was read at 450 nm by use of a Varioskan Flash microplate reader.

Monocyte adhesion assay
THP-1 monocytes (American Type Culture Collection, Manassas, USA) were used in the adhesion assay as described previously [24]. Briefly, THP-1 cells (5 × 10⁵ cells/ml) were labeled with a fluorescent dye BCECF-AM (10 μM; Beyotime) in serum-free medium for 45 min at 37°C with frequent agitation. After stimulation, the HBEpiCs monolayers were washed before adding BCECF-AM-loaded THP-1 cells. After incubation for 45 min at 37°C, unbound monocytes were removed by washing monolayers three times with PBS, followed by fixation and mounting with a glass coverslip. Bound monocytes were quantified by counting the cells under a fluorescent microscope.

Immunofluorescence
HBEpiCs were fixed in 4% paraformaldehyde and permeabilized in PBS containing 0.1% Triton X-100. After being blocked with BSA for 30 min, samples were incubated with rabbit anti-NF-κB p65 (1 : 100; Cell Signaling) antibodies at 4°C overnight. Alexa 488-conjugated goat anti-rabbit IgG (1 : 500; Jackson ImmunoResearch, West Grove, USA) was used as the secondary antibody. A drop of Prolong gold antifade reagent with DAPI (Vector Laboratories, Burlingame, USA) was used to seal the coverslip. Images were acquired by a laser scanning confocal microscope (LSM 710; Zeiss, Oberkochen, Germany). Data were analyzed by Image-Pro Plus 6.0.

Statistical analysis
Data are expressed as the mean ± SD. Statistical analysis was performed by Student’s t-test for two groups of data and by one-way analysis of variance for multiple comparisons.

Figure 1. Relative IL-8 secretion and VCAM-1 expression with hyperstretch at various time points in HBEpiCs. After HBEpiCs were stimulated by hyperstretch for 0, 4, 8, 12, 16, and 24 h, IL-8 secretion was determined by ELISA and VCAM-1 expression levels of mRNA and protein were assessed by RT-PCR and western blot analysis, respectively. (A) IL-8 secretion by ELISA. (B) Quantification of RT-PCR results of VCAM-1 mRNA expression. (C,D) Western blot analysis of VCAM-1 protein. Values are expressed as the mean ± SD from three independent experiments. *P < 0.05 vs. control. Control, cells under static.
SPSS for Windows v16.0 (SPSS Inc., Chicago, USA) was used for statistical analysis. A $P$ value < 0.05 was considered statistically significant.

Results

Time-dependent regulation of IL-8 and VCAM-1 by hyperstretch in HBEpiCs

HBEpiCs were stimulated by hyperstretch for various time points. Hyperstretch induced a time-dependent regulation of IL-8 and VCAM-1 expression. Compared with cells under static condition, 24 h hyperstretch could significantly increase the mRNA and protein expression of VCAM-1, and the enzyme-linked immunosorbent assay (ELISA) results also showed that IL-8 secretion was up-regulated by hyperstretch stimulation ($P < 0.05$; Fig. 1). We stimulated HBEpiCs by hyperstretch for 24 h in the subsequent experiments.

Hyperstretch induced superoxide production and increased DNA damage in HBEpiCs

We investigated the oxidative stress induced by hyperstretch in cells. Superoxide anion production was evaluated by DHE staining. There was almost no superoxide production in cells under static, while hyperstretch stimulation could significantly induce oxidative stress, which was the main cause of DNA damage ($P < 0.05$; Fig. 2A,B). Then, we evaluated the effect of hyperstretch on DNA damage in HBEpiCs. The comet assay is a simple and sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell. As shown in Fig. 2C,D, in the static cells, there was almost no DNA in the tail, while

Figure 2. Hyperstretch induced oxidative stress and DNA damage, and increased PARP-1 expression and activity HBEpiCs were stimulated by hyperstretch for 24 h, superoxide production was evaluated by DHE staining, and DNA damage was assessed by comet assay. PARP-1 expression and activity were also determined. (A,B) Hyperstretch increased superoxide anion production. (C,D) Hyperstretch induced DNA damage. (E–G) Hyperstretch increased PARP-1 expression and activity. Values are shown as the mean ± SD. *$P < 0.05$ vs. control; Control, cells under static.
hyperstretch could increase the DNA content in the cellular tail, which suggested that hyperstretch could induce DNA damage in HBEpiCs.

