Preconditioning of physiological cyclic stretch inhibits the inflammatory response induced by pathologically mechanical stretch in alveolar epithelial cells

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Abstract. The aim of the present study was to investigate the effects of preconditioning of physiological cyclic stretch (CS) on the overexpression of early pro-inflammatory cytokines [including tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-8] during the inflammatory response induced by pathologically mechanical stretch in lung epithelial cells, and to determine its molecular mechanism of action. Cells were subjected to 5% CS for various durations (0, 15, 30, 60 and 120 min) prior to 6 h treatment with pathological 20% CS. In a separate experiment, cells were preconditioned with physiological 5% CS or incubated with a nuclear factor (NF)-κB inhibitor, pyrroldine dithiocarbamate (PDTC). The expression levels of inflammatory mediators were measured using reverse transcription-quantitative polymerase chain reaction. NF-κB was quantified using western blot analysis. Preconditioning with physiological 5% CS for 30, 60 and 120 min was demonstrated to significantly attenuate the release of pathologically mechanical stretch-induced early pro-inflammatory cytokines (TNF-α, IL-1β and IL-8) in alveolar epithelial cells ($P<0.05$) and significantly reduce the expression of NF-κB ($P<0.05$). Peak suppression was observed in cells preconditioned for 60 min. In the second set of experiments, it was demonstrated that mechanical stretch-induced release of TNF-α, IL-1β and IL-8 was significantly inhibited by both PDTC pretreatment and 5% CS pretreatment alone (all $P<0.05$). Furthermore, significant inhibition was also observed when both 5% CS pretreatment and PDTC pretreatment was used on mechanical stretch-induced cells ($P<0.05$), which was markedly greater than the inhibition induced by either pretreatment alone. The present findings suggest that preconditioning with physiological 5% CS is able to inhibit the inflammatory response induced by pathologically mechanical stretch in alveolar epithelial cells. These anti-inflammatory effects are induced, at least in part, by suppressing the NF-κB signaling pathway.

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Introduction

Mechanical ventilation (MV) is typically used for treating patients with acute respiratory failure in intensive care units and is also a crucial element used in general anesthesia (1). However, the misuse of MV may also induce damage known as ventilator-associated lung injury (VALI) (2). It is generally accepted that VALI is an excessive, uncontrolled, inflammatory, response within the lung (3). Previous studies have suggested that the release of pro-inflammatory cytokines have a central role in VALI (4,5). Nuclear factor (NF)-κB is required for maximal transcription of various cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-8, and IL-1β (6,7). Furthermore, it has been suggested that inhibitors of NF-κB function may be useful as anti-inflammatory agents (8).

Wolfson *et al* (9) have recently demonstrated that pathological 18% cyclic stretch (CS) increased expression of the late pro-inflammatory cytokine high mobility group box protein 1 (HMGB1), whereas physiological cyclic stretch (5% CS) attenuated oxidative- and lipopolysaccharide-induced increases in HMGB1 expression via a signaling pathway with critical involvement of the transcription factor, signal transducer and activator of transcription 3 (STAT3). A recent study by the present authors demonstrated that preconditioning with physiological cyclic stretch has beneficial effects on mechanical stretch-induced alveolar epithelial cell apoptosis and barrier dysfunction via regulation of Rac and Rho activities (10). Furthermore, another recent study by the present authors demonstrated that preconditioning with physiological 5% CS is able to reduce the expression of HMGB1 induced by pathologically mechanical stretch through the IL-6/STAT3 pathway associated with upregulated suppressor of cytokine signaling 3 expression (11). However, to the best

of our knowledge, few studies have assessed the effects of preconditioning with physiological cyclic stretch on early pro-inflammatory cytokines, including TNF-α, IL-1β and IL-8, induced by pathologically mechanical stretch in lung epithelial cells. Therefore, it was hypothesized that preconditioning with physiological CS may attenuate TNF-α, IL-1β, and IL-8 expression and release induced by pathologically mechanical stretch.

A549 cells have been characterized as a typical alveolar epithelial cell line with many features specialized for alveolar epithelial cells and therefore may be considered as an ideal culture model for alveolar epithelial cell type II in research. The present study aims to investigate the preconditioning effects of physiological CS on pathologically mechanical stretch-induced increases in TNF-α, IL-1β and IL-8 expression by using human alveolar epithelial cell line A549, and to elucidate the mechanisms of these effects.

