Respiratory Physiology & Neurobiology 242 (2017) 108–116

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Cyclic mechanical stretch-induced oxidative stress occurs via a NOX-dependent mechanism in type II alveolar epithelial cells

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ARTICLE INFO

Keywords:
Cyclic mechanical stretch
Oxidative stress
Alveolar epithelial cell injury
NADPH oxidase
Ventilator induced lung injury

ABSTRACT

Cyclic mechanical stretching (CMS) of the alveolar epithelium is thought to contribute to alveolar epithelial injury through an increase in oxidative stress. The aim of this study was to investigate the mechanisms of CMS-induced oxidative stress in alveolar epithelial cells (AECs). A549 cells were subjected to CMS, and the levels of 8-isoprostane and 3-nytrotyrosine were measured. Twenty-four hours of CMS induced a significant increase in the levels of 8-isoprostane and 3-nytrotyrosine. Although CMS did not increase the xanthine oxidase activity or the mitochondrial production of reactive oxygen species, it upregulated the expression of nicotine adenine dinucleotide phosphate oxidase (NOX) 2, 4, 5 and DUOX2. The NOX inhibitors DPI and GKT137831 significantly attenuated CMS-induced oxidative stress. Furthermore, the measurement of annexin V/propidium iodide by flow cytometry showed that CMS induced late-phase apoptosis/necrosis, which was also attenuated by both DPI and GKT137831. These data suggest that CMS mainly induces oxidative stress, which may lead to cell injury by activating NOX in AECs.

1. Introduction

Alveolar epithelial injury is a central event in the pathogenesis of various inflammatory and fibrotic pulmonary diseases (Kuwano, 2007; Manicone, 2009). The nonphysiological mechanical stretching of the alveolar epithelium during mechanical ventilation is known to be associated with the initiation of alveolar epithelial injury, which leads to the pathogenesis of ventilator-induced lung injury (VILI) (Lionetti et al., 2005). Radical scavengers, such as pentoxifylline, amifostine, N-acetylcysteine, and apocynin have shown protective effects against VILI in animal models and oxidative stress has been suggested to play a major role in VILI (Chiang et al., 2012; Chiang et al., 2011; Fu et al., 2011; Smalling et al., 2004).

Oxidative stress (the imbalance between the production of reactive oxygen species [ROS] and intracellular defense mechanisms) has been shown to be associated with various pulmonary diseases (Park et al., 2009). Since oxidative stress has been shown to be increased in type II AECs undergoing cyclic mechanical stretch (CMS) (Chapman et al., 2005; Jafari et al., 2004; Penuelas et al., 2013; Upadhyay et al., 2003), it is hypothesized that repetitive CMS-induced oxidative stress in type II AECs may contribute to pathogenic alveolar epithelial injury. However, the precise mechanisms of CMS-induced oxidative stress in type II AECs remain to be elucidated.

The production of superoxide anions, a major, physiologically-significant ROS, is mediated by nicotine adenine dinucleotide phosphate (NAD[P]H) oxidase (NOX), xanthine oxidase (XO) and mitochondrial production (Birben et al., 2012). A combination of a NOX-dependent mechanism and mitochondrial production was found to be the source of CMS-induced superoxide in a human bronchial epithelial cell line (16HBE) (Chapman et al., 2005); however, the source of superoxide in type II AECs is unclear. Thus, we investigated whether oxidative stress is increased in type II AECs undergoing CMS, and—if so—which of the ROS-generating mechanisms are responsible for the oxidative stress. We also investigated whether CMS-induced oxidative stress contributes to cell injury in type II AECs.

2. Materials and methods

2.1. Chemicals and reagents

Mouse anti-human NOX2, 4 and dual oxidase (DUOX) 2 and rabbit anti-human NOX5 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). A mouse monoclonal anti-β-actin antibody (clone AC-74) and diphenyleneiodonium chloride (DPI; a nonspecific inhibitor of all NOX isoforms) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). GKT137831 (a small-

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http://dx.doi.org/10.1016/j.resp.2017.04.007
Received 9 December 2016; Received in revised form 18 April 2017; Accepted 18 April 2017
Available online 22 April 2017

molecule NOX1/4 dual-inhibitor) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Radioimmunoprecipitation assay (RIPA) buffer was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Cell culture

The human A549 alveolar epithelial cell line, which was obtained from American Type Culture Collection (#CCL-185; Manassas, VA, USA), was used for all of the experiments. A549 cells, which were derived from an individual with alveolar cell carcinoma, have been extensively used to assess the function of type II AECs because they retain many of the characteristics of normal type II AECs (Lieber et al., 1976). The A549 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 culture medium with 10% fetal bovine serum, penicillin (100 μg/ml) and streptomycin sulphate (250 μg/ml; Wako Pure Chemical Industries, Ltd.) in a humidified incubator at 37 °C with 5% CO_2.

