


Terbutaline alleviates the lung injury in the neonatal rats exposed to endotoxin: Potential roles of epithelial sodium channels

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Abstract

Intrauterine inflammation generates inflammatory mediators that damage the developing bronchoalveolar epithelium, resulting in neonatal lung injury. Lung fluid transport disorders are the main reasons for the development of pulmonary edema, an important pathology of lung injury. Previous studies suggested that epithelial sodium channels (ENaCs) play an important role in lung fluid transport. Here, we investigated whether changes in the expression of ENaCs were observed when neonatal rat lung injury was induced by maternal exposure to endotoxin. We also examined the therapeutic effect of terbutaline nebulizer inhalation on this injury. The results showed that maternal exposure to endotoxin increased the levels of TNF- α and IL-1 β in bronchoalveolar lavage fluid, suppressed α -, β -, γ -ENaC in the neonatal rat lung, and resulted in the formation of pulmonary edema on postnatal days 1 and 7. Terbutaline up-regulated the expression of β - and γ -ENaC in the distal lung after 7 days of treatment. The potential signal molecules cAMP, PKA, and CREB expressions were increased after terbutaline treatment. In summary, maternal exposure to endotoxin decreased the expression of ENaCs in neonatal rats which, in turn, may exacerbate pulmonary edema. Inhalation of the β 2-adrenergic receptor agonist terbutaline improved lung liquid clearance. By increasing the expression of sodium ion channels, the effective removal of alveolar fluid provides a new way for the prevention and treatment of neonatal lung injury.

KEYWORDS

endotoxin, epithelial sodium channels, lung injury, neonatal rat, terbutaline

Keyu Lu and Xu Chen contributed equally to this work.

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1 | INTRODUCTION

Neonatal respiratory failure, one of the most critical illnesses in intensive care units, is characterized by a high mortality and serious residual effects.¹ The primary causes of neonatal respiratory failure are respiratory distress syndrome (43.9%), pneumonia/sepsis (21.7%), transient respiratory insufficiency (14.7%), transient tachypnea (8.1%), and meconium aspiration syndrome (7.0%).²⁻³ Exposure to intrauterine inflammation or infection is a key perinatal factor.⁴ Intrauterine infections can induce a large number of pro-inflammatory mediators and cytokines. This may lead to a fetal inflammatory response syndrome by triggering the fetal innate immune system.⁵⁻⁶ This system may then activate alveolar inflammatory cells contributing to fetal lung injury.⁷⁻⁸

The obstruction of gas exchange caused by pulmonary edema is an important pathological basis of lung injury.⁹ If a newborn is in a period of lung fluid transition, a delay or obstruction of lung fluid transport may result in pulmonary edema.¹⁰ Therefore, understanding the mechanism by which intrauterine inflammation influences the mechanisms of liquid movement in the newborn lung is an important area of study.

Epithelial sodium channels (ENaCs), the primary mechanism of pulmonary alveolar liquid transport, and function by moving sodium ions through the apical membrane into lung epithelial cells where it is then extruded by the ATPase pump. This creates an osmotic gradient resulting in the net clearance of fluid from the airspace.¹¹ Many studies have shown that an appropriate number and function of alveolar ENaCs is crucial for lung fluid transport.^{10,12} In contrast, inflammatory mediators can significantly stifle the expression of alveolar ENaCs in vivo.¹³ But, it is still unclear whether intrauterine infection affects the expression of ENaCs in neonatal rats.

The current study was designed to further research the basis of abnormal lung fluid transport when neonatal lung tissue is injured by an intrauterine infection. We first established an optimal animal model of intrauterine infection-induced lung injury in neonatal rats by using the intraperitoneal injection of lipopolysaccharides (LPS) on day 18 of gestation. Second, we investigated changes in the expression of ENaCs in this model and explored the therapeutic effect of terbutaline nebulizer inhalation. This study may provide new clues for the prevention and treatment of neonatal respiratory disease.

2 | MATERIALS AND METHODS

2.1 | Animals and materials for the experiment

Male (300-350 g) and female (230-280 g) Sprague Dawley rats were purchased from the Laboratory Animal Center of Nanjing Medical University. Animal experiments were approved by the Laboratory Animal Ethics Committee of Nanjing Medical University (IRB: IEC of Children's Hospital Affiliated Nanjing Medical University, permit number: 201801173-1). Rats were housed under a temperature- and humidity-controlled environment, and specific pathogen-free conditions, and were granted free access to food and water. All operations conformed to the Guide for the Care and Use of Laboratory

Animals. LPS (*Escherichia coli* serotype O55:B5) was purchased from Sigma-Aldrich (St. Louis, MO). Terbutaline (β_2 -adrenergic receptor agonist) was purchased from AstraZeneca (Stockholm, Sweden).

