

## THE WNK4/SPAK PATHWAY STIMULATES ALVEOLAR FLUID CLEARANCE BY UPREGULATION OF EPITHELIAL SODIUM CHANNEL IN MICE WITH LIPOPOLYSACCHARIDE-INDUCED ACUTE RESPIRATORY DISTRESS SYNDROME

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**ABSTRACT**—With-No lysine Kinases (WNKs) have been newly implicated in alveolar fluid clearance (AFC). Epithelial sodium channels (ENaCs) serve a vital role in AFC. The potential protective effect of WNK4 in acute respiratory distress syndrome (ARDS), mediated by ENaC-associated AFC was investigated in the study. A model of lipopolysaccharide (LPS)-induced ARDS was established in C57BL/6 mice. WNK4, Sterile 20-related proline-alanine-rich kinase (SPAK), small interfering RNA (siRNA)-WNK4 or siRNA-SPAK were transfected into mouse lung or primary alveolar epithelial type II (ATII) cells. AFC, bronchoalveolar lavage fluid and lung histomorphology were determined. The expression of ENaC was determined to investigate the regulation of AFC by WNK4-SPAK signaling pathway. Activation of WNK4-SPAK signaling improved lung injury and survival rate, with enhanced AFC and reduced pulmonary edema via the upregulation of ENaC in ARDS. In primary rat ATII cells, gene-silencing by siRNA transfection reduced ENaC expression and the level of WNK4-associated SPAK phosphorylation. Immunoprecipitation revealed that the level of neural precursor cell-expressed developmentally downregulated gene 4 (Nedd4-2) binding to ENaC was decreased as a result of WNK4-SPAK signaling. The present study demonstrated that the WNK4/SPAK pathway improved AFC during LPS-induced ARDS, which is mainly dependent on the upregulation of ENaC with Nedd4-2-mediated ubiquitination.

**KEYWORDS**—Alveolar fluid clearance, acute respiratory distress syndrome, epithelial sodium channel, With-No lysine Kinases, Sterile 20-related proline-alanine-rich kinase

### INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a critical syndrome resulting in a severe form of pulmonary disease. ARDS remains a high mortality of 40% in past 10 years (1–3). ARDS is characterized by severe and persistent hypoxemia with protein-rich pulmonary edema, resulting in a disruption of oxygen delivery from the alveoli to the blood (4). According to the Berlin definition, mild ARDS is defined by 200 mm Hg  $< PaO_2/FiO_2$  ratio  $< 300$  mm Hg and positive end-expiratory pressure  $\geq 5$  cmH<sub>2</sub>O (5).

ARDS can progress rapidly within 72 h, with the clinical features of respiratory distress, severe hypoxemia, and bilateral alveolar infiltrates that cannot be explained by cardiogenic reasons (6). The primary ARDS-associated pathology is lung injury-induced alveolar-capillary barrier dysfunction, which increases epithelial permeability and diffused alveolar edema (7). The delayed clearance of fluid in the alveolar space is associated with poor outcome (8). Therefore, effective resolution of pulmonary edema is vital for improving the prognosis of patients with ARDS. Alveolar fluid clearance (AFC), achieved by balancing the lung fluid content, is markedly impaired in ARDS (9).

The epithelial sodium channel (ENaC), specifically expressed on alveolar type II cells, consists of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits (10). ENaC is the rate-limiting step in alveolar edema reabsorption, and the driving force for AFC. It is widely accepted to be the key channel involved in regulating the water-sodium balance in AFC (11).  $\alpha$ -ENaC gene-knockout or low expression levels of  $\beta$ -ENaC or  $\gamma$ -ENaC have a close relationship with retarded AFC (12–14). Therefore, sufficient expression levels of ENaC are essential for AFC.

The With-No lysine Kinases (WNKs) are serine/threonine protein conserved in multicellular organisms, defined by their lack of a conserved lysine residue in subdomain II (15). The Sterile 20-related proline-alanine-rich kinase (SPAK) belongs to the germinal center kinase group VI family. WNK4, one of WNK family members, activates the downstream of SPAK by phosphorylation of residue Thr233 of the T-loop, and Ser373 of the S-motif (16). WNK4 and SPAK are widely distributed in the lungs (17). WNK-SPAK pathway has been proved to

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serve a principal role in water and salt homeostasis (18,19). Although WNK4 serves a prominent function in AFC (20), the mechanism of the regulation of ENaC by WNK4-SPAK pathway remains unknown. In the study, WNK4-SPAK signaling on ENaC-mediated AFC was investigated in lipopolysaccharide (LPS)-induced ARDS.

## MATERIALS AND METHODS

### Constructs

Full-length cDNAs encoding mouse WNK4 and SPAK were previously generated (21,22). cDNAs subcloned into a pCMV-Myc vector (Stratagene; Agilent Technologies Inc., Santa Clara, CA) were linearized using *MluI* and purified by the PCR Purification kit (Qiagen, Valencia, CA). WNK4 and SPAK gene-silencing was achieved by double-stranded siGENOME SMARTpool oligonucleotides (GE Healthcare Dharmacon, Inc., Lafayette, CO). Intratracheal delivery of the gene or its corresponding small interfering RNA (siRNA) was performed as described previously (23,24).

### Animal model and treatment groups

Male C57BL/6 mice (20 g–25 g) were obtained from Chongqing Medical University (Chongqing, China) between 8 and 10 weeks of age. Mice were given food and water under a humidity- and temperature-controlled environment with a 12 h light/dark cycle. Mice were anaesthetized by sodium pentobarbital (intraperitoneal injection) at a dose of 50 mg/kg. A mouse model of ARDS was performed by intratracheal administration of lipopolysaccharide (LPS) (5 mg/kg, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in 50  $\mu$ L sterile PBS (25). After administration of LPS, pCMV-Myc-WNK4 or pCMV-Myc-SPAK, and the corresponding siRNAs (75  $\mu$ g–100  $\mu$ g, pipetted in 100  $\mu$ L saline) were directly into the mouse's throat by inspiration, then keeping the mouth closed for 2 h. The control group received an equivalent volume of sterile water transfected with nontargeting pCMV-Myc vector or siRNA. The mice were randomly divided into the following groups: Control, ARDS (LPS), WNK4 (LPS + WNK4), SPAK (LPS + SPAK), siRNA-WNK4 (LPS + siRNAWNK4), siRNA-SPAK (LPS + siRNA-SPAK) and siRNA-WNK4 + siRNASPAK (LPS + siRNA-WNK4 + siRNA-SPAK). Mice were sacrificed and lung tissue was collected 24 h after LPS administration. The study was approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University. All procedures were performed according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

### Primary cell culture and transfection

Alveolar epithelial type II (AII) cells were isolated from lung tissue of C57BL/6 mice by elastase digestion with incubation in 5% CO<sub>2</sub> and 95% air at 37°C (26). In brief, the pulmonary artery was cannulated, and the lungs were perfused with normal saline after mice were killed. Then, the fresh lungs were injected 1% lowmelting-point agarose to prevent the isolation of Clara cells and upper airway epithelial cells. The lungs were transferred on ice, rinsed with saline, and placed in 5 mL dispase to digest at room temperature for 60 min with gentle rocking. The lung suspension was filtered through sterile nylon mesh. After centrifugation, final cells were gently suspended in Dulbecco's modified Eagle's medium (10% fetal bovine serum, 0.1 mg/mL streptomycin, and 100 U/mL penicillin), counted using a hemocytometer. After 60% to 75% confluence was reached, then cells were harvested by trypsinization. Cells ( $9 \times 10^6$ ), mixed with 7 pmol to 10 pmol of siRNA/ $10^6$  cells, were transfected by Lipofectamine<sup>TM</sup> reagent (Invitrogen, Waltham, MA). After transfection, cells were plated onto cell plates at a density of  $3 \times 10^6$  cells, then harvested for experiments 48 h later.

