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Lidocaine pretreatment up-regulates aquaporin-5 expression in primary alveolar epithelium type II cells injured by lipopolysaccharides

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Abstract:Acute respiratory distress syndrome (ARDS) or acute lung injury (ALI) is associated with decreased aquaporin-5 (AQP5) expression. Lipopolysaccharides (LPS) can decrease AQP5 expression. The effects and mechanisms of lidocaine pretreatment on primary alveolar epithelium type II (AEC II) cells injured by LPS were investigated. Primary AEC II cells were isolated from rats previously injured with LPS as an ALI model. The groups of cells were evaluated: 1) pretreated with lidocaine (2, 20, 200μg/ml) and/or Infliximab, an anti-TNF-α neutralizing antibody, 2) uninjured cells; 3) solvent pretreated injured cells and 4) untreated injured cells as controls. TNF-α levels were evaluated by ELISA. AQP5 expression was determined by mRNA and protein expression (q-PCR and western blot).The release of TNF-α was increased significantly in AEC II cells following LPS injury. The release of TNF-α was decreased by 33%-100% as a result of lidocaine pretreatment in a dose-dependent fashion. This decrease was accompanied by up-regulated AQP5 expression in LPS injured AEC II cells, and Infliximab can greatly block AQP5 expression in LPS injured AEC II cells pretreated with lidocaine. Lidocaine pretreatment (2-200μg/ml) of LPS injured AEC II cells results in a decrease in TNF-α release, then up-regulates AQP5 expression, which maybe involved in the mechanism of its effects on AEC II cells injured by LPS.

Keywords: Lidocaine, aquaporin-5,alveolar epithelium type II cells, acute lung injury, tumor necrosis factor alpha.

INTRODUCTION

Diffuse inflammatory lung injury with increased pulmonary edema and the rapid onset of hypoxemic respiratory failure are characteristics of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), which is the more severe form of ALI (E. Ortiz-Diaz, *et al*., 2013; Bellani *et al*., 2016). Increasing evidence shows that aquaporins (AQPs), a group of membrane transporters related to water permeability, such as AQPl, AQP3, AQP4, AQP5, AQP8 and AQP9 are expressed in different regions of the lungs and have different physiological functions (Ishibashi K, *et al*., 2009), which are involved in the edema displayed in the pathological process of ALI/ARDS (Pires-Neto, *et al*., 2016; Blondonnet, *et al*., 2017).

The AQPs are critical in maintaining the pulmonary fluid balance under physiological and pathological conditions. As an example of their varied functions, AQPl mainly transports water from the bronchial to perivascular tissues, while AQP5 clears water from alveolar cavities to the perivascular tissues (Matthay, 2014). The expression of AQPl, AQP3, AQP4, and AQP5 have been shown to be down-regulated in lipopolysaccharides (LPS) induced alveolar epithelium type II (AEC II) cells, a model which serves to emulate acute lung injury. More specifically, the expression levels of AQP5 is reduced to a greater degree than AQP3 and AQP4 (Sun, *et al*., 2014; Alige, *et al*., 2017). Of note, AQP5 plays a major role in regulating fluid balance in the lung and may be involved in the pathogenesis of pulmonary edema in AEC II cells. The decline in the clearance of excessive fluid related to pulmonary edema has been observed in AQP5 knock out mice, resulting in excessive fluid accumulation in alveolar cavities and mesenchyme (Pires-Neto *et al*., 2016; Ware and Matthay, 2001), up-regulation of AQP5 expression maybe involved in the mechanism of improvement of alveolar cavities in ALI animal model established byLPS (Hasan, *et al*., 2014).

