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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