**Hyperstretch increased PARP-1 expression and activity in HBEpiCs**

PARP-1 can be activated by DNA breaks and participates in a variety of cellular processes. After HBEpiCs were stimulated by hyperstretch for 24 h, we examined the effect of hyperstretch on PARP-1 expression and activity. Compared with the static cells, PARP-1 expression was significantly increased by hyperstretch, and PARP-1 activity was also up-regulated ($P < 0.05$; Fig. 2E–G).

Silencing of PARP-1 attenuated IL-8 secretion and VCAM-1 expression

We used PARP-1 siRNA in the following experiment. To ensure that PARP-1 had been effectively inhibited in target cells, we analyzed PARP-1 expression after incubation with small interfering RNA (siRNA). Compared with the control, PARP-1 expression was significantly reduced by siRNA, while the negative control (NC) of siRNA had almost no effect on PARP-1 protein expression (Fig. 3A,B). Considering the critical role of the inflammatory factors in asthma, we measured the effect of PARP-1 silencing on IL-8 secretion and VCAM-1 expression in HBEpiCs. Hyperstretch stimulation markedly increased IL-8 secretion and VCAM-1 expression when compared with static cells. However, after...
PARP-1 silencing, IL-8 secretion and VCAM-1 expression were significantly reduced ($P < 0.05$; Fig. 3C–E). The NC of PARP-1 siRNA had no significant effect on the expression of inflammatory cytokines, which suggested that PARP-1 was indispensable in hyperstretch-induced inflammatory cytokines expression.

**PARP-1 silencing reduced monocytes adhesion to HBEpiCs**

Asthma is a complex inflammatory disease involving the critical action of adhesion of inflammatory cells to airway epithelial cells. To evaluate whether PARP-1 silencing could affect monocyte adhesion to HBEpiCs, THP-1 cells were incubated with hyperstretch-stimulated HBEpiCs. As shown in Fig. 4, compared with cells under static condition, stimulation of HBEpiCs with hyperstretch resulted in a significant increase in THP-1 cell adherence; while pretreatment of HBEpiCs with PARP-1 siRNA before hyperstretch stimulation resulted in a significant decrease in monocyte adhesion. The NC of PARP-1 siRNA had no effect on monocytes adhesion.

**PARP-1 silencing inhibited NF-κB translocation**

It has been demonstrated that NF-κB plays a critical role in inflammatory response. So we wondered whether the negative effect of PARP-1 silencing was associated with defective NF-κB activation. The results showed that NF-κB p65 was mostly cytoplasmic before hyperstretch stimulation, but its localization quickly changed to the nucleus with stimulation. After PARP-1 silencing, p65 remained primarily cytoplasmic after hyperstretch treatment (Fig. 5). These results suggested that PARP-1 takes part in hyperstretch-induced inflammatory mediator expression, partly through inducing the nuclear translocation of NF-κB.

**Discussion**

Asthma is a disease of airway dysfunction that inflammatory response plays an important role in the pathogenesis of this disease. Lung biopsies of patients with asthma provided compelling pathological evidence that inflammatory processes take part in the lung parenchyma [25,26]. In asthma patients, the lung suffered more stretch than that during...
normal respiration [9], and the hyperstretch aggravated the airway obstruction in asthma by inducing myogenic and pulmonary inflammatory responses [27]. However, the mechanism of hyperstretch-induced pulmonary inflammatory responses remains unclear. In the present study, we investigated the mechanisms by stimulating HBEpiCs with 24 h hyperstretch. We found that hyperstretch could induce IL-8 secretion and VCAM-1 expression in a time-dependent manner. Hyperstretch induced oxidative stress and DNA damage, and increased the PARP-1 expression and activity. Silencing of PARP-1 reduced inflammatory cytokines expression and monocytes adhesion, partly through the inhibition of NF-κB translocation, which showed that PARP-1 plays a fundamental role in hyperstretch-induced inflammation response.