### Lung wet/dry ratio

Pulmonary edema was evaluated by calculation of wet/dry (W/D) ratio. Primarily weighing the wet weight of the isolated right lungs, then they were dried in an oven at 80°C for 48 h. The lungs were weighed again to obtain the dry weight.

### Bronchoalveolar lavage

Bronchoalveolar lavage fluid (BALF) was acquired as described previously (27). The left lung was isolated and bronchoalveolar lavage was conducted; instillation and aspiration was repeated three times with 1 mL sterile saline. More than 90% BALF was collected from each mouse and centrifuged at 500g for

10 min. Protein concentration in BALF was assessed by a protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). The cells were resuspended in 50  $\mu$ L PBS with Wright-Giemsa staining (KeyGen Biotech Co., Nanjing, China). Neutrophil counts and total cell were determined using a hemocytometer.

### Mice survival curve

To evaluate the survival of LPS-induced ARDS mice with transfection of WNK4 or siRNA-WNK4 and SPAK or siRNA-SPAK. Survival rates were recorded in a murine model of ARDS by transfection of WNK4 or siRNAWNK4 and SPAK or siRNA-SPAK for 72 h respectively (n = 10 per group). A Kaplan–Meier plot was constructed to compare survival rates between groups.

### Evaluation of lung injury

The left lung was harvested with 4% paraformaldehyde fixation and paraffin wax embedded. Paraffin-embedded tissue was cut into 5  $\mu$ m section by hematoxylin and eosin staining sequentially (Richard-Allan Scientific; Thermo Fisher Scientific, Inc.) for microscopic analysis. For each mouse, histological lung injury was evaluated in five random fields. Lung injury score was evaluated including alveolar congestion and hemorrhage, alveolar wall thickening, neutrophils infiltration and hyaline membrane formation by a semiquantitative scoring system based on lung histopathology (28).

### Alveolar fluid clearance

AFC was quantified based on the change in alveolar fluorescein isothiocyanate (FITC)-conjugated albumin (Sigma-Aldrich, Millipore, MA) concentration (29). The isolated lungs were incubated in a heating pad at 37°C with 100% oxygen inflation by continuous positive airway pressure at 7 cm H<sub>2</sub>O. Warm saline containing FITC-conjugated albumin at 12.5 mL/kg body weight was instilled into the lungs for 2 min. A 100  $\mu$ L alveolar fluid was aspirated at 1 and 15 min after instillation. The aspirate was centrifuged at 3,000g for 10 min. The supernatant was measured in duplicate. AFC was calculated by:  $AFC = (C_f - C_i) / C_f \times 100$  (C<sub>i</sub>: initial concentration of the aspirate at 1 min, C<sub>f</sub>: final concentration of the aspirate at 15 min.)

### Immunofluorescence

Lung tissues were incubated with 4% paraformaldehyde in 0.5% Triton X-100 permeabilization and 10% goat serum blocking for 30 min. The sections were incubated with anti- $\alpha$ -ENaC antibody (1:400), anti- $\beta$ -ENaC antibody (1:400), anti- $\gamma$ -ENaC antibody (1:400) and primary antibodies targeting WNK4 (1:100) or SPAK (1:50) overnight at 4°C. Alexa Fluor 594-labeled secondary antibodies (Thermo Fisher Scientific, Inc.) or Alexa Fluor 488 antibody (BIOSS) was incubated for 1 h at 37°C. The nuclei were stained with DAPI (1:2,000; Sigma-Aldrich; Merck KGaA). A confocal laser-scanning microscope (TCS-SP2, Leica Microsystems, Germany) was used for image capture. All images were analyzed by Image-Pro 6.0 software (Media Cybernetics, Rockville, MD).

### Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was prepared using TRIzol<sup>®</sup> reagent (Invitrogen) and quantified by a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, Inc.). A total of 1  $\mu$ g RNA was used as a template for cDNA amplification, using the HiScript 1st Strand cDNA Synthesis Kit (Vazyme, Piscataway, NJ). RT-qPCR was performed with SYBR<sup>®</sup> Green (Takara, Otsu, Japan). The relative gene expression was determined by the comparative Ct (2<sup>- $\Delta\Delta$ Ct</sup>) method (CFX manager software; Bio-Rad Laboratories, Hercules, CA). The primer sequences are as follows:  $\alpha$ -ENaC forward, 5'-GCTCAACCTTGACCTAGACCTTG-3', and reverse, 5'-CGCCTGTCTTCACGCTTGT-3';  $\beta$ -ENaC forward, 5'-GTTCTGCTTACGCTGCTCTTC-3', and reverse, 5'-GTCCTGGTGGTG TTGCTGTG-3';  $\gamma$ -ENaC forward, 5'-TGCTGTGAGTGACCTCCTGAC-3', and reverse, 5'-TTCCGCTCCGACCAGTGAA-3'; GAPDH forward, 5'-CAAGGTCATCCATGACAACTT-3', and reverse, 5'-GGCCATCCACAGTCTTCTGG-3'.

### Western blotting and immunoprecipitation

The membrane proteins of the lung tissues and cells were obtained using a Protein Extraction kits (KeyGen Biotech Co.). Fifty micrograms of protein were separated using SDS–PAGE, and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with primary antibodies overnight at 4°C after blocking with 5% nonfat milk. Then the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 2 h. A total of 500  $\mu$ g total protein from cells was immunoprecipitated with the indicated antibodies at 4°C overnight, and subsequently incubated on protein

A/G-agarose (Santa Cruz Biotechnology) beads with gentle rotation for 4 h at 4°C. After washing in lysis buffer for four times, the samples were separated by SDS-PAGE and transferred to PVDF membranes for neural precursor cell-expressed developmentally downregulated gene 4 (Nedd4-2; Abcam, Cambridge, MA) blot. Using the enhanced chemiluminescence method (EMD Millipore, Billerica, MA), protein blot was analyzed by Bio-Rad One software (Bio-Rad Laboratories). The following primary antibodies were used:  $\alpha$ -ENaC (1:1,000),  $\beta$ -ENaC (1:1,000), and  $\gamma$ -ENaC (1:1,000) were purchased from StressMarq Biosciences, Inc. (CN, Canada). Phospho-SPAK (Ser373) (1:1,000) and WNK4 (1:1,000) antibodies were purchased from Sigma-Aldrich. Total SPAK (1:1,000) were purchased from Cell Signaling Technology, Inc.

