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Preconditioning effects of physiological cyclic stretch on pathologically mechanical stretch-induced alveolar epithelial cell apoptosis and barrier dysfunction ☆

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ABSTRACT

Background: We aim to investigate the effects of preconditioning of physiological cyclic stretch on the alveolar epithelial cell apoptosis induced by pathologically mechanical stretch and barrier dysfunction and how these effects are linked to differential expression of small GTPases Rac and Rho mRNA.
Methods: Pulmonary alveolar epithelial cells were subjected to different treatments of cyclic stretch (CS) at 5% and 20% elongation, respectively. Cells maintained in normal cell culture were used as negative control. On the other hand, cell apoptosis and Rac/Rho activities in cells with or without preconditioning of physiologically relevant magnitudes of CS (5% CS) with different durations (0, 15, 30, 60 and 120 min) in prior to 6-h treatment with pathological CS stimulation (20% CS) were compared and measured.
Results: Pathological CS could cause a significant increase in apoptosis rate, which is considered to be associated with the repression of Rac mRNA and activation of Rho mRNA. In contrast, physiological 5%-CS preconditioning suppressed cell apoptosis and induced nearly complete monolayer recovery with fewer actin stress fibers and paracellular gap formation. Consistent with differential effects on cell apoptosis and epithelial cell integrity, physiological CS preconditioning enhanced expression of Rac mRNA but inhibited Rho activation.
Conclusions: Physiological CS preconditioning has an inhibitory effect on cell apoptosis while exerts a stimulatory impact on epithelial cell recovery via regulation of Rac and Rho activities.

1. Introduction

Mechanical ventilation (MV) is essential for patients with acute respiratory failure, maintaining blood gas levels following a repression in respiratory function. However, ventilator-induced lung injury (VILI) and pulmonary edema have been established as significant potential risks in patients receiving mechanical ventilation, leading to high rates of morbidity and mortality [1].

It is widely known that pathologically mechanical stimulation could directly exert its effect via increasing the vascular epithelial and endothelial permeability, leading to changes in the cell monolayer barrier integrity and compromising barrier functions [2,3]. Moreover, MV may expose the lungs, and particularly the alveoli, to over distension that could cause volutrauma. It is evitable to be followed by a release of inflammatory cytokines, activating subsequent signaling pathways and inducing cell apoptosis that propagate VILI to a large extent. However, measurements of alveolar epithelial distension in the mechanically ventilated lungs remains to be established and determined due to complexity of local pulmonary distension patterns. The significance of the interactions between the pathological over distension of the functional alveoli and the lung inflammation induced by MV has been only recently recognized [4]. To investigate the mechanisms by which mechanical ventilation injures the lungs, it is critical to identify new therapeutic targets via establishing cell culture models in vitro.

It has been revealed that VILI caused by biomechanical forces was able to trigger a series of signaling mechanisms including activation of signaling kinases, ion channels, small GTPases, inflammatory cytokines, and gene expression. Small GTPases Rac, Rho, and Cdc42 have been suggested to play essential regulatory roles in cell motility, cytoskeletal remodeling [5,6] and cell barrier function

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[7–12] via activation of its downstream effector kinases, which involves PAK, mDia, Rho-kinase, [6,13–15], and nonenzymatic cytoskeletal and cell adhesion effectors such as Arp-2/3 complex, cortactin, N-WASP, paxillin, and PKL/GIT2 [6,13,16,17]. Stimulation of small GTPases Rac and Rho signaling are suggested to be essential in endothelial recognition in endothelial cell (EC) cultures exposed to physiologically and pathologically relevant cyclic stretch (CS) magnitudes [18]. Birukova and her colleagues had shown that pathologically relevant levels of CS could stimulate the activation of thrombin-induced small GTPases Rho while physiologically relevant levels of CS could promote Rac activation, which implied a critical role in the pulmonary endothelial cell recovery phase [4]. Moreover, they also demonstrated that synergistic protective effects of HGF and physiologically relevant levels of CS were able to activate the Rac-mediated signaling pathways and its expression to a large extent. Recent studies published had showed that molecular mechanisms of small GTPases Rac and Rho were of profound importance in the regulation of lung endothelial permeability induced by physiologically relevant levels of CS and its corresponding agonists [19]. And Rho signaling is believed to play an important role in mediating cell apoptosis. Wang et al. provided experimental evidence that Rho pathway was involved in diabetic-induced cellular apoptosis in the kidney glomeruli by downstream effector of Rho [20] and in mediating high glucose-induced apoptosis in cultured podocytes [21]. However, whether lung epithelial cells exposed to preconditioning of physiologically regimen of CS could contribute to the regulation of lung epithelial cell permeability and cell apoptosis is rarely reported and remains to be fully established and characterized.