Materials and methods

Cell culture. The A549 human type II-like alveolar epithelial cell line was obtained from the cellular immunity laboratory of the Medical College of Yangzhou University (Yangzhou, China). A549 cells were cultured in Dulbecco's Modified Eagle's Medium-Ham's F12 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified cell incubator in an atmosphere of 5% CO_2 at 37˚C.

Cell deformation. Cells were stretched using a Flexercell Tension Plus FX-4000T system (Flexcell International Corp., Burlington, NC, USA) equipped with a loading station, according to the manufacturer's protocol. These deformations were selected as previously described (12). Cells were seeded at $2.0x10^5$ cells/cm^2 on type I collagen-coated flexible bottom BioFlex plates (Flexcell International Corp.) and were then subjected to various different regimens. Initially, to examine the potential protective effects of 5% CS and define the optimum preconditioning time for 5% CS, cells were subjected to CS of 5% elongation for various durations (0, 15, 30, 60 and 120 min) at a frequency of 15 cycles/min. Cells were subsequently exposed to CS of a higher magnitude (20% elongation) with the same frequency for 6 h. The sham group consisted of $2.0x10^5$ cells/cm^2 and did not receive any treatment. In a second set of experiments, cells were incubated with 100 μM pyrroldine dithiocarbamate (PDTC) for 1 h at 37˚C, which is a specific NF-κB inhibitor, or preconditioned with 5% CS for 60 min, or were exposed to both stimuli, prior to 6 h treatment with pathological CS stimulation (20% CS). The sham group did not receive any treatment. The dosages of PDTC were determined according to a recent study (13). The duration of 5% CS preconditioning in the second set of experiments was determined according to the first set of experiments.

Reverse transcription-quantitative polymerase chain reaction. Following exposure to CS, total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reverse transcription was conducted using a Reverse Transcription System (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. A total of 2 μg RNA was mixed with 1 μl stem-loop reverse transcription primers and RNase free ddH_2O in a total volume of 12 μl and incubated at 65˚C for 5 min followed by being chilled on ice for 5 min, then mixed with 4 μl 5x reaction buffer, 2 μl dNTP mixture, 1 μl RNase Inhibitor and 1 μl M-MuLV reverse transcriptase (Promega Corporation) in a final volume of 20 μl. The reactions were performed at 25˚C for 5 min, followed by 42˚C for 60 min and 70˚C for 5 min. cDNA were stored at -80˚C for subsequent assessments. A TaqMan® Gene Expression assays (Applied Biosystems, Thermo Fisher Scientific, Inc.) and a 7900HT Fast Real-Time PCR system (Thermo Fisher Scientific, Inc.) were used to quantify the mRNA expression levels of TNF-α, IL-8 and IL-1β, according to the manufacturer's protocol. Primers for TNF-α, IL-8 and IL-1β were designed and synthesized by Shanghai Shenggong Biological Engineering Technology Service Ltd. (Shanghai, China). Primer sequences were as follows: TNF-α, forward, 5'-CCTGTGAGGAGGACGAAC-3' and reverse, 3'-AAGTGGTGGTCTTGTTGC-5'; IL-1β, forward, 5'-GGAGAATGACCTGAGCAC-3' and reverse, 3'-GACCAGACATCACCAAGC-5'; IL-8, forward, 5'-TCT GCACCCACTTTTCCTTG-3' and reverse, 3'-AACCTT CCTGCTGCTTCTGA-5'; and β-actin, forward, 5'-GTGACG TTGACATCCGTAAAGA-3' and reverse, 3'-GCCGGACTC ATCGTACTCC-5'. The amplified expression of TNF-α, IL-8 and IL-1β was normalized to β-actin expression. PCR conditions were as follows: Initial denaturation at 95˚C for 5 min; 40 cycles of denaturation at 95˚C for 5 sec and extension at 60˚C for 30 sec; and denaturation at 95˚C for 15 sec, extension at 72˚C for 10 sec, and a final denaturation at 95˚C and 15 sec. The relative expression of TNF-α, IL-8 and IL-1β in the experimental group compared with the control group was calculated using the 2ΔΔCq method (14).