2.2 | Animal model and interventions

Adult male and female rats were used to mate. Pregnant Sprague Dawley rats of gestation day 18 were divided randomly into control, LPS, and LPS + terbutaline groups (pregnant rats, $n = 10$ in each group). Pregnant rats in the LPS and LPS + terbutaline groups were injected intraperitoneally with 0.7 mg/kg LPS. The control group received an equal volume of saline. These pregnant rats gave birth to newborn rats. In the group of LPS + terbutaline, neonates from LPS-treated-mother were given terbutaline inhaled once each day and continued for 7 days in an enclosed container from the day of birth (10 mg/kg/d). Lung tissues were collected from neonatal rats on P1 and P7 in different groups. Eight neonates were used in each group at each time point ($n = 8$). Bronchoalveolar lavage fluid (BALF) and lung tissues were stored at -70°C before analysis. The rats were sacrificed 24 h after the last terbutaline exposure by a lethal intraperitoneal injection of 50 mg/kg pentobarbital.

2.3 | Lung histology

The right upper lung lobes were fixed in 10% formalin for 24 h. The tissues were then dehydrated and embedded in paraffin. Slides from the anterior upper and posterior lower lobes were stained with hematoxylin and eosin, and then scored using a semiquantitative scoring system by a pediatric pathologist (S.C.S.) blinded to the treatment group. Edema, alveolar and interstitial inflammation, alveolar and interstitial hemorrhage, atelectasis, necrosis, and hyaline membrane formation were each scored on a 0- to 4-point scale: no injury = score of 0; injury in 25% of the field = score of 1; injury in 50% of the field = score of 2; injury in 75% of the field = score of 3; and injury throughout the field = score of 4. Quantitative morphometric measurements were performed on slides obtained from anterior upper and posterior lower lobes of the studied animals, using a modified method as previously described.¹⁴

2.4 | Measurement of extravascular fluid in lung tissue

Lungs were isolated for the determination of wet-to-dry weight ratios to evaluate pulmonary edema. Briefly, the lower lobe of the right lung of the rat was cut out and the wet weight of the lung lobe was measured. After baking in 80°C oven for 72 h, the dry weight of the lung lobe was measured after no change of the mass, and the lung tissue wet-to-dry was calculated. Lungs were not perfused before collection. After the collection, the right main bronchus was ligated and the left lung was perfused with saline.

2.5 | Secretion of TNF- α and IL-1 β in BALF

Bronchoalveolar lavage was performed using a catheter inserted into the trachea through a tracheotomy. Lavage fluid was centrifuged at 1000g at

4°C for 5 min to remove cell debris. The lavage fluid was then mixed with 1 mL of saline. TNF- α and IL-1 β were analyzed by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN).

2.6 | RNA extraction and quantitative real time (qRT)-PCR analysis for α -, β -, and γ -ENaC

Total RNA was extracted from lung tissues with TRIzol (Invitrogen, Burlington, ON, Canada), then quantitated on a spectrophotometer. qRT-PCR amplification was performed in an ABI PRISM 7500 System (Applied Biosystems, Foster City, CAA). The Primer sequences were as follows: TNF- α : forward: 5'-AGTGC GGG ACCCATCAGGCA-3'; reverse: 5'-GCAGTGTGGGGCAGCGTT-3'; IL-1 β : forward: 5'-TGCTCGTGTCTGACCCAT-3'; reverse: 5'-CAGGGTGGGTG TCCGTCTT-3'; α -ENaC: forward: 5'-CAGCAACAATCCCAAGTG-3'; reverse: 5'-TCCACCCAGAGGAGTATGT-3'; β -ENaC: forward: 5'-CACACCACCTCCAGATAACAAT-3'; reverse: 5'-CCCAATCCTGTCT-TACAACA-3'; γ -ENaC forward: 5'-CTAGGCTGATGCTCCACGAG-3'; reverse: 5'-CCAGCAGCACCCCAATAGAA-3'; cAMP: forward: 5'-CAACCAGCAGTCTTTGGACA-3'; reverse: 5'-TCCTTCACTCGGAAC CTCAC-3'; PKA: forward: 5'-AGCCAAAGCCAAGGAAGATT-3'; reverse: 5'-AGCATCACTCGCCCAAAG-3'; CREB: forward: 5'-CAGA-CAACCAGCAGAGTGGGA-3'; reverse: 5'-TACAGTGGGAGCAGATGAC G-3'; GAPDH: forward: 5'-TCAGTGCCGCGCTCGTCTCAT-3'; reverse: 5'-TGACCAGCGGCCAATACGG-3'. The relative quantitation for PCR signals was compared among groups after normalizing to the intensity of GAPDH as an internal reference. Reverse transcription reaction conditions were 25°C for 10 min, 55°C for 30 min, 85°C for 5 min and 4°C for 5 min. Polymerase chain reactions comprised pre-denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s and polymerization at 72°C for 30 s. Real-time PCR data were analyzed by the ABI StepOne software supplied by Applied Biosystems.