### Statistical analysis

All data were expressed as the mean  $\pm$  SD. The differences between two groups were using Student's *t* test. The differences between multiple groups were using one-way analysis of variance (ANOVA).  $P < 0.05$  was considered as a significant difference. Statistical analysis was performed by GraphPad 5.0 prism software (GraphPad Software, CA).

## RESULTS

### Transfection of WNK4, SPAK, and their associated siRNAs were determined in vivo and in vitro

The successful transfection of WNK4, SPAK, siRNAWNK4, and siRNA-SPAK in alveolar epithelial cells of mouse lung tissue

were determined by western blotting (Fig. 1 and Fig. S1, <http://links.lww.com/SHK/B459>) and immunofluorescence (Fig. 2 A and Fig. S2, <http://links.lww.com/SHK/B460>). Transfection of WNK4, SPAK, siRNA-WNK4, and siRNA-SPAK were localized in the alveolar epithelial cells which was identified in the previous study (24). The successful transfection of WNK4, SPAK, siRNA-WNK4, and siRNA-SPAK in ATII cells were determined by western blotting (Fig. 1 and Fig. S1, <http://links.lww.com/SHK/B459>).

### WNK4/SPAK signaling alleviates lung edema and accelerates AFC in LPS-induced ARDS

To clarify the effect of WNK4/SPAK pathway on alveolar-capillary barrier function, W/D ratio and AFC were determined. The W/D ratio was significantly decreased by transfection of WNK4 or SPAK in LPS-induced ARDS, while the WNK4 or SPAK-induced decrease in the W/D ratio was blocked by siRNA-mediated knockdown of WNK4 or SPAK, compared with the control ( $P < 0.05$ ; Fig. 2B). AFC was significantly increased with WNK4 or SPAK transfection, but was inhibited by siRNA-silencing of WNK4 or SPAK ( $P < 0.05$ ; Fig. 2C). Compared with ARDS group, the W/D ratio and AFC were

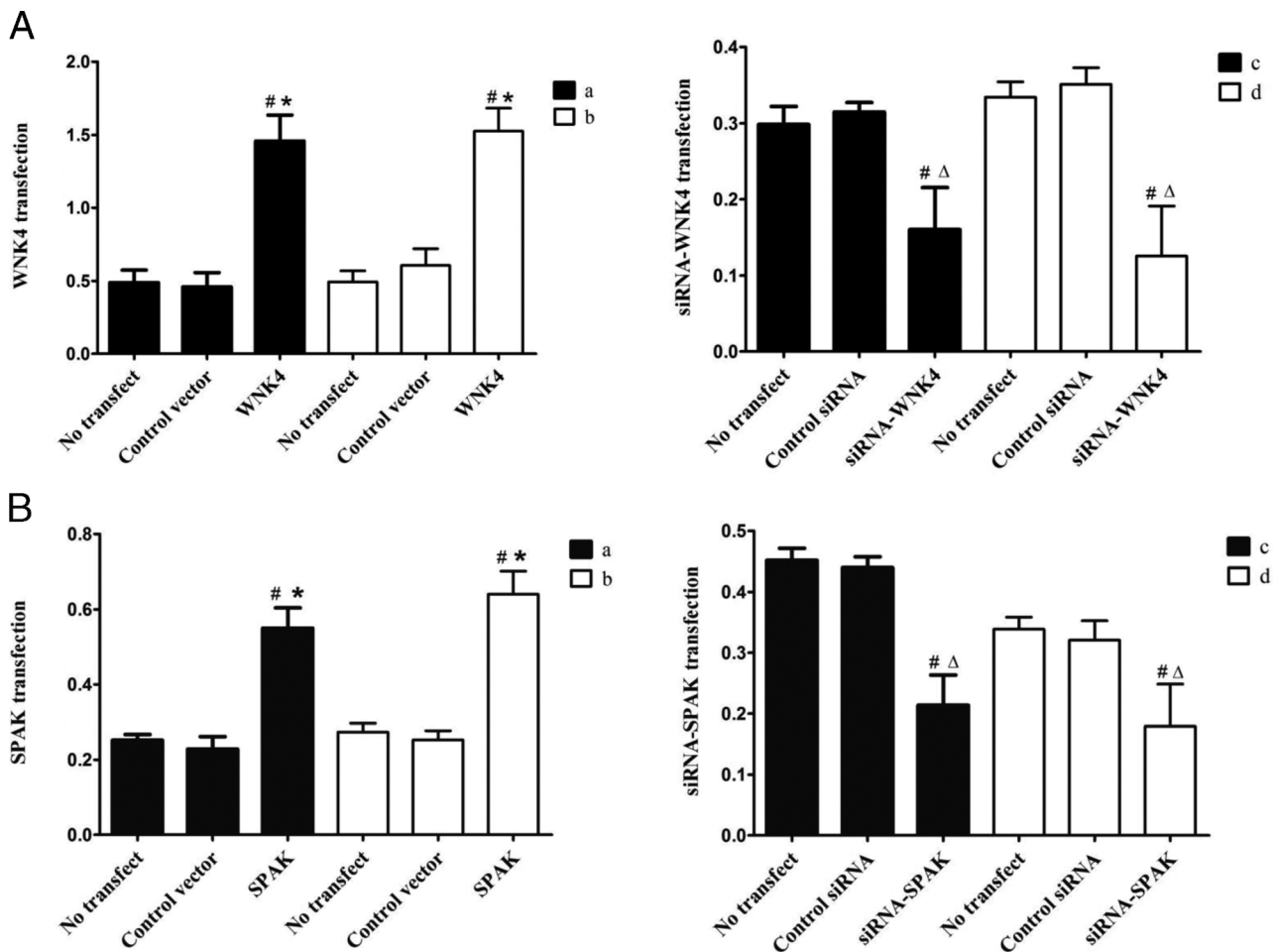
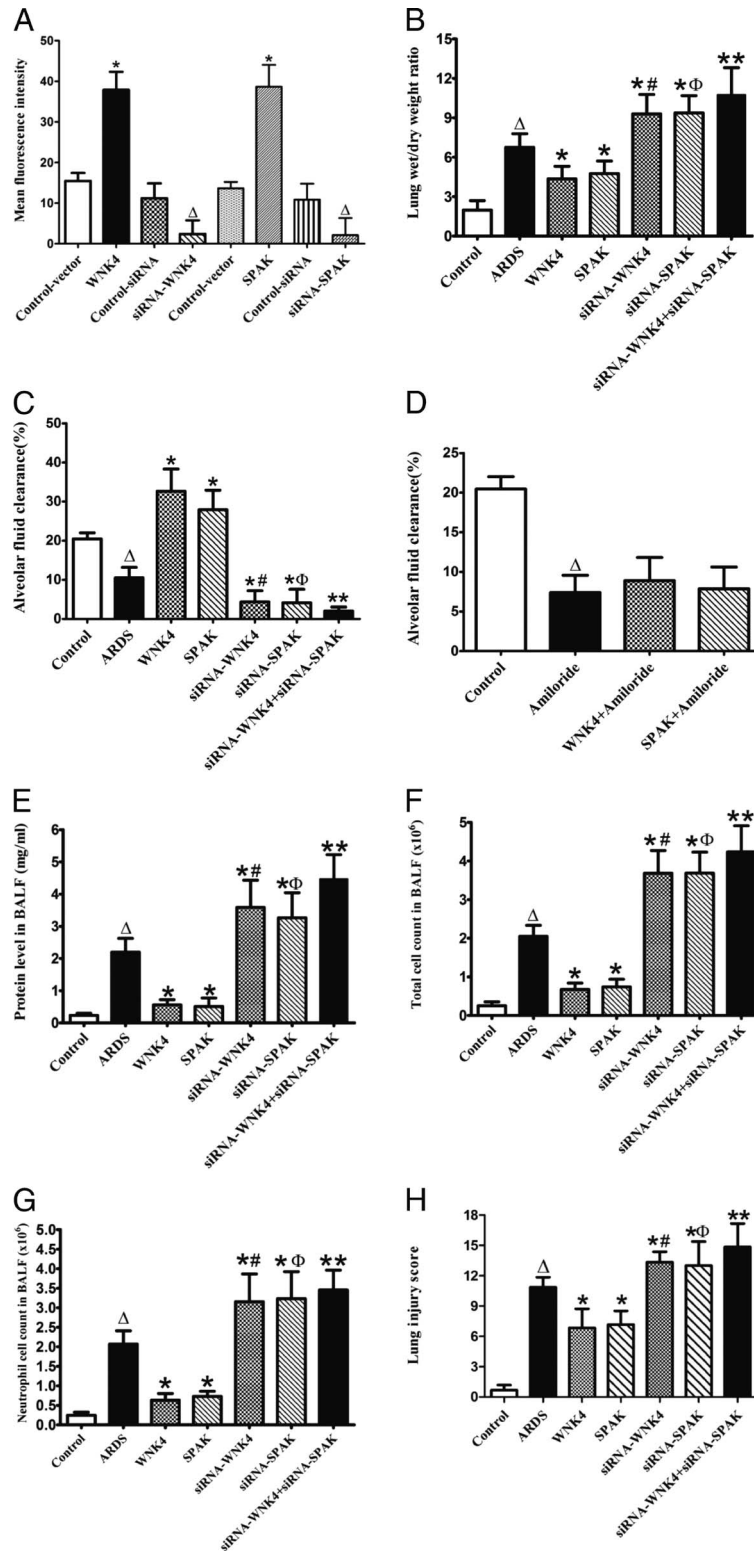