The up-regulation of adhesion molecules on the surfaces of BEpiCs can result in enhanced adhesion of leukocytes and increased expression of chemokines in affected tissues, which is an important cause of the initiation and progression of asthma [28]. Surface adhesion molecules, such as VCAM-1 and intercellular adhesion molecule-1, are known to be involved in this process [29]. It is possible that suppression of adhesion between respiratory epithelial cells and inflammatory cells could lead to attenuation of asthma [30]. In the present study, we found that hyperstretch stimulation could increase the inflammatory cytokines expression and result in the increased monocytes adhesion. The adhesive monocytes could release various chemotactic factors and lead to a vicious cycle. Our results suggested that in addition to all kinds of allergens, mechanical stretch could also induce the inflammatory response of respiratory epithelial cells.

PARP-1 is an abundant nuclear enzyme that can be activated by DNA strand breaks and functionally linked to DNA repair [31]. Once activated, PARP-1 plays a critical role in DNA repair under moderate stress [32]. However, extensive activation of PARP-1 depletes cellular NAD⁺ and its precursor ATP, leading to irreversible energy crisis and cell death [33]. PARP-1 activation is associated with the pathogenesis of numerous diseases, including energetic failure, diabetes, cerebral ischemia, and angiogenesis [34,35]. It has also been demonstrated that PARP-1 is activated by allergen challenge in an animal model of asthma, and PARP-1 inhibition prevents infiltration of inflammatory cells into the lungs after allergen exposure [36]. PARP-1 deficiency inhibited IL-5 expression through calpain-dependent degradation of STAT-6 in allergen-induced asthma [17]. Increased PARP-1 expression and activity were associated with inflammation but not goblet cell metaplasia in murine models of allergen-induced airway inflammation [37]. However, the effect of mechanical stretch on PARP-1 during asthma is still unclear. Our in vitro study showed that hyperstretch could induce
oxidative stress and DNA damage as assessed by DHE and comet assay, and increase PARP-1 expression and activity. PARP-1 silencing reduced hyperstretch-induced inflammation response, including inflammatory cytokines expression and monocytes adhesion. Our results confirmed the previous studies which revealed that PARP inhibition with PARP inhibitor PJ34 suppressed inflammatory cell migration [18].

In this study, we also tried to investigate the underlying mechanisms. Increasing evidence showed that NF-κB plays an essential role in the expression of inflammatory mediators in asthma [38,39]. A previous study has revealed that PARP-1 inhibition by inhibitor 3-AB can inhibit NF-κB activation and IL-8 expression induced by H2O2 [19]. The NF-κB family consists of the members p50, p52, p65 (Rel A), c-Rel, and Rel B, which form a variety of homodimers and heterodimers [40]. The NF-κB dimers locate in the cytoplasm in an inactive form bound to an inhibitory protein known as IkappaB (IκB). Various stimulations mediate the activation of NF-κB pathways, which can activate the IκB kinase (IKK) complex by phosphorylation [41]. Activated IKK complex then phosphorylates IκB, resulting in their degradation and the activation of NF-κB. Then, NF-κB is translocated to the nucleus and binds to the promoter or the enhancer regions of specific genes to initiate transcription [42,43]. In the present study, we found that PARP-1 played an essential role in NF-κB activation. Under static condition, NF-κB resides in the cytoplasm, while hyperstretch could induce NF-κB activation and translocate it to the nucleus. However, PARP-1 silencing suppressed NF-κB activation and nuclear translocation.

In summary, our results showed that mechanical stretch could activate pro-inflammatory transcription factor NF-κB via a DNA damage/PARP-1 pathway, then initiated the expression of several inflammatory mediators. The increased expression of these pro-inflammatory mediators might contribute to the pathogenesis of asthma, which needs further investigation by in vivo experiments.

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References