2.7 | Western blot analysis for α -, β -, and γ -ENaC

Proteins were obtained with a membrane protein extraction kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions and stored at -80°C before analysis. Proteins were quantified with a BCA kit (Beyotime, Shanghai, China). Equivalent amounts of sample were loaded and separated on 10% SDS-PAGE gels, and electrotransferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA). Membranes were blocked in Tris-buffered saline with 5% nonfat milk for 2 h at room temperature, and then incubated with the anti- α -ENaC, anti- β -ENaC, or anti- γ -ENaC (1:150, Santa Cruz Biotechnology, Dallas, TX) and anti- β -actin antibodies (1:1000, Santa Cruz Biotechnology) overnight at 4°C. Membranes were then incubated with an IgG alkaline horseradish peroxidase-labeled secondary antibody (1:10000, Zhongshan Golden Bridge, Beijing, China) at 37°C for 1 h. An enhanced chemiluminescence kit (EMD Millipore) and ChemiDoc XRS gel imaging system were used for image analysis. The relative abundance of proteins was

quantified by Image Lab Software after normalization to β -actin levels in the same sample.

2.8 | Immunohistochemistry/immunofluorescence for α -ENaC

Frozen tissue slices were treated with 3% hydrogen peroxide to quench endogenous peroxidases and incubated with an anti- α -ENaC antibody at 4°C overnight (1:20, Santa Cruz Biotechnology). Tissues were then incubated with a biotinylated secondary antibody (Sigma-Aldrich) and stained with 3,3'-diaminobenzidine (Sigma-Aldrich) for microscopic observation.

2.9 | Statistical analysis

All data are presented as means \pm standard deviation. Statistical analyses were performed by one-way analysis of variance (ANOVA) and Student's *t*-test using SPSS 17.0 software (IBM, Chicago, IL). *P* values less than 0.05 were considered significant.

3 | RESULTS

3.1 | Terbutaline reduced the intrauterine inflammation in the lung tissues from neonatal rats treated with endotoxin

Pathology can directly reflect the degree of tissue damage. Lung tissues from neonatal rats in the LPS group were significantly injured with the presence of intra-alveolar exudates, edema, and inflammatory cell infiltration, compared with the control group. Inhalation of terbutaline using a nebulizer significantly decreased the lung damage caused by LPS (Figures 1A and 1B).

Lung water content is a valid indicator of pulmonary edema. Lung water contents were assessed by measuring lung wet/dry weight ratios. Compared with the control group, the wet/dry ratios of the LPS group were significantly increased on both days 1 and 7. Meanwhile, terbutaline prevented this increase. (Figure 1C) In summary, terbutaline reduced the intrauterine inflammation in the lung tissues from neonatal rats treated with endotoxin.

3.2 | Terbutaline decreased the BALF levels of TNF- α and IL-1 β after LPS treatment

The inflammatory cytokines such as TNF- α and IL-1 β are produced by fetal immune cells owing to infection of the amniotic cavity. TNF- α and IL-1 β can be used as a marker for predicting the severity of inflammatory response. The levels of both TNF- α and IL-1 β proteins in BALF from the LPS groups were significantly higher than in the control group. Terbutaline decreased the BALF levels of TNF- α and IL-1 β proteins compared with the LPS group on both P1 and P7. As expected, the expression of TNF- α and IL-1 β mRNA was increased in the LPS group compared to the control group on P1 and P7. And the