FIG. 1. Mouse lung transfected with WNK4 (A-a), or siRNA-WNK4 (A-c) and SPAK (B-a) or siRNA-SPAK (B-c) were determined 24 h after transfection by western blotting. Type II alveolar epithelial cell transfected with WNK4 (A-b) or siRNA-WNK4 (A-d) and SPAK (B-b) or siRNA-SPAK (B-d) were determined by western blotting. The band intensity was quantitated by normalized for GAPDH. Three independent experiments from each group were performed. The images were included in Figure S1, <http://links.lww.com/SHK/B459>. Data are presented as mean  $\pm$  SD # $P < 0.05$  vs. no transfect; \* $P < 0.05$  vs. control vector;  $\Delta P < 0.05$  vs. control siRNA.



**FIG. 2. Mouse lung transfected with WNK4, SPAK, siRNA-WNK4, and siRNA-SPAK were determined by immunofluorescence (green).** A, The images were included in Figure S2, <http://links.lww.com/SHK/B460>. Transfection of WNK4, SPAK, and siRNA on pulmonary edema and AFC in LPS-induced ARDS in mice (n = 5 per group). B, Lung W/D ratio 48 h after LPS or saline intratracheal instillation. C, AFC 48 h after LPS or saline intratracheal instillation. Albumin solution containing amiloride ( $5 \times 10^{-4}$  M) were injected into the alveolar spaces (D). Protein concentrations (E), total cells (F), and neutrophil cells (G) in BALF in a murine model of ARDS. H, Lung injury scores were utilized for the quantitative analysis of lung histopathologic damage (n = 6 per group). Lung injury score as follows: 0 = no injury; 1 = slight injury (25%); 2 = moderate injury (50%); 3 = severe injury (75%); and 4 = very severe injury (almost 100%). The results were graded from 0 to 4 for each item. The four variables were summed to represent the lung injury score from 0 (no damage) to 16 (maximum damage). Pulmonary morphology with H&E staining in LPS-induced ARDS (original magnification 200 $\times$ ). A representative figure from each group is shown. The images were included in Figure S3, <http://links.lww.com/SHK/B461>. Data are presented as mean  $\pm$  SD  $^{\Delta}P < 0.05$  vs. control; \* $P < 0.05$  vs. ARDS; # $P < 0.05$  vs. WNK4;  $\Phi P < 0.05$  vs. SPAK; \*\* $P < 0.05$  vs. ARDS.

significantly altered during cotransfection of siRNA-WNK4 and siRNA-SPAK, ( $P < 0.05$ ; Fig. 2, B and C). Also, amiloride, a sodium channel inhibitor, inhibited AFC following transfection of WNK4 or SPAK (Fig. 2D).

#### **WNK4/SPAK signaling decreases protein levels, and neutrophil infiltration into the BALF in ARDS**

To determine the effect of WNK4/SPAK pathway on alveolar-capillary barrier permeability *in vivo*, total protein concentrations and neutrophil counts in BALF were analyzed. The protein content and neutrophil counts in the BALF of ARDS mice were significantly decreased with transfection of WNK4 or SPAK, compared with the ARDS group ( $P < 0.05$ ; Fig. 2, E–G). Effect of WNK4 and SPAK was abolished by siRNA-WNK4 and siRNA-SPAK respectively ( $P < 0.05$ ; Fig. 2, E–G). Compared with the ARDS group, the protein content and neutrophil infiltration in BALF were significantly different by cotransfection with siRNA-WNK4 and siRNA-SPAK ( $P < 0.05$ ; Fig. 2, E–G).