Western blotting. Western blotting was performed as previously described (11). In brief, the harvested lung tissue was weighed, homogenized in RIPA Lysis Buffer (cat. no. P0013B; Beyotime Institute of Biotechnology, Haimen, China) and centrifuged at 12,000 x g and 4˚C for 15 min. Total protein concentration in lung tissue homogenates was assessed using a BCA protein assay kit (cat. no. P0009; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Equal amounts (20 μl) of protein were separated by 10% SDS-PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 1.2% Tween-20) at room temperature for 1 h. Membranes were incubated with the following primary antibodies: Anti-inhibitor against NF-κB (cat no. ab16502; 1:300; Abcam, Cambridge, USA), phosphorylated (p)-NF-κB (cat no. 93H1:1:300; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-β-actin antibody (cat no. AA128; 1:500; Beyotime Institute of Biotechnology) at 4˚C for 12 h. Subsequently, membranes were incubated at room temperature for 1 h with the secondary antibody conjugated to horseradish peroxidase (cat. no. A0208; 1:200; Beyotime Institute of Biotechnology).

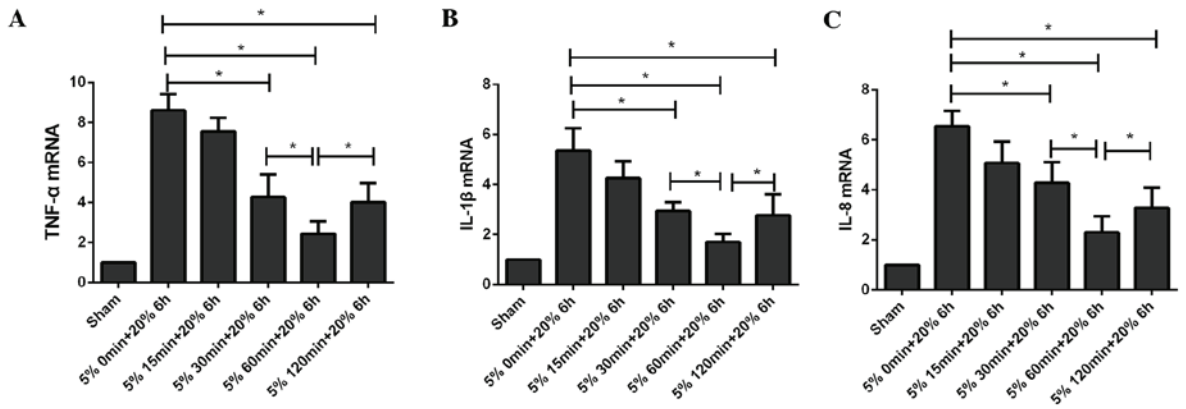

Figure 1. The effect of preconditioning with 5% CS on the expression of inflammatory mediators in lung epithelial cells prior to pathological 20% CS. The expression of the inflammatory mediators (A) TNF-α, (B) IL-8 and (C) IL-1β mRNA was assessed by reverse transcription-quantitative polymerase chain reaction. Data are expressed as the mean ± standard deviation from three independent experiments. *P<0.05. CS, cyclic stretch; TNF, tumor necrosis factor; IL, interleukin.

Protein bands were visualized using enhanced chemiluminescence with an ECL Western Blotting System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The intensity was quantified using Image J 1.8.0 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are presented as the mean ± standard deviation. All experiments were performed a minimum of three times unless otherwise stated. Differences between two groups were tested with unpaired Student's t-test. One-way analysis of variance followed by post hoc analysis with Bonferroni method was applied for multiple-group comparisons. P<0.05 was considered to indicate a statistically significant difference. Data were analyzed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA).

Results

CS reduces inflammatory mediators in lung epithelial cells. To examine the effect of physiological 5% CS on the release of inflammatory mediators, TNF-α, IL-1β and IL-8 levels were quantified by RT-qPCR (Fig. 1). Preconditioning with physiological 5% CS for 30, 60 and 120 min significantly inhibited TNF-α, IL-1β and IL-8 release (all P<0.05). Furthermore, the greatest suppression was observed in cells preconditioned at 5% CS for 60 min.

CS reduces NF-κB activation in lung epithelial cells. NF-κB activation was analyzed via western blotting to investigate the molecular mechanisms responsible for mediating the anti-inflammatory effects of preconditioning with physiological 5% CS (Fig. 2). Following exposure to 20% CS for 6 h, NF-κB phosphorylation was significantly increased compared with the sham group. Preconditioning with physiological 5% CS for 30, 60 and 120 min significantly reduced this phosphorylation level (all P<0.05). The greatest suppression of NF-kB activation was observed following 5% CS stimulation for 60 min.