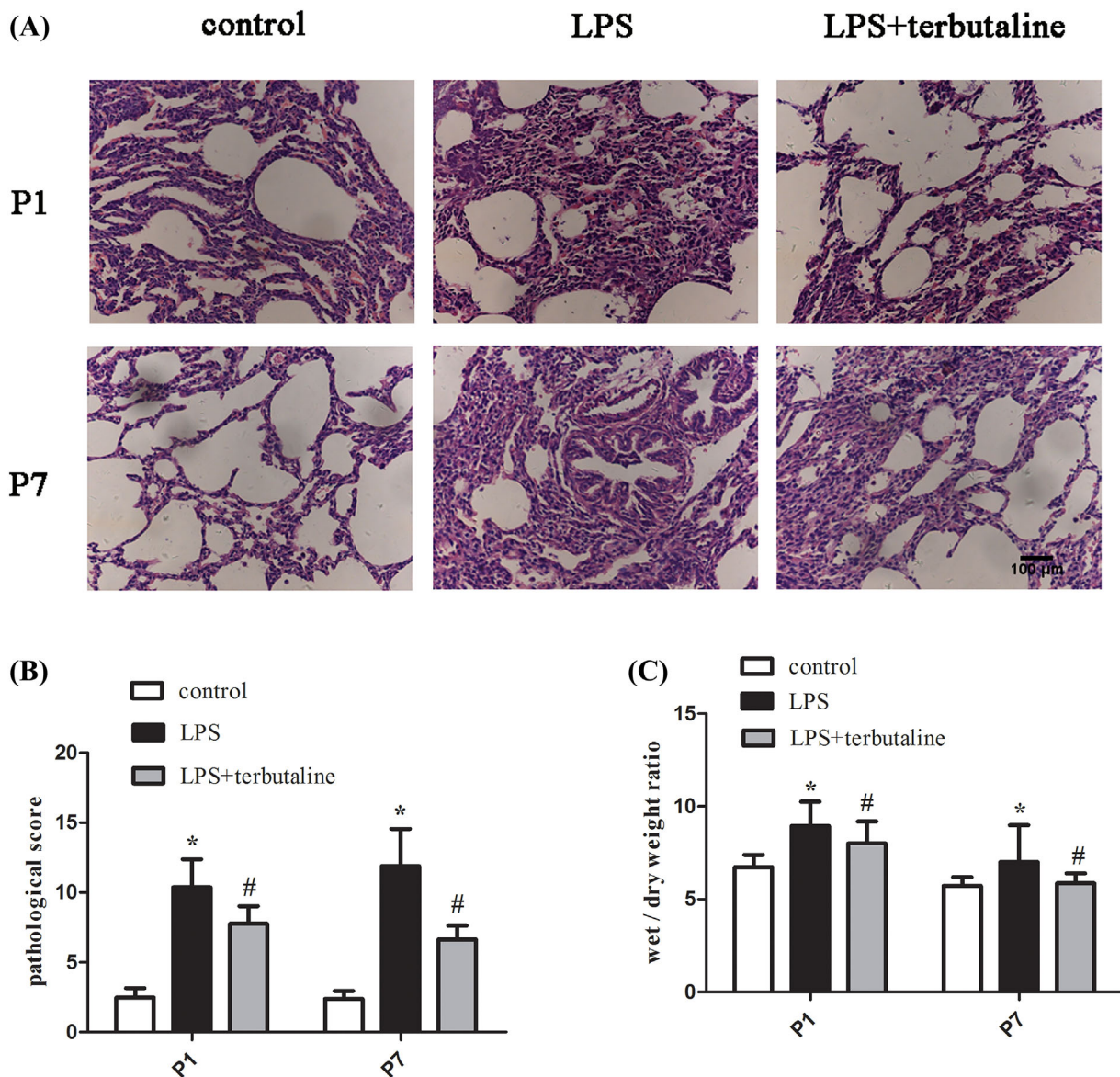


FIGURE 1 Terbutaline reduced the intrauterine inflammation in the lung tissues from neonatal rats treated with endotoxin. A and B, Effects of terbutaline on LPS-induced lung histological alterations and pathological score after LPS challenge on P1 and P7 (H&E stain, magnification $\times 200$). C, Effects of terbutaline on wet/dry ratios in neonatal lungs on P1 and P7 after LPS challenge. Lung wet/dry weight ratios in the terbutaline group were significantly lower than those in the LPS group. Data are presented as means \pm SD. * $P < 0.05$ compared with the control group, # $P < 0.05$ compared with the LPS group, $n = 8$ in each group

expression of TNF- α and IL-1 β mRNA was decreased in the terbutaline group compared to the LPS group on P1 and P7 (Figures 2).

3.3 | Terbutaline reversed the effects of LPS on ENaC expression

The expression of α -, β - and γ -ENaC mRNA was decreased in the LPS group compared to the control group on P1 and P7. Terbutaline had no effect on the expression of any ENaC compared to the LPS group on P1. However, when terbutaline was inhaled for 7 days, the expression of β - and γ -ENaC mRNA was significantly increased from the LPS group. α -ENaC mRNA was not changed on P7, although its expression

remained the highest of the three genes in each treatment group at the same times.

Content of α -ENaC on the cell membrane determine the rate of transfer. Protein levels of α -, β -, and γ -ENaC in lungs from the LPS group were significantly lower than in the control group on both P1 and P7, while protein levels of α -, β -, and γ -ENaC in lungs from the terbutaline group were significantly higher than in the LPS group on P7. And terbutaline partially improved the protein levels of α -EnaC on P1. But there had no changes of the expression of β -, and γ -ENaC between the LPS group and the terbutaline group on P1. These data indicated that maternal exposure to endotoxin may decrease the expression of α -, β -, and γ -ENaC in neonatal rat lungs and that terbutaline can inhibit this effect (Figures 3).