Lidocaine, a local anesthetic and a cell membrane stabilizer, is widely used in nerve blocks and as an anti-arrhythmic. The multiple beneficial effects has been reported, including reducing injury from the inhalation of hydrochloric acid, hyperoxia injury, damage from IV endotoxin, acute severe pancreatitis, ischemia-reperfusion injury by inhibiting neutrophil aggregation, and the superoxide anion respiratory burst seen in ALI (Matthay, 2014). Lidocaine preconditioning can lead to a reduction in the expression of AQP_4 and subsequently reduce brain edema in rats with focal cerebral ischemia-reperfusion, which leads to a protective effect on the brain (Hart and Pail, 2000). A decrease of AQP5 in human bronchial epithelial cells and salivary gland acinar cells induced by lidocaine pretreating has been explored (Mezzasoma *et al*, 2013; Yamamura *et al*., 2012). TNFα is a cytokine, which

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is involved in inflammation and upregulated in the acute phase reaction. It has been reported TNFα is one of the main proinflammatory cytokine initially released in LPS-induced acute lung injury (Rhea, *et al*., 2013), the protective effect was occurred when lidocaine pretreated on LPS-induced ALI in rats through NF-κB and p38 MAPK signaling pathway (Chen, *et al.*, 2018). However, research related to the effects of lidocaine pretreatment on pulmonary fluid transport in ALI is very limited. This study investigates the effects of lidocaine on alveolar type II epithelial cells challenged by LPS, and we used TNFα as an indicator of LPS-induced injury and lidocaine anti-inflammation treatment.

MATERIALS AND METHODS

Sprague–Dawley (SD) rats were purchased from Xiangya Animal Laboratories (Changsha, Hunan, China). All animal surgeries used in this study were approved by Central South University Animal Use and Care Committee. Trypsin, type I collagenase was purchased from Sigma-Aldrich (Roche, MO, USA). DEXTRAN 40 was from obtained from Amersham Biosciences (Piscataway, NJ, USA). Nylon meshes (120 and 200 μm) were purchased from Tetko (Elmsford, NY, USA). DNase and LPS were obtained from Sigma (Shanghai, China). Infliximab (anti-TNF-α neutralizing antibody) and lidocaine were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco RBL (Grand Island, NY, USA). Trizol reagent was bought from Invitrogen Life Technologies (Carlsbad, CA, USA). The First Strand cDNA Synthesis kit and SYBR Green PCR Master Mix kit were obtained from MBI Fermentas, Inc. (Vilnius, Lithuania). Aquaporin 5 and GADPH antibodies were got from Abcam (Santa Cruz, UK). Finally, the TNF- assay kits were from Beyotime Biotechnology (Jiangsu, China).

Primary AEC II cells isolation

Primary AEC II (pAEC II) cells were separated from rat lungs, as previously described (Jiwang, *et al*., 2004; Jinle, *et al*., 2017.). AEC II cell samples were collected from three different rats, weight about 200g, however, they shared the same genotype. Rats were anesthetized with 3mL/kg 10% pentobarbital sodium solution. Heparin 200 UI/kg was given to each rat. The heart and lungs of the rats were exposed and a needle was inserted into the pulmonary artery through the right ventricle and the pulmonary artery was flushed with PBS. The lungs were removed, followed by 8 to 10 cycles of alveolar lavage with phosphate-buffered saline (PBS) at37°C. Next, the lungs were digested by instilling a 12 ml mixture of trypsin and type I collagenase (concentration of trypsin is 0.1%) for 10 min at 37 °C; this process was repeated two times. After chopping down the lung tissue, the suspension was mixed with 100μg/ml DNase I, incubatedat 37°Cfor 5 min with gentle rotation, filtered, then centrifuged for 10 min at 12000 rpm/ min. The cells were then incubated in two 100 mm Petri dishes (coated with 1.5 mg rat IgG/dish) sequentially at 37°C, for 1h each. Unattached cells were centrifuged for 9 min at 900rpm/min and resuspended with DMEM/F12 with10% FBS at a concentration of 1-2× 10^6cells/ml in 6-wellplates. After 48h, the supernatants were discarded to remove any remaining macrophages. The resulting cells were cultured to use for the detection of viability, cell yield, and purity evaluated by the following methods.