Comparison of the inhibitory effects of CS and NF-κB signal pathway inhibition on pathologically mechanical stretch-induced inflammatory responses. Following the

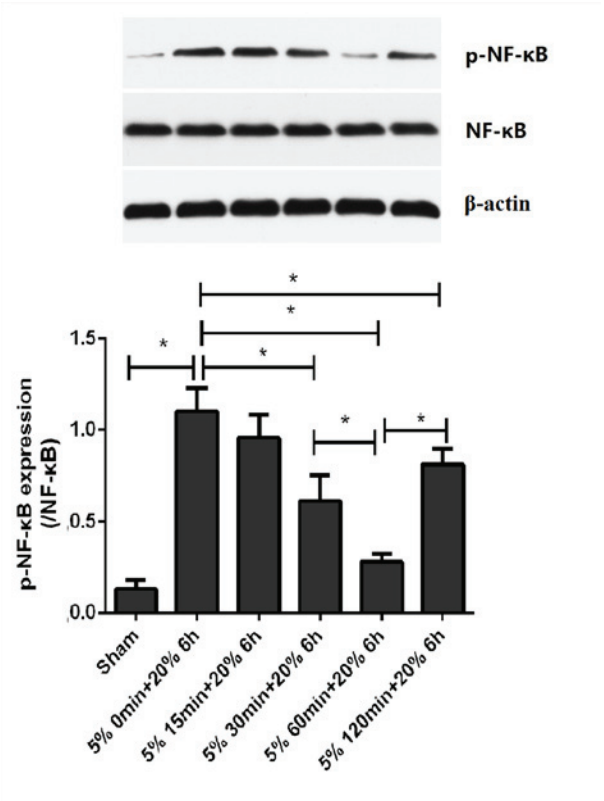

Figure 2. Preconditioning with physiological 5% CS prior to pathological 20% CS may reduce NF-κB activation in lung epithelial cells. NF-κB activation was assessed by western blotting. Data are expressed as the mean ± standard deviation from three independent experiments. *P<0.05. CS, cyclic stretch; NF, nuclear factor; p, phosphorylated.

observation that CS suppressed both the pathologically mechanical stretch-triggered activations of NF-κB, the relative contribution of inhibiting these signaling pathways to the anti-inflammatory action of CS was determined. PDTC was used to specifically block NF-κB activity. Cells were exposed to 5% CS, PDTC or PDTC and 5% CS for 60 min, followed by stimulation with 20% CS for 6 h (Fig. 3). In cells exposed to 20% CS, it was demonstrated that mechanical stretch-induced release of TNF-α, IL-1β and IL-8 was significantly inhibited

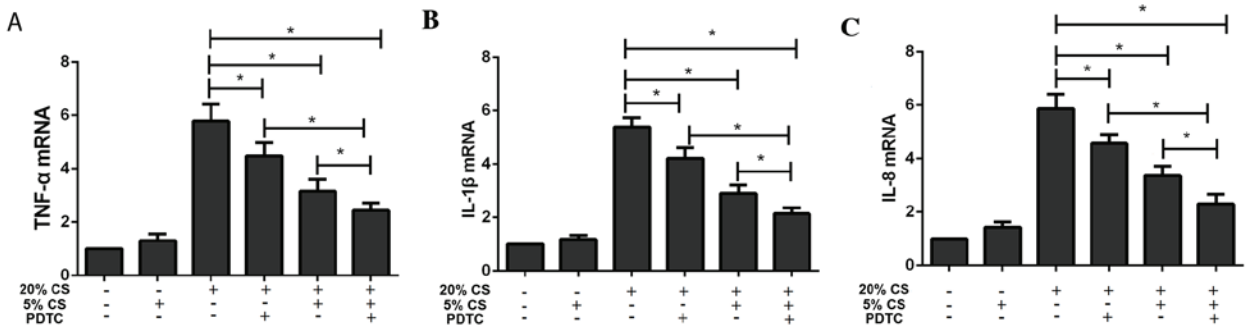

Figure 3. Administration of an NF-κB inhibitor suppressed release of pathologically mechanical stretch-induced release of inflammatory mediators in lung epithelial cells. The mRNA expression of the inflammatory mediators (A) TNF-α, (B and C) IL-1β and IL-8 was assessed via reverse transcription-quantitative polymerase chain reaction. Data are expressed as the mean ± standard deviation from three independent experiments. *P<0.05. NF, nuclear factor; TNF, tumor necrosis factor; IL, interleukin; CS, cyclic stretch; PDTC, pyrroldine dithiocarbamate.