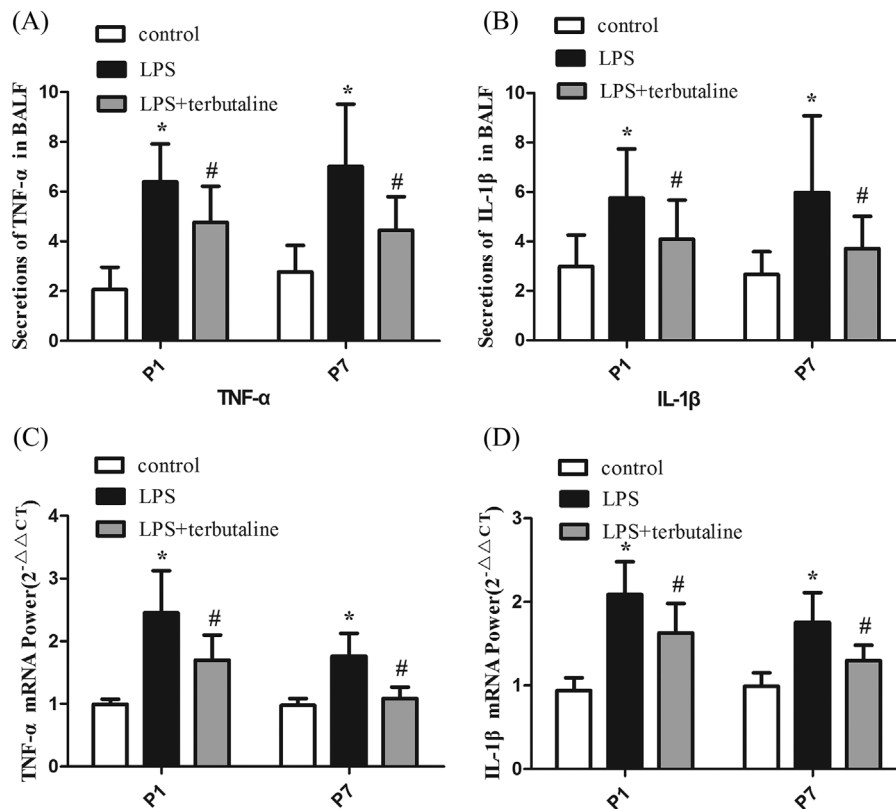


FIGURE 2 Terbutaline decreased the BALF levels of TNF- α and IL-1 β after LPS treatment. A, Effects of terbutaline on the levels of TNF- α on P1 and P7 in neonatal rat BALF after LPS treatment. B, Effects of terbutaline on the levels of IL-1 β on P1 and P7 in neonatal rat BALF after LPS treatment. C and D, The expression of TNF- α and IL-1 β mRNA decreased after terbutaline treatment. Data are presented as means \pm SD. * $P < 0.05$ compared with the control group, # $P < 0.05$ compared with the LPS group, $n = 8$ in each group

3.4 | The up-regulation of α -ENaC protein in the alveolar epithelium by terbutaline treatment

Immunohistochemical and immunofluorescence analyses were used to determine the distribution of α -ENaC in neonatal rat lungs on P1 and P7. The expression of α -ENaC was localized specifically to the alveolar epithelium at both times. The number of cells expressing α -ENaC was significantly decreased in the LPS group. However, terbutaline treatment restored the expression of α -ENaC (Figures 4).

3.5 | Terbutaline increased cAMP, PKA, and CREB in the neonatal lungs treated with endotoxin

Compared to the control group, the expression of cAMP, PKA, and CREB mRNA was decreased in the LPS group on P1 and P7. And, compared to the LPS group, the expression of cAMP, PKA and CREB mRNA was significantly increased in the terbutaline group on P7, but had no changes on P1 (Figures 5).

4 | DISCUSSION

Maternal exposure to endotoxin causes acute lung injury in neonatal rats by stimulating an excessive inflammatory reaction.¹⁵ In the current study, neonatal rat lung tissues were injured significantly by

treatment of the pregnant dams with LPS with the presence of widened alveolar septa, narrowed alveolar lumens, and inflammatory cell infiltration into pulmonary interstitial and intraalveolar spaces. This indicated that this treatment successfully established an optimal animal model of lung injury after maternal intrauterine infection in neonatal rats.

Current research on pulmonary edema induced by intrauterine inflammation is mainly focused on examining changes in alveolar vascular permeability induced by inflammatory factors.^{16–17} Studies looking at the effect of re-absorption disturbances on lung fluid balance are rare. Clinical studies have shown that the timely clearance of excess lung fluid plays a decisive role in the prognosis of lung disease. In particular, it is important for newborns to rapidly and effectively reabsorb lung liquids during the transition period. Therefore, the current study explored the effect of intrauterine inflammation on alveolar fluid transport in newborn rats.

