Alcohol inhibits alveolar fluid clearance through the epithelial sodium channel via the A2 adenosine receptor in acute lung injury

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Abstract. Chronic alcohol abuse increases the risk of mortality and poor outcomes in patients with acute respiratory distress syndrome. However, the underlying mechanisms remain to be elucidated. The present study aimed to investigate the effects of chronic alcohol consumption on lung injury and clarify the signaling pathways involved in the inhibition of alveolar fluid clearance (AFC). In order to produce rodent models with chronic alcohol consumption, wild-type C57BL/6 mice were treated with alcohol. A2a adenosine receptor (AR) small interfering (si)RNA or A2bAR siRNA were transfected into the lung tissue of mice and primary rat alveolar type II (ATII) cells. The rate of AFC in lung tissue was measured during exposure to lipopolysaccharide (LPS). Epithelial sodium channel (ENaC) expression was determined to investigate the mechanisms underlying alcohol-induced regulation of AFC. In the present study, exposure to alcohol reduced AFC, exacerbated pulmonary edema and worsened LPS-induced lung injury. Alcohol caused a decrease in cyclic adenosine monophosphate (cAMP) levels and inhibited α-ENaC, β-ENaC and γ-ENaC expression levels in the lung tissue of mice and ATII cells. Furthermore, alcohol decreased α-ENaC, β-ENaC and γ-ENaC expression levels via the A2aAR or A2bAR-cAMP signaling pathways *in vitro*. In conclusion, the results of the present study demonstrated that chronic alcohol consumption worsened lung injury by aggravating pulmonary edema and impairing AFC. An alcohol-induced decrease of α-ENaC, β-ENaC and γ-ENaC expression levels by the A2AR-mediated cAMP pathway may be responsible for the exacerbated effects of chronic alcohol consumption in lung injury.

Introduction

Alcohol abuse and alcohol-related problems have been significant public health issues in a number of countries for several decades (1). Chronic alcohol consumption not only affects the central nervous system, but adversely affects other organs, including the lung (2). Compared with non-alcoholic individuals, patients with a history of alcohol abuse have enhanced susceptibility to lung injury. This includes a 2-4-fold risk of developing acute respiratory distress syndrome (ARDS), which may affect hospitalization costs, the incidence of intensive care unit-related mortality and poor patient outcomes (3-5). During the coronavirus (COVID-19) pandemic, there has been growing concern over the impact of excessive alcohol consumption in patients with COVID-19 and alcohol use disorder (6). Chronic alcohol consumption reduces the immunity of patients to viral and bacterial infections, which may increase the infection and mortality rates of COVID-19 (7). ARDS is a severe form of respiratory failure that is life threatening, which has demonstrated an overall mortality rate of 45% in patients that have been admitted to hospital since 2010 (8). To the best of the authors' knowledge, the molecular mechanisms underlying the association between alcohol abuse and ARDS have yet to be fully elucidated and no specific therapies are currently available to treat or decrease the risk of lung injury in patients suffering from alcoholism.

ARDS, which develops from acute lung injury (ALI), is characterized by alveolar-capillary barrier injury, resulting in the accumulation of protein-rich fluid in the alveolar spaces that impairs gas exchange. This leads to severe hypoxemia and acute respiratory failure (9). Resolution of ALI/ARDS requires clearance of excess edema fluid in the alveolar spaces and repair of the alveolar-capillary barrier (10). Alveolar edema is resolved by active salt and water transport through epithelial sodium channels (ENaC) which provide a driving force to remove edema fluid from the alveolar spaces (11,12). ENaC is a multimeric protein composed of three homologous

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Abbreviations: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; AFC, alveolar fluid clearance; AR, adenosine receptor; BALF, bronchoalveolar lavage fluid; cAMP, cyclic adenosine monophosphate; COVID-19, corona virus disease 2019; ENaC, epithelial sodium channel; ELISA, enzyme-linked immunosorbent assay; LPS, Lipopolysaccharide; PCR, polymerase chain reaction

Key words: epithelial sodium channel, alcohol, adenosine receptor, alveolar fluid clearance, acute respiratory distress syndrome

subunits, α, β and γ (13). ENaC is the rate-limiting step in the resolution of lung edema; mice lacking α-ENaC, β-ENaC or γ-ENaC genes are unable to clear alveolar edema fluid, which indicates the important function of ENaC in alveolar fluid clearance (AFC) (14-16).

Alcohol ingestion increases the systemic levels of extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter (17,18). From a previous study, regulation of ENaC expression by adenosine occurs via the A2 adenosine receptor (AR) pathway (19). However, to the best of the authors' knowledge, the association between alcohol and AFC and the role of ENaC in ALI have yet to be fully elucidated. The present study aimed to determine the role of alcohol in AFC inhibition and the role of A2 AR signaling through the ENaC in lipopolysaccharide (LPS)-induced lung injury.

Materials and methods

Animal experiments and materials. Adult female C57BL/6 mice (n=66; Chongqing Medical University; weight, 20-25 g; age, 8-10 weeks) and male Sprague-Dawley rats (n=27; Chongqing Medical University; weight, 200-240 g; age, 5-7 weeks) were used in the present study in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animals were housed under specific pathogen-free conditions in a temperature (18-25°C)-and humidity (40-60%)-controlled environment with a 12-h light/dark cycle and given free access to food and water. All surgery was performed under anesthesia with sodium pentobarbital and all efforts were made to minimize suffering. The study was approved by the Ethics Committee of The Second Affiliated Hospital of Chongqing Medical University (approval no. 2019-009). Ethyl alcohol (C_2H_5OH) was purchased from North-South Chemical, China. LPS (*Escherichia coli* serotype O111:B4) was purchased from Sigma-Aldrich (Merck KGaA). The α-ENaC antibody (cat. no. PA1-920A) was purchased from Thermo Fisher Scientific, Inc. and β-ENaC (cat. no. YT1551) and γ-ENaC (cat. no. YT5032) antibodies were purchased from ImmunoWay Biotechnology Company. AR antibodies (cat. nos. PA1-042 and PA5-72850) were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). CGS-21680 (a specific agonist of A2aAR), Bay 60-6583 (a specific agonist of A2bAR) and cyclic adenosine monophosphate (cAMP) inhibitor [(R)-Adenosine, cyclic 3',5'-(hydrogenphosphorothioate) triethylammonium] were purchased from Tocris Bioscience.

Animal models. Mice were administered alcohol intraperitoneally (i.p.; 4 g/kg; 5% v/v in sterile water) for the first week of the experiment until 20% v/v alcohol was reached. Beginning on the fourth week, mice were administered with 5% incremental doses at each subsequent week. Mice remained on a 20% v/v alcohol diet for an additional four weeks to establish a chronic alcohol consumption model (20,21). This approach had previously been reported to mimic alcohol abuse in a mouse model for 11 weeks (22). The control group of mice were administered an equivalent volume of sterile water. The alcohol and control mice were given the same diet. Mice were anesthetized by intraperitoneal administration of sodium

pentobarbital (Sigma-Aldrich; Merck KGaA) at a dose of 50 mg/kg. The ALI model was established by LPS i.p. injection (10 μg/g) three days after the establishment of the chronic alcohol consumption model. The vital signs and behaviors of mice were monitored in every 1-2 days. Mice were sacrificed and the blood and lung tissue were collected 24 h after LPS administration. Then, mice were sacrificed by exsanguination from the bleeding of carotid artery (mortality was confirmed by disappearance of breath and nerve reflex as well as muscle relaxation).

Primary cell culture and treatment. Rats were sacrificed by exsanguination at the carotid artery. Alveolar epithelial type II (ATII) cells were isolated from male Sprague-Dawley rats by elastase digestion of lung tissue and incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C as previously described (23-25). Cells were cultured in DMEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA), 100 U/ml penicillin and 0.1 mg/ml streptomycin. Freshly isolated alveolar epithelial cells were treated with 0.20% v/v ethanol for 72 h. Cells were subsequently incubated with CGS-21680, Bay 60-6583 or cAMP inhibitor for subsequent western blotting experiments.