Our study aims to evaluate the preconditioning effects of physiological CS on pathologically mechanical stretch-induced alveolar epithelial cell apoptosis and cell barrier dysfunction by using human alveolar epithelial cell line A549, which was derived from human lung adenocarcinoma cells. A549 cells have been characterized as a typical alveolar epithelial cell line with many features specialized for alveolar epithelial cells and therefore could be considered as an ideal culture model for alveolar epithelial cell typeII in research [22]. Most importantly, the activation of small GTPases Rho and Rac expression at mRNA level caused by the MV-induced cell apoptosis and subsequent over distension is another objective of our study, which may be associated with cell barrier dysfunction and could the provide vital information to have a thorough understanding of the molecular mechanisms of MV-induced VILJ and therefore identify novel therapeutic biomarkers for the restoration of pulmonary epithelial cell monolayer integrity.

2. Methods

2.1. Cell culture

Human typeII-like alveolar epithelial cells (A549 cells) were obtained from cellular Immunity laboratory of Tongji Medical College, Huazhong University of Science and Technology. Cells were cultured and maintained in DMEM-F12 medium (Hyclone) containing 10% fetal bovine serum (FBS, GIBCO-BRL) with 1% penicillin–streptomycin (GIBCO-BRL) in humidified incubator at 37 °C with 5% CO_2.

2.2. Cell deformation

Cell deformation was stretched with the Flexercell Tension PlusTM FX-4000T system (Flexcell International, USA) equipped with a loading station, which is designed to provide uniform strain to the cultured cells. The vacuum pressure is controlled by the computer, allowing cell monolayers to receive different levels of elongation [23]. These deformations were selected as previously described. Briefly, cells were seeded at 2.0×10^5 cells/cm^2 on type I collagen-coated flexible bottom BioFlex plates (Flexcell international) and allowed to reach 50% confluence after 24 h. Then the culture was changed into serum-deprived in DMEM-F12 medium in each plate and the experimental plates with EC monolayers were mounted onto the Flexecell system. Cells were then subjected to different regimens. Firstly, A549 cells were exposed to CS of 20% elongation for different duration (0–6 h) with a frequency of 15 cycles/min, investigating the effect of cyclic-deformed duration on cell apoptosis and mRNA expression of Small GTPases Rac and Rho. Secondly, cells were subjected to CS of 5% elongation for specialized duration (0 min, 15 min, 30 min, 60 min, and 120 min, respectively) with a frequency of 15 cycles/min. After that, cells were exposed to CS of a higher magnitude (20% elongation) with the same frequency for 6 h to compare and examine the potential protective effects of 5% CS on cell apoptosis and cell barrier dysfunction.

2.3. Detection of apoptosis

To investigate the amplitude-dependent effects of CS on cell apoptosis, the cells after CS exposures were stained with FITC-conjugated annexinV and propidium iodide (PI) following manufacturer's instructions (KeyGEN Biotech Co. Ltd, China) and was analyzed by flow cytometry (Beckman Coulter Co, USA).

2.4. F-actin staining and image analysis

After exposures to CS, cells were washed twice with PBS and fixed in 4% paraformaldehyde solution in PBS for 10 min at room temperature, permeabilized twice with 0.1% Triton X-100 in PBS for 5 min. F-actin filaments staining was performed with phalloidin-FITC diluted in 3% BSA in PBS for 60 min followed by staining with Actin-Trakcer Green (Beyotime, Shanghai, China). After the immunostaining, the elastic membranes of the wells with cells were excised and mounted onto large coverslips and then the slides were analyzed by fluorescent microscopy equipped with a Nikon video-imaging system (Nikon Instech Co., Japan). All images were acquired randomly with ×40 objective with consistent intensity setting and selected for next analysis. For each experimental condition at least 10 microscopic fields from different areas of plate (both central and peripheral) were analyzed using Image J software (version 1.43J).