Alveolar ENaCs are the key limiting step in the transport of lung fluid.^{18–19} According to the selectivity of ENaCs to sodium ions, they are divided into high-select (HSC) and non-high-select (NSC) channels.²⁰ In the neonatal transition period, the alveolar epithelium is transformed to increase the absorption of lung fluid through the up-regulated ENaCs. Specifically, NSCs are gradually transformed into HSCs thereby increasing the influx of Na⁺, ultimately leading to the concomitant uptake of Cl⁻ and a reversal of lung fluid flow from the

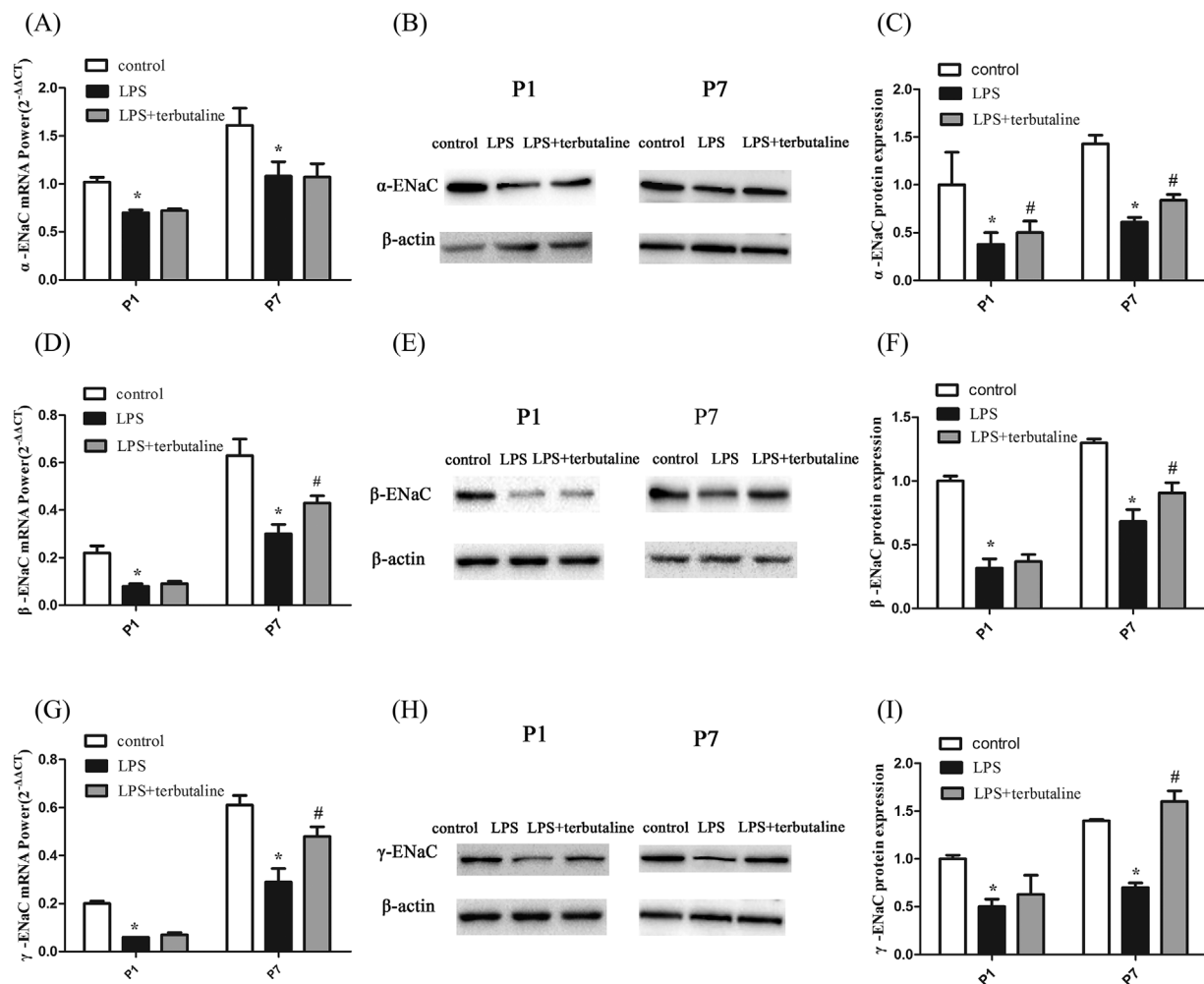


FIGURE 3 Terbutaline reversed the effects of LPS on ENaC expression. Effects of terbutaline on mRNA transcription levels of the alveolar epithelial sodium channel (ENaC) in neonatal rat lungs on P1 and P7 after LPS treatment (A, α -ENaC; D, β -ENaC; G, γ -ENaC). Effects of terbutaline on protein expression levels of the cell membrane alveolar epithelial sodium channel (ENaC) in neonatal rat lungs on P1 and P7 after LPS treatment (B and C, α -ENaC; E and F, β -ENaC; H and I, γ -ENaC). Data are presented as means \pm SD. * $P < 0.05$ compared with the control group, # $P < 0.05$ compared with the LPS group, $n = 8$ in each group