Small interfering (si)RNA transfection. siRNA targeting A2aAR or A2bAR and the negative control were obtained from Ambion (Thermo Fisher Scientific, Inc.) The following pairs of primers were used: A2a AR siRNA forward, 5'-GAC GGGAACUCCACGAAGATT-3' and reverse, 5'-UCUUCG UGGAGUUCCCGUCTT-3'; A2b AR siRNA forward, 5'-ACC TCAACCGAGACTTCCGCT-3' and reverse, 5'-CAAAAA AACCGAGACTTCCGC-3'; Negative control siRNA forward, 5'-GAAUUAAUUAAAGAUGGCCCG-3' and reverse, 5'-UCA UCGAAGUUAUAGGGAUAC-3'. Intratracheal delivery of specific siRNA for A2aAR or A2bAR was performed as previously described (26). Briefly, 100 μg A2aAR siRNA or A2bAR siRNA (in 100 μl saline) was administered directly into the throat of each mouse and the mouth was immediately kept closed for 2 h to ensure delivery of siRNA to the lung. The control group were transfected with non-targeting siRNA in an equivalent volume of sterile water. Cells (9x10⁶) mixed with 100 nmol/l siRNA/10⁶ cells were transfected using Lipofectamine® reagent incubated for 15 min at room temperature (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following transfection, cells were incubated with culture medium (DMEM with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin) and plated onto cell culture plates at a seeding density of 3x10⁶ cells. After 48 h transfection, cell lysates were prepared for subsequent western blotting.

cAMP accumulation assay. Lung tissues were homogenized in 6% trichloroacetic acid (1 ml trichloroacetic acid/100 mg tissue), centrifuged at 1,000 x g and 4°C for 15 min and extracted with water-saturated diethyl ether. Cells were seeded into 96-well plates in 100 μl DMEM. After 24 h, the medium was removed and cells were washed three times with 100 μl DMEM containing 50 mM HEPES (pH 7.4). Cells were subsequently incubated with ethanol at 37°C in 5% CO_2.

The concentration of cAMP in lung and alveolar epithelial cells was evaluated using the cAMP Biotrak Enzyme Immunoassay (Amersham Biosciences; Cytiva). according to the manufacturer's protocol.

Pulmonary edema evaluation. The ratios of wet lung weight to dry lung weight were calculated as a measure of pulmonary edema. The lungs of mice were filled with 100 μl saline via intratracheal instillation. The lungs were excised and weighed 30 min following instillation. The lungs were subsequently dried at 60°C for 48 h and weighed again to obtain the wet:dry weight ratios.

Bronchoalveolar epithelial permeability assessment. Bronchoalveolar epithelial permeability was determined by bronchoalveolar lavage fluid (BALF)/serum fluorescence as previously described (27). Briefly, mice were injected with fluorescein isothiocyanate-conjugated dextran 4000 (FD4; Sigma-Aldrich; Merck KGaA) solution in PBS (10 mg/kg) via the internal jugular vein 24 h after LPS-induced lung injury. BALF was collected by instillation of saline into the lungs three times. The serum was collected from the internal jugular vein by centrifugation at 1,500 x g and 4°C for 5 min. The concentrations of FD4 in BALF and serum were determined by a spectrofluorometer (Beckman Coulter, Inc.).

Measurement of inflammatory cytokines. An endotracheal catheter was inserted into the lungs following instillation of saline five times. BALF was collected following five rounds of extraction and centrifuged at 4°C for 15 min at 500 x g. The supernatant was used to assay TNF-α (cat. no. MTA00B) and IL-6 (cat. no. M6000B) using ELISA kits (R&D Systems, Inc.) according to the manufacturers' protocols.

Lung injury evaluation. The right lungs were harvested and fixed using 10% neutral buffered formalin for 24 h. The fixed tissues were embedded in paraffin and sectioned at 5 μm. Sections were mounted onto glass slides for further staining with hematoxylin and eosin. Briefly, the slices were soaked with 80-100% ethanol, followed by 0.6% hematoxylin (15 min at room temperature) and 1.0% eosin (5 min at room temperature) dyeing, then dehydrated with 80-100% ethanol. Lung injury was evaluated for intra-alveolar exudate, interstitial edema, alveolar hemorrhage and inflammatory cell infiltration using the lung injury scoring system (28). Assessment of histological lung injury was performed by grading as follows: A, neutrophils in the alveolar space; B, neutrophils in the interstitial space; C, hyaline membranes; D, proteinaceous debris filling the airspaces; and E, alveolar septal thickening. The sum of each of the five independent variables was calculated and then normalized to the number of the fields evaluated (total score: 0-2).

AFC measurement. The rate of AFC was measured by Evans's blue-labeled albumin as previously described (29,30). Briefly, after mice were completely sedated with diazepam (5 mg/kg; i.p.) and pentobarbital (50 mg/kg; i.p.), a tracheostomy tube was inserted. Mice were ventilated at a rate of 200 breaths/min with a tidal volume of 150 ml and inspired oxygen concentration of 100%. Physiological saline solution (5 ml/kg) containing 5% albumin and 0.15 mg/ml Evans blue dye (Sigma-Aldrich; Merck KGaA) was instilled into the tracheostomy tube. Mice were ventilated for 30 min after alveolar fluid was aspirated. The concentration of Evans blue-labeled albumin in the injected and aspirated solutions were measured using a spectrophotometer (Beckman Coulter, Inc.). AFC was calculated as follows: AFC=[(Vi-Vf)/Vi] x100, to obtain a percentage, where Vf is (Vi x Pi)/Pf. V represents the injected (i) volume and final (f) volume of alveolar fluid. P represents the i and f concentrations of Evans blue-labeled 5% albumin solution.

Immunohistochemistry. Tissue slices were blocked with BSA (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature and incubated with α-ENaC (1:200), β-ENaC (1:300) and γ-ENaC (1:200) antibodies at 4°C for 24 h. Subsequently, tissues were incubated in biotinylated anti-rabbit IgG (1:400; cat. no. sc-2491; Santa Cruz Biotechnology, Inc.) for 30 min at 37°C, followed by avidin-biotin-peroxidase complex (Sigma-Aldrich; Merck KGaA) for 30 min at 37°C and stained with 3,3'-diaminobenzidine (Sigma-Aldrich; Merck KGaA) for 5 min at room temperature. Normal rabbit isotype IgG (1:400; cat. no. sc-2027; Santa Cruz Biotechnology, Inc.) was used as a negative control. The number of positive cells was counted using a light microscopy imaging system (magnification, x400; Olympus Corporation).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from lung and alveolar epithelial cells (3×10^6) using the RNA isolation kit (Takara Bio, Inc.) according to the manufacturer's protocol. The following pairs of primers were used: α-ENaC forward, 5'-GCTCAACCTTGACCTAGA CCTTG-3' and reverse, 5'-CGCCTGTTCTTCACGCTTGT-3'; β-ENaC forward, 5'-GTTCCTGCTTACGCTGCTCTTC-3' and reverse, 5'-GTCCTGGTGGTGTTGCTGTG-3'; γ-ENaC forward, 5'-TGCTGTGAGTGACCTCCTGAC-3' and reverse, 5'-TTCCGCTTCCGACCAGTGAA-3'; and GAPDH forward, 5'-CAAGGTCATCCATGACAACTT-3' and reverse, 5'-GGC CATCCACAGTCTTCTGG-3'. RNA was reverse transcribed into cDNA using the HiScript 1st Strand cDNA Synthesis kit (Vazyme Biotech Co., Ltd.). qPCR was performed using a SYBR Green PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR amplification reaction was conducted at 94°C for 60 sec, followed by 30 cycles at 94°C for 30 sec, then 53°C (α-ENaC), 53°C (β-ENaC), 55°C (γ-ENaC) or 55°C (β-actin) for 30 sec and finally 72°C for 60 sec. PCR amplification were performed using a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). For each experiment, the expression levels of the indicated genes were normalized to endogenous GAPDH expression levels and calculated using the $2^{-\Delta\Delta Cq}$ method (31). A total of 3-6 repeats was performed.

