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Contribution of concentration-sensitive sodium channels to the absorption of alveolar fluid in mice

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ABSTRACT

The concentration-sensitive sodium channel (Na_c) is activated by an increase in the extracellular sodium concentration. Although the expression of Na_c in alveolar type II epithelial cells (AEC II) has been reported previously, the physiological role of Na_c in the lung has not been established. We characterized Na_c expression and examined amiloride-insensitive sodium transport mediated by Na_c in mouse lung. Immunofluorescence studies revealed that Na_c did not colocalize with either aquaporin 5 or cystic fibrosis transmembrane conductance regulator, but partially colocalized with the epithelial sodium channel γ-subunit. Immunoelectron microscopy studies showed that Na_c localized at the basolateral membrane of pulmonary microvascular endothelial cells (PMVECs). Na_c mRNA and protein were expressed in PMVECs isolated from the lungs of mice. Image analysis indicated that sodium influx into the alveolar wall was dependent on increases in extracellular sodium concentration. We conclude that Na_c expressed in PMVECs and AEC II contributes to the reabsorption of sodium via an amiloride-insensitive pathway during alveolar fluid clearance.

1. Introduction

Alveolar fluid balance is maintained by ion and fluid transport mechanisms. Pulmonary edema is caused by an imbalance between the influx of fluid into the alveolar space and alveolar fluid clearance (AFC). Decrease in AFC contributes to the development of pulmonary edema in patients with acute respiratory distress syndrome (Morty et al., 2007; Ware and Matthay, 2001). Active sodium transport across the alveolar epithelium is a driving force for AFC (Wang et al., 2013). Epithelial sodium channels (ENaCs) located at the apical membrane of the alveolar epithelium are involved in active sodium transport during AFC. There is considerable evidence that the amiloride-sensitive ENaC-mediated pathway contributes to the reabsorption of sodium and fluid from the alveolar space (Dobbs and Johnson, 2007). However, amiloride-insensitive sodium channels, such as cyclic nucleotide-gated channels and other unidentified sodium channels, might also be involved. In adult guinea pigs, adult rats, and postnatal sheep, 55–70% of lung fluid reabsorption occurs via amiloride-insensitive sodium channels (Fronius, 2013; Junor et al., 1999; Norlin et al., 1998, 2001).

The concentration-sensitive sodium channel (Na_c), also known as Na_x or NaG, is an amiloride-insensitive sodium channel, which opens in response to increases in the extracellular sodium concentration ($[Na^+]_o$) above 150 mM, but not in response to increased osmolarity of the extracellular solution (Hiyama et al., 2002). Na_c belongs to the voltage-gated sodium channel family, but it is not a voltage-dependent channel: the amino acid sequence of the putative voltage-sensing site of Na_c differs from that of other voltage-gated sodium channels (Goldin et al., 2000; Ogata and Ohishi, 2002). Na_c is present in the lung, uterus, heart, dorsal root ganglia, peripheral nerve, and circumventricular organs (Felipe et al., 1994; Gautron et al., 1992; George et al., 1992; Watanabe et al., 2000). Previous reports have indicated that Na_c functions as a sensor of sodium levels in the brain (Shimizu et al., 2007). Although Na_c is expressed in alveolar epithelial type II cells (AEC II) in mouse lungs, its physiological functions in the lung are not yet known (Watanabe et al., 2002).

We hypothesize that Na_c, as one of the amiloride-insensitive sodium channels, contributes to the activity of amiloride-insensitive pathways during AFC. We carried out a series of experiments on mouse lung to test this hypothesis. We quantified changes in Na_c expression during mouse lung development, examined Na_c subcellular localization, and assessed sodium

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concentration-dependent sodium influx into the alveolar walls in the presence of amiloride.

2. Methods

2.1. Animals

All experiments were conducted according to the Guidelines for the Care and Use of Experimental Animals of Kindai University. Embryonic day (E) 15- to 12-week-old male ICR mice were used in this study. Late fetal and neonatal mice were killed by decapitation under hypothermic anesthesia. Mice at 2–12 weeks of age were killed by cervical dislocation under anesthesia with chloral hydrate (520 mg/kg, intraperitoneally).

2.2. Quantitative RT-PCR

Total RNA from lung tissue was isolated with ISOGEN (Nippon Gene, Tokyo, Japan) and treated with a DNA-free kit (Invitrogen, Carlsbad, CA) to remove genomic DNA contamination. RT was performed using a TaKaRa RNA PCR kit (TaKaRa Bio, Shiga, Japan). Real-time quantitative PCR was performed on a Chrome 4 Real-Time PCR Detector (Bio-Rad), using SYBR Premix Ex Taq (TaKaRa Bio). The thermal profile included an initial step of 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s with two steps. The primers used in our study were as follows. Na_c primers: 5′-CCTTCGAGCAC TAAGAGTTCTG-3′ (forward) and 5′-GCCAGCAAATAACTGCACTC-3′ (reverse); αENaC primers: 5′-CATCAACCTCAATTCGGAC-3′ (forward) and 5′-AAGCGTCTGTTCCGTGATG-3′ (reverse); βENaC primers: 5′-TCCATGGGCTTCAAGACG-3′ (forward) and 5′-CTGGTGGTGTTGCTGTGG-3′ (reverse); γENaC primers: 5′-ATGGGAGGCAGTGAGTATGG-3′ (forward) and 5′-CTTGGCTCCAGTGGATGAC-3′ (reverse); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers: 5′-TGCACCACCAACTGCTTAG-3′ (forward) and 5′-GGATGCAGGGATGATGTTC-3′ (reverse); β-actin primers: 5′-TCCATCATGAAGTGTGACGT-3′ (forward) and 5′-GAGCAATGATCTTGATCTTCAT-3′ (reverse).

