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Research article

Role of mitochondrial hydrogen peroxide induced by intermittent hypoxia in airway epithelial wound repair *in vitro*

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ABSTRACT

The airway epithelium acts as a frontline barrier against various environmental insults and its repair process after airway injury is critical for the lung homeostasis restoration. Recently, the role of intracellular reactive oxygen species (ROS) as transcription-independent damage signaling has been highlighted in the wound repair process. Both conditions of continuous hypoxia and intermittent hypoxia (IH) induce ROS. Although IH is important in clinical settings, the roles of IH-induced ROS in the airway repair process have not been investigated. In this study, we firstly showed that IH induced mitochondrial hydrogen peroxide (H_2O_2) production and significantly decreased bronchial epithelial cell migration, prevented by catalase treatment in a wound scratch assay. RhoA activity was higher during repair process in the IH condition compared to in the normoxic condition, resulting in the cellular morphological changes shown by immunofluorescence staining: round cells, reduced central stress fiber numbers, pronounced cortical actin filament distributions, and punctate focal adhesions. These phenotypes were replicated by exogenous H_2O_2 treatment under the normoxic condition. Our findings confirmed the transcription-independent role of IH-induced intracellular ROS in the bronchial epithelial cell repair process and might have significant implications for impaired bronchial epithelial cell regeneration.

1. Introduction

The airway epithelium acts as a frontline barrier against various environmental insults [1]. Therefore, the process of airway epithelial cell repair after lung injury is critical for the restoration of lung homeostasis, which is involved in various components, such as growth factors, cytokines, and reactive oxygen species (ROS) [2]. Recently, in the process of wound repair, the role of ROS as transcription-independent damage signaling (e.g. calcium ion waves and hydrogen peroxide [H_2O_2] gradients) has been highlighted [3]. This signaling immediately initiates the structural and functional changes of cytoskeleton, followed by cell shape changes, which integrate transcription-dependent signaling (e.g. receptor tyrosine kinase signaling, nuclear factor-kappa B, and hypoxia-inducible factor [HIF]) [2–4]. ROS are generated endogenously in the organelles and by intracellular enzymes (e.g. mitochondria, nicotinamide adenine dinucleotide phosphate oxidases [NOXs], and xanthine oxidase) or exogenously in interactions with environmental agents [5]. And, among ROS, extracellularly produced H_2O_2 is

Abbreviations: ANOVA, analysis of variance; BEBM, bronchial epithelial cell basal medium; BEGM, bronchial epithelial cell growth medium; DMEM, Dulbecco's modified Eagle's medium; DPI, diphenyleneiodonium; DUOX, dual oxidase; FBS, fetal bovine serum; H_2DCF-DA, 2′, 7′-Dichlorodihydrofluorescein diacetate; HGF, hepatocyte growth factor; HIF, hypoxia-inducible factor; H_2O_2, hydrogen peroxide; IH, intermittent hypoxia; MitoTEMPO, (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)−2-oxoethyl), triphenylphosphonium chloride monohydrate; NOX, nicotinamide adenine dinucleotide phosphate oxidase; O2-, superoxide anion; PEG, polyethylene glycol; PHBE, primary human bronchial epithelial; ROCK, Rho-kinase; ROS, reactive oxygen species; SEM, standard error of the mean

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incorporated into cells by simple diffusion and across water cannels, the aquaporins [6].

Both conditions of continuous hypoxia and intermittent hypoxia (IH) induce ROS. The IH condition reveals oxygen concentration change between low and baseline levels and is clinically important such as in obstructive sleep apnea [7]. The intracellular roles of IH-induced ROS have been focused on transcription-dependent signaling through nuclear factor-kappa B and HIF [8,9]. The association between IH and wound repair is studied only in a vascular endothelial cell; its migration was accelerated through the IH-induced activation of HIF-1 and its downstream proteins, N-myc downstream regulated gene 1 and CRK-I/II [10,11]. Therefore, the roles, particularly as transcription-independent signaling, of IH-induced ROS in the processes of airway injury and repair have been unknown.

The repair process of airway epithelial cells is consist of cell migration, which involve the constant restructuring of the actin cytoskeleton, followed by cell proliferation. The process of cell migration is regulated by three members of Rho GTPases, RhoA, Rac1 and Cdc42 [12–14]. Rho GTPases act as molecular switches that cycle between GTP-bound (active) and GDP-bound (inactive) forms [15]. The exchange between GTP and GDP is modulated by ROS in a transcription-independent manner because these Rho GTPases, particularly RhoA and Rac1, have conserved redox-sensitive motifs [16–19]. The altered functions of those oxidized motifs in the airway epithelial cells have not been investigated.