Western blotting. Mouse lung tissue was perfused with PBS and placed on ice with 1 ml lysis buffer and 1 ml extraction buffer (Nanjing KeyGen Biotech, Co., Ltd.). Alveolar epithelial cells were washed in ice-cold PBS and solubilized in 1 ml lysis buffer. The lysates were cleared by centrifugation for 10 min at 4°C and 14,000 x g All samples were prepared for quantification of the target proteins by using a protein extraction kit (Nanjing KeyGen Biotech Co., Ltd.) according
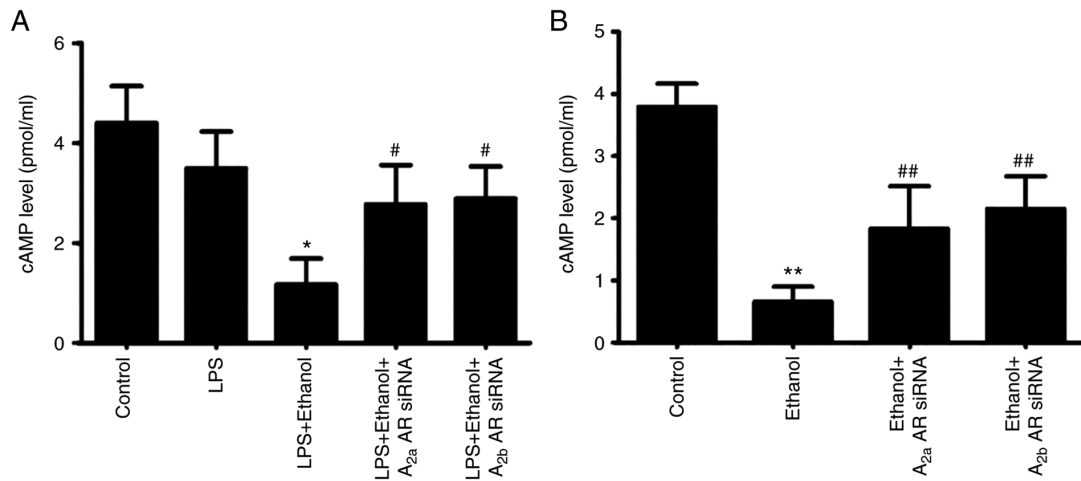
Figure 1. Accumulation of cAMP was induced by ethanol following A2aAR siRNA or A2bAR siRNA transfection in (A) the lung tissue of mice and (B) alveolar epithelial cells (n=3 per group). Data are presented as the mean ± standard deviation based on three independent experiments. *P<0.01 vs. LPS; #P<0.05 vs. LPS + ethanol; **P<0.05 vs. control; ##P<0.05 vs. ethanol. cAMP, cyclic adenosine monophosphate; siRNA, small interfering RNA; LPS, lipopolysaccharide.
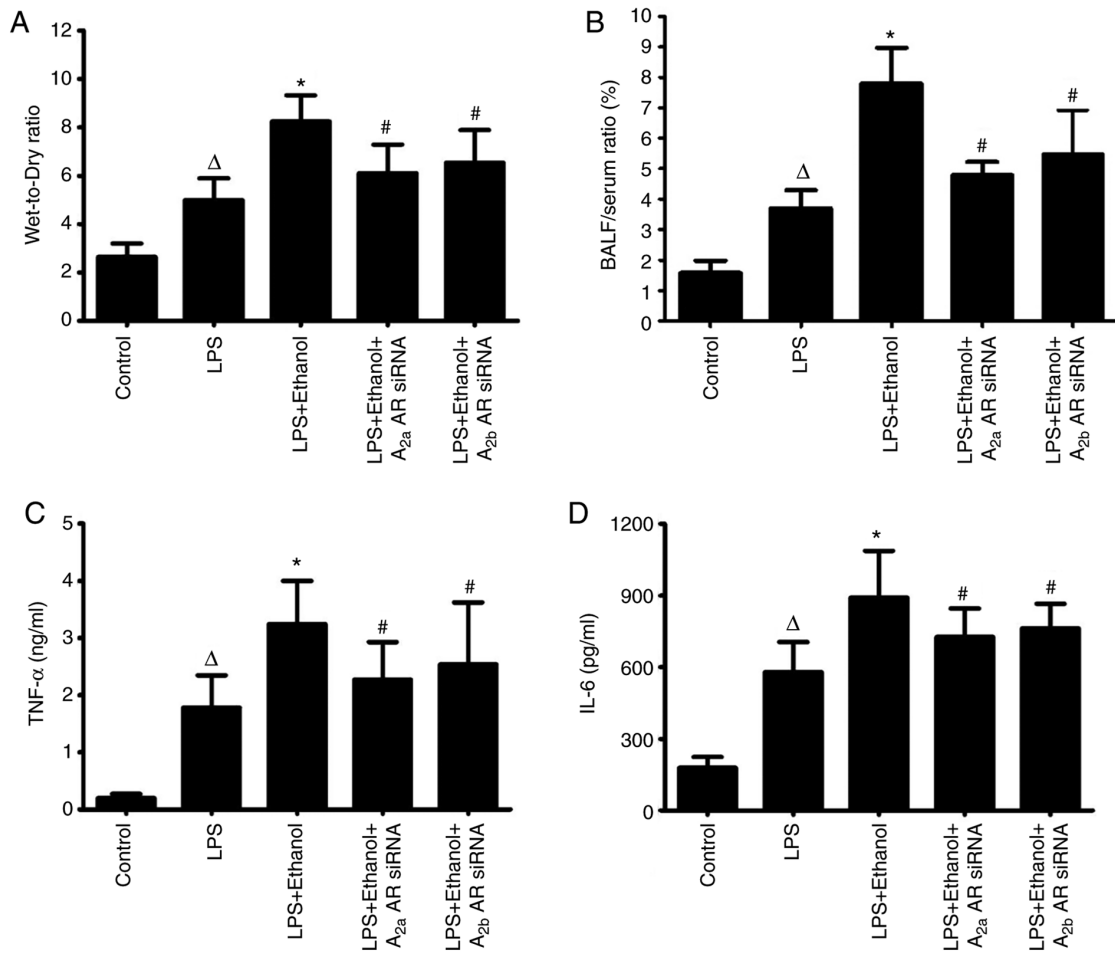

Figure 2. Effects of ethanol on pulmonary edema, bronchoalveolar epithelial permeability and inflammatory cytokines in LPS-induced lung injury. (A) Effects of ethanol on pulmonary edema in LPS-induced lung injury (n=6 per group). (B) Effect of ethanol on the pulmonary epithelial barrier in LPS-induced lung injury (n=6 per group). Bronchoalveolar epithelial permeability was determined by the fluorescent ratio of BALF to serum. Levels of (C) TNF-α and (D) IL-6 in BALF were investigated using ELISA kits (n=6 per group). Data are presented as the mean ± standard deviation. ΔP<0.05 vs. control; *P<0.01 vs. LPS; #P<0.05 vs. LPS+ ethanol. LPS, lipopolysaccharide; BALF, bronchoalveolar lavage fluid.

to the manufacturer's instructions. The concentration of each protein sample was determined using a BCA protein assay kit. Samples containing equal amounts (75-100 µg) of proteins