2.5. RNA extraction and RT-PCR

Cell pellets were obtained and total RNA was extracted according to the manufacture's protocol. Primers for RhoA and RAC were designed and synthesized by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China). The primer sequences used to amplify RhoA and RAC were shown as follows in 5–3′ direction, RhoA sense GATGGAGCTTGTGGTAAGA and RhoA antisense AAACTATCAGGGCTGTCG; RAC-1 sense CATCACCTA TCCGCAGGGTC and RAC-1 antisense GACAGGACCAAGAACGAGGG. The amplified expression of RhoA and Rac transcript was normalized to β-actin expression. Cycle threshold ($^{\Delta\Delta}$Ct) values were calculated for each experimental group, indicating the amount of template cDNA available in each reaction and therefore the Rho and Rac1 expression levels can be expressed by the values of $2^{-\Delta\Delta CT}$.

2.6. Data analysis

Statistical analysis was carried out and data are presented as mean ± standard error (SE) combining three independent

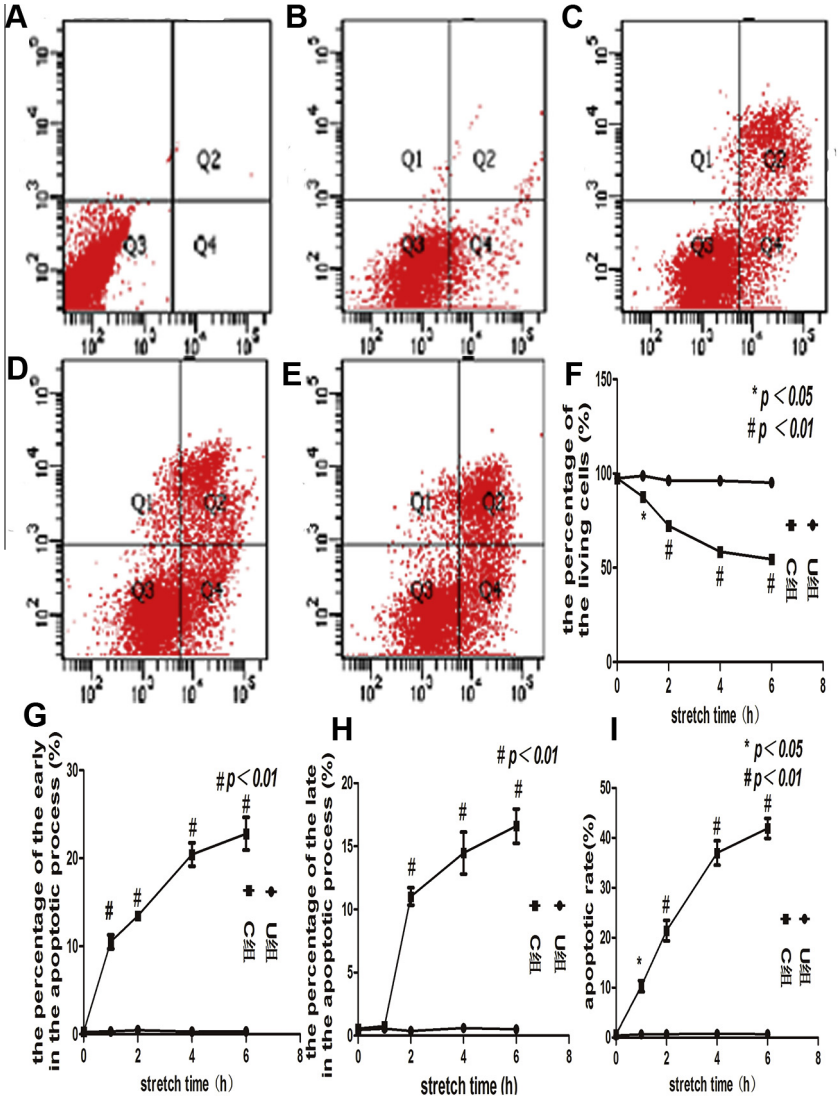

Fig. 1. Time-dependent effect of mechanical stretch on apoptosis by Annexin V and PI staining (A–E). The percentages of different types of cells were shown in the F–I, corresponding to live cells, cells at the early phase, cells at the late phase of apoptosis and the necrotic cells, respectively. Aoptotic rate increased as the time went, which was peaked at 6 h (I). The percentage of the late in the apoptotic process was not changed in monolayer at 1 h compared with unstretched monolayer (H). Values are means ± SE from ⩾3 independent experiments ($^{*}p < 0.05$, $^{\#}p < 0.01$). U, unstretch.