2.3. Immunoblotting

A rabbit anti-Na_c antibody was produced against a synthetic peptide corresponding to amino acids 852–866 of rat Na_c (Hiyama et al., 2002). Mouse lung tissue was homogenized in a solution containing 50 mM Tris-HCl, 100 mM NaCl, 10% glycerol, 1% Triton X-100, and a protease-inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) on ice. The homogenate was centrifuged at 14,000*g* for 15 min at 4 °C and the supernatant was mixed with an equal volume of 2× SDS sample buffer. The protein concentration was determined using an RC DC Protein Assay kit (Bio-Rad) with bovine serum albumin (BSA) as the standard. Proteins were resolved by electrophoresis in 6–10% SDS-polyacrylamide gels. The proteins resolved by SDS-PAGE were blotted onto Immobilon-P membranes (Millipore, Billerica, MA). The membranes were pre-incubated with blocking buffer (5% non-fat dry milk, 0.05% Tween-20 in PBS) for 30 min at 25 °C and then with 0.08 μg/mL anti-Na_c antibody diluted with blocking buffer at 4 °C overnight. A rabbit anti-actin antibody (Sigma-Aldrich, St. Louis, MO) was used as the loading control. Primary antibodies were detected with a horseradish peroxidase-conjugated anti-rabbit IgG (1:5000), using an enhanced chemiluminescence kit (PerkinElmer, Waltham, MA) and X-ray film. The signal intensities were quantified using NIH Image-J software (Schneider et al., 2012).

2.4. Measurement of lung water content (LWC)

Lungs removed from mice at different developmental stages were immediately weighed to obtain the wet weight. Then, they were incubated at 80 °C in a drying oven overnight, and subsequently reweighed to obtain the dry weight. The water content was calculated as the ratio of the wet weight to the dry weight (Barker et al., 1998).

2.5. Preparation of frozen sections

Mice were killed and perfused with PBS via the right ventricle to remove blood from the lung. To prepare tissue for immunofluorescence studies using anti-aquaporin 5 (AQP5) antibody (sc-9891; Santa Cruz Biotechnology, Santa Cruz, CA), a mixture of 2 mL Tissue-TEK OCT compound (Sakura Finetek, Tokyo, Japan) with PBS (1:1) was infused via the trachea into the lungs. Lung tissues were then frozen in liquid nitrogen and stored at −80 °C. To prepare the tissue for in situ hybridization, immunohistochemistry, and immunofluorescence (with antibodies other than anti-AQP5), the lungs were fixed by perfusion with 4% paraformaldehyde solution in PBS. Fixed lung tissues were equilibrated in PBS containing 30% sucrose, embedded in OCT compound, and stored at −80 °C. Frozen lung tissues were sliced into 5-μm-thick sections. Cryosections prepared from lung tissues frozen in liquid nitrogen were fixed in 100% methanol at −20 °C for 10 min. To enhance antigen retrieval, the sections stained with anti-cystic fibrosis transmembrane conductance regulator (CFTR) (sc-8909; Santa Cruz Biotechnology) and anti-γENaC (13943-1-AP; Proteintech, Chicago, IL) antibodies were heated in a microwave in the presence of 50 mM glycine-HCl (pH 3.5, with 0.01% EDTA) twice for 2 min each.

2.6. Immunohistochemistry

To denature endogenous peroxidases, the sections were treated with PBS containing 0.3% H_2O_2. Then, the sections were incubated with blocking solution (PBS containing 2% BSA and 2% normal donkey serum) for 30 min at room temperature. Anti-Na_c and anti-surfactant protein C (SPC) (sc-7706; Santa Cruz Biotechnology) antibodies were diluted with blocking solution (anti-Na_c, 0.5 μg/mL; anti-SPC, 2 μg/mL) and incubated with the sections at 4 °C overnight. Anti-Na_c- and anti-SPC-stained sections were incubated with a goat anti-rabbit IgG biotinylated secondary antibody (1:200) and a rabbit anti-goat IgG biotinylated secondary antibody (1:200) for 1 h at room temperature, respectively. Biotinylated secondary antibodies were visualized using a VECTASTAIN ABC Kit (Vector Laboratories, Burlingame CA) and a Histofine DAB substrate kit (Nichirei, Tokyo, Japan). The sections were counterstained with Methyl Green.

2.7. Immunofluorescence

The sections were treated with PBS containing 0.3% Triton X-100 and incubated with blocking solution (PBS containing 2% BSA and 2% normal donkey serum) for 30 min at room temperature. Primary antibodies were diluted with blocking solution (anti-Na_c, 0.5 μg/mL; anti-AQP5, 4 μg/mL; anti-SPC, 2 μg/mL; anti-CFTR, 4 μg/mL; anti-γENaC, 5 μg/mL) and incubated with the sections at 4 °C overnight. After incubation, the sections were washed thrice with PBS and incubated with a CF488 donkey anti-rabbit IgG (1:1000) and a CF633 donkey anti-goat IgG (1:1000) for 1 h at room temperature. To double stain with rabbit anti-Na_c and anti-γENaC antibodies, after the incubation with an anti-γENaC antibody, the sections were incubated with a goat anti-rabbit IgG biotinylated secondary antibody (1:200) for 1 h at room temperature and then fixed with 2% paraformaldehyde for 10 min. The sections were incu-