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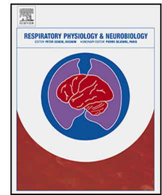

Impaired self-healing capacity in airway epithelia lacking aquaporin-3

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ABSTRACT

In this study, we utilized AQP3-knockout mice as the in vivo model and AQP3-knockdown human bronchial epithelial cells (HBECs) as the in vitro model. Airway injury was experimentally induced by intra-tracheal injection of naphthalene. HE staining, transmission and scanning electron microscope were performed to evaluate self-healing capacity in vivo. Transwell and wound-healing assays were performed to evaluate epithelial cell migration in vitro. We found that both the airway epithelial cells of AQP3-knockout mice and AQP3-knockdown HBECs exhibited an obviously impaired self-healing capacity with defective epithelial cell migration through AQP3-facilitated glycerol transport. In addition, glycerol supplementation could largely correct defective injury healing and epithelial cell migration. For the first time, we found evidence for distinct defects in AQP3-deficient airway epithelial cell migration. Mechanistic analysis showed AQP3-facillitated glycerol transport plays a role in airway epithelial self-healing after injury.

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1. Introduction

The lung is constantly subjected to harmful exposures, such as inhaled toxic substances, particulate matter, viral or bacterial infections, and autoimmune reactions that cause injury to the airway and alveolar epithelium. The epithelial barrier plays a significant role in a number of airway diseases, including asthma, where an impaired epithelial barrier function makes the airway susceptible to damage. Currently, existing therapies fail to reverse the course of disease but instead only focus on improving symptoms (Lambrecht and Hammad, 2012). Adjunct therapies targeting repair of airway epithelium are needed.

Aquaporins (AQPs) are a family of transmembrane channel proteins responsible for transporting water and other small solutes between and into cells (Rojek et al., 2008; Verkman, 2005). AQP3 belongs to the so-called aquaglyceroporin group of AQPs, which transport both water and the humectant glycerol (Boury-Jamot et al., 2009). AQP3 is expressed in various tissues such as the skin, kidney, respiratory tract, and gastrointestinal tract. AQP3 is known to be necessary for urine concentration in the kidney. AQP3-facilitated water and glycerol transport is essential for the healing of cutaneous wounds and the hydration and elasticity of skin. Recent data implicate AQP3-facilitated water and glycerol transport in cell migration, proliferation, differentiation, lipid metabolism and barrier formation (Hara-Chikuma and Verkman, 2008a).

In the lung and airways, fluid transport across epithelial and endothelial barriers occurs during alveolar fluid movement, airway hydration, and submucosal gland secretion. AQP3 is localized in basal epithelial cells in large airways and throughout the nasopharynx (King et al., 1997; Nielsen et al., 1997). Understanding its contribution to the self-healing capacity of airway epithelium following injury is limited. The present study aimed to investigate the involvement and potential mechanism of AQP3 in the self-healing capacity of airway epithelium.

2. Materials and methods

2.1. Transgenic mice and animal husbandry

$AQP3^{-/-}$ mice (BALB/c genetic background) were maintained at pathogen-free facilities in Fudan University, Shanghai, China. Litter mates of six to eight week old $AQP3^{+/+}$ and $AQP3^{-/-}$ mice were used in the experiments. 5% glycerol was used for 3 days before naph-

Abbreviations: AQP, aquaporin; HBEC, human bronchial epithelial cell; SEM, scanning electron microscopy; TEM, transmission electron microscopy; siRNA, small interfering RNA.

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thalene treatment in the glycerol supplement study. This study was approved by Ethic Committee of Zhongshan Hospital, Fudan University and was carried out in accordance with the National Institutes of Health guide for the care of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Naphthalene treatment

At six to eight weeks of age, mice were anesthetized to undergo treatment. Naphthalene (Sigma Chemical Co., St Louis, MO) was dissolved at a concentration of 30 mg/ml and administered to mice (275 mg/kg) by intratracheal injection. Animals were sacrificed on 2 h, 24 h and 7 days following Naphthalene exposure (Van Winkle et al., 2002; Xing et al., 2012).

2.3. Cell culture

Human bronchial epithelial cells (HBECs) were obtained from Lonza (Lonza, Basel, Switzerland). Cells were cultured in Dulbecco's modified Eagle's medium with or without 10% fetal bovine serum (FBS, Hyclone) at 37 °C in a 5% CO2, 95% air environment in humidified incubators.

2.4. Scanning electron microscopy (SEM)

The collected samples were immediately rinsed with jet streams of physiological saline to remove mucus, followed by serial fixation with 3% glutaraldehyde and 1.5% osmium tetroxide. They were then dehydrated in a graded series of alcohol, immersed in amylacetate, and dried with liquid carbon dioxide at the critical point. The dried samples were fixed on the stage under a dissecting microscope and subjected to ion-spattering coating with Au-Pd. Samples were examined and photographed under a scanning electron microscope (Hitachi Ltd, Tokyo, Japan).