experimental repeats. All experiments were performed a minimum of three times unless otherwise stated. Stimulated samples were compared with the controls by unpaired Student's *t*-test. One-way analysis of variance (ANOVA) followed by post hoc analysis with Bonferroni *t*-test was applied for multiple-group comparisons (GraphPad Prism 5.0 software). $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Effects of CS on cell apoptosis

Annexin V binding and PI staining was used for cell apoptosis analysis. Cells negative for PI and annexin V were regarded as viable cells (bottom left quadrant of Flow Cytometry). PI-negative cells binding annexin V were defined as cells at an early phase in the apoptotic process (bottom right quadrant of Flow figure). Moreover, cells staining with annexin V and PI were classified as cells at late phase in apoptosis (top right quadrant of Flow Cytometry). AnnexinV-negative staining cells binding PI were considered as necrotic cells (top left quadrant of Flow Cytometry figure).

Flow cytometry plots were very useful to analyze the annexin V binding and PI staining status in lung EC cells at the same time and a series of the representative plots of the flow cytometry analysis were presented (Fig. 1A–F). As is shown, the percentages of different types of cells were shown in the Fig. 1F–I, corresponding to live cells, cells at the early phase, cells at the late phase of apoptosis and the necrotic cells, respectively. Compared with the condition of monolayer without stretch, the level of apoptosis was enhanced as increased time duration, which was peaked at 6 h. But no significant change was observed regarding the percentage of cells at the late phase during apoptosis at 1 h duration as compared with the control group (without CS treatments). The densitometric data obtained from five independent experimental repeats were also presented. These results had demonstrated that high-magnitude CS (20% elongation) could further propagate the progressive death of alveolar epithelial cells, which was opposite to the phenomena observed in cells under static conditions. These results were also highly consistent with clinical observations and data acquired from animal model studies.

To further clarify the effects of preconditioned CS (5%) on cell apoptosis, cells exposed to 2 h of 5% CS were treated with