In this study, we hypothesized that IH-induced intracellular ROS accumulation would impair airway epithelial cell repair by altering the activities of the aforementioned Rho GTPases. Thus, we performed *in vitro* experiments to evaluate the effects of IH on bronchial epithelial cell repair using a wound scratch assay, that measures cell migration [20].

2. Material and methods

2.1. Reagents

Apocynin and diphenyleneiodonium (DPI) were purchased from Cayman Chemicals Co. (Ann Arbor, MI, USA). Catalase-polyethylene glycol (PEG) was purchased from Sigma (St. Louis, MO, USA). Hepatocyte growth factor (HGF) and H_2O_2 were purchased from Wako (Osaka, Japan). Rhodamine-phalloidin Alexa Fluor 488, anti-vinculin antibody, and MitoTEMPO ((2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)−2-oxoethyl) triphenylphosphonium chloride monohydrate) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). 2′, 7′-Dichlorodihydrofluorescein diacetate (H_2DCF-DA) was purchased from AnaSpec (Fremont, CA, USA). Anti-Cdc42 antibody was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Horseradish peroxidase-conjugated mouse IgG was purchased from Bio-Rad (Tokyo, Japan). Anti-rabbit Alexa 594, penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Life Technologies (Carlsbad, CA, USA). Bronchial epithelial cell basal medium (BEBM) and bronchial epithelial cell growth medium (BEGM) Bulletkit were purchased from Lonza (Walkersville, Maryland, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Primary bronchial epithelial cell isolation and cell cultures

Primary human bronchial epithelial (PHBE) cells were isolated from patients who underwent surgical lung resection for the treatment of lung cancer. The patients were all never smokers with normal pulmonary function and had no history of pulmonary diseases. The cells were isolated from the portions of bronchus that were not involved in tumor according to our previous method [21]. This procedure was performed with the ethical approval of the Kyoto University Graduate School and Faculty of Medicine Ethics Committee (689) and informed consent was obtained from all subjects. PHBE cells were cultured between passages 1 and 3 in BEBM supplemented with BEGM Bulletkit and 1% penicillin-streptomycin.

The human bronchial epithelial cell line, BEAS-2B (American Type Culture Collection, Manassas, Virginia, USA), was cultured between passages 52 and 62 in DMEM containing, 1% penicillin-streptomycin and 10% FBS.

Before all treatments, the cells reaching confluence were starved for 24 h in starvation medium: 99% BEBM with 1% BEBM supplemented with BEGM Bulletkit for PHBE cells and with 0.1% FBS for BEAS-2B cells.

2.3. In vitro IH protocol

BEAS-2B cells or PHBE cells were exposed to a normoxic or IH condition. The normoxic condition was maintained at atmospheric oxygen concentrations (21% O_2, 5% CO_2 with the balance as N_2). The IH condition was set to alternating cycles of normoxia and hypoxia (1% O_2, 5% CO_2 with the balance as N_2) using a custom-designed incubation chamber attached to an external O_2-CO_2-N_2 computer-driven controller (O_2 programmable control, 9200E SP, Wakenyaku Co., Ltd, Kyoto, Japan). In this chamber, the actual O_2 concentration above the cell monolayer could be monitored using a fiberoptic O_2 probe (Oxford Optronix, Oxford, UK) (Supplemental. Fig. S1A). To determine the effects of a custom-designed incubation chamber on results, the sham condition was maintained at atmospheric oxygen concentrations in this chamber.

2.4. In vitro wound scratch assay

Wounds of ~ 500 μm were created by scraping the confluent BEAS-2B cell monolayer or PHBE cell monolayer using a sterile P-200 pipette tip to create an *in vitro* bronchial epithelial injury according to a previous method [20]. Images of the wound closure were acquired at 16 points and at 0, 8, 12 and 16 h post-wounding using a conventional microscope (Olympus, Tokyo, Japan). The area of each wound at each time point was measured using the National Institutes of Health Image J software. The percentage of wound repair relative to the initial wound size (0 h) was calculated using the following equation: % wound repair relative to 0 h $= 100 \times (1 - \text{wound area}_{tx}/\text{wound area}_{t0})$.

2.5. Cell scatter assay

This assay was performed as described previously [22]. BEAS-2B cells were seeded at a density of 2×10^4/ml in 35-mm dishes. After 36–48 h, cells were starved for 24 h. The cell aggregates were stimulated with 30 ng/ml HGF.

2.6. Assessment of cell death and the cell cycle

Before these analysis, BEAS-2B cells were subjected to a normoxic or IH condition for 8 h post-wounding. Analysis of apoptosis/necrotic cell death was performed using a PE Annexin V Apoptosis Detection Kit I (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. The cell cycle was analyzed by measuring the cellular DNA content following cell staining with propidium iodide. Apoptotic/necrotic cells and cellular DNA content were quantified using a FACS Calibur Flow Cytometry System (Becton Dickinson, Franklin Lakes, NJ, USA).