# Dexmedetomidine alleviates pulmonary edema through epithelial sodium channel (ENaC) via the PI3K/Akt/Nedd4-2 pathway in LPS- induced acute lung injury

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## Abstract

Background:  Pulmonary edema is a hallmark in acute lung injury(ALI). Researchers have also revealed that dexmedetomidine (Dex) alleviate pulmonary edema following ALI, but the mechanism is unclear.The alveolar epithelial sodium channel (ENaC)-mediated alveolar fluid clearance (AFC) plays an important role in reducing pulmonary edema. In this study, we attempted to investigate the effect of Dex on ENaC in modulating AFC and its mechanism. Methods:  LipopolysacchAride (LPS) was used to induce ALI in rat and alveolar epithelial cell injury in A549 cell. The rats were randomly allotted into the following groups: control, LPS, LPS+Dex, LPS+Dex+LY294002 (n = 6 per group). In vitro, cells (1×10 6 cells/cm 2 ) were subcultured in six-well plates, then cells were allotted into the following groups: control, LPS, LPS+Dex, LPS+Dex+LY294002. Results: In vivo, Dex markedly reduced pulmonary edema induced by LPS through promoting AFC.Moreover, Dex prevented LPS-induced downregulation of α-, β- and γ-ENaC expression. In A549 cells stimulated with LPS, Dex attanuated LPS-mediated cell injury and the downregulation of α-, β- and γ-ENaC expression. Howere, all of which was blocked by PI3K inhibitor LY294002,suggesting that the protective role of Dex is PI3K dependent. Additionaly, Dex increases the expression of phosphorylated Akt and reduces the expression of Need4-2 in vivo and vitro, while the LY294002 reverses the effect of Dex, indicating that Dex activates the PI3K/Akt/Nedd4-2 signaling pathway. C onclusio ns: Dex alleviates pulmonary edema by promoting AFC, and the mechanism is partly related to up-regulation of ENaC expression via PI3K/Akt/Nedd4-2 signaling pathway.


## Background

Acute lung injury (ALI) is characterized by acute, diffuse and inflammatory lung injury. Clinically, ALI manifests as severe respiratory distress and intractable hypoxemia. At present, lung protective ventilation support is beneficial to ALI patients [1,2], but there is no effective drug therapy. Despite the efforts made to cure ALI, but its clinical mortality has remained high in the past decades [3]. Pulmonary edema is the central link to the pathogenesis of ALI, which is associated with alveolar epithelial injury and impaired alveolar fluid clearance (AFC) [4]. Studies have shown that maximum AFC has lower mortality and requires less mechanical ventilation time [5]. Therefore, the timely and effective removal of excess liquid from alveoli are key goals in the treatment of ALI.

The clearance of alveolar water is related to ENaC in alveolar epithelial cells [6,7]. Sodium ions ($Na^+$) are actively transported into cells through ENaC, and then $Na^+$ is pumped into the interstitium through the action of Na, K-ATPase, which leads to an osmotic gradient that drives the transfer of water into the interstitium and into the blood circulation through aquaporin (AQP) [8]. Current research suggests that pulmonary edema may be attributed to the flammatory response during ALI. Several inflammatory cytokines, including TNF-α and IL-1β may affect ENaC expression [9-11]. Many studies have indicated that inhibiting the release of inflammatory cytokines may promote AFC by increasing the expression of ENaC[12]. So, inhibition of inflammatory response may be beneficial for upregulating ENaC expression and reducing pulmonary edema.

Dexmedetomidine (Dex), a highly selective $\alpha_2$-adrenegicreceptor ($\alpha_2$AR) agonist, can reduce pulmonary edema through inhibiting the production of inflammatory cytokines, including TNF-α, IL-1β and IL-6 in ALI model[13-16]. Consistent with these studies, our previous studies have found that Dex can attanuate pulmonary edema in LPS-induced ALI, and improve the $PaO_2$, reduce the inflammatory response. Further research found that: Dex increase the expression of aquaporin1 (AQP1) and aquaporin5 (AQP5) in lung tissue [17], indicating that the effect of Dex in reducing pulmonary edema may be related to the promotion of AFC. As 90% of water transport resistance comes from ENaC-mediated $Na^+$ transport, it is reasonable to speculate that Dex may alleviate pulmonary edema by stimulating AFC through the upregulation of ENaC expressionedema.

Previous studies have shown that the continuous stimulation of the PI3K/Akt signaling pathway promotes $Na^+$ absorption in epithelial cells [18].Other studies have found that insulin and RvD1 can activate the PI3K/Akt signaling pathway, upregulate the expression of ENaC and promote AFC [19,20]. Furthermore, Akt increased ENaC activity by inducing Nedd4-2 expression, thereby increasing $Na^+$absorption [21]. Recent studies have found that Dex reduces LPS-induced ALI by activating the PI3K/Akt signaling pathway [15].These results promote us to hypothesize that effect of Dex on ENaC may be related to PI3K/Akt/ Need4-2 signaling pathway.

Collectively, this study was designed to investigate whether Dex can reduce pulmonary edema by promoting AFC in LPS-induced ALI in rats. Additionally, we studied the effect of Dex on ENaC expression and the role of PI3K/Akt/Need4-2 signaling in these effects.

## Materials And Methods

### Drugs and Reagents

LPS (Escherichia coli 055:B5), Dexmedetomidine, Evans blue, CCK8 was obtained from Sigma (St. Louis, MO, USA). LY294002 was obtained from MCE (Monmouth Junction, NJ,USA).Cytokines (TNF-α, IL-1β, IL-6, IL-10) and myeloperoxidase (MPO) enzyme-linked immunosorbent assay (ELISA) detection kits were obtained from Jianglai Biotechnology (Shanghai, China). A549 cells were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). LDH assay kit, anti- αENaC antibody, anti-β ENaC antibody, anti-γ ENaC, HRP labelled goat anti-rabbit IgG antibody were purchased from Abcam (Cambridge, UK). Anti-p-Akt antibody , anti-Akt antibody , Anti-Nedd4-2, and anti-β-actin antibody were purchased from CST (Boston, MA, USA).

### Animals

The Medical Faculty Ethics Committee of Shenzhen People's Hospital , Shenzhen, China, approved all the animal procedures and care. The animal experiments complied with the Guidelines for the Care and Use of Laboratory Animals from the NIH. SPF grade male Wistar rats (6 weeks old, weighing 180 ~ 220 g) were purchased from Guangdong Medical Animal Experiment Center (Guangzhou, China). Animal handling was conducted according to the requirements of the animal protection committee of the Second Clinical Medical College Of Jinan University. All animals were housed in an air-conditioned room under a 12-hours dark/light cycle and were granted free access to water and food.

### Experimental protocols

The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p. injection). The skin of the left thigh was disinfected with ethanol, the skin was cut open, the femoral vein was exposed, a 24# trocar was inserted and fixed, and then LPS (20mg/kg) was injected ( approximately 10 minutes) to induce ALI. After LPS or saline injection, the neck skin was disinfected, the trachea was exposed and a homemade tracheal catheter was inserted. At 8 h after LPS administration, all the rats were sacrifificed. The study consisted of two parts. The fifirst part was the survival study, for which 10 animals were used in each group.The survival rate of rat was recorded every 2 h for 3 days after LPS administration, and determine the dosage of Dex.

In the second part, the rats were divided into four groups (n=6 per group): the control group, LPS group, LPS+Dex group, and LPS+Dex+ LY294002 group. The rats in the control group were intravenously administered 0.9% normal saline (5 ml/kg). The rats in the LPS group were intravenously administered 20 mg/kg LPS. The rats in the LPS+Dex group were intravenously administered 20 mg/kg LPS and then intraperitoneally administered 100 μg/kg Dex. The rats in the LPS+Dex+LY294002 group were intravenously administered 3mg/kg LY294002 30 min prior to LPS injection and then administered 100 μg/kg Dex.

### Histopathological studies

The lower lobe of the right lung was taken and fixed with 10% neutral formaldehyde for 24h, embedded in paraffin and stained with H&E for light microscopy analysis. Histological lung injury was scored based on alveolar edema, pulmonary capillary congestion, neutrophil infiltration, and the thickness of the alveolar septum in five random fields in a blind manner using light microscopy. Lung sections were scored as 1 (no or very slight pathological changes), 2 (slight pathological changes), 3 (moderate pathological changes), or 4 (severe pathological changes). Evaluation scores were added to the total injury score.