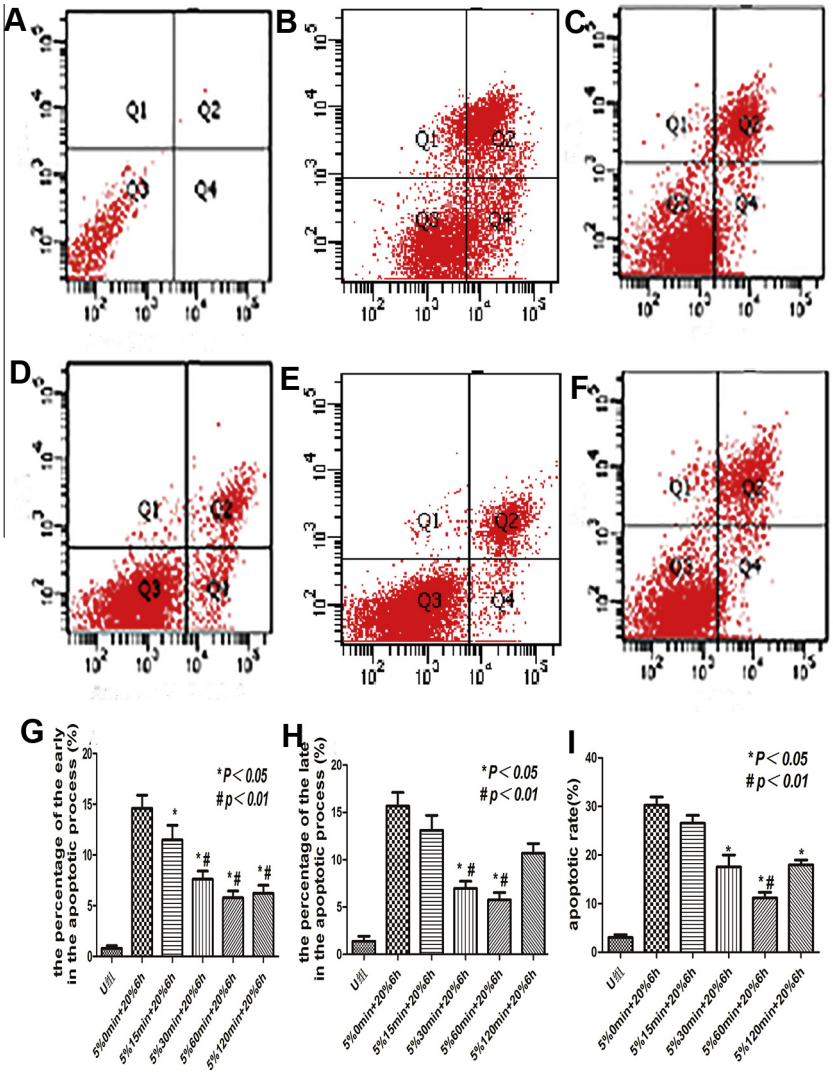

Fig. 2. The effect of preconditioning on apoptosis by Annexin V and PI staining (Fig. 1A–F). The percentage of the late in the apoptotic process was not changed in monolayer preconditioned with 5% elongation at 15 min and 120 min compared with monolayer only stretched to 20% elongation (H). The apoptotic rate of cells decreased at 30 min, 60 min, 120 min in monolayer preconditioned with 5% elongation compared with monolayer only stretched to 20% elongation (I). Suppression of cell apoptosis peaked with preconditioned at 60 min. Values are means ± SE from ⩾3 independent experiments ($^{*}p < 0.05$, $^{\#}p < 0.01$). UNT, unstretch.

pathologically CS relevant levels (20% CS elongation) for another 6 h and further flow cytometry analysis was performed and demonstrated in Fig. 2A–F. As shown in Fig. 2I, the number of apoptotic A549 cells exposed to pathologically relevant level (20% elongation) revealed a significant increase as compared to that in cells under 5% CS preconditioning for 30, 60 and 120 min. Furthermore, suppression of cell apoptosis was observed at the highest level in cells preconditioned at 5% CS for 60 min, where apoptosis was decreased by 21.46% ($p < 0.01$).

3.2. Effects of CS on Rac and RhoA mRNA

A549 cells were subjected to CS treatments at different levels followed by measurements of Rac and RhoA mRNA expressions using RT-PCR as described in Section 2. A549 cells exposure to 20% CS enhanced Rho mRNA expression by both 4 h and 6 h of CS stimulation (Fig. 3A) without obvious change monitored at 1 h and 2 h. On the other hand, the expression of Rac mRNA was repressed in cells with pathological 20% CS exposure at duration ranged from 1 h to 6 h (Fig. 3B). A549 cells stimulated with physiological 5% CS precondition at corresponding duration of 0–120 min were used as positive controls for Rac and RhoA activation, respectively. Comparison of Rac and RhoA mRNA expressions activated by 5% CS precondition and 20% CS stimulation is summarized in Fig. 3C and D. Preconditioning of physiological 5% CS significantly reduced RhoA mRNA expression level to a large extent, as compared to that in cells exposed to only pathological 20% CS. Moreover, the most obvious suppression of Rho mRNA expression was obtained in response of 5% CS stimulation for 60 min, which was 1.56 ± 0.21-fold ($p < 0.01$) higher in positive control cells (Fig. 3C). In contrast, a significant difference in activating the Rac mRNA expression level was also presented but showed only at 30 and 60 min of 5% CS precondition (Fig. 3D). Fig. 3C and D also indicated that 5% CS alone would induce significantly regulate both levels of Rho and Rac mRNA expressions, which would be used as the controls for other treatments.

3.3. F-actin staining and gap formation

We tested the hypothesis that preconditioning of physiologically relevant magnitude of CS (5% CS) has potential repressive effect on cells in the presence of pathological 20% CS in terms of A549 barrier dysfunction. It had been demonstrated that cells exposed to CS revealed cytoskeletal arrangement in pulmonary