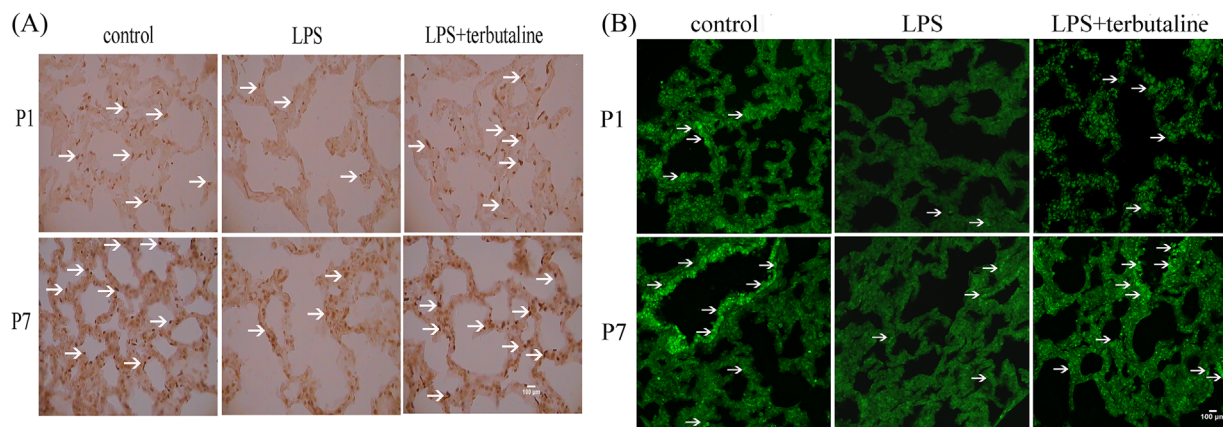


FIGURE 4 Terbutaline upregulated α -ENaC in the alveolar epithelium. A, immunohistochemistry, brown yellow particles as positive expression; B, immunofluorescence stain, bright green particles as positive expression, magnification $\times 200$. The arrow pointed to the positive-stained cells

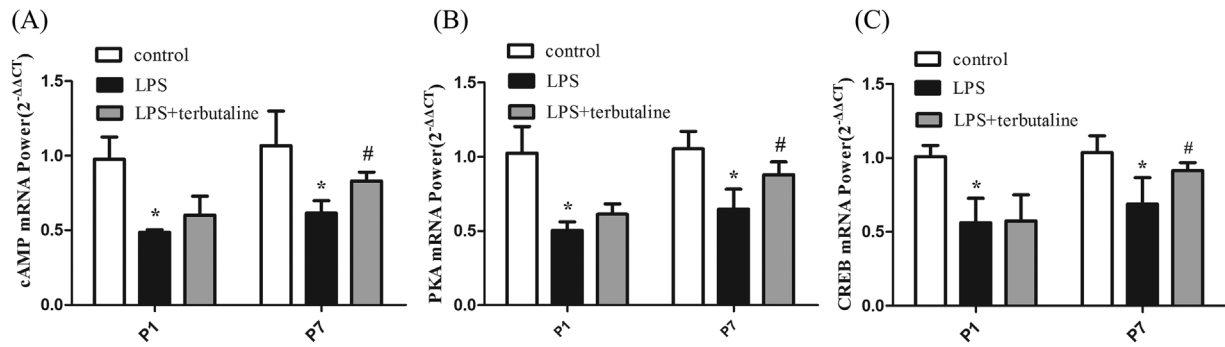


FIGURE 5 Terbutaline increased cAMP, PKA, and CREB in the neonatal lungs treated with endotoxin. A, cAMP; B, PKA; C, CREB. Data are presented as means \pm SD. * $P < 0.05$ compared with the control group, # $P < 0.05$ compared with the LPS group, $n = 8$ in each group

intrauterine period. The newborn establishes normal breathing after these processes are complete.²¹

Previous animal experimental results showed that α -ENaC-deficient mice developed respiratory distress and died within 40 h after birth. This indicated that ENaC deficiency was associated with the formation of pulmonary edema.²² Low pulmonary expression of ENaCs may cause lung fluid transport disorders, thereby contributing to the development of lung injury.²³ However, the relationship between the pulmonary edema induced by intrauterine inflammation and the expression of ENaCs is unknown. In order to better understand the mechanism of lung liquid clearance, the current study used an animal model of intrauterine inflammation to examine the mechanism of pulmonary edema in newborn rats.