Light and electron microscope

Proliferating cells were examined daily by phase contrast light microscope (Leica, Wetzlar, Germany), after the pAEC II cells were isolated and cultured for 48h. Cells were fixed with 4% neutral-buffered formalin and stained with haematoxylin-eosin, for transelectron microscopy. Microscope studies were conducted in the electron microscopy department of the Modern Analysis and Testing Center, Central South University, Changsha, Hunan, China.

Evaluation of cell viability and Purity

pAEC II viability was measured by trypan blue dye exclusion. The purity of pAEC II were determined by the modified alkaline phosphatase staining or immunocytochemistry with monoclonal anti-SP-A antibodies. At least 500 cells were counted for purity evaluation.

The dyeing for alkaline phosphatase (ALP)

The pAEC II cells were mounted onto glass cover slips and cultured for 48h. They were then washed with TBS, fixed according to the manufacturer's instructions for the BCIP/NBT kit for phosphatase detection, and kept in dark for 20 minutes. This was followed by nuclear fixation, dyeing, gradient dehydration, and dry mount preparations.

Immunohistochemistry for SP-A

The pAEC II cells were mounted onto glass cover slips and cultured for 60h, and fixed according to SABC kit instructions. SP-A monoclonal antibodies (1:100) were added. SP-A expression in the pAEC II cells was observed through an inverted microscope.

Established the ALI model in vitro

The pAEC II cells were seeded in culture plates and cultured in DMEM-F12, supplemented with 2mM 1-glutamine, 100 UI/ml penicillin, 100μg/ml streptomycin, 10% FCS, 10mM HEPES and 3.6 mg/ml glucose for 24 h to allow attachment, at 37˚C, 5% CO2. Cells were then cultured in 0.1% FCS containing medium for 24 h. Subsequently, cells were injured with LPS (1μg/ml) in phosphate buffered saline. Uninjured cells were cultured under normal conditions as a control. For lidocaine

studies, cells were pretreated with lidocaine at concentrations of 2μg/mL, 20μg/ml or 200μg/ml for 30min respectively, before being exposured to LPS (1μg/ml) for 4 h. Lidocaine or ethyl alcohol (the solvent for lidocaine) with uninjured cells were cultured respectively as controls. To assess the effects of TNF-α release on the down regulation of AQP5 expression in mRNA and protein, cells were pretreated with lidocaine (2μg/ml, 20μg/ml or 200μg/ml) combined with Infliximab at 1 μg/ml. After pretreatment with these inhibitors for 30min, cells were injured with LPS for 4 h to assess AQP5 gene expression by real-time polymerase chain reaction and protein production was assessed by western blot.

Quantitative polymerase chain reaction (qPCR)

Following LPS and/or lidocaine treatment, total RNA was extracted from the pAEC II cells by using Trizol reagent and quantified by detecting the absorbance at 260nm. cDNA was reverse transcripted from RNA using a Reverse Transcriptase kit, then utilized for qPCR. mRNA expression was quantitative analyzed with the Power SYBR Green PCR Master Mix kit by the ABI 7300 real-time PCR system, the primers as follow: AQP-5 primers, sense, Rat-AQP-5-cctcatcttcgtcttctttg-3; anti-sense, Rat-AQP-5-cctattaagagggccagagt-3; GAPDH primers, sense, Rat-GAPDH- 5-ctcatgaccacagtccatgc-3; anti-sense, Rat-GAPDH-5-ttcagctctgggatgacctt-3. The PCR amplification profiles underwent denaturating at 95˚C for 4 min, followed by 40 cycles of denaturating at 95˚C for 30 sec and annealing at 55˚C for 30 sec. For each sample, the amplification reactions, were carried out 4 times. In all qRT-PCR reactions, GADPH for rats was used as a reference gene. The relative gene expression is defined as ΔCp value (ΔCp = (Cp GADPH)-(Cp gene of interest)). Log fold changes were defined as ΔΔCp = ΔCp (treatment)-ΔCp (Control) [19].