### Measurement of cytokines

After the rats were sacrificed, the main bronchus were exposed. The right bronchus was ligated, and a homemade tracheal catheter was inserted into the main bronchus. Then, 2 mL cold phosphate-buffered saline (PBS) was infused into the left lung and extracted three times. The bronchoalveolar lavage fluid ( BALF) was centrifuged at 1200×g for 10 min at 4°C. The supernatant was separated into aliquots and stored at -70°C. An aliquot of BALF supernatant was used to assay the levels of TNF-α, IL-1β, IL-6, IL-10 by ELISA according to the manufacturer's instructions.

### MPO activity assay in lung tissues

The middle lobe of the right lung was removed immediately after the animals were exsanguinated. Then, the activity of MPO in the lung tissue was determined by ELISA according to the manufacturer's instructions.

### Arterial oxygen tension ($PaO_2$) assay

Arterial blood (0.5 ml) was extracted from the right common carotid artery before the rat was sacrificed for blood gas analysis, and the $PaO_2$ was measured with a blood gas analyzer.

### Measurement of lung wet/dry(W/D) weight ratio

At the end of the experiments, we immediately removed the upper lobe of the right lung, and precisely measured the lung wet weight. After that, we placed the lung tissues in a 75°C constant temperature oven for 24 h, and measured the lung dry weight. Finally, the W/D ratio was calculated to evaluate the extent of pulmonary edema.

### Measurement of alveolar fluid clearance (AFC)

The AFC was determined by Evans blue- tagged albumin concentration .The 5% bovine serum albumin perfusion solution labeled with Evans blue was injected (5ml/kg) into the left lung via the trachea, and 2 ml oxygen was injected to facilitate distribution. Rats were ventilated with 100% oxygen, positive end expiratory pressure was kept at 2~3 cm $H_2O$ during the baseline period to maintain lung tension. These tissue units were wrapped with plastic wrap and then incubated in a 37°C water bath for 1 hour. The alveolar fluid was immediately aspirated, and labeled albumin was measured by a spectrophotometer at a 620nm. AFC was calculated based on the following formula: AFC (%) = $[(C_f - C_i) / C_f] \times 100\%$, where Ci represents the concentration of injected Evans blue-labeled 5% albumin and $C_f$ represents the final concentration of Evans blue-labeled 5% albumin.

### Cells culture and treatment

A549 cells were seeded in culture dishes at a density of $1\times 10^6$ cells/$cm^2$ and cultured in a 5% $CO_2$ and 95% air atmosphere in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum, 0.1 mg/ml streptomycin, and 100 U/ml penicillin. The culture medium was changed every two days. For all experiments, cells were subcultured in six-well plates. Once the cells reached 80% confluence, they were serum-starved for 24 h. Following starvation, the cells were treated as follows: (1) the cells in the control group were treated with PBS for 12 hours; (2) the cells in the LPS group were treated with LPS (1 μg/ml) for 12 hours; (3) the cells in the LPS+Dex group were treated with LPS(1 μg/ml) and the Dex (10 μM) for 12 hours; (4) the cells in the LPS+Dex+ LY294002 group were treated with LY294002 (10 μM) 30 min prior to LPS (10 μM) administration and were then treated with Dex (10 μM) for 12 hours .

### Cell viability assay

CCK8 assay was performed to measure cell viability.The cells (100μl/well) were cultured in a 96-well plate for 24 hours. Then the cells were incubated with 1 μg/ml LPS for 12 h in the absence or presence of Dex, following the 10μl CCK8 solution was added to each well, the culture plate was incubated in the

incubator for 2 hours under 5% CO2 at 37˚C, and the absorbance at 490nm was measured with a microplate reader.

### LDH activity assay

The cells(100 μl/ well) were cultured in a 96-well plate for 24 hours. Then the cells were incubated with 1 μg/ml LPS for 12 h in the absence or presence of Dex,The supernatant was collected to measure lactate dehydrogenase (LDH) activity by using LDH Assay kit according to the manufacturer's instructions. The absorbance at 490nm was measured with a microplate reader

### Immunohistochemistry analyses

Lung tissues were fixed in 10% neutral formaldehyde solution, and paraffin tissue sections were produced. Paraffin sections were then baked overnight in a 60°C oven, dewaxed with dimethyl benzene, dehydrated with in gradient ethanol solutions, repaired with 500 ml EDTA antigen repair solution, treated with a drop of 50 μl 3% hydrogen peroxide solution at room temperature for 20 min to block the activity of endogenous peroxidase and rinsed with TBS 3 times (3 min each time). Then, 5% normal goat serum solution was added at room temperature for 20 min, and the superfluous liquid was discarded without washing. Diluted primary antibody was added, and the tissues were incubated at room temperature for 30 min and washed with TBS 3 times (3 min each time). Secondary antibody (biotinylated goat anti-rabbit IgG) was added, and the tissues were incubated at room temperature for 30 min and washed with TBS 3 times (3 min each time). After that, the cells were stained with 3,3̓-diaminobenzidine for 3~5 min. PBS was used instead of primary antibody for the negative control group. The average optical densities (AODs) of α-, β-, γ-ENaC were measured by an imaging analysis system .

### Western blotting analysis in rat lung tissues and A549cells

Proteins were obtained with RIPA lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, and leupeptin) and PMSF. The protein concentrations of the supernatants were determined by using a BCA protein assay kit . The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. Tris buffer solution containing 5% skim milk powder was used to seal the membrane for 1 h at room temperature. The membrane was blocked with PBS containing 0.05% Tween 20 and washed with PBST 5 times (5 min each time). Then, the membrane was incubated overnight at 4°C with the following primary antibodies: α-, β-, and γ-ENaC, Akt, p-Akt, Need4-2 and β-actin.The membrane was incubated with horseradish peroxidase (HRP)-labeled goat anti- rabbit antibody at room temperature for 3h, and the membrane was washed with PBST 5 times(5min each time). Finally, the bands were visualized using an enhanced chemiluminescence kit (ECL) by UVP gel imaging system. The band intensities were analyzed by Image J Software .

### Statistical analysis

All data are expressed as the mean ± standard deviation (SD). The significance of the differe- nces among the four groups was tested using one-way ANOVA followed by a least significant difference (LSD) multiple comparison test. Survival analysis was done using the Kaplan-Mei- er method, and comparisons between groups were made using the log rank test. A two-side *p* value less than 0.05 was considered statistically significant.

## Results

### Dexmedetomidine increased survival rate in LPS- induced ALI

To determine the therapeutic effect of Dex on ALI, we observed the effect of Dex on survival rate in LPS-induced ALI in rats. As shown in figure 1, Dex has a dose-dependent effect on survival rate in rats with ALI , and 100μg/kg of Dex has the best therapeutic effect Therefore, 100μg/kg Dex was used in vivo.

### Dexmedetomidine alleviated LPS-induced ALI

To evaluate whether Dex can alleviate LPS-induced ALI, we first evaluated pulmonary histological alterations by HE staining. In the control group, the lung structure was intact, the alveolar cavity was clear, and was free from inflammatory cell infiltration. Compared with control, LPS induced significant changes in lung injury, namely interstitial edema, alveolar septum thickening, a large number of inflammatory cells infiltration, as evidenced by an increase in lung injury score. Dex treatment significantly alleviated the pathological alterations induced by LPS (Fig 2A). Subsequently, we measured the lung injury score and MPO, and the results showed that the LPS group had the highest lung injury score and MPO levels, Dex treatment reduced lung injury score(Fig 2B) and MPO levels (Fig 2C). However, the Dex-induced protection against lung injury was blocked by treatment with LY294002, sugesting that Dex provides a protective role against LPS-induced ALI in a PI3K dependent manner.

### Dexmedetomidine reduced the inflammatory response in LPS-induced ALI

LPS induces neutrophil activation, which in turn releases inflammatory cytokines that damage alveolar epithelial cells, leading to increased pulmonary edema. We next analyzed the effect of Dex on the concentration of TNF-α、IL-1β、IL-6 and IL-10 in bronchoalveolar lavage (BALF). Compared with the control group, the concentration of TNF-α,IL-1β、IL-6 and IL-10 in BALF was increased in the LPS group, while Dex alleviated the concentration of TNF-α (Fig 3A), IL-1β (Fig 3B), IL-6 (Fig 3C) and increased the concentration of IL-10 ( (Fig 3D). However, the PI3K inhibitor LY294002 partially reverses these effect of Dex .