2.5. Transmission electron microscopy (TEM)

The samples were double-fixed with 1.5% osmium tetroxide and 3% glutaraldehyde, dehydrated in a graded series of alcohol, embedded in Epon 812, and then cut into 60 nm ultra-thin sections and stained with uranyl acetate and lead citrate. Sections were examined and photographed under a transmission electron microscope (Philips, Hillsbro, USA).

2.6. Immunohistochemistry

The tracheae were fixed in paraformaldehyde for 12 h, and then cut into 4 μm-thick transverse sections. Sections were blocked with 1% BSA for 30 min, incubated in AQP3 primary antibody (Abcam Inc., Cambridge, MA) overnight, and then incubated with secondary antibody (Sigma Chemical Co., St Louis, MO) after three washes. Sections were examined and photographed under a Nikon A1R confocal laser scanning microscope system (Nikon Corp., Tokyo, Japan).

2.7. Small interfering RNA (siRNA) transfection

SiRNA transfections were performed according to the manufacturer's protocol. Briefly, in 6-well plates using Lipofectamine^{TM} 2000 with three different sequences of siRNA duplexes targeting AQP3 (Supplementary Table 1) and RNA negative control (GenePharma, shanghai, China), 3 μl of Lipofectamine^{TM} 2000 and 60 pmol of each siRNA were transfected in triplicate, except for ratio-dependent effect studies where several ratios of Lipofectamine^{TM} 2000/siRNA were tested to optimize the efficacy of transfection. Cells were prepared for experiments either 48 h or 72 h after transfection.

2.8. Migration assay

In the transwell (Corning Inc., Corning, NY) assay, HBECs at the concentration of 5×10^5/ml in serum-free medium were seeded in the upper chamber, while medium containing 10% FBS was added to the lower chamber. Cells migrated through the permeable membrane at 48 h were fixed and stained with Giemsa, and then counted under microscope. In the wounding-healing assay, the wound track was made by scraping the monolayer-adherent cells with a sterilized pin. Cell migration into the cell-free area was assessed using an inverted light microscope and migration distances were measured at 0, 24 and 48 h in the absence or presence of glycerol (20 ng/ml) (Sigma Chemical Co., St Louis, MO).

2.9. Measurement of glycerol and ATP content

Trachea homogenates (3500 g, 10 min, 4 °C) were assayed for glycerol and ATP concentration. For glycerol measurement, we uesd Triglyceride Determination Kit (Catalog Number TR0100, Sigma). As shown in manufacturers' instructions, the procedure of colorimetric assay kit involved enzymatic hydrolysis by lipase of the triglycerides to glycerol and free fatty acids. The glycerol produced was then measured by coupled enzyme reactions. The increase in absorbance at 540 nm was directly proportional to triglyceride concentration of the sample. For ATP measurement, we uesd ATP Bioluminescence Assay Kit HS II (Catalog Number 11699709001, Sigma). As shown in manufacturers' instructions, the determination of ATP used the ATP dependency of the light emitting luciferase catalyzed oxidation of luciferin.

2.10. Statistical analysis

Data was expressed as mean ± standard errors. For the statistical analysis, 0.05 was set as the P value for significance. Normal distribution was assumed. The data of two groups were analyzed by t-test. The data of three groups were analyzed by ANOVA.

3. Results

3.1. Impaired in vivo airway epithelial repair in AQP3$^{-/-}$ mice

Immunohistochemistry showed AQP3 expression in the airway epithelium of wild-type (AQP3$^{+/+}$) mice but not in AQP3$^{-/-}$ mice (Fig. 1A). AQP3 was most abundant in the plasma membranes of basal epithelial cells. We then measured healing after airway injury induced by naphthalene in AQP3$^{+/+}$ and AQP3$^{-/-}$ mice. Fig. 1B shows impaired airway epithelial repair after 7 days of airway injury in AQP3$^{-/-}$ mice. At 2 h after naphthalene treatment, airway epithlial cells had a more rounded appearance in both AQP3$^{+/+}$ and AQP3$^{-/-}$ group. Many of the epithlial cells had exfoliated from the airway basement membrane, leaving extremely attenuated squamated cells. At 7 days after naphthalene treatment, airway was gradually covered by squamated cells to rapidly restore some barrier protection in AQP3$^{+/+}$ group. Newly regenerated epithlial cells comprised most of the airway epithelium in AQP3$^{+/+}$ mice. In contrast, fewer Clara cells regeneration were shown in AQP3$^{-/-}$ mice.

The effects of AQP3 on airway epithelial ultrastructure were assessed by SEM and TEM. At 2 h after injury, SEM showed no morphologic differences in cilia between the AQP3$^{+/+}$ group and AQP3$^{-/-}$ groups (Fig. 2A and B). At 24 h after injury, disruption and deciduation of ciliated epithelial cells were seen in both the AQP3$^{+/+}$ and AQP3$^{-/-}$ groups (Fig. 2C and D). 7 days after injury, repair of the

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