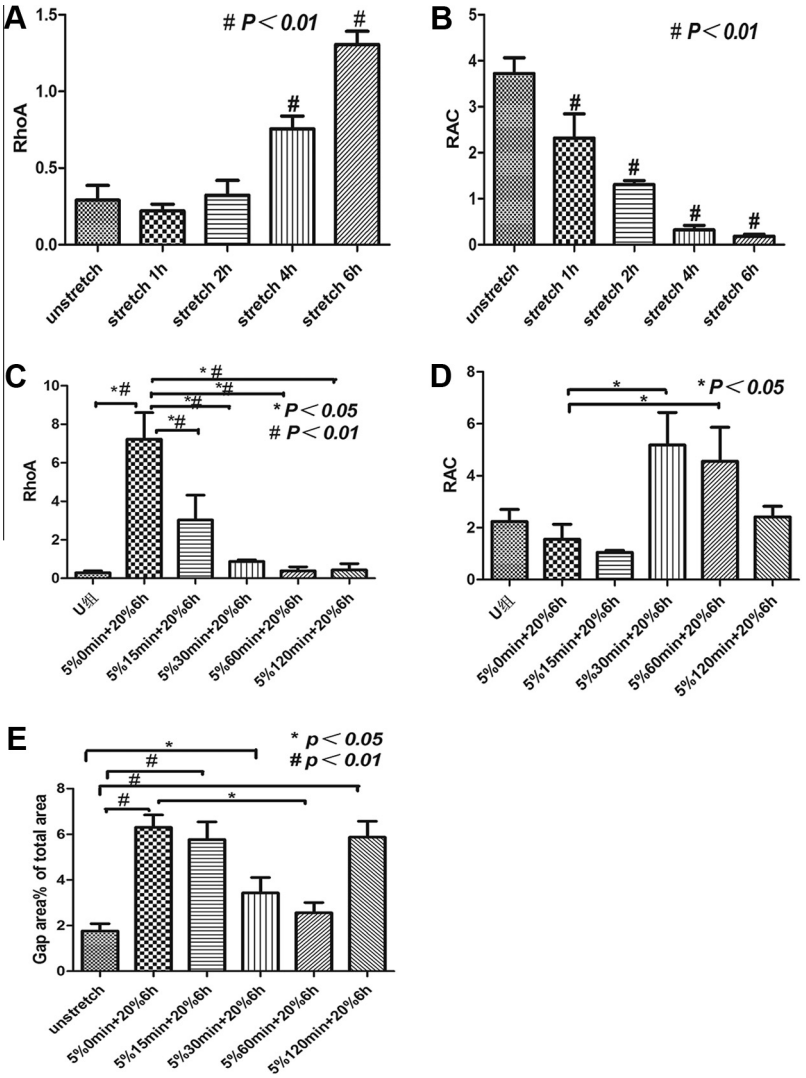

Fig. 3. The expression of RhoA and Rac mRNA was dependent on stretch. Cells exposure to 20% CS increased Rho mRNA expression at 4 h and 6 h of CS stimulation (A), but did not affect Rho activity at 1 h and 2 h. Rac activation was reduced at 1–6 h of CS exposure and dependent on stretch time (B). (C) Indicated physiological CS levels reduced significantly lower levels of Rho activation, as compared to only pathological CS. (D) Showed there was significant difference about increasing the expression of Rac mRNA only at 30 and 60 min of preconditioning. Values are means ± SE from ⩾3 independent experiments ($^{*}p < 0.05$, $^{\#}p < 0.01$). UNT, untreated.

endothelial cell [4], which was also presented in A549 cells. The paracellular gap formation in Fig. 4A was only observed in A549 cells exposed to pathologically relevant CS levels (20% elongation) in comparison with cells exposed to unstretched monolayer (Fig. 4B). In the meantime, F-actin remodeling and paracellular gap formation was examined by F-actin staining of monolayer. Cell cytoskeletal arrangement were found in A549 cells exposed to 5% CS, which was determined and characterized by circumferential F-actin rim and few central stress fibers oriented in a perpendicular direction to the main distension vector (Fig. 4C–F). A dramatic reduction in the level of paracellular gap formation was observed in cells preconditioned at physiologically relevant stretch (5% elongation) lasted from 15 min to 60 min as compared to cells exposed to pathological 20% elongation (Fig. 4C–E). Furthermore, nearly complete disappearance of paracellular gap formation was demonstrated in cells preconditioned for 60 min at 5% CS.

Analysis on the effects of precondition of physiological relevant CS showed that pre-incubation of 5% CS stimulated the formation of stress fiber bundles with larger paracellular gaps observed in this case. In contrast, more central stress fibers with less pronounced peripheral F-actin staining were found in cells exposed to only 20% CS without prior 5% CS treatment. As demonstrated in Fig. 5, monolayer preconditioned at 5% CS showed a 3.6-fold reduction in the paracellular gap formation after combining treatments of 60-min 5% CS with 6-h 20% CS, as compared to cells subjected to 6-h 20% CS only (Fig. 3E). But no significant changes in the gap area were obtained in the 5% CS-preconditioned cells for other time showed as compared to cells exposed to only 20% elongation.