Lung water content is a valid indicator of pulmonary edema. Our results show that the water content of lung tissue from neonatal rats exposed to intrauterine inflammation during the stage of late gestation was increased significantly compared to normal newborn animals. This indicated that intrauterine inflammation may cause fluid retention in neonatal rat lung leading to lung edema.

The expression of TNF- α and IL-1 β mRNA in neonatal rat lung and the levels of TNF- α and IL-1 β proteins in BALF of the LPS group were significantly higher than the control group. Moreover, the expression of α -, β -, γ -ENaC mRNA, and protein, were decreased in the LPS compared to the control groups on P1 and P7. Dagenais and coworkers found that inflammatory mediators can significantly stifle the expression of α -, β -, and γ -ENaC mRNA and protein in alveolar epithelial cells, and that the decrease was related to the concentration of inflammatory mediators.¹³ Our study showed that the expression of ENaCs was significantly decreased when neonatal rats were exposed to intrauterine inflammation that caused lung injury. Combined with previous research, these findings suggested that intrauterine inflammation may induce pulmonary inflammation of perinatal rats through the release of a large number of inflammatory mediators. These mediators could reduce the expression of alveolar ENaCs and may lead to abnormal clearance of fetal lung liquids, thus inducing neonatal respiratory failure.

Up-regulation of the expression of sodium channels may promptly remove excess alveolar liquid. This suggests a new approach for the prevention and treatment of neonatal lung injury.^{24–25} In adults, several

agents have been found to stimulate ENaCs in pulmonary epithelia via different pathways.²⁶ These include transmitters interacting with G protein-coupled receptors, circulating hormones, and reactive oxygen species. A previous study suggested that epinephrine was the most efficient drug to upregulate Na⁺ transport.²⁷ The effect of epinephrine on ENaCs is mediated mainly through β 2-receptors, which has been shown that can upregulate ENaCs expression by cAMP-PKA pathway.^{28–29} The binding of a β 2-receptor agonist to the cell membrane β 2-adrenergic receptor increases cellular cAMP concentrations that further activate cAMP-dependent protein kinase A (PKA).³⁰ PKA activation then increases the number and/or function of ENaCs³¹ by inserting already synthesized ENaCs into the cell membrane²⁷ and upregulating the transcription and translation of the ENaC gene.³⁰ Terbutaline is a widely used β 2-receptor agonist in clinical.

Based on these findings, we chose inhaled terbutaline as a likely treatment for neonatal lung injury. The results show that terbutaline treatment of neonatal rats improved the pathological score, lung wet-to-dry ratio, and the levels of TNF- α and IL-1 β in BALF of neonates from dams treated with LPS. These data implied that the inhalation of terbutaline may reduce neonatal rat lung injury and pulmonary edema by decreasing intrapulmonary effusion.

The results of qRT-PCR analyses indicated that 7 days of inhaled terbutaline could up-regulate the pulmonary expression of the β - and γ -ENaC mRNA, but had no effect on α -ENaC mRNA. Regulation of the expression of ENaC subunits may involve terbutaline-induced changes in HSCs and NSCs in the membrane. As NSCs transform into HSCs, there are changes in the biochemical characteristics of the channels, thereby increasing the efficiency of lung fluid transport.³² The mechanism of this effect may involve terbutaline inducing an intracellular pool α -ENaC to return to the surface of the cell membrane, or by reducing the degradation of α -ENaC at the cell membrane. Both mechanisms may increase the number of NSCs on the cell membrane and increase lung water transport thereby improving the efficiency of lung liquid clearance.

We also found that after inhaling 7 days terbutaline, the expression of cAMP, PKA and CREB mRNA in the terbutaline group was significantly increased compared to the LPS group. As well as after the exposure to the intrauterine inflammatory environment, the expression of cAMP, PKA and CREB mRNA in the LPS group was

significantly decreased compared to the control group. This result suggested that terbutaline may regulate the cAMP-PKA-CREB pathway and reduce the inflammatory response through increasing ENaCs expression, which was consistent with previous studies.^{27–30} However, although the decrease of lung water content may be due to an increase of α -ENaC on the cell membrane, further experiments are needed to confirm this supposition. This can be achieved by detecting Na^+ currents through the patch clamp technique in vitro. This is also a limitation of the current study.

5 | CONCLUSION

In summary, the expression of α -, β -, γ -ENaC mRNA, and protein, was decreased in neonatal rat lung tissues following maternal endotoxin exposure that caused intrauterine inflammation. These changes were accompanied by lung injury and pulmonary edema. Inhalation of the β 2-adrenergic receptor agonist terbutaline improved lung liquid clearance in the neonatal rats with lung injury. This therapeutic effect appeared to be mediated by the up-regulation of ENaCs, and cAMP-PKA pathway may be involved with the terbutaline's effect on ENaC expression.

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CONFLICT OF INTEREST

There are no potential conflicts of interest.

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