were separated by 10% SDS-PAGE and transferred to PVDF membranes using a semi-dry transfer apparatus (Bio-Rad Laboratories, Inc.). After blocking with 5% skimmed milk
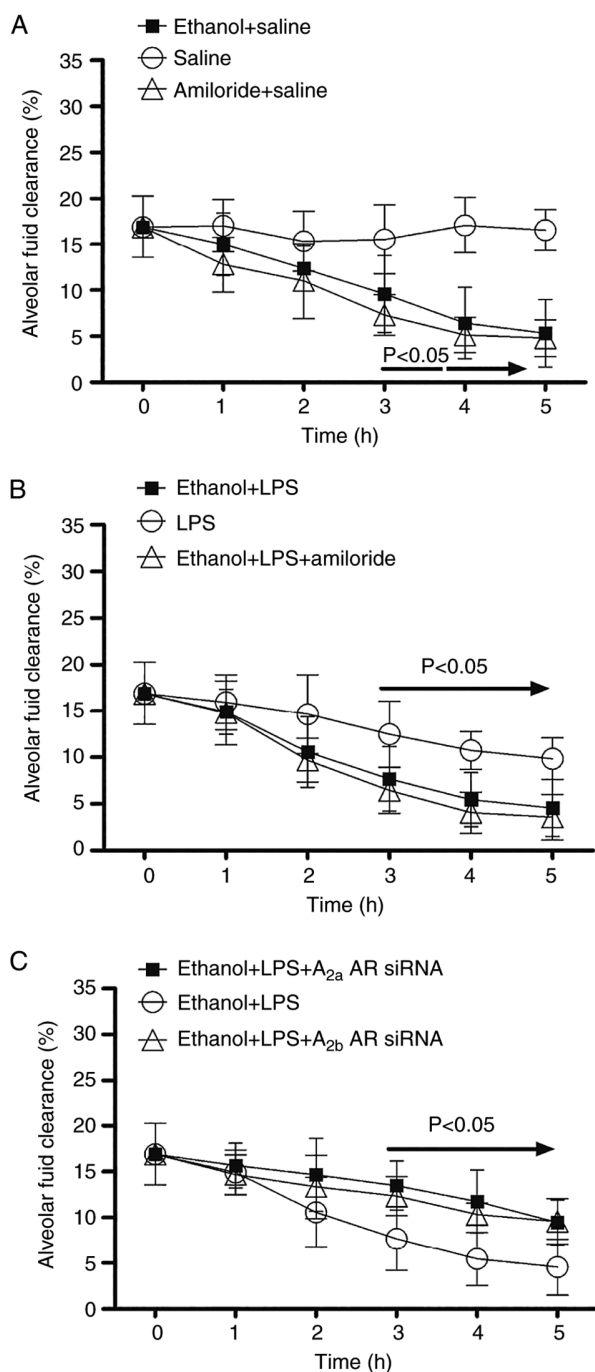
Figure 3. Effects of ethanol on AFC in LPS-induced lung injury (n=7 per group). (A) AFC in mice after ethanol ingestion or amiloride treatment, as indicated by the solid line. P<0.05 vs. mice treated with saline. (B) AFC in mice treated with ethanol or ethanol and amiloride following LPS-induced lung injury, as indicated by the solid line. P<0.05 vs. mice treated with LPS. (C) AFC in mice with LPS-induced lung injury treated with ethanol, following co-instillation of A2aAR or A2bAR small interfering RNA, as indicated by the solid line. P<0.05 vs. mice treated with LPS. Albumin solution containing amiloride ($5x10^{-4}$ M) was injected into the alveolar spaces. Data are presented as mean ± standard deviation. AFC, alveolar fluid clearance; LPS, lipopolysaccharide; AR, adenosine receptor.

in TBS containing 0.05% Tween-20 at room temperature, the membranes were incubated with α-ENaC antibody (1:1,000), β-ENaC antibody (1:1,000), γ-ENaC antibody (1:1,000) and β-actin (1:500) at 4˚C. Following the primary incubation, membranes were incubated with an HRP-conjugated

secondary antibody (1:5,000; cat. no. bs-0295M; BIOSS, Inc.) at room temperature and protein bands were detected using an Enhanced Chemiluminescence method (Nanjing KeyGen Biotech, Co., Ltd.). The protein bands were visualized using a UVP Gel imaging system (Analytik Jena AG) and analyzed by Quantity One software (version 4.4, Bio-Rad Laboratories, Inc.).

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical differences between multiple groups were analyzed using a one-way ANOVA followed by Tukey post hoc test. Single comparisons were performed using a Student's unpaired t-test. Statistical analysis was performed using GraphPad 5.0 Prism software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Alcohol inhibits cAMP accumulation in vivo and in vitro. The concentration of cAMP was significantly decreased in cells with LPS-induced injury following chronic alcohol ingestion, but the effect of alcohol on cAMP concentration was inhibited by co-instillation of A2aAR or A2bAR siRNA (Fig. 1A). Following incubation with alcohol, the concentration of cAMP was also significantly decreased in alveolar epithelial cells. The ethanol-induced decrease in cAMP concentration was significantly blocked by A2aAR or A2bAR siRNA (Fig. 1B).

Alcohol aggravates lung pulmonary edema in LPS-induced lung injury. Wet:dry lung weight ratio was significantly increased in mice with LPS-induced lung injury following alcohol ingestion. Following co-instillation of A2aAR or A2bAR siRNA, the wet: dry lung weight ratio was significantly decreased, indicating the alleviation of pulmonary edema in LPS-induced lung injury (Fig. 2A).

Alcohol disrupts the pulmonary epithelial barrier and increases the release of inflammatory mediators in LPS-induced lung injury. The pulmonary epithelial barrier was disrupted in mice with LPS-induced lung injury following alcohol ingestion, with a significantly high bronchoalveolar epithelial permeability. However, the bronchoalveolar epithelial permeability was decreased by A2aAR or A2bAR siRNA in LPS-induced lung injury following chronic alcohol treatment. This indicated the potential role of alcohol in altering the function of the pulmonary epithelial barrier (Fig. 2B). The levels of TNF-α and IL-6 in the BALF were markedly increased in LPS-induced lung injury compared with the control group. Alcohol ingestion significantly contributed to the release of inflammatory mediators during LPS-induced lung injury. The level of these inflammatory mediators was decreased following co-instillation of A2aAR or A2bAR siRNA (Fig. 2C and D).

Alcohol inhibits AFC in LPS-induced lung injury. Following alcohol ingestion or amiloride treatment, the rate of AFC in mice was significantly decreased at 3 h, compared with mice with saline treatment (Fig. 3A). Mice with LPS-induced lung injury treated with alcohol or a combination of alcohol
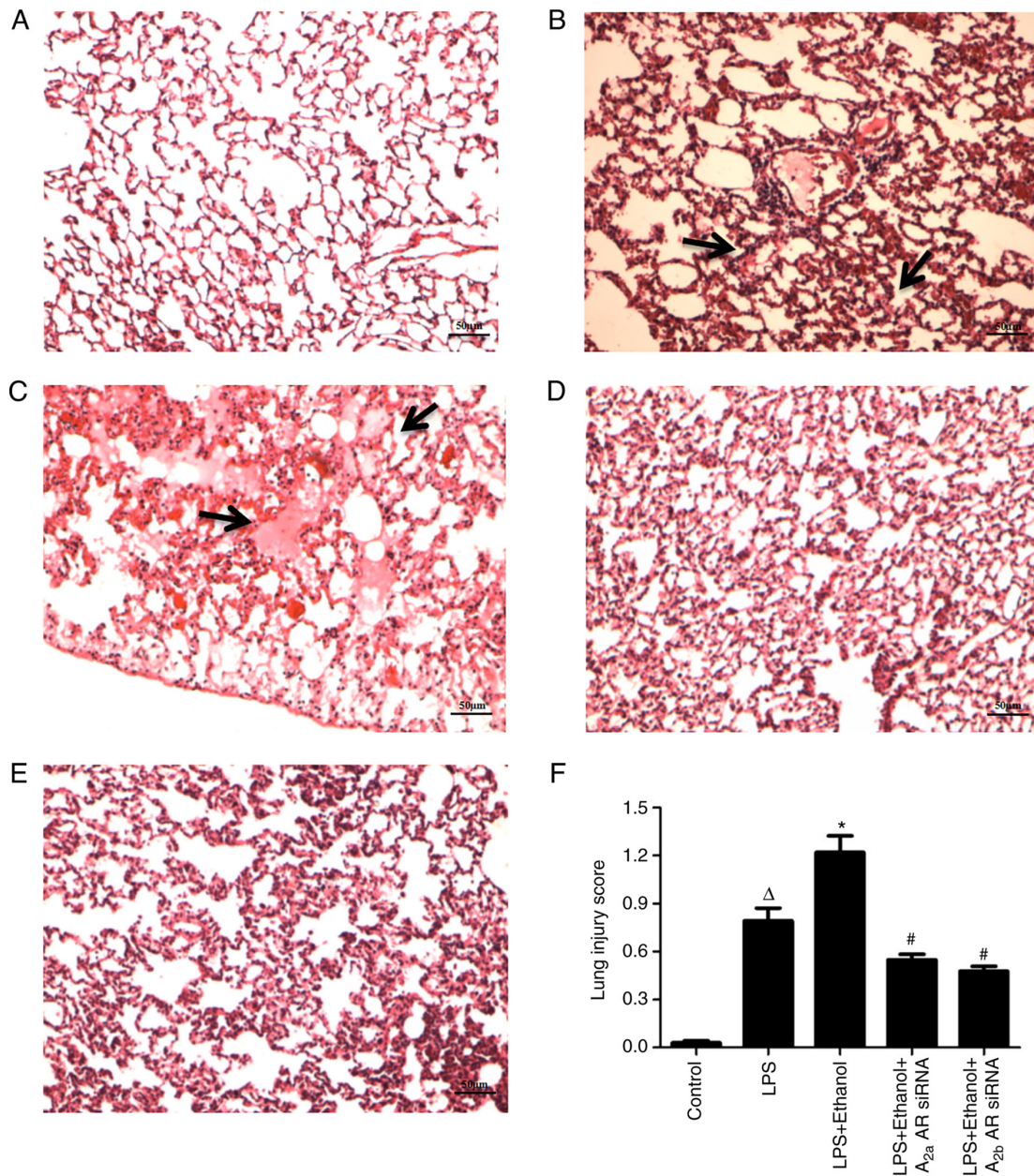
Figure 4. Effects of ethanol on pulmonary morphology in LPS-induced lung injury (n=7 per group). (A) Control group containing normal lung tissues from mice. (B) Tissues from the LPS group showing intra-alveolar exudate, inflammatory cells and red blood cell infiltration in the alveolar spaces (arrow). (C) LPS + ethanol group, with marked intra-alveolar exudate, edema with proteinaceous debris filling the airspaces and inflammatory cell infiltration in the alveolar spaces (arrow). (D) LPS + ethanol + A2aAR siRNA group, with alveolar edema and inflammation alleviated by A2aAR siRNA transfection. (E) LPS + ethanol + A2bAR siRNA group, with alveolar edema and inflammation relieved by A2bAR siRNA transfection. (F) Lung injury score. Five random areas were examined (magnification, x200). Data are presented as the mean ± standard deviation. $^{\Delta}$P<0.05 vs. control; *P<0.05 vs. LPS; $^{\#}$P<0.01 vs. LPS+ ethanol. LPS, lipopolysaccharide; siRNA, small interfering RNA; AR, adenosine receptor.