### Dexmedetomidine alleviated pulmonary edema and promotes alveolar fluid clearance in LPS-induced ALI

Pulmonary edema is a landmark event in ALI, and it is the main cause of hypoxemia. We calculated the effect of Dex on the lung W/D ratio in an LPS-induced ALI. Compared with the control group, the W/D ratio was increased in the LPS group,while Dex treatment significantly alleviated the W/D ratio (Fig4A). Decreased alveolar fluid clearance(AFC) is an important mechanism that leads to pulmonary edema in ALI. We also examined the effect of Dex on AFC.

Compared with the control group, the AFC was decreased in the LPS group, while Dex treatment increased the AFC. (Fig 4B). However, LY294002, a PI3K inhibitor, partly prevented the protective effects of Dex.These results indicated that Dex attenuates pulmonary edema in a PI3K dependent manner.

**Dexmedetomidine improved $PaO_2$ in LPS-induced ALI**

Hypoxemia leads to reduced oxygenation and induces organ injury. In this experiment, we calculated the effect of Dex on the $PaO_2$. Compared with the control group, $PaO_2$ levels were decreased in the LPS group, while Dex treatment significantly increased the $PaO_2$. (Fig 5).

**Dexmedetomidine increased the expression of ENaC in LPS-induced ALI**

To clarify that Dex stimulates AFC by increasing the expression of ENaC, the expression levels of α-, β-, and γ-ENaC in rat lung tissues were detected by western blotting analysis. Furthermore, immunohistochemistry analysis was employed to assess α-, β-,and γ-ENaC levels in rat lung tissues.

Compared with the control group, the expression levels of α-, β-, γ-ENaC were downreg-

ulated in rat lung tissues in the LPS group, while Dex treatment increased the expression of α–ENaC(Fig 6A), β-ENaC(Fig 6B), γ-ENaC(Fig 6C). However, the PI3K inhibitor partially prevented the protective effects of Dex.

Immunohistochemical analysis was used to determine the expression of α-, β-, γ-ENaC in the rat lungs. Positively immunostained cells appeared brown. Compared with the control group, the expression of α-, β-, γ-ENaC was decreased in the LPS group, but increased in the LPS+Dex group compared with the LPS group (Fig 7A-F). However, the PI3K inhibitor partially prevented the protective effects of Dex. These results indicate that Dex acts through PI3K to increase ENaC expression.

**Dose and time dependency of LPS regulated ENaC expression in A549cells**

To determine the relationship between the dose and the stimulation time of LPS and the expression of ENaC, we first stimulated A549 cells with different concentrations of LPS (0, 0.5, 1, 5 ug/ml) and detected the expression of α-ENaC through western blotting. The results showed that the expression of α-ENaC was dose-dependent, and the dose of 1ug/ml produced the most significant effect. Accordingly, the LPS (1ug/ml) was used in subsequent cell experiments. (Fig.8A)

To determine the expression of α-ENaC in A549 cells at different times, cells were stimulated with 1ug/ml LPS for 0, 6, 12, 24 and 48 h. The results showed that the expression of α-ENaC decreased at 6, 12, 24 h, and the decrease was the most obvious at 12 h. Therefore, in the subsequent cell experiments, the LPS stimulation time was 12 hours.(Fig.8B)

**Dexmedetomidine increased the cell viability and decreased LDH activity in LPS- stimulated A549 cells**

We evaluated the effects of Dex on cell viability and LDH activity. A549cells were treated with Dex at different concentrations (0.1, 1, 10, 100μM) in the presence of LPS (1 μg/ml) for 12h. The results showed that both 10μm and 100μm Dex could effectively increase cell activity(Fig 9A) and decrease LDH activity(Fig 9B) in the presence of LPS. Therefore, Dex (10μm) was used in subsequent cell experiments.

**Dexmedetomidine upregulated the expression of ENaC in LPS-stimulated A549 cells**

To further clarify that Dex stimulates AFC by increasing the expression of ENaC, we detected the expression levels of α-, β-, γ-ENaC in A549 cells. Compared with the control group, the expression levels of α-, β-, γ-ENaC were downregulated in A549 cells in the LPS group, while Dex increased the expression of α-ENaC (Fig10A), β-ENaC (Fig10B), γ-ENaC (Fig 10C). However, the PI3K inhibitor partially prevented the effects of Dex .

**Dexmedetomidine activated the P13K/Akt/ Nedd4-2 signaling pathway in vivo and in vitro**

To study whether Dex regulates the expression of ENaC through PI3K/Akt/Nedd4-2 signaling pathway, the phosphorylated Akt and Nedd4-2 were measured by western blotting. Compared with the control group, The protein level of phosphorylated Akt was decreased in LPS group in vivo and vitro, while Dex increase the phosphorylated Akt expression (Figure 11A and B). Compared with the control group, The protein level of Nedd4-2 was increased in LPS group in vivo and vitro, while Dex decrease the Nedd4-2 expression (Figure11 C and D). However, PI3K inhibitor LY294002 reversed these effects of Dex, indicating that Dex activates the PI3K/Akt/Nedd4-2 signaling pathway.

## Discussion

Our study showed that LPS induced lung tissue injury, which was characterized by increased neutrophil infiltration in lung tissue, alveolar structure destruction and interstitial edema. Dex treatment reduced lung histopathological injury in rats. Hypoxemia is primarily caused by pulmonary edema in patients with ALI. Our data sugested that Dex alleviated pulmonary edema and improved hypoxemia in LPS induced-ALI. Moreover, impaired AFC was observed in the LPS group and Dex increased AFC. These data indicated that Dex alleviated LPS-induced ALI through alleviate pulmonary edema and improve hypoxemia. In the current study, we also confirmed that the LY294002, a PI3K inhibitor, reversed the protective effect of Dex, suggesting that these effects of Dex are PI3K dependent. Our previous studies showned that Dex reduces pulmonary edema by increasing aquaporin (AQP) expression in LPS-induced ALI[17]. Howere, ENaC is thought to be the rate-limiting factor for reabsorption of pulmonary edema. The ENaC is mainly composed of the three homologous subunits (α-, β- and γ-ENaC), which are expressed in the alveolar epithelial cells. The α subunit is necessary in the transport of sodium ions, while the β and γ subunit promotes channel activity. Recent studies have found that up-regulated ENaC expression promotes AFC and reduces pulmonary edema in ALI animal models.[22]. Clinical trials have

also shown that inhalation of ENaC activator significantly reduces the aextravascular lung water index (EVLWI) in ARDS patients [23]. In this experiment, we observed that the ENaC expression was low in the LPS group, while Dex increased α-, β- and γ-ENaC expression in LPS induced ALI and LPS stimulated A549 cells. However, the PI3K inhibitor LY249002 blocked these effects of Dex. In summary, our results suggest that Dex reduce pulmonary edema through stimulating AFC by increasing the expression of ENaC, and this effect is PI3K- dependent.

Studies have shown that the expression of the inflammatory cytokines TNF-α and IL-1β is increased significantly in ALI [24,25]. Our previous studies also confirmed that TNF-α and IL-1β concentrations in lu ng tissues are significantly increased in LPS-induced ALI models, while Dex reduces TNF-α and IL-1β concentrations [17].It is generally believed that the inflammatory response not only destroys the alveolar capillary membrane barrier and increas-

es lung permeability, but also reduces the absorption of alveolar water. Studies have shown that TNF-α, IL-1β can inhibit the expression of ENaC [10,26]. Our study showed that Dex reduced the TNF-α, IL-1β, IL-6 concentrtation and increased IL-10 concentration in BALF, suggesting that Dex may be benificial to regulate the balance of inflammatory responses. The previous studies demonstrated that the PI3K/Akt pathway plays a crucial role in the attenuation of inflammation [27,28]. In this study, these beneficial effects were abrogated by PI3K inhibitor (LY294002), indicating that anti-inflammatory effect of Dex is PI3K dependent.These results suggest that Dex may increase the expression of ENaC by preventing excessive inflammatory responses in LPS-induced ALI.