2.3. The cell stretching model

The cyclic mechanical stretching experiments were performed using an ST-140 cell stretcher system (Strex Inc., Osaka, Japan). Before cell seeding, the silicone chambers, STB-CH-10 (Strex Inc.) were coated with human plasma fibronectin (Thermo Fisher Scientific Inc.). After the coating process, A549 cells were seeded in the chambers at a density of 1.0×10^6 cells/chamber, and were cultured for 16 h before each stretching experiment. The chambers were subsequently attached to a stretcher system, and cyclic uniaxial stretch (15% elongation, 20 cycles/min) was applied for varying durations in a humidified incubator at 37 °C with 5% CO_2. A 15% linear strain was determined with reference to previous reports investigating oxidative stress in A549 cells undergoing CMS; this surface area change corresponds to 60% of the total lung capacity (Jafari et al., 2004; Penuelas et al., 2013). After the A549 cells were elongated by ≥20% through 24 h of CMS, the cells detached from the chamber. In the NOX inhibitor experiments, DPI (1 μM) or GKT137831 (5 μM) were added to the culture medium at 1 h or 16 h before stretch stimulation, respectively. After treatment with NOX or DPI, the proliferation of the A549 cells was measured using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). The concentrations of these inhibitors were determined to be within ranges that did not affect the proliferation of A549 cells (data not shown).

2.4. Trypan blue staining

The CMS-induced change in cell viability was analyzed by trypan blue staining. Before and after stretch stimulation, the A549 cells were trypsinized and mixed with a ten-fold volume of 0.4% trypan blue solution (Thermo Fisher Scientific Inc.). The percentage of viable cells was calculated as the number of dead cells (stained) versus the total number of cells.

2.5. The quantification of 8-isoprostane and 3-nytrotyrosine using EIA and ELISA

After stretch stimulation, the cell supernatant was recovered, and the A549 cells were washed with phosphate-buffered saline (PBS). Whole-cell lysate was prepared in RIPA buffer. The concentration of 8-isoprostane, a marker of the oxidation of lipids by ROS, was measured in the cell supernatant by a competitive enzyme immunoassay using a commercially available kit (Cayman Chemical Co.) according to the manufacturer's protocol. Similarly, the concentration of 3-nytrotyrosine, a well-established marker of oxidative stress-induced protein damage, was measured in whole-cell lysate using a commercially available 3-nytrotyrosine ELISA kit (Abcam, Cambridge, England).

2.6. The measurement of the xanthine oxidase (XO) activity

XO is a complex molybdoflavoenzyme, which is recognized as the terminal enzyme of purine catabolism, catalyzing the hydroxylation of hypoxanthine to xanthine and then uric acid. XO has also been noted to produce hydrogen peroxide and superoxide. After stretch stimulation, the XO activity in A549 cell lysate was measured using a commercially available Xanthine Oxidase Fluorometric Assay Kit (Cayman Chemical Co.) according to the manufacturer's instructions.

2.7. The measurement of the mitochondrial ROS production

After stretch stimulation, the mitochondria-induced ROS levels were measured in A549 cells using MitoSOX Red staining (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. MitoSOX Red mitochondrial superoxide indicator is a novel fluorogenic dye that allows for the highly selective detection of superoxide in the mitochondria of live cells. The stained cells were quantified using a BD FACSCanto II flow cytometer (BD biosciences). Signals from 1.0×10^6 cells were acquired for each sample.

2.8. The quantitative real-time RT-PCR

After stretch stimulation, total RNA was extracted from A549 cells using ISOGEN reagents with Spin Columns (Nippon Gene, Tokyo, Japan) and converted to complementary DNA using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan), according to the manufacturer's protocol. A quantitative real-time RT-PCR was performed using the TaqMan method and an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems Japan, Ltd., Tokyo, Japan). The TaqMan Gene Expression Assay that was used to detect the 7 NOX isoforms (NOX1-5 and DUOX1-2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems Japan, Ltd., and THUNDERBIRD Probe qPCR Mix was purchased from TOYOBO. The relative expression levels of all of the target mRNAs in the original samples were normalized to the expression levels of GAPDH mRNA.

2.9. Western blotting

After stretch stimulation, whole-cell lysate was prepared for the immunoblotting experiments in RIPA buffer. The protein concentrations were determined by a BCA Protein Assay Kit (Thermo Fisher Scientific Inc.) using bovine serum albumin as a standard. Samples (8 μg of total protein/lane) were separated by 4–20% sodium dodecyl sulphate-polyacrylamide gel electrophoresis under reducing conditions, and then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membrane were blocked in Tris-buffered saline (0.15 M NaCl, 0.05 M Tris-HCl [pH 8.0], and 0.05% [vol/vol] Tween 20) containing 5% skim milk and incubated with the indicated antibodies at the dilutions recommended by the manufacturer. An anti-β-actin antibody was used to confirm equal protein loading. After incubation with horseradish peroxidase-conjugated secondary antibodies, immunoreactive bands were detected with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions, and an ImageQuant LAS4000mini system (GE Healthcare Life Sciences, Piscataway, NJ, USA).

2.10. The measurement of the NOX activity in A549 cells

The NOX activity in A549 cells was measured using a lucigenin chemiluminescence assay as described in previous reports (Lin et al., 2012; Parinandi et al., 2003). After stretch stimulation, A549 cells were gently scraped and centrifuged at 1200 rpm for 5 min at 4 °C. The cell pellet was resuspended in 400 μl of ice-cold RPMI-1640 culture