and amiloride demonstrated a significantly decreased AFC at 3 h compared with the control group (Fig. 3B). Following co-administration of A2aAR or A2bAR siRNA, the rate of AFC was significantly increased at 3 h in mice with LPS-induced lung injury following alcohol ingestion compared with the control group (Fig. 3C).

Alcohol aggravates LPS-induced lung injury. The lung tissue was significantly injured, as indicated by the presence of intra-alveolar exudate and inflammatory cell infiltration following LPS treatment (Fig. 4B-F). The presence of marked intra-alveolar edema with proteinaceous debris filling the airspaces and inflammation

infiltration in the alveolar spaces was significantly upregulated following alcohol administration in the LPS-induced ALI model (Fig. 4C-F). Lung injury was significantly attenuated by A2aAR or A2bAR siRNA (Fig. 4D-F). The pathobiology of lung injury was quantified using the lung injury score, which was determined for each group (Fig. 4F).

Alcohol decreases ENaC localization in LPS-induced lung injury. Immunohistochemical analysis was used to determine the lung distribution of α-ENaC (Fig. 5Aa-f), β-ENaC (Fig. 5Ba-f) and γ-ENaC (Fig. 5Ca-f) in the lung tissue of mice. The number of cells expressing α-ENaC, β-ENaC or
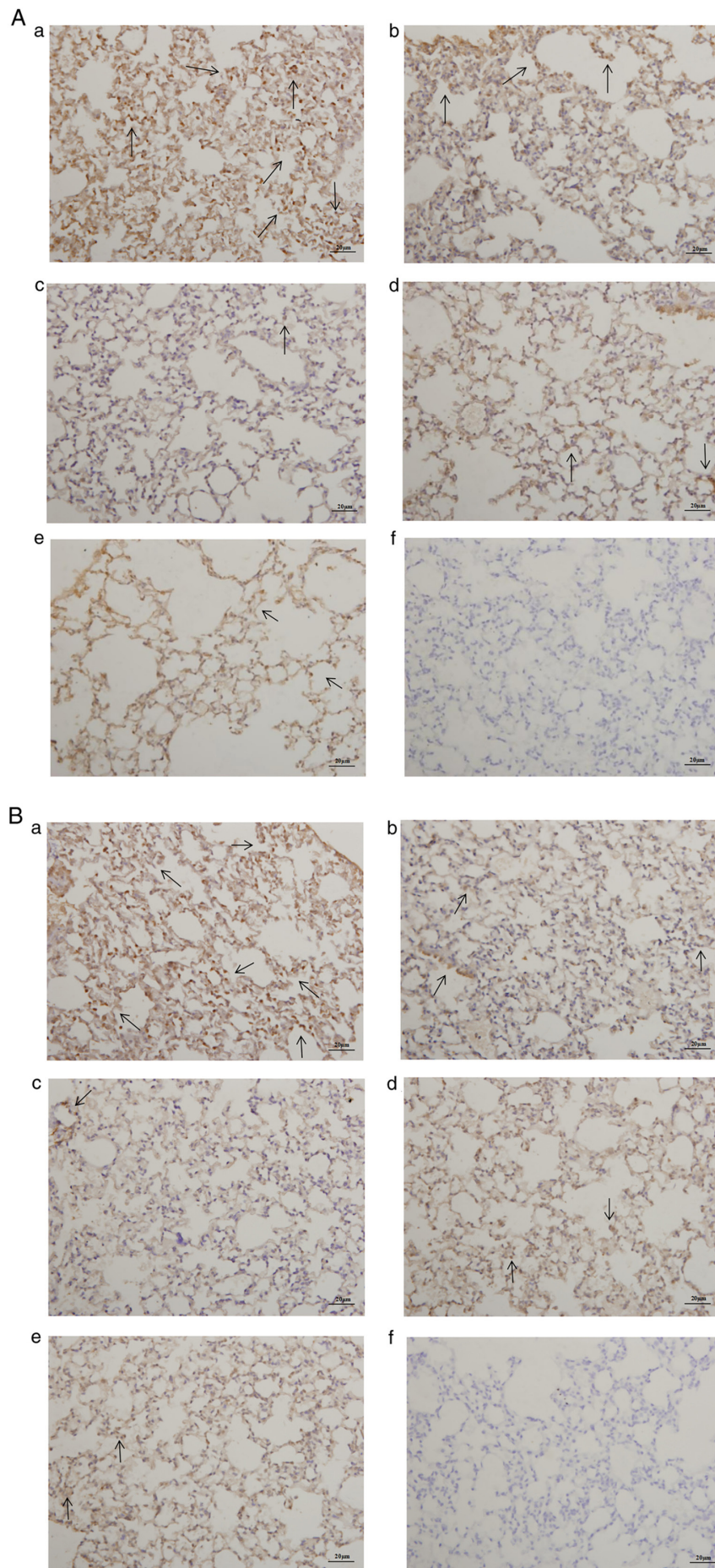
Figure 5. Continued.