by both PDTC pretreatment and 5% CS pretreatment alone (all P<0.05). Furthermore, significant inhibition was also observed when both 5% CS pretreatment and PDTC pretreatment were performed to the mechanical stretch-induced cells (P<0.05), which was markedly greater than the inhibition induced by either pretreatment alone. These results indicate that NF-κB activation is associated with the release of inflammatory mediators from A549 cells exposed to 20% elongation, that the inhibition of NF-κB signal pathways is a mechanism for the anti-inflammatory effects of preconditioning with 5% CS, and that the effects of 5% CS pretreatment may not be completely removed by blocking NF-κB signaling pathway.

Discussion

The major finding of the present study is that preconditioning with physiological 5% CS is able to attenuate the release of the pathologically mechanical stretch induced early pro-inflammatory cytokines TNF-α, IL-1β and IL-8 in alveolar epithelial cells. Furthermore, it was also demonstrated that these anti-inflammatory effects are caused by suppressing the NF-κB signaling pathways.

Mechanical stretch can have notable effects on alveolar epithelial cell function that alter lung development and induce pathological conditions (12,15,16). It is also known that patients ventilated with high tidal volumes are at risk for VALI and a higher mortality rate (12,17). Although it is unclear precisely how mechanical stretch may induce lung injury, mechanisms that promote the release of inflammatory mediators including TNF-α, IL-1β, IL-8 and HMGB 1 been implicated (8,12,18). An administration of drugs with anti-inflammatory features has shown a decrease in VILI (12,19,20).

Serum levels of TNF-α, IL-1β, IL-8 peak early during sepsis (21) and pathologic CS is known to upregulate early pro-inflammatory cytokines, including IL-6, IL-1β and IL-8 (18). Pro-inflammatory cytokines that are released early, such as TNF-α and IL-1β, may also upregulate the expression of HMGB1, which is a late mediator of sepsis (22). The present results demonstrated that TNF-α, IL-1β and IL-8 were decreased markedly by preconditioning with 5% CS administration, suggesting that preconditioning with 5% CS provides potential protection from the inflammatory response induced by pathologically mechanical stretch in alveolar epithelial cells. In addition, the peak suppression of inflammatory response was observed in cells preconditioned for 60 min, which was consistent with previous findings by the present authors (10).

It has been suggested that NF-κB serves an important role in the regulation of inflammatory response that is the hallmark of VALI (23). Furthermore, mechanical stretching has been demonstrated to induce activation of NF-κB in lung fibroblasts and A549 cells (18,24). Activation of the NF-κB signaling pathway may be a key mechanism by which excessive stretch results in cellular activation, inflammation, and injury (25). In the present study, the expression of p-NF-κB in cells preconditioned at physiologically relevant amplitude CS (5% elongation) for 30, 60 and 120 min was determined and revealed obvious reductions. NF-κB is strongly suggested to be associated with regulating this anti-inflammatory effect. In accordance with these findings, to further clarify the regulatory role of NF-κB, cells were pre-incubated with an NF-κB inhibitor and/or preconditioned with 5% CS for 60 min and subsequently stimulated with 20% CS for 6 h. The present findings suggest that preconditioning with 5% CS significantly inhibited the activation of NF-κB. Therefore, the present study supports the hypothesis that preconditioning with 5% CS inhibits the NF-κB signaling pathways and regulates the release of pro-inflammatory cytokines.

In conclusion, it was demonstrated that preconditioning with physiological 5% CS may attenuate the release of the pathologically mechanical stretch induced early pro-inflammatory cytokines, TNF-α, IL-1β and IL-8, in alveolar epithelial cells. Furthermore, it was also demonstrated that these anti-inflammatory effects are caused, at least in part by suppressing the NF-κB signaling pathway. The present study provides an experimental basis for using preconditioning with MV to reduce ventilator-induced lung injury.

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