Western blotting analysis

pAEC II cells were lysed in T-PER lysis buffer (Thermo Fisher Scientific, Germany), containing proteinaseand phosphatase inhibitors (Roche, Switzerland). Protein concentration was evaluated by the BCA assay (Thermo Fisher Scientific, Germany). The protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane.It were blocked with 5% milk powder in TBST at room temperature for 1h, incubated with the primary antibody of AQP-5 (diluted 1μg/ml) or GADPH (diluted 1:1,000) (both rabbit polyclonal antibodies) at 4˚C overnight, then incubation with the secondary antibodies (goat anti-rabbit, diluted 1:5,000) at room temperature for 1h. The detection was conducted using an ECL kit. The results were normalized to GADPH expression.

ELISA

Supernatants were taken from the cultures of the pAEC IIcells. According to the manufacturer's instructions of the enzyme-linked immunosorbent assay (ELISA), secreted TNF-α was assessed.

STATISTICAL ANALYSIS

Data were expressed as means ± standard deviation of the mean. One-way ANOVA was used for analyses involving a single factor, whereas two-way ANOVA was used for analyses involving two factors. Differences were considered significant when $P<0.05$.

RESULTS

Identification of pAEC II

Phosphatase staining was applied as a marker for pAEC II identification (fig. 1A and B). It was shown that the cytoplasm was rich in purple particles of different sizes, which was the specific staining for ALP in the cells isolated from rats, observed. Immunostaining using anti-SP-A, was shown in (fig. 1C and D). The cells were grown in island-like and fused, SP-A brown granules, the special marker of pAEC II, were be seen in the cytoplasm through light microscope.

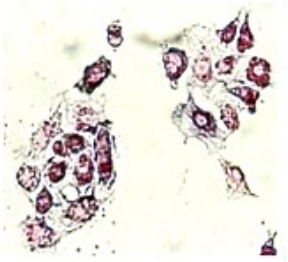

FIG 1A

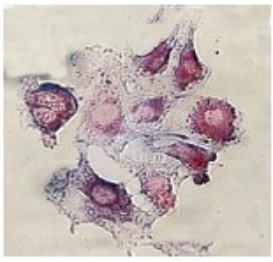

FIG 1B

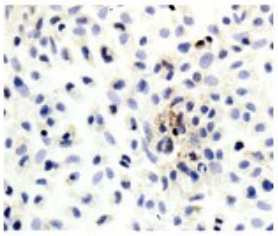

FIG 1C

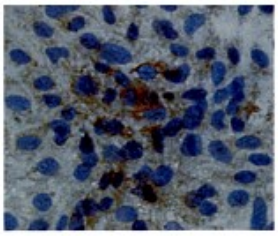

FIG 1D

Fig. 1: AEC II identification. A and B: Purified AEC II were mounted onto glass cover slips and couture for 48h, the dyeing for Phospharate enzyme, it was shown one field in different size. C and D: Purified AEC II cells were mounted onto glass cover slips and coutured for 60h, fixed with paraformaldehyde, and labeled with polyclonal anti-rabbit antibodies for SP-A. A and C: Scale bar: 200×; B and D: Scale bar: 400×.

Morphology of Isolated pAEC II cells

The cells adhered to the walls and grew in a circular or polygonal shape. Nucleoli were seen clearly through light microscopy and there were a large number of small particles in the cytoplasm, which were fused gradually

after 24 hours, and at 48 hours fused as a monolayer. After six to seven days, the particles in the cytoplasm decreased significantly, followed by the cells losing their original shape. Overall, the cells were round or polygonal (fig. 2A, B).