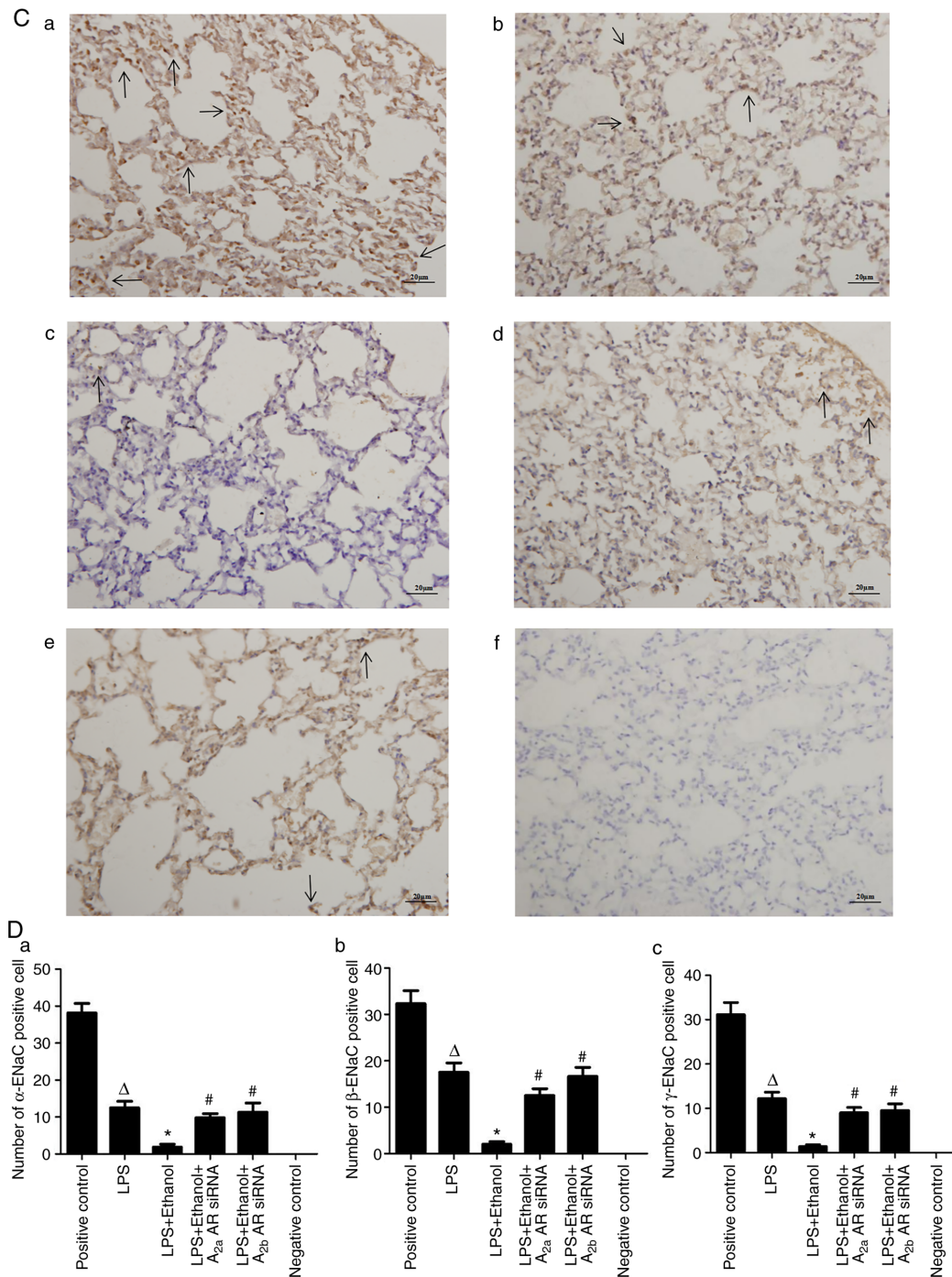Figure 5. Effects of ethanol on lung localization of (A) α-ENaC, (B) β-ENaC and (C) γ-ENaC in LPS-induced lung injury (n=6 per group). (a) Positive control group. (b) LPS group. (c) LPS+ ethanol group. (d) LPS + ethanol + A2aAR siRNA group. (e) LPS + ethanol + A2bAR siRNA group. (f) Negative control group. (D) Number of positive cells for (a) α-ENaC (b) β-ENaC and (C) γ-ENaC expression was counted in five random high-power fields of each section and the average was calculated. Cells stained brown were positive, indicated by the arrow (magnification, x400). Data are presented as the mean ± standard deviation. $^{\Delta}$P<0.05 vs. positive control; *P<0.05 vs. LPS; $^{\#}$P<0.05 vs. LPS + ethanol. ENaC, epithelial sodium channel; LPS, lipopolysaccharide; AR, adenosine receptor; siRNA, small interfering RNA.

γ-ENaC were significantly decreased in LPS-induced lung injury following alcohol administration, compared with those with ALI alone. However, the number of cells expressing α-ENaC, β-ENaC or γ-ENaC were significantly increased by A2aAR or A2bAR siRNA (Fig. 5Da, Db and Dc).

Alcohol inhibits ENaC mRNA and protein expression levels in LPS-induced lung injury. In vivo, the mRNA and protein

expression levels of α-ENaC, β-ENaC and γ-ENaC in the lung tissue of mice were significantly decreased in LPS-induced lung injury following chronic alcohol treatment. However, the mRNA expression levels of α-ENaC, β-ENaC and γ-ENaC were significantly increased by A2aAR or A2bAR siRNA (Figs. 6A and 7A). *In vitro,* the mRNA and protein expression levels of α-ENaC, β-ENaC and γ-ENaC were significantly decreased following alcohol treatment, but the effect of alcohol