4. Conclusions

Our study aims to evaluate the effects of CS-preconditioning at physiological relevant magnitude (5% elongation) on pathological 20% CS-induced cell apoptosis and barrier dysfunction as well as the molecular mechanism underlying these effects. Our data exhibited that high-magnitude CS (20% elongation) had stimulated epithelial cell apoptosis, comprising barrier function and aggregating cell damage as time went. In contrast, CS preconditioning at physiological amplitudes (5% elongation) effectively minimized cell apoptosis caused by 20% CS treatment to a large extent and promoted cell barrier recovery, which suggested to be closely associated with the regulatory roles of small GTPases of RhoA and Rac.

Apoptosis is essential in alveolar type II cells in ALI and ARDS [24]. Tschumperlin DJ showed that the percentage of dead cells

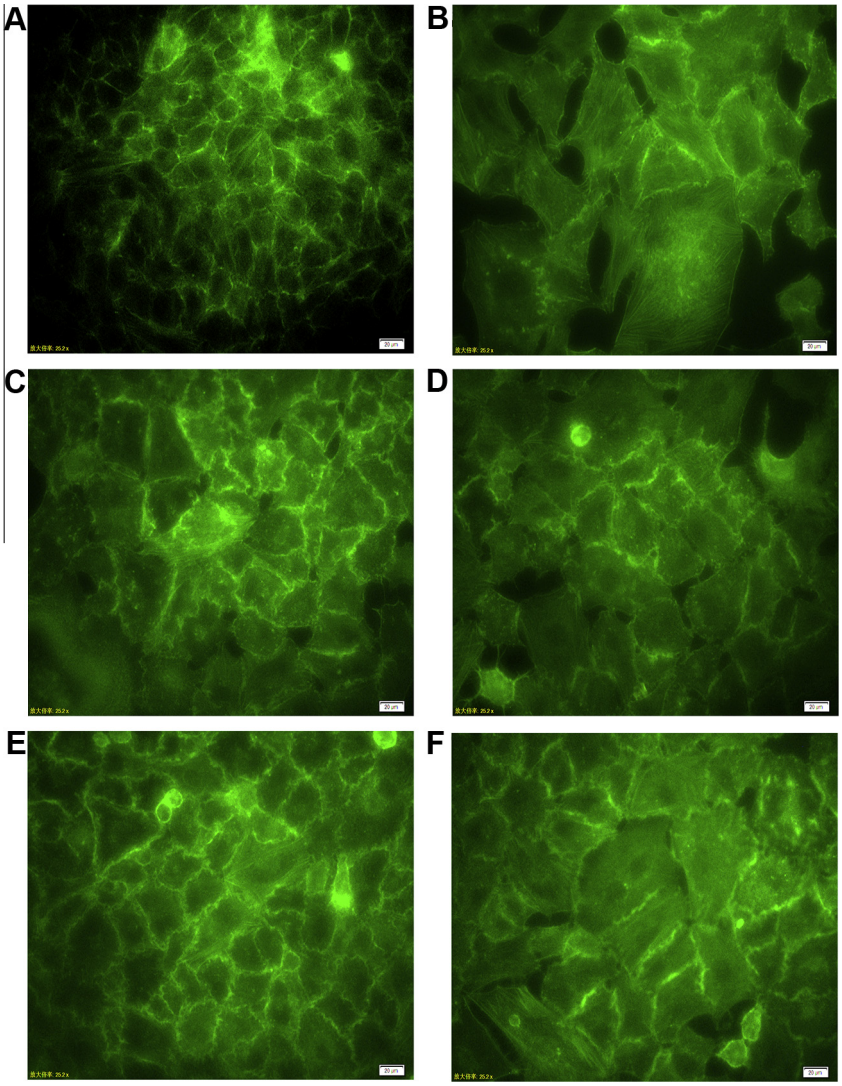

Fig. 4. The effect of preconditioning on cells barrier compromise, (B) was only observed in A549 cells exposed to pathologically relevant CS levels (20% elongation) in comparison with cells exposed to unstretched monolayer (A). Monolayer exposed to precondition about physiologically relevant CS levels (5% elongation) from 15 min to 60 min indicated a dramatic reduced in the level of paracellular gap formation compared with cells only stretched with 20% elongation (C–F). Nearly complete disappearance of paracellular gaps was demonstrated at 60 min of precondition (E). Fig. 3E it demonstrated monolayer preconditioned at 5% CS showed a 3.6-fold reduction in the gap area after 60 min CS, as compared to only stretch with 20% elongation. But precondition of 5% CS with other time showed no change compared in the gap area with only 20% elongation. Data are means ± SD of 5 independent experiments ($^{*}p < 0.05$, $^{\#}p < 0.01$).

increased dramatically after stimulated with deformation ranged from 0.5% to 72.0% [25]. In addition, a rapid transient increase of apoptosis in rat alveolar type II cells was observed after short-term CS stimulation (22% elongation at 0.05 Hz), resulting in nuclear condensation and generation of DNA fragments [24]. However, results obtained from recent study found that physiologic CS could inhibit apoptosis in vascular endothelium cultures in comparison with pathologic CS [18]. Additionally, He Yang reported that RhoA/ROCK signaling pathway was involved in mediating high glucose-induced apoptosis in cultured podocytes [21].

In this study our results demonstrated that pathological CS (20% elongation) could be able to promote RhoA mRNA expression in a time-dependent control as well as increase apoptosis rate progressively. Both the apoptosis rates and the expression of RhoA mRNA in cells preconditioned at physiologic relevant amplitude CS (5% elongation) after 30 min, 60 min and 120 min, respectively, were determined and showed obvious reductions (Figs. 2 and 3). These data together implied that CS-preconditioning at physiologic amplitudes would present an inhibitory effect on cellular apoptosis and RhoA is strongly suggested to be involved in regulating this pathway. Therefore further studies are of profound importance to be investigated to further clarify the regulatory the regulatory role of Rho A.