Our study also revealed that Dex plays a protective role by reducing pulmonary edema through increasing ENaC-mediated AFC. Howere, its mechanism is not clear. In recent years, preliminary studies have been conducted on the relevant signaling pathways that can cause changes in ENaC expression, including the glucocorticoid receptor signaling pathway [29] and the $\beta_2$-adrenergic receptor agonist-mediated CAMP-PKA signaling pathway [30,31], but these pathways are not ideal for clinical treatment and remain controversial. The lipid kinase PI3K generates phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which is a second messenger that facilitates the translocation of AKT to the plasma membrane. At the membrane, AKT is phosphorylated and plays an important role in processes such as cell proliferation, differentiation, survival and apoptosis. Previous studies have shown that the activation of PI3K/Akt pathway decreases pulmonary edema by up-regulating the expression of the ENaC in LPS- induced ALI [32,33]. Experimental studies revealed that Stimulation of LPS reduced the expression of p-Akt [34]. Our study showed that Dex inhibition the reduction of p-Akt expression in LPS-induced ALI and LPS-stimulated A549 cells. Howere, a PI3K inhibitor (LY294002) markedly prevented Dex-induced the level of phosphorylated Akt and the expression of α-, β-, γ- ENaC. These results confirmed that Dex acts through activating PI3K/Akt to increase ENaC expression .

Nedd4-2 is a ubiquitin-protein ligase that mediates ubiquitination and degradation of ion channel proteins. The carboxyl terminals of the α, β and γ subunits of ENaC have PY modules, and the ww domain of Nedd4-2 is bound to the PY modules of ENaC, thereby promoting ubiquitination of ENaC and reducing the expression of ENaC on the cell membrane. Recent studies have shown that degradation of ENaC mediated by ubiquitination of Nedd4-2 is involved in the occurrence and development of acute pulmonary edema [35]. In addition, the Akt/Nedd4-2 pathway plays an important role in regulating ENaC [36]. Akt attenuates ENaC ubiquitination by phosphorylating Nedd4-2, thereby increasing ENaC expression.In this study, we found that Dex decreased the expression of Nedd4-2. However, the beneficial effects of Dex were inhibited by LY294002 in vivo and vitro, indicating that PI3K-dependent activation of Nedd4-2 may be involved in the upregulation of ENaC expression.

## Conclusions

In summary, our study demonstrates that Dex alleviates pulmonary edema in LPS -induced ALI. This protective effect is attributed to promote AFC through the upregulation of the ENaC expression via activating the PI3K/Akt/Nedd4-2 signaling pathway. Our results provide new insight into alleviating pulmonary edema in LPS-induced ALI and suggests a new therapeutic use of Dex for patients with ALI.

## Abbreviations

ALI: Acute lung injury; ENaC:alveolar epithelial sodium channel;AFC:alveolar fluid clearance; LPS: LipopolysacchAride; AQP: aquaporin; TNF-α: tumor necrosis factor-α; IL-1β: interleukin-1β; $\alpha_2$AR: $\alpha_2$-adrenegicreceptor; PI3K:phosphatidylinositol 3-kinase; Akt: protein kinase B; BALF: bronchoalveolar lavage fluid; ELISA:enzyme-linked immunosorbent assay; MPO: myeloperoxidase; $PaO_2$:Arterial oxygen tension;W/D:wet/dry; LDH: lactate dehydrogenase;

## Declarations

### Ethics approval and consent to participate

No human participants, human data or human tissues were included in this study. ALL animal experiments complied with the Guidelines for the Care and Use of Laboratory Animals from the NIH; ALLanimal handling was conducted according to the requirements of the animal protection committee of the Second Clinical Medical College of Jinan University.

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets used and /or analyzed during the current study are available from the correspon-

ding author on reasonable request.

### Competing interests

The authors have declared no competing financial interests.

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### Authors' contributions

Yuan-Xu Jiang, Xue-Ping Zhang, and Ming-Zhu Xia worked on the experimental design. Jing Xu performed the histological and immunohistochemistry examination of the lung. Yuan-Xu Jiang, Wen-Jie Yang, Qi- ang Huang, and Yuan-Xu Jiang conducted the experiments, analyz-

ed the data, and interpreted the data. Yu- an-Xu Jiang, Ming-Zhu Xia drafted the article. The authors read and approved the final manuscript.

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## Figures

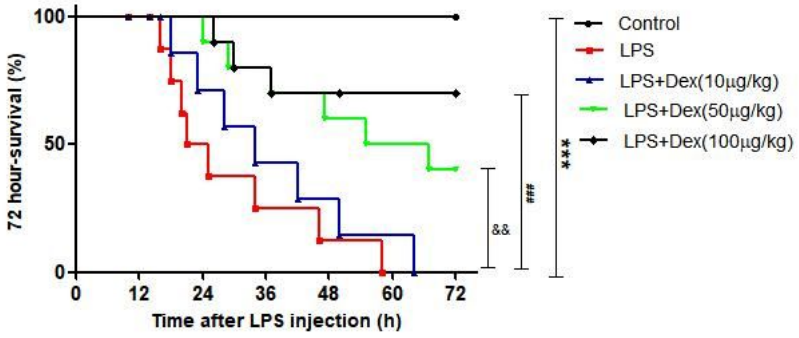


**Figure 1**

Effect of Dex on LPS-induced survival rate in ALI rats. The rats were immediately intraperitoneally injected with Dex (10, 50 or 100 μg/kg) after intravenous injection of LPS (20mg/kg). Survival was monitored during 72 hours.Data were presented with means ±SD. n = 10, ***P < 0.001，###P < 0.001，&&P < 0.01.