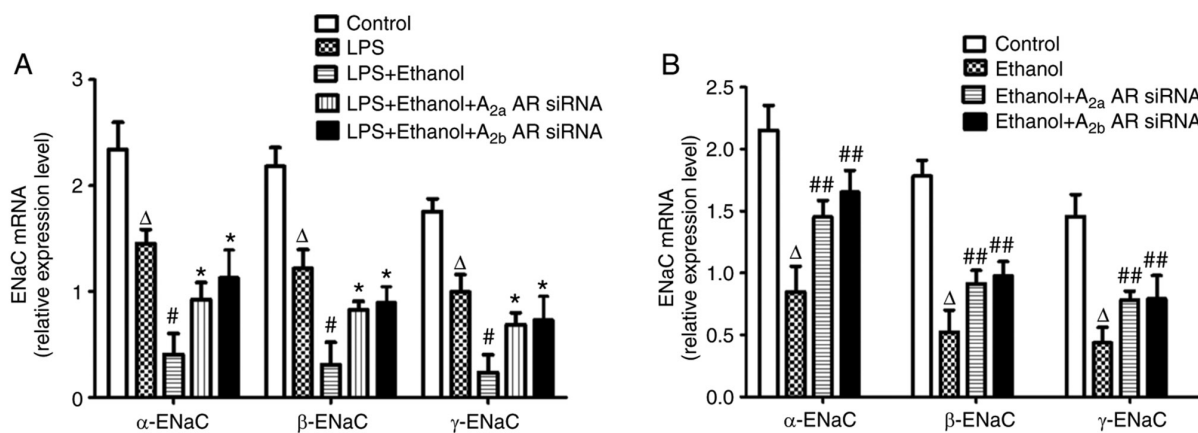Figure 6. Quantification of α-ENaC, β-ENaC and γ-ENaC gene expression using reverse transcription-quantitative PCR. (A) α-ENaC, β-ENaC and γ-ENaC mRNA expression levels were determined in the lung tissue of mice with LPS-induced lung injury following alcohol treatment (n=6 per group). (B) α-ENaC, β-ENaC and γ-ENaC mRNA expression levels were determined in alveolar epithelial cells following ethanol treatment. Values were normalized to endogenous GAPDH expression levels and were presented as the relative expression levels. The data represent three independent experiments. Data are presented as the mean ± standard deviation. $^{\Delta}$P<0.05 vs. control; $^{\#}$P<0.05 vs. LPS; *P<0.05 vs. LPS + ethanol; $^{\#\#}$P<0.05 vs. ethanol. ENaC, epithelial sodium channel; LPS, lipopolysaccharide.
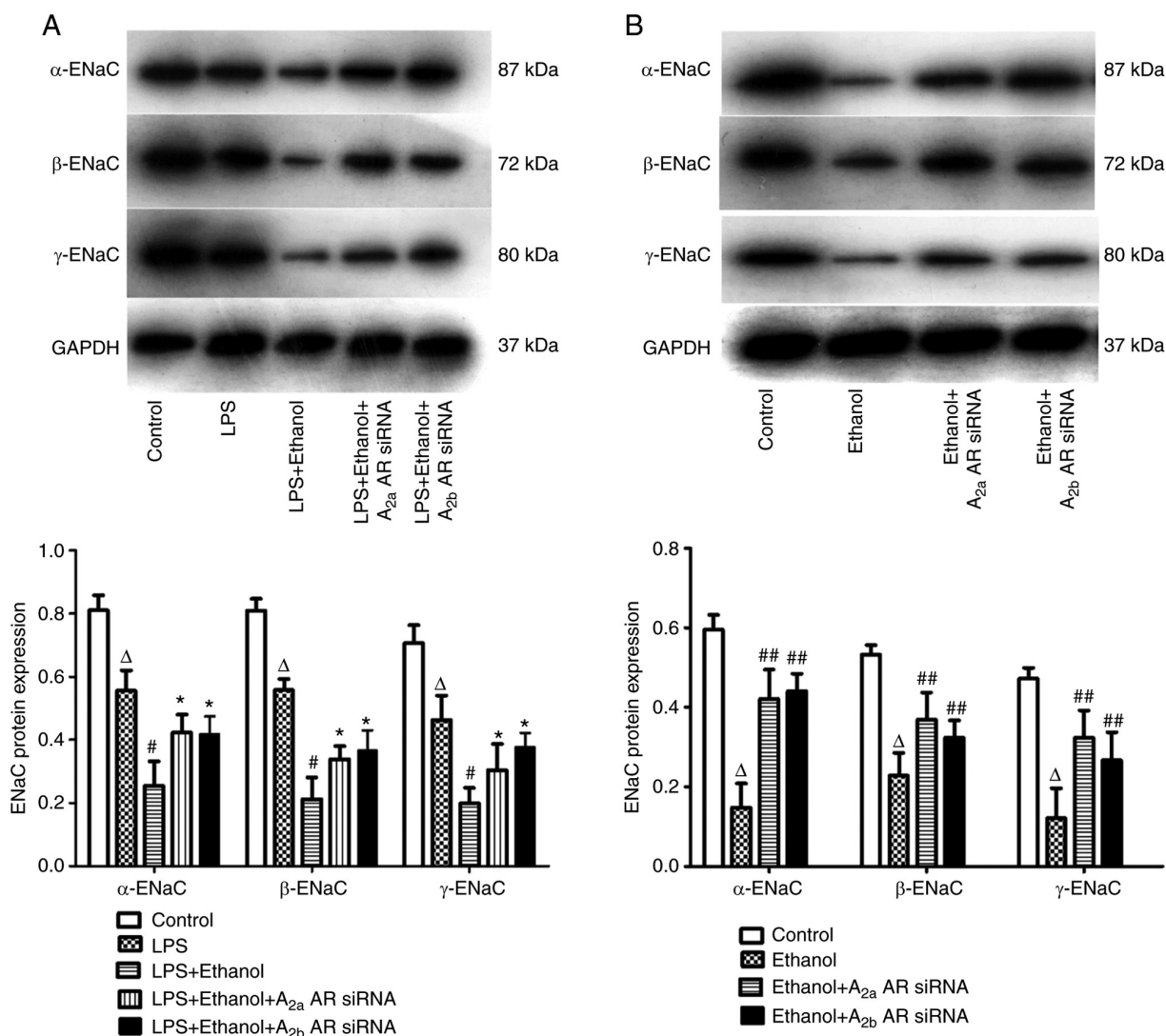
Figure 7. Determination of α-ENaC, β-ENaC and γ-ENaC protein expression levels using western blotting. (A) α-ENaC, β-ENaC and γ-ENaC protein expression levels were detected in the lung tissue of mice with lipopolysaccharide-induced lung injury following alcohol treatment (n=6 per group). (B) α-ENaC, β-ENaC and γ-ENaC protein expression levels were detected in alveolar epithelial cells following ethanol treatment. The data represent three independent experiments. The band intensities of α-ENaC, β-ENaC and γ-ENaC were normalized to GAPDH. Data are presented as the mean ± standard deviation. $^{\Delta}$P<0.05 vs. control; $^{\#}$P<0.05 vs. LPS; *P<0.05 vs. LPS + ethanol; $^{\#\#}$P<0.05 vs. ethanol. ENaC, epithelial sodium channel.
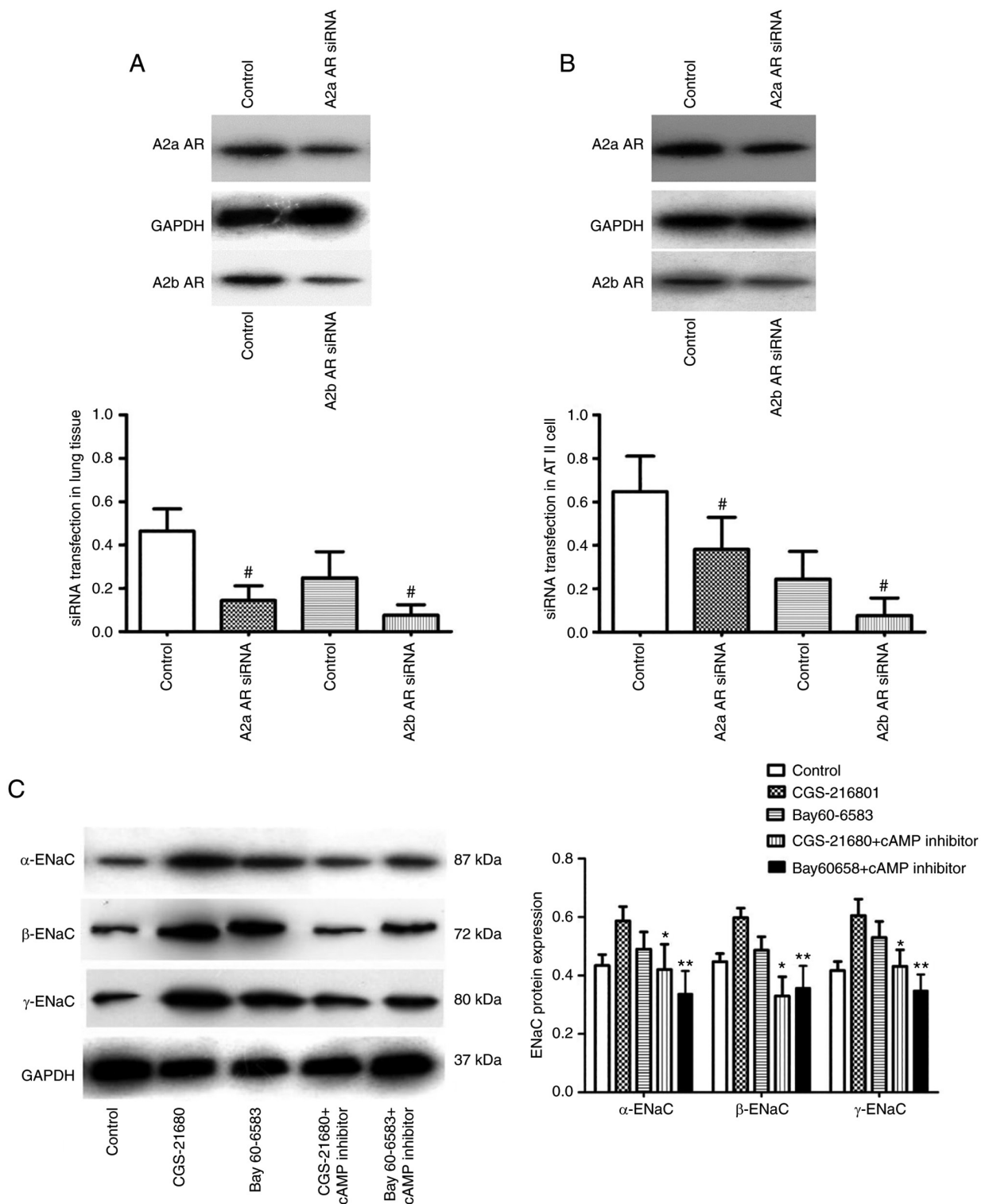
Figure 8. Effects of siRNA-mediated knockdown of A2aAR and A2bAR in (A) lung tissue of mice or (B) alveolar epithelial cells. A2aAR and A2bAR expression levels were determined following siRNA transfection or the transfection agent alone by western blot analysis. (C) Alveolar epithelial cells were incubated with 100 μmol/l CGS-21680, or 100 μmol/l Bay 60-6583 for 8 h prior to cAMP inhibitor treatment (500 nmol/l) for 15 min, to detect α-ENaC, β-ENaC and γ-ENaC protein expression levels. The data represent three independent experiments (n=3 per group). The band intensities of α-ENaC, β-ENaC and γ-ENaC were normalized to GAPDH. Data are presented as the mean ± standard deviation. [#]P<0.05 vs. control. [*]P<0.05 vs. CGS-21680; [**]P<0.05 vs. Bay 60-6583. siRNA, small interfering RNA; AR, adenosine receptor; ENaC, epithelial sodium channel; cAMP, cyclic adenosine monophosphate.

on ENaC mRNA expression levels was prevented by A2aAR or A2bAR siRNA (Figs. 6B and 7B).