#### **WNK4/SPAK signaling alleviates lung injury in LPS-induced ARDS**

LPS promoted neutrophil infiltration, thickened alveolar septum, alveolar edema and hemorrhage, as indicated by an increase in lung injury score (Fig. 2H and Fig. S3, <http://links.lww.com/SHK/B461>). Lung injury was alleviated by WNK4 or SPAK transfection, as illustrated by a decrease in the lung injury score (Fig. 2H). However, siRNA-WNK4 or siRNA-SPAK transfection significantly aggravated pathological injury induced by LPS (Fig. 2H).

#### **WNK4/SPAK signaling decreases mortality in mice with LPS-induced ARDS**

Transfection of WNK4 or SPAK significantly increased the survival of mice with ARDS, compared with the control ( $P < 0.05$ , Fig. 3, A and B), but this effect was significantly blocked by siRNA-WNK4 or siRNA-SPAK ( $P < 0.05$ , Fig. 3, A and B).

#### **Effect of WNK4/SPAK signaling on the localization of ENaC to the lung in LPS-induced ARDS**

The lung distribution of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -ENaC were determined by immunofluorescence (Fig. S4, <http://links.lww.com/SHK/B462>). As a control,  $\alpha$ -ENaC,  $\beta$ -ENaC, and  $\gamma$ -ENaC were localized on the alveolar epithelium in normal mice. The distribution of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -ENaC was markedly decreased in ARDS group.  $\alpha$ -ENaC,  $\beta$ -ENaC, and  $\gamma$ -ENaC was significantly increased by WNK4 or SPAK transfection, while the immunostaining of  $\alpha$ -ENaC,  $\beta$ -ENaC, and  $\gamma$ -ENaC were decreased following siRNA-WNK4 and/or siRNA-SPAK transfection (Fig. 4, A–C).

#### **WNK4/SPAK signaling increases the expression of ENaC *in vivo* and *in vitro***

To further clarify the effect of WNK4/SPAK pathway on ENaC-associated AFC, the mRNA and protein expressions of  $\alpha$ -ENaC,  $\beta$ -ENaC, and  $\gamma$ -ENaC were determined. The expressions of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -ENaC were significantly increased

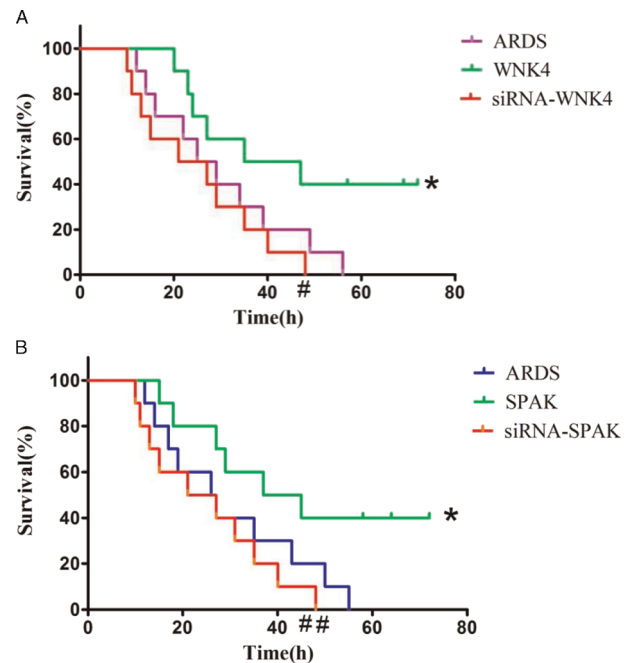


FIG. 3. Animal survival in mice with LPS-induced ARDS. Survival was evaluated for 72 h by transfection of WNK4 or siRNA-WNK4 (A) and SPAK or siRNA-SPAK (B) in a murine model of ARDS. Results are presented as a Kaplan–Meier plot ( $n = 10$  per group). \* $P < 0.05$  vs. ARDS; # $P < 0.05$  vs. WNK4; ## $P < 0.05$  vs. SPAK.

following transfection of WNK4 or SPAK in LPS-induced ARDS, while siRNA-WNK4 and/or siRNA-SPAK blocked the expression of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -ENaC (Fig. 5, A and B). In primary alveolar epithelial cells, transfection of WNK4 or SPAK significantly increased the expression of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -ENaC, but this effect was inhibited by siRNA-WNK4 and/or siRNA-SPAK administration (Fig. 5, C and D). This indicated that AFC was achieved by the upregulation of ENaC expression via WNK4/SPAK signaling. There is no change between control vector and control siRNA of WNK4 or SPAK *in vivo* (Fig. 5, E and F). Also, the expression of WNK4 and SPAK were decreased in LPS-induced ARDS (Fig. 5, G and H).

#### **Activation of the WNK4/SPAK pathway inhibits Nedd4-2 in ENaC regulation**

Western blotting and immunoprecipitation were performed to further investigate whether the regulation of ENaC by WNK4/SPAK pathway was associated with Nedd4-2, a negative regulator for ENaC function (30). The protein expression of phosphorylated SPAK was markedly increased in mouse lung and AII cells following WNK4 transfection ( $P < 0.05$ , Fig. 6). However, the protein expression of phosphorylated SPAK was significantly decreased in mouse lung and AII cells following transfection of siRNA-WNK4 ( $P < 0.05$ , Fig. 6). The detection of Nedd4-2 immunocomplexes using immunoprecipitation with  $\alpha$ -,  $\beta$ -, and  $\gamma$ -ENaC antibodies, revealed a similar sized band to that observed in western blotting, while no such band was illustrated with the control antibody. This revealed that Nedd4-2 interacted with  $\alpha$ -ENaC,  $\beta$ -ENaC, and  $\gamma$ -ENaC under basal conditions (Fig. 7A). The levels of coimmunoprecipitated