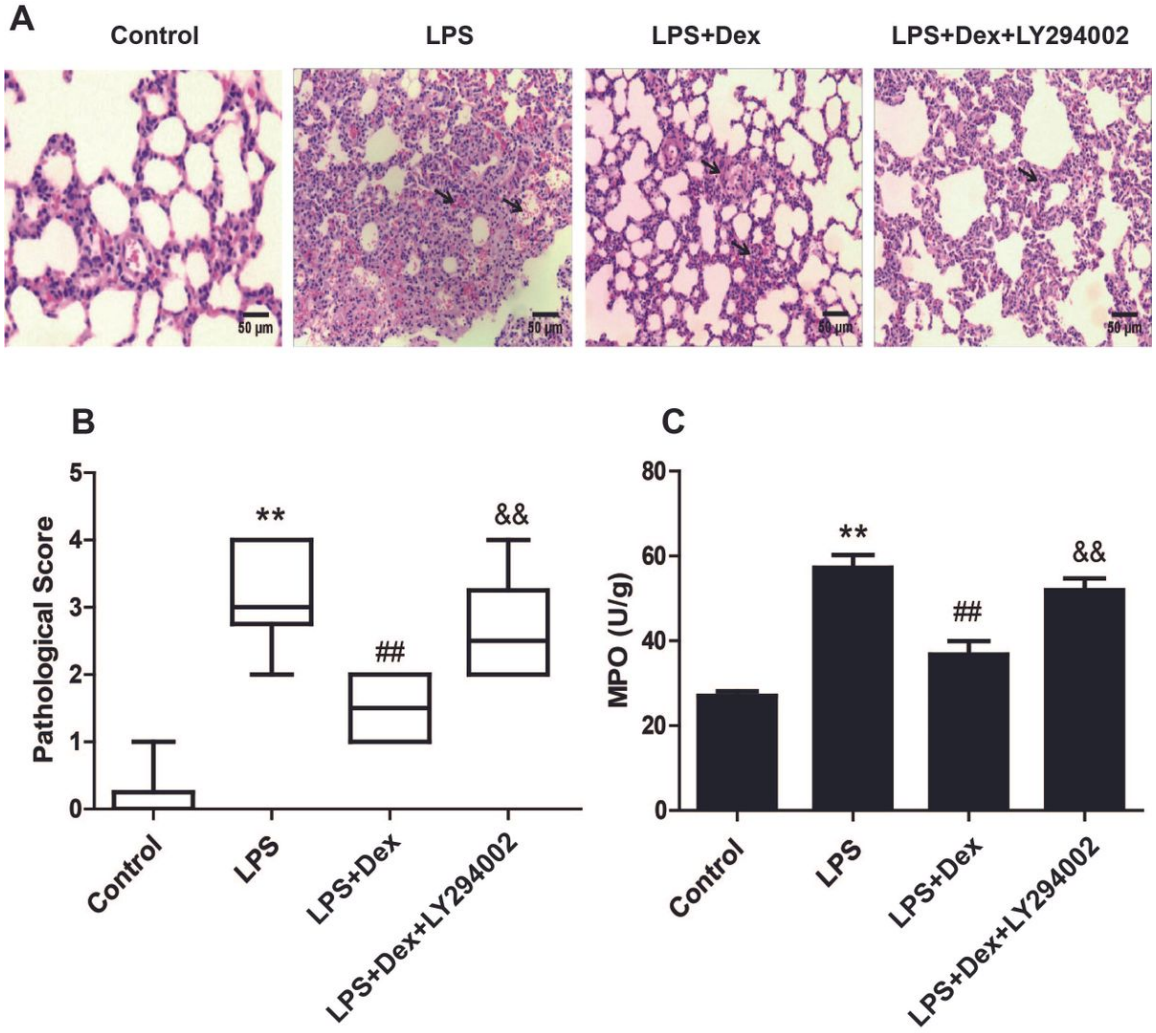


Figure 2

Effect of Dex on LPS-induced ALI. In a rat model of LPS (20 mg/kg)-induced ALI, the rats were treated with Dex (100μg/kg). To investigate whether the protective effect of Dex is PI3K dependent, LY294002 (3 mg/kg), a PI3K inhibitor, was given 30 minutes prior to LPS administration. Eight hours after, the rats were sacrificed by bloodletting, a the lung tissues were assessed by histopathology (H&E staining, magnification, 200×), b the lung injury score, c MPO level. Data were presented with means ±SD. n = 6, **P < 0.01，##P < 0.01，&&P < 0.01.

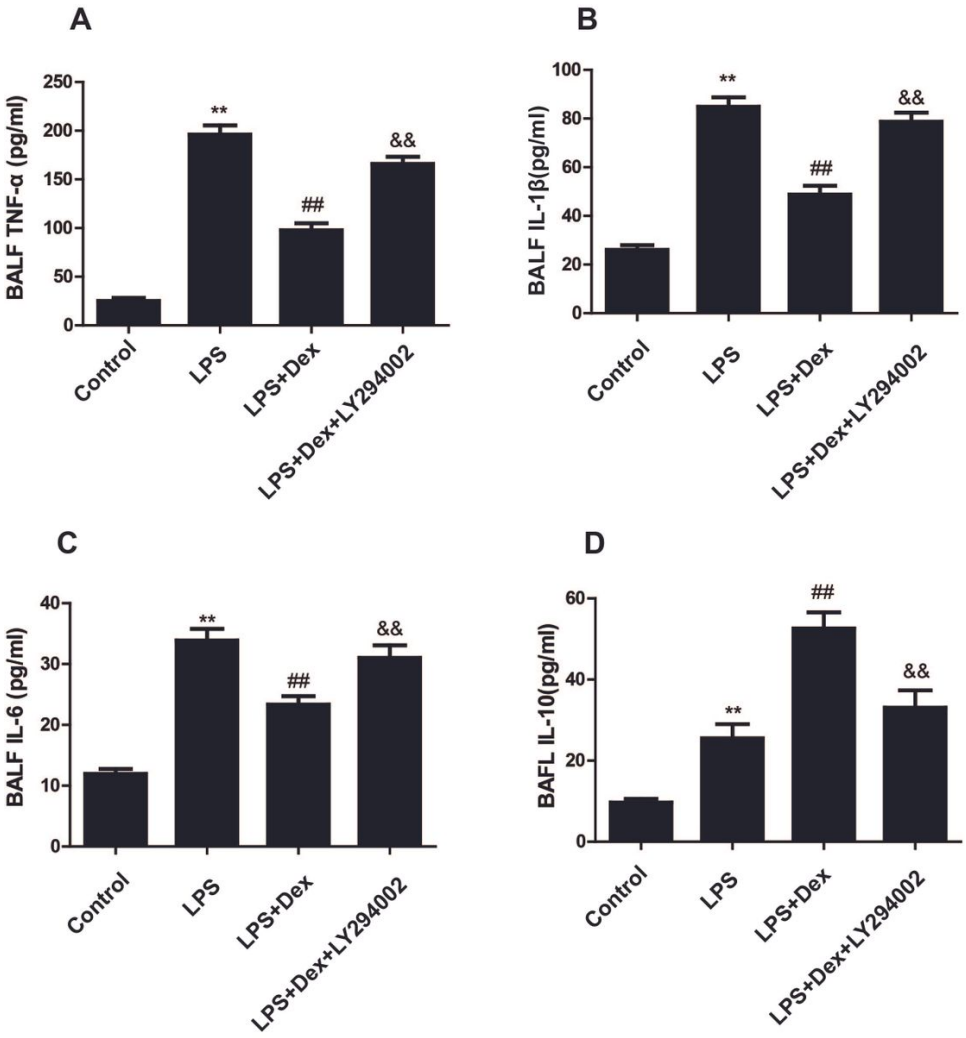


Figure 3

Effect of Dex on inflammatory response in LPS-induced ALI. In a rat model of LPS (20 mg/kg)-induced ALI, the rats were treated with Dex (100μg/kg). To investigate whether the protective effect of Dex is PI3K dependent, LY294002 (3 mg/kg), a PI3K inhibitor, was given 30 minutes prior to LPS administration. Eight hours after, the rats were sacrificed by bloodletting. Bronchoalveolar lavage fluid was collected to determine the concentrations of TNF-α (a), IL-1β (b), IL-6 (c) and IL-10 (d) in each group by ELISA. Data were presented with means ±SD. n = 6, **P < 0.01 ##P < 0.01 &&P < 0.01.