Actin cytoskeleton remodeling is one of the main reasons that lead to epithelial barrier destruction [26–28]. Several studies had demonstrated that cyclic stretch is crucial in initiation of cell actin remodeling [19,29]. Moreover, the significant involvement of small GTPases Rac and Rho in cell motility and cytoskeletal remodeling via activating downstream effectors such as kinases PAK, mDia, Rho-kinase and so on are also suggested in several published studies [5,6]. Barrier dysfunction induced by actin remodeling was closely linked to activation of Rho expression and down-regulation of Rac expression. And GTPases RhoA/Rac imbalance is an important mechanism for triggering endothelial cell barrier destruction [8,9,11]. As a consequence, we aim to establish and characterize the underlying mechanism of preconditioning effects on pathologically mechanical stretch-induced alveolar epithelial cell barrier dysfunction.

As shown in Section 3, we had demonstrated that pathological relevant amplitude of CS (20% elongation) stimulated Rho activation whereas repressed Rac activation to a relatively low level, leading to the paracellular gap formation. By contrast, preconditioning of physiological CS (5% elongation) was beneficial in suppressing the activation of Rho. Furthermore, the peak suppression of Rho mRNA expression and minimization of paracellular gap formation were observed in cells preconditioned for 60 min, which was consistent with the maximum time needed for obtain the recovery of cell monolayer integrity. The precondition of physiological CS (5% elongation, 60 min) was able to exert protective effects on maintain the cell barrier integrity, which was mediated by the inhibition of Rho mRNA expression. On the other hand, physiological CS preconditioning (5% CS, 15 min, 30 min, and 120 min) had no obvious effects on the gap formation as compared with cells exposed to only 20% CS for 6 h. These results also implied that the cell monolayer integrity restoration was closely related to the duration of physiological CS preconditioning.

Based on the results obtained in this study, we had demonstrated that precondition of physiological CS has a crucial role in protecting and restoring barrier integrity via inducing RhoA/Rac-mediated mechanisms, which promote the restoration of cell monolayer integrity. In contrast, 6-h CS at high-magnitude (20% elongation) along would repress Rac activities whereas enhance Rho activation, propagating barrier disruption and dysfunction. Moreover, precondition of physiological CS had been revealed to have an inhibitory effect on apoptosis and the underlying mechanism has to be further clarified and established.

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Disclosures

The above authors have no conflicts of interest to disclose.

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