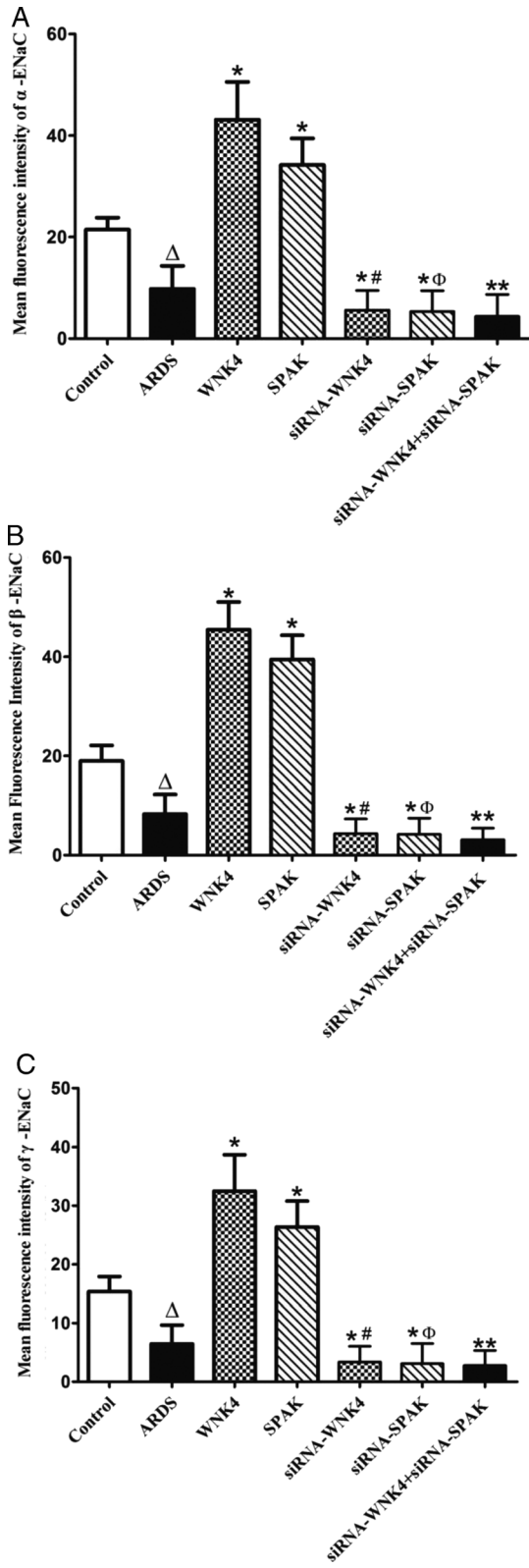


FIG. 4. The location and expressions of  $\alpha$ -ENaC,  $\beta$ -ENaC, and  $\gamma$ -ENaC in mouse lung with LPS-induced ARDS were examined by immunofluorescence staining (n = 6 per group). The immunofluorescence images were included in Figure S4, <http://links.lww.com/SHK/B462>. Data are presented as mean  $\pm$  SD.  $\Delta P < 0.05$  vs. control; \* $P < 0.05$  vs. ARDS; # $P < 0.05$  vs. WNK4;  $\Phi P < 0.05$  vs. SPAK; \*\* $P < 0.05$  vs. ARDS.

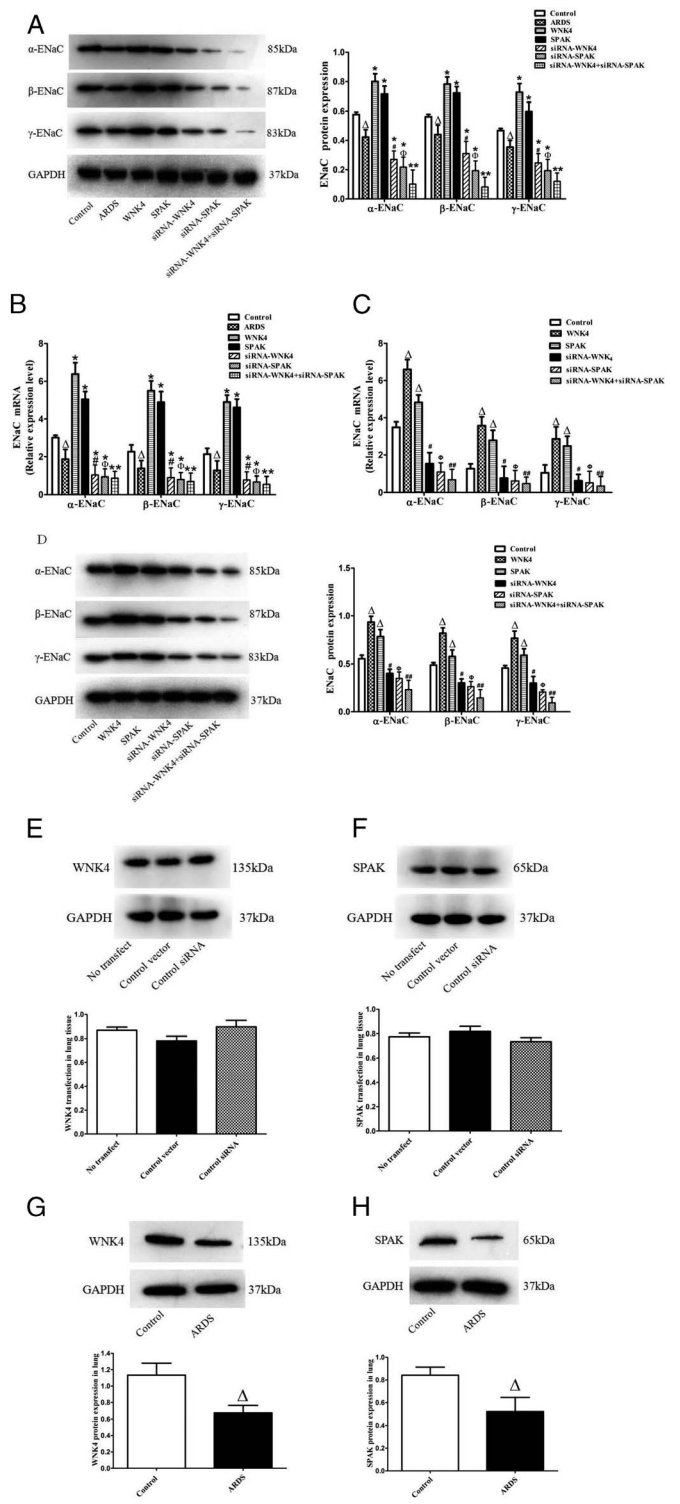


FIG. 5. The protein and mRNA expression levels of  $\alpha$ -ENaC,  $\beta$ -ENaC, and  $\gamma$ -ENaC in mouse lung with LPS-induced ARDS determined by western blotting (A) and real-time PCR (B) with transfection of WNK4 or SPAK and siRNA (n = 6 per group). The mRNA and protein expression levels of  $\alpha$ -ENaC,  $\beta$ -ENaC, and  $\gamma$ -ENaC in alveolar epithelial type II cells determined by real-time PCR (C) and western blotting (D) with transfection of WNK4 or SPAK and siRNA (three independent experiments for each group). Control vector and control siRNA of WNK4 (E) or SPAK (F) in mouse lung was determined by western blotting (n = 3 per group). The lung expression of WNK4 (G) or SPAK (H) in LPS-induced ARDS was determined by western blotting (n = 3 per group). The band intensity of  $\alpha$ -ENaC,  $\beta$ -ENaC, and  $\gamma$ -ENaC were quantitated by normalized for GAPDH. Data are presented as mean  $\pm$  SD.  $\Delta P < 0.05$  vs. control; \* $P < 0.05$  vs. ARDS; # $P < 0.05$  vs. WNK4;  $\Phi P < 0.05$  vs. SPAK; \*\* $P < 0.05$  vs. ARDS; ### $P < 0.05$  vs. control.