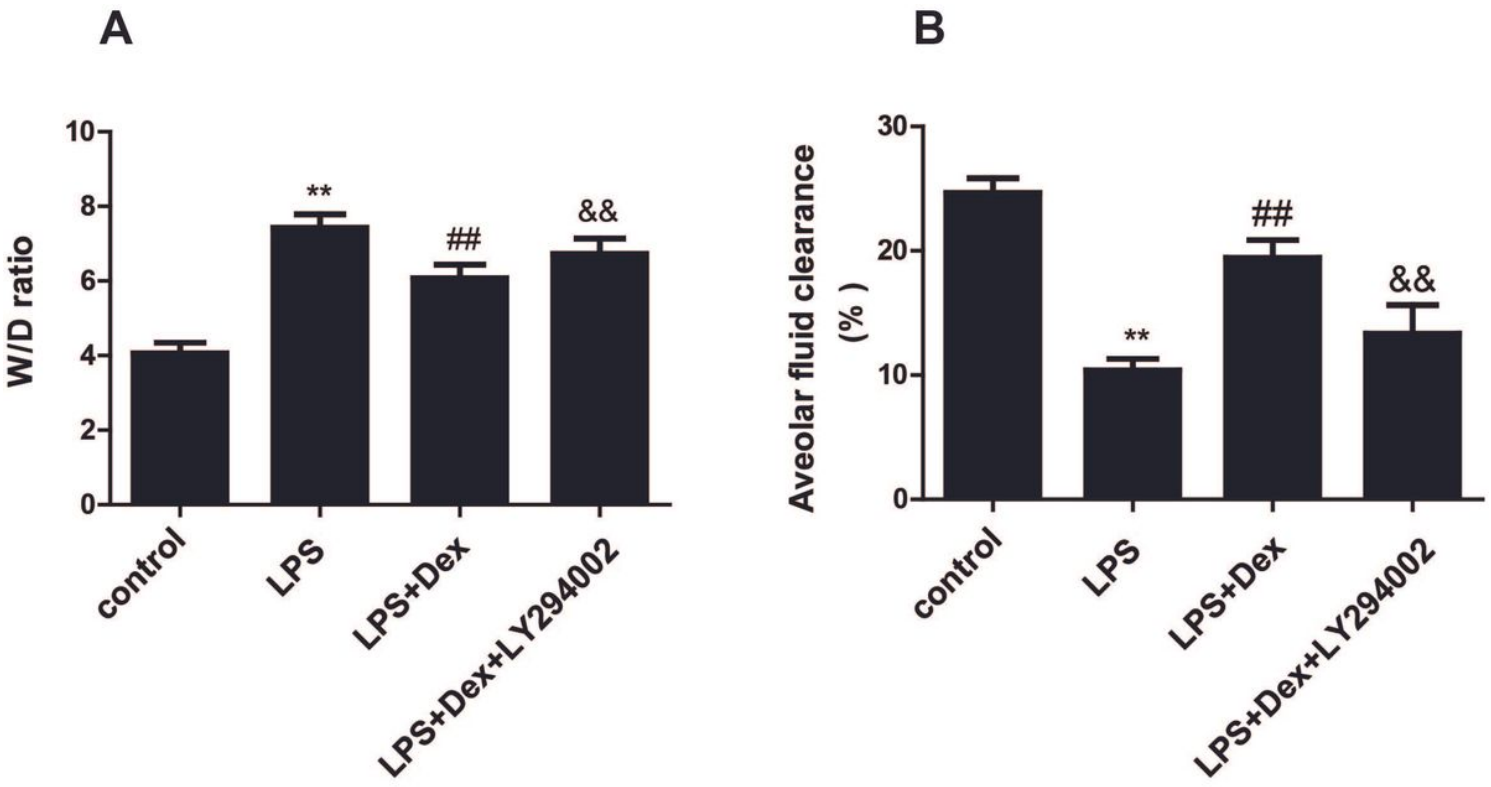


Figure 4

Effect of Dex on pulmonary edema in LPS-induced ALI. In a rat model of LPS (20 mg/kg)-induced ALI, the rats were treated with Dex (100μg/kg). To investigate whether the protective effect of Dex is PI3K dependent, LY294002 (3 mg/kg), a PI3K inhibitor, was given 30 minutes prior to LPS administration. Eight hours after, the rats were sacrificed by bloodletting. a The right lung tissue was harvested to measure W/D ratio；b 5% albumin solution containing Evans blue–labeled albumin (5 ml/kg) was instillated into the left lung through a tracheostomy and the AFC was calculated. Data were presented with means ±SD. Data were presented with means ±SD. n = 6, **P < 0.01；##P < 0.01；&&P < 0.01.

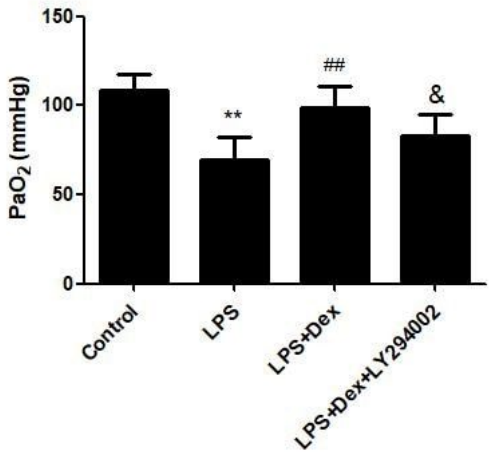


Figure 5

Effect of Dexmedetomidine on PaO2. Effect of Dex on pulmonary edema in LPS-induced ALI. In a rat model of LPS (20 mg/kg)-induced ALI, the rats were treated with Dex (100μg/kg). To investigate whether the protective effect of Dex is PI3K dependent, LY294002 (3 mg/kg), a PI3K inhibitor, was given 30 minutes prior to LPS administration. Before the rats sacrifice, 0.5ml blood was taken from the common carotid artery to detect the PaO2. Data were presented with means ±SD. Data were presented with means ±SD. n = 6, **P < 0.01；##P < 0.01；&P < 0.05.

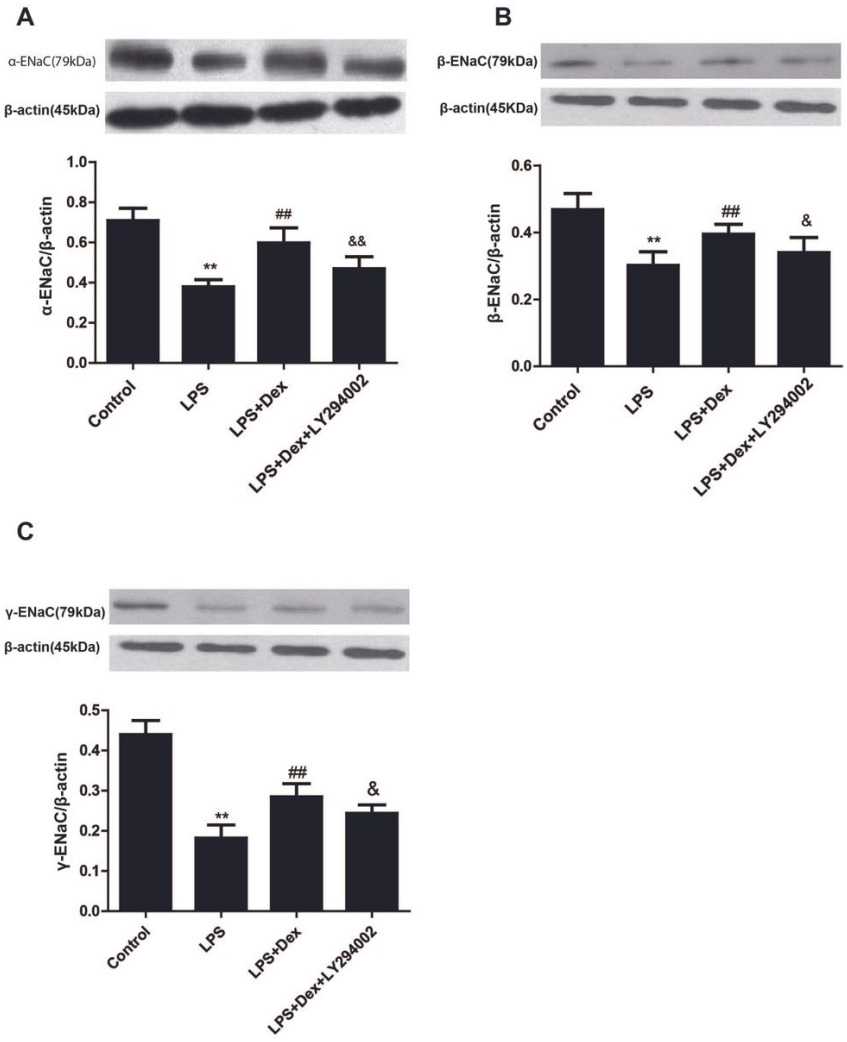


**Figure 6**

Effect of Dex on the expression of ENaC in LPS-induced ALI. In a rat model of LPS (20 mg/kg)-induced ALI, the rats were treated with Dex (100μg/kg). To investigate whether the protective effect of Dex is PI3K dependent, LY294002 (3 mg/kg), a PI3K inhibitor, was given 30 minutes prior to LPS administration. Eight hours after, the rats were sacrificed by bloodletting,The left lung tissue was harvested to assess the levels of α-ENaC (a) , β-ENaC (b) and γ-ENaC (c) by Western blotting. Data were presented with means ±SD. Data were presented with means ±SD. n = 6, **P < 0.01，##P < 0.01，&P < 0.05, &P < 0.05.