Alcohol regulates ENaC protein expression levels by the A2aAR or A2bAR-mediated cAMP pathway. The transfection efficiency of A2aAR or A2bAR siRNA was determined *in vivo* and *in vitro*. The successful transfection of A2aAR and A2bAR siRNA was determined in the lung tissue of mice and ATII cells by western blot analysis (Fig. 8A and B). The protein expression level of α-ENaC was significantly increased

following treatment with CGS-21680 or Bay 60-6583 in alveolar epithelial cells incubated with alcohol. The protein expression levels of α-ENaC, β-ENaC and γ-ENaC were significantly decreased by the cAMP inhibitor following incubation with CGS-21680 or Bay 60-6583 (Fig. 8C).

Discussion

A number of clinical studies have demonstrated the association between chronic alcohol abuse and ARDS (32-35) and chronic alcohol consumption has been implicated as a positive risk factor in the development of ARDS. Chronic alcohol abuse is associated with poor outcomes in patients with ARDS; however, the mechanisms underlying the effects of alcohol in ARDS remain unclear (36). The mechanistic associations between alcohol and ARDS may contribute to alveolar epithelial dysfunction through a number of mechanisms, including via systemic effects on vascular tone and fluid retention as well as inducing the apoptosis of alveolar epithelial cells (37). Furthermore, alveolar epithelial dysfunction is an important factor in the decrease of AFC in ARDS (10). In the present study, the association between chronic alcohol abuse and AFC was the key focus.

It is well known that alcohol is absorbed and transported throughout the body in an unbound and unaltered state, such as in the lungs where it achieves the high concentrations of alcohol without being immediately metabolized (38). The consequence of chronic ethanol ingestion was investigated in mice exposed to LPS, a well-recognized murine model of ALI. The lungs may be particularly susceptible to damage due to their delicate architecture, numerous resident inflammatory cells and a high degree of vascularization (39). It is well known that the intestine contains an enormous potential reservoir of bacteria and bacterial products for systemic infections and sepsis following injury (40,41). The results of previous animal studies demonstrated that post-burn intestinal permeability was exacerbated when intoxication preceded the injury, leading to the greater translocation of bacteria and bacterial products, such as LPS, into the lung (42,43). LPS stimulation produces pro-inflammatory cytokines, such as TNF-a and IL-6, that recruit polymorphonuclear neutrophils into the injured lung and contribute to the pathogenesis of ALI and ARDS (44,45). In addition, activated neutrophils transmigrate across the endothelial surface into the lung by the release of reactive oxygen species, resulting in alveolar capillary barrier damage and interstitial and alveolar edema following adherence to the lung endothelium (46). In the present study, alcohol administration was accompanied by an increase in pulmonary edema and a reduction in the AFC. A large number of inflammatory mediators were released during alcohol administration following LPS-induced lung injury. The evaluation of the pulmonary epithelial barrier and the histological lung injury score revealed the effects of chronic alcohol on ALI. However, inhibition of A2aAR or A2bAR attenuated alcohol-induced lung injury, which suggested the involvement of the A2aAR or A2bAR pathway.

Though the ingestion of alcohol was found to regulate AR via changes in the systemic levels of adenosine, the association of A2aAR or A2bAR and alcohol had not yet been elucidated (47). AFC is an important procedure involving the removal of edema fluid from the alveolar spaces via the ENaC in ALI/ARDS (48). The AR is made up of seven-transmembrane spanning receptors, otherwise known as G-protein coupled receptors and four AR subtypes; A_1, A_{2a}, A_{2b} and A_3 have been identified in lung tissue (49). The important contribution of A2aAR or A2bAR signaling has been implied in AFC (49-51). The results of the present study demonstrated that chronic alcohol consumption decreased AFC in ALI, while A2aAR or A2bAR siRNA inhibited the effect of alcohol on AFC. The results illustrated that alcohol impaired AFC via A2aAR or A2bAR. Furthermore, amiloride, a sodium channel inhibitor, blocked alcohol-induced AFC, supporting the hypothesis that ENaC participates in ALI following chronic alcohol ingestion. Therefore, the association between ENaC and alcohol was further investigated in the present study. In vivo, the expression levels of α-ENaC, β-ENaC and γ-ENaC were decreased in ALI following alcohol administration, but were increased by the intervention with A2aAR or A2bAR siRNA. In vitro, alcohol administration decreased the expression levels of α-ENaC, β-ENaC and γ-ENaC and the aforementioned decrease was inhibited by A2aAR or A2bAR silencing. The results demonstrated that ENaC was inhibited by A2aAR or A2bAR in ALI following alcohol administration.

cAMP is a ubiquitous second messenger derived from adenosine triphosphate that serves important regulatory roles in diverse biological processes, including learning and memory (52). cAMP is generated via the binding of a ligand to the membrane-bound G protein-coupled receptors. Ethanol alters the cAMP signaling pathways by G protein-related adenylyl cyclase activity in animal models as well as in model cell culture systems (53). In the present study, alcohol reduced cAMP levels both in vivo and in vitro, which was consistent with the findings of previous studies (54,55). By inhibition of A2aAR or A2bAR, the systemic cAMP levels were increased in the present study. Furthermore, the expression levels of α-ENaC, β-ENaC and γ-ENaC were decreased by a cAMP inhibitor following A2aAR or A2bAR siRNA administration. Activation of A2aAR or A2bAR enhanced ENaC activity by elevating cAMP levels to promote AFC in ALI (50,51,56). Collectively, these results confirmed that alcohol inhibited ENaC through the A2aAR or A2bAR-mediated cAMP pathway. Therefore, A2aAR or A2bAR may serve as potential therapeutic targets for the treatment of ALI following chronic alcohol consumption.

In addition, previous studies have investigated the mechanism underlying alcohol in pulmonary immune dysfunction (57,58) and alveolar epithelial dysfunction (37,59) during the development of ARDS. The association between chronic alcohol consumption and AFC in ARDS remains to be elucidated. Dada et al (47) reported that alcohol reduced AFC via a mechanism that involved the downregulation of the sodium potassium pump via the AMP-activated protein kinase pathway in hypoxia-induced lung injury. In another study, chronic alcohol consumption leads to the altered regulation of α-ENaC in the alcohol-induced damaged lung without ARDS (60). In the present study, alcohol inhibited AFC and ENaC through the A2aAR or A2bAR-mediated cAMP pathway. However, previous studies have reported that ethanol activated ENaC by increasing the release of intracellular reactive oxygen species

via the increased production of the metabolic product, acetaldehyde, in A6 distal nephron cells. This stimulation of ENaC was due to tissue differentiation, acute ethanol exposure and high concentrations of ethanol (61,62). Thus, further investigation is required to determine the effects of ethanol on ENaC in the whole organism.

In conclusion, the results of the present study demonstrated that alcohol worsened LPS-induced lung injury in mice, further aggravating pulmonary edema and inhibiting AFC. Alcohol prevented the expression of ENaC-associated proteins via the A_2AR-mediated cAMP pathway. The results further indicated that therapies which target A2aAR and/or A2bAR may be beneficial for the treatment of alcohol abuse-associated ARDS. In future studies, clinical research will be performed to investigate the outcome in alcoholic patients with ARDS.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WD, DQ and DXW participated in the design of the study. WD and JH performed the animal study including the animal model, cAMP assay, pulmonary edema, bronchoalveolar epithelial permeability, lung histopathology and AFC measurement. WD and XMT performed the cell culture and transfection experiment. CYL performed the immunocytochemistry and RT-qPCR. JT performed the western blotting. DQ performed the data collection and statistical analysis. WD interpreted the data and drafted the manuscript. DXW contributed to the critical revision of the manuscript. WD, DQ and DXW confirm the authenticity of all the raw data. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University (approval no. 2019-009). All animal experiments were conducted according to relevant national and international guidelines.

Patient consent for publication

Not applicable

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