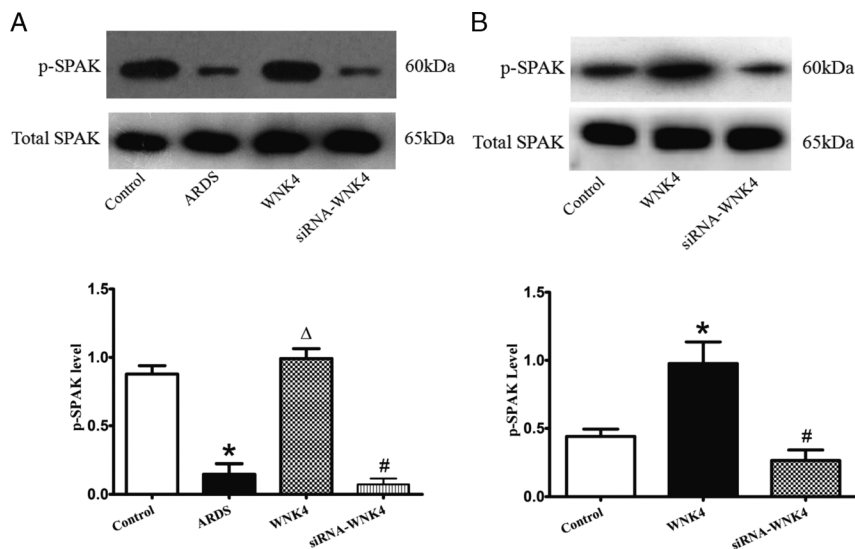


FIG. 6. The expression of phosphorylated SPAK (p-SPAK) in mouse lung (A) and AT II cells (B) by transfection of WNK4 or siRNA-WNK4 were determined by western blotting. Three independent experiments from each group were performed. Data are presented as mean  $\pm$  SD. \* $P < 0.05$  vs. control; # $P < 0.05$  vs. WNK4;  $\Delta P < 0.05$  vs. ARDS.

Nedd4-2 were significantly decreased by transfection of WNK4 or SPAK, while siRNA-WNK4 and siRNA-SPAK significantly blocked this decrease (Fig. 7B). These findings indicated that WNK4/SPAK pathway was in regulation of ENaC by inhibition of Nedd4-2.

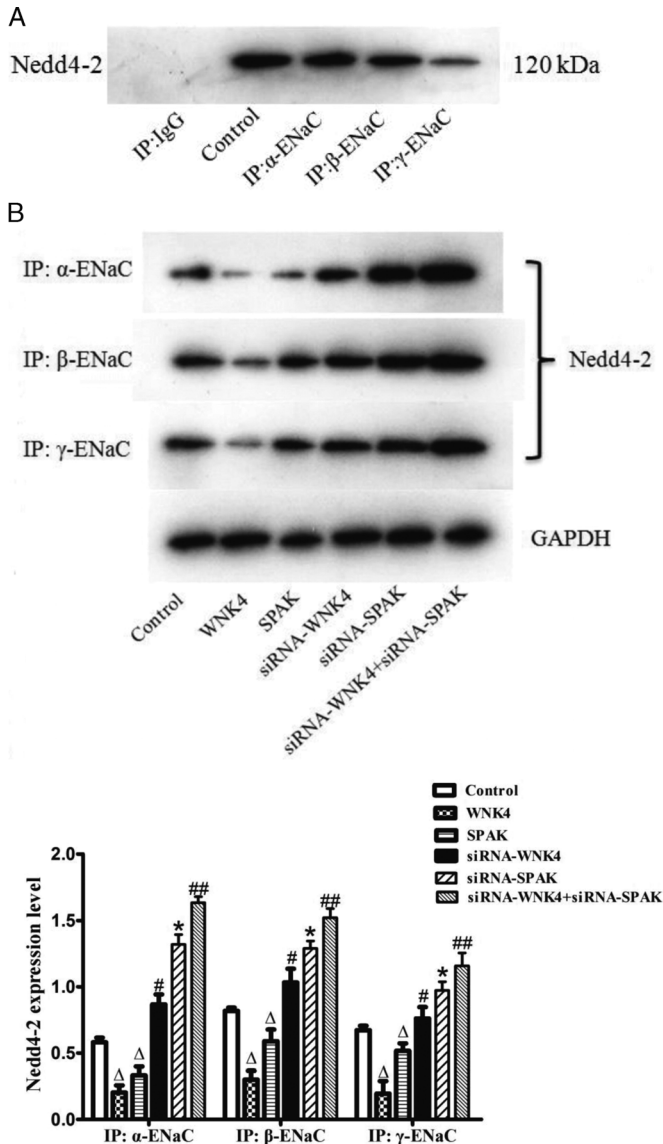
## DISCUSSION

The present study demonstrated that the WNK4/SPAK pathway enhanced ENaC-mediated AFC in LPS-induced ARDS, which may have potential clinical implications for the treatment of the disease (Fig. 8).

An intrapulmonary model of ARDS was established by intratracheal injection of LPS into mice. LPS imitates the effects of a gram-negative bacterial infection to induce ARDS in animals (31). It achieves this by stimulating macrophages and other immune cells to produce inflammatory cytokines; this result in the recruitment of neutrophils into the lung epithelium, and the subsequent release of proinflammatory mediators, which are recognized as primary investigators in the development and pathogenesis of ARDS (32,33). Activated neutrophils immigrate into lung endothelium across the endothelial surface by producing reactive oxygen species that contribute to alveolar-capillary barrier injury (34). Exposure to LPS results in lung injury, including alveolar edema with hyaline membrane formation, alveolar septa thickening, neutrophil infiltration, and alveolar hemorrhage. In the present study, lung injury was observed with lung histological damage and alveolar-capillary barrier dysfunction indicated by increases in BALF protein concentrations, W/D ratios and decrease in AFC, which was representative of the alveolarcapillary barrier injury and accumulation of pulmonary edema, the hallmarks of ARDS in humans (35).

It is well established that AFC is an important process of edema fluid clearance, which in ARDS, is facilitated by ENaCs (36). WNK4, a regulator of ion channels, achieves its effect by