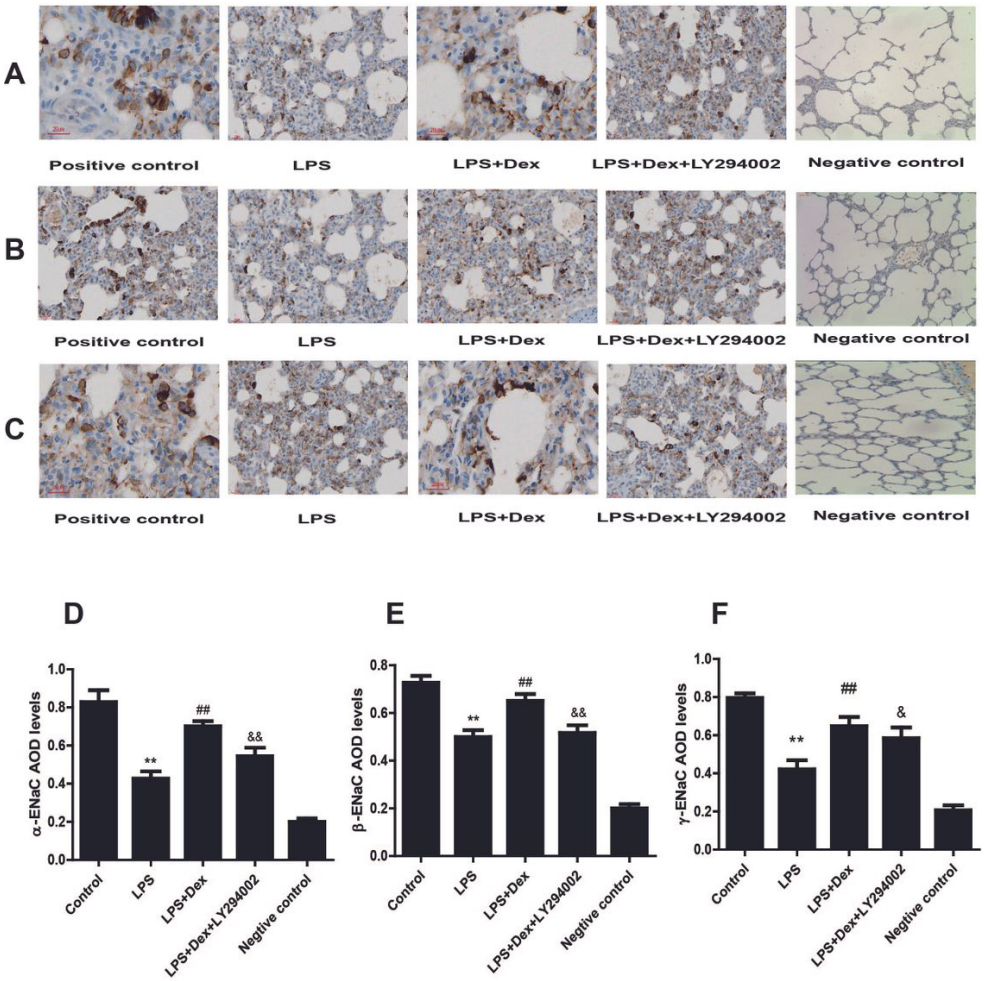


Figure 7

Effect of Dex on the expression of ENaC in LPS-induced ALI. In a rat model of LPS (20 mg/kg)-induced ALI, the rats were treated with Dex (100μg/kg). To investigate whether the protective effect of Dex is PI3K dependent, LY294002 (3 mg/kg), a PI3K inhibitor, was given 30 minutes prior to LPS administration. Eight hours after, the rats were sacrificed by bloodletting,The left lung tissue was harvested to assess the levels of α-ENaC (a) , β-ENaC (b) and γ-ENaC (c) by immunohistochemistry. Densitometric quantifification of the level of α-ENaC (d) , β-ENaC (e) and γ-ENaC (f) Data were presented with means ±SD. Data were presented with means ±SD. n = 6, **P < 0.01，##P < 0.01，&&P < 0.05, &P < 0.05.

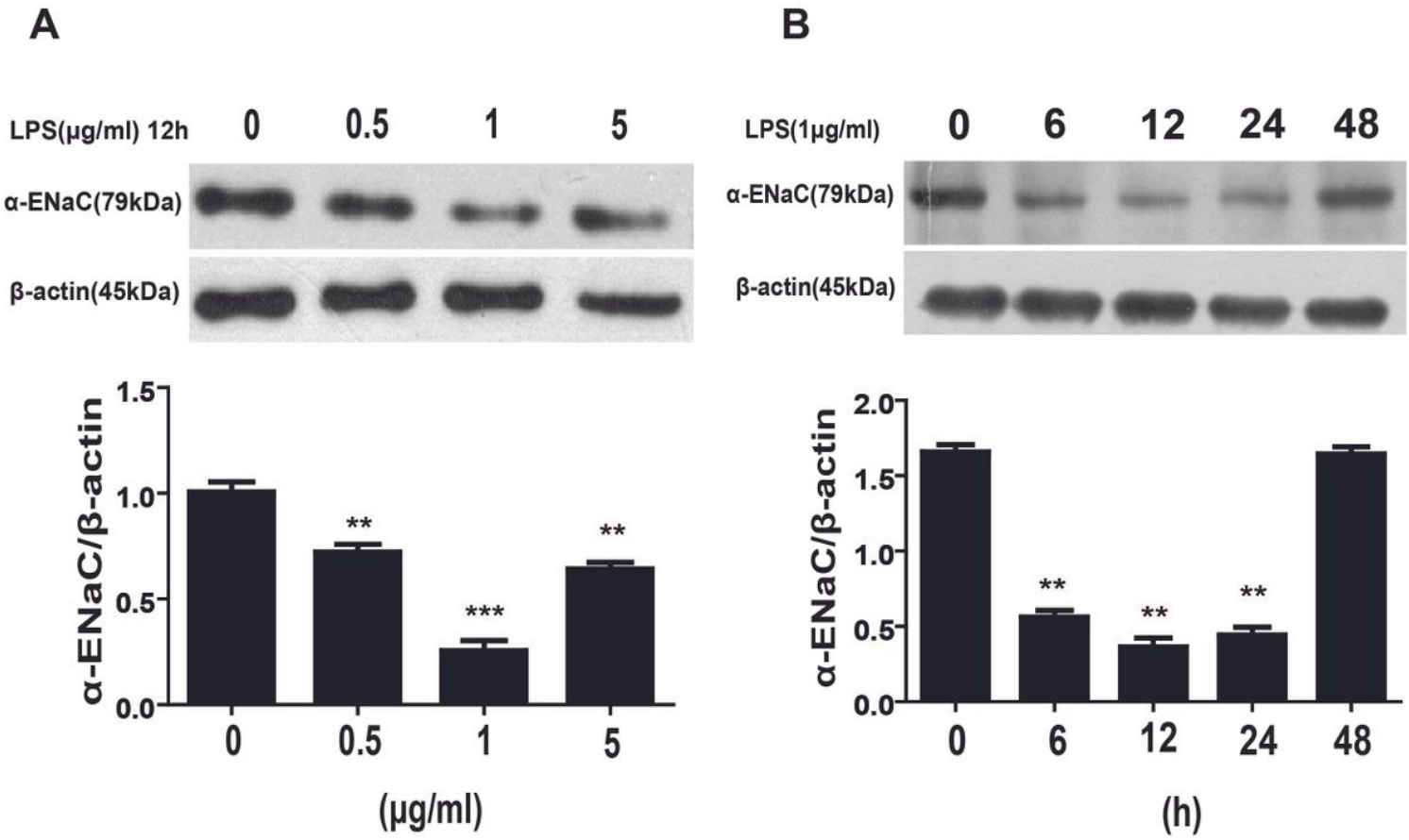


Figure 8

Effect of LPS on α-ENaC expression in vitro. A549 cells were stimulated by LPS at different concentrations for 12 hours, and the expression of α-ENaC (a) was detected by Western blotting. 1ug/ml LPS stimulated A549 cells, and Western blotting was used to detect the expression of α-ENaC (b) at different times. Data were presented with means ±SD. n = 6, **P < 0.01，***P < 0.001.