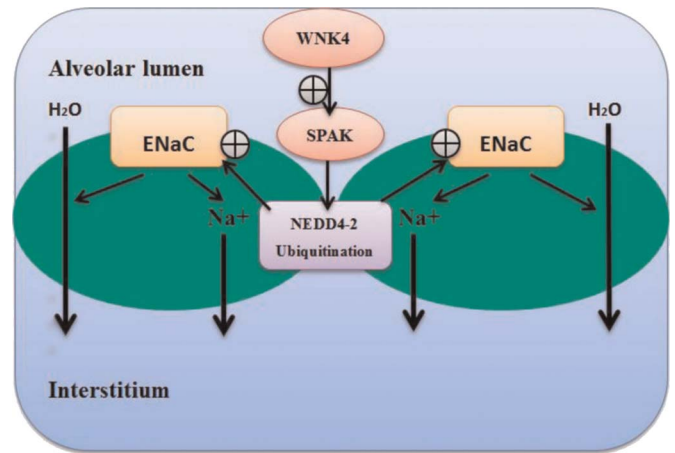
upregulation of ENaC in the renal tubules, through the phosphorylation of SPAK (37,38). SPAK also serves a fundamental role in epithelial transport (17), which is regulated by WNK4 (39). In the present study, the expression of WNK4 and SPAK were decreased in LPS-induced ARDS. Lung injury was alleviated by WNK4 or SPAK transfection, resulting in an increase in AFC, a decrease in pulmonary edema, an improvement in survival rate and a low histological lung injury score in mice with LPS-induced ARDS; transfection of siRNA-WNK4 or siRNA-SPAK inhibited this effect. Importantly, the protein content and neutrophil in BALF were significantly increased by cotransfection of siRNA-WNK4 and siRNA-SPAK, indicating decrease in AFC and increase in lung injury by inhibition phosphorylation of SPAK was inhibited by siRNA-WNK4 *in vivo* and *in vitro*. The results indicated that WNK4/SPAK pathway contributed to the attenuation of lung injury, potentially by means of an AFC-associated mechanism. The present study also revealed that amiloride inhibited AFC stimulated by WNK4 or SPAK transfection, supporting the fact that WNK4/SPAK enhanced AFC through ENaC activation. Therefore, WNK4/SPAK signaling was essential for the maintenance of the lung water volume balance, as previously reported (20,40). The association between ENaCs and the WNK4/SPAK pathway was further investigated. *In vivo*, the expression levels of  $\alpha$ -ENaC,  $\beta$ -ENaC, and  $\gamma$ -ENaC were increased by activation of the WNK4/SPAK pathway, but decreased by WNK4/SPAK gene silencing in LPS-induced ARDS. *In vitro*, the expression levels of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -ENaC were also inhibited by WNK4/ SPAK gene-silencing in ATII cells, which was consistent with the results *in vivo*. The expression levels of  $\alpha$ -ENaC,  $\beta$ -ENaC, and  $\gamma$ -ENaC were significantly decreased by cotransfection of siRNA-WNK4 and siRNA-SPAK. The results implied the gene transcription activity and protein expression were potential correlation with WNK4/SPAK. WNK4/ SPAK gene silencing possibly modified the functions of other kinases known to regulate ENaC, resulting in the decreased mRNA expression (19,41). Furthermore, SPAK phosphorylation was



**FIG. 7. Association of Nedd4-2 with α-ENaC, β-ENaC, and γ-ENaC.** A, Protein from AT II cells lysate was immunoprecipitated with anti-α-ENaC, anti-β-ENaC, anti-γ-ENaC antibodies or goat anti-rabbit IgG (serving as a negative control). Levels of coimmunoprecipitated Nedd4-2 were determined by western blotting. B, Transfection of WNK4, SPAK, siRNA-WNK4, or siRNA-SPAK on Nedd4-2 in coimmunoprecipitated α-ENaC, β-ENaC, and γ-ENaC. Data are normalized for the amount of Nedd4-2 immunoprecipitated and expressed as fold of the control. Relative expression levels of ENaC expressed as fold of the control. Three independent experiments from each group were performed. Data are presented as mean ± SD. <sup>Δ</sup>*P* < 0.05 vs. control; <sup>#</sup>*P* < 0.05 vs. WNK4; <sup>\*</sup>*P* < 0.05 vs. SPAK; <sup>##</sup>*P* < 0.05 vs. control.

blocked by siRNA-WNK4 transfection. These results confirmed that WNK4/SPAK signaling promoted AFC by upregulating ENaC expression in LPS-induced ARDS, which contrasted with previous findings suggesting that WNK4 reduced ENaC expression in A6 cells (42).

Moreover, neutrophil in the alveolar edema fluid may induce downstream inflammatory and immune responses in the development of ARDS (9). Maintenance of AFC by ENaC can protect alveolar epithelial barrier from proinflammatory cytokines including interleukin (IL)-1b, IL-8, tumor necrosis factor



**FIG. 8. Activation of WNK4-SPAK pathway improved AFC by upregulation of ENaC with Nedd4-2-mediated ubiquitination in LPS-induced ARDS.**

(TNF)α, and TGFβ1 (43). In the study, neutrophil and total cells in BALF were decreased by ENaC-mediated AFC via WNK4/SPAK pathway. These results suggest that upregulation of ENaC expression can alleviate inflammation process or alter the inflammatory pathway by activation of WNK4/SPAK pathway.

ENaC is regulated by numerous different kinases, including serine/threonine-protein kinase isoforms, protein kinase A, protein kinase C, and AMP-activated protein kinase (41). PY motifs in the COOH-terminal domains of ENaC interacted with WW domains of Nedd4-2, a negative regulator of ENaC expression (44, 45). As a result of Nedd4-2 binding to these motifs, ENaC is degraded by ubiquitination, resulting in fewer channels expression (46,47). The expression of Nedd4-2 in coprecipitated α-ENaC, β-ENaC, and γ-ENaC were decreased by WNK4/SPAK gene transfection, while the levels of Nedd4-2 were increased by WNK4/SPAK gene-silencing. These results indicated that the inhibitory effect of WNK4/SPAK on Nedd4-2 binding to ENaC, which was increased ENaC expression both in vitro and in vivo. In addition, cotransfection of siRNA WNK4 and siRNA-SPAK significantly increased the expression of Nedd4-2 as well as inhibited the expression of α-ENaC, β-ENaC, and γ-ENaC compared with transfection of siRNA-WNK4 or siRNA-SPAK alone in ATII cells. The phosphorylation of SPAK may provide a binding site or activate downstream kinases associated with ENaC regulation, through inhibition of the WW domains of Nedd4-2 (40,48). These findings first suggested the WNK4/SPAK pathway upregulated ENaC expression mediated by inhibition of Nedd4-2. The results were consistent with the report suggesting that WNK4 stimulates ENaC activity in HEK 293 cells (49). However, Yu et al. (42) indicated that WNK4 signaling regulated ENaC independently of Nedd4-2-mediated-ENaC in distal nephron cells. This contrary effect might be due to a single nonselective cation channel observed in ENaC-expressing A6 cells rather than traditional ENaC channels in ATII cells. Also, cell culture in different conditions may contribute to the different observations. The study probably more accurately indicated the regulation of ENaC by WNK4 in lung because of native cells expressing endogenous ENaC. However, additional research is still required to investigate the potential mechanisms involved.



## CONCLUSIONS

The study first demonstrated that the WNK4/SPAK pathway serves a crucial role in regulating all subunits ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -) of ENaC that have a close relationship with AFC during LPS-induced ARDS. *In vitro* study, the upregulation of  $\alpha$ -ENaC,  $\beta$ -ENaC, and  $\gamma$ -ENaC expression was mediated by the WNK4-associated activation of SPAK in a potentially mechanism of inhibition of Nedd4-2. These novel findings may provide a new sight for the clinical treatment of AFC in ARDS.

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