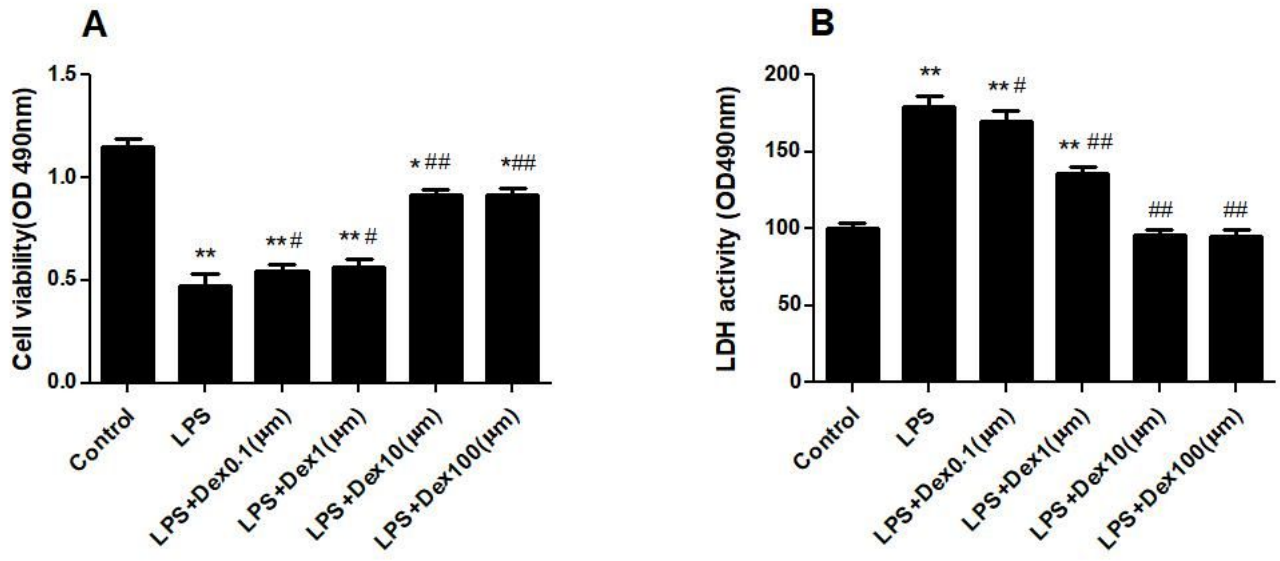


Figure 9

Effect of Dex on cell viability and LDH activity in vitro. A549 cells stimulated by LPS, following that thecells were treated with different concentrations of Dex (0.1,1,10,100μm) for 12 hours. (B) Effects of different concentrations of Dex on LDH activity in A549 cells induced by LPS for 12 hours. The cell viability and LDH activity were detected . Data were presented with means ±SD. n = 6, *P < 0.05, **P < 0.01，#P < 0.05，##P < 0.01.

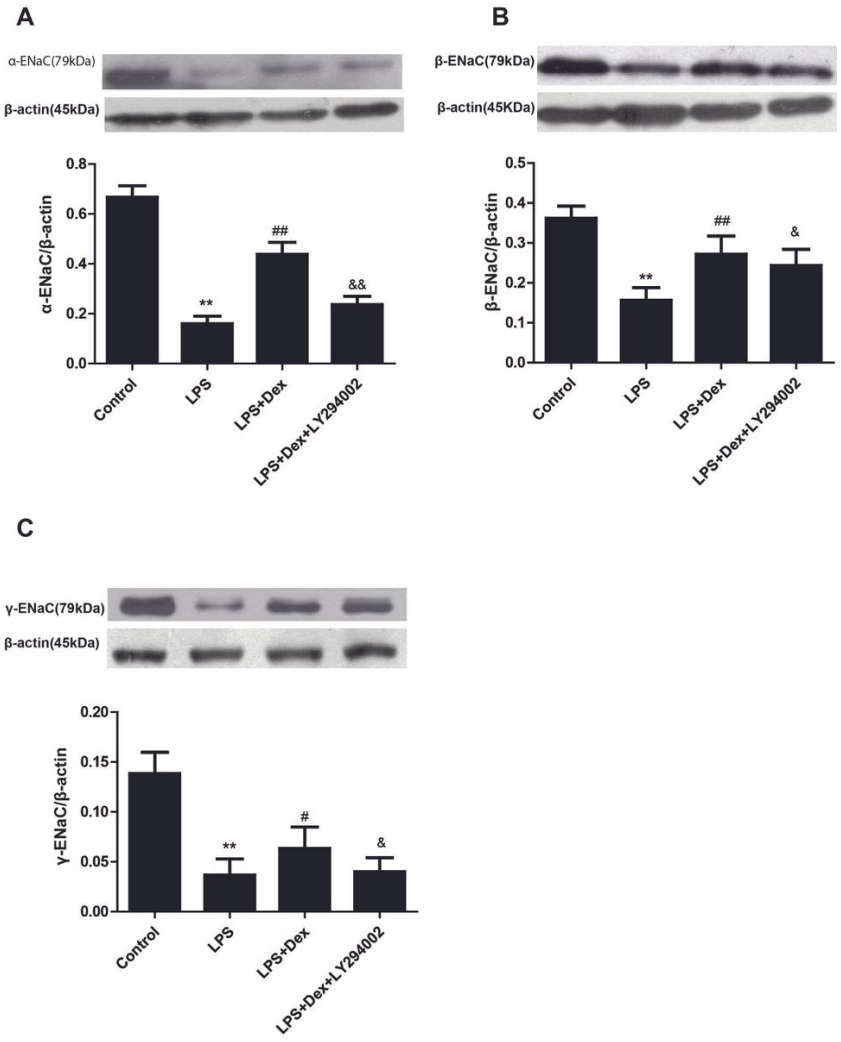


Figure 10

Effect of Dex on the expression of ENaC in vitro. A549 cells were treated with LY294002 (10 μM) 30 min prior to LPS (1μg/ml) administration and were then treated with Dex (10 μM). The protein expression of α-ENaC (a), β-ENaC (b), γ- ENaC (c) were assessed by western blotting 12 hours after LPS treatment. Data were presented with means ±SD. n = 6, **P < 0.01␣#P < 0.05␣##P < 0.01␣&P < 0.05␣&&P < 0.01.

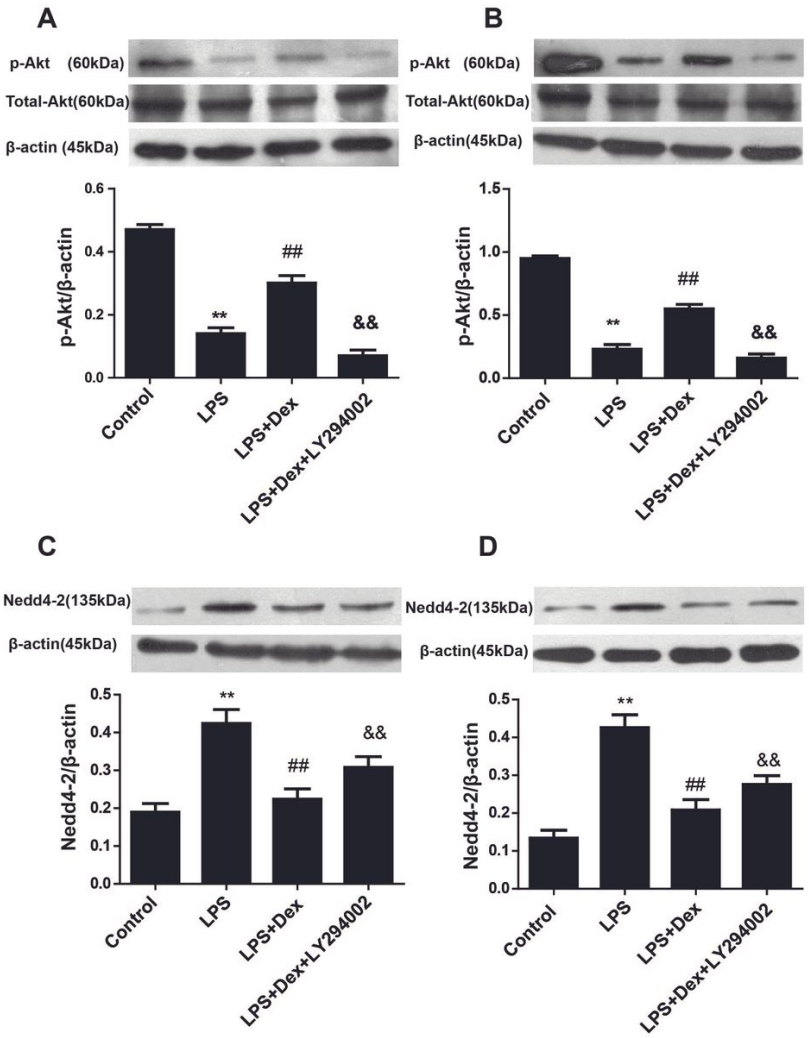


Figure 11

Effect of Dex on the exptression of the phosphorylated Akt and Nedd4-2 in vivo and vitro. A549 cells were treated with LY294002 (10 μM) 30 min prior to LPS (1μg/ml) administration and were then treated with Dex (10 μM). Western blotting was used to assess the levels of phosphorylated Akt in LPS-induced ALI (a) and LPS-stimulated A549 cells ( b) . Western blotting was used to assess the levels of Nedd4-2 in LPS-induced ALI (c) and LPS-stimulated A549 cells( d) . Data were presented with means ±SD. n = 6, **P < 0.01，##P < 0.01，&&P < 0.05.