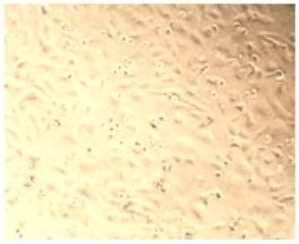

FIG 2A

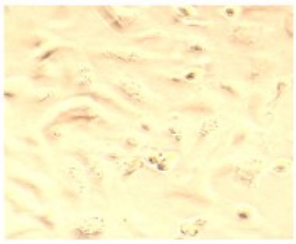

FIG 2B

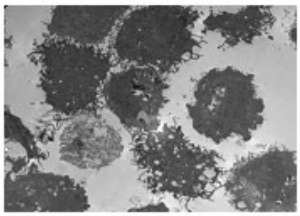

FIG 2C

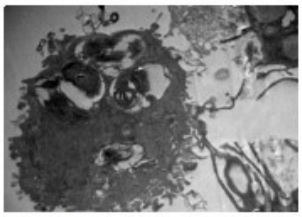

FIG 2D

Fig. 2: A and B: Morphology features of purities of primary AEC II cells. A: Scale bar: 200×; B: Scale bar: 400×. C and D: Electron microscopy images of primary AEC II cells. Original magnifications: (C) AEC II cells 10000×; (D) AEC II cells 15000×.

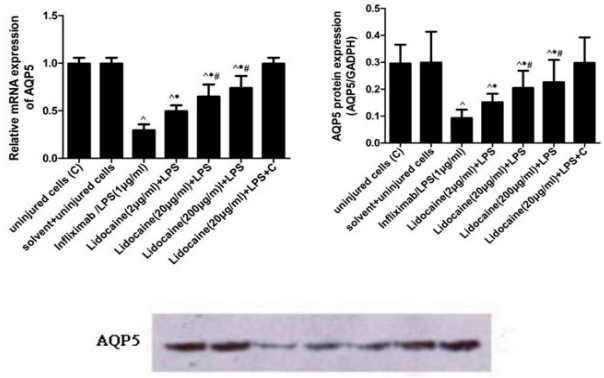

Fig. 3: Lidocaine pretreatment restored aquaporin (AQP)-5 expression in primary AEC II cells injured by LPS. Levels of AQP-5 mRNA(A) and protein (B, C) in LPS-injured primary AEC II cells pretreated with different dose of lidocaine or not, and controls. Values are reported as means ± standard deviations. ^P<0.05, versus uninjured cells; *P<0.05, versus LPS(1μg/ml); #P<0.05, Lidocaine (20μg/ml+LPS) versus Lidocaine (2μg/ml+LPS); Lidocaine (20μg/ml+LPS) versus Lidocaine (200μg/ml+LPS).

The isolated AEC II had typical features of AEC II seen *in vivo* lungs was revealed through transmission electron microscopy analysis, such as Golgi apparatus, mitochondria, abundant microvilli on the surface of the cells, and lamellar bodies of various sizes in the cytoplasm (fig. 2 C, D).

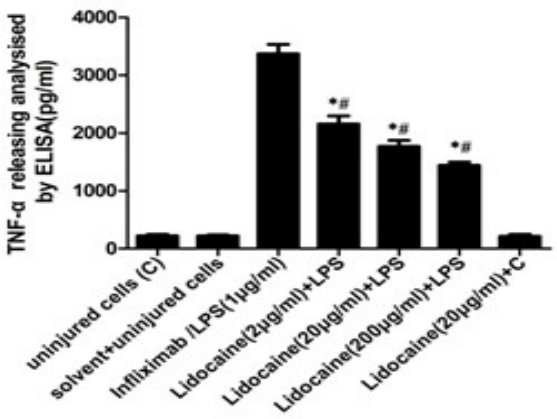

Fig. 4: TNF-α releasing was inhibited by lidocaine pretreatment. Measurement of TNF-α levels in uninjured, untreated cells (control group), LPS-injured cells, lidocaine treated uninjured cells and LPS-injured cells pretreated with lidocaine (2, 20, 200μg/ml, n = 4-6 for the time-point). Levels of cytokines were normalized to those of total protein. As no difference was observed between controls; Values are reported as means ± standard deviations. *P<0.05; versus LPS (1μg/ml); #P<0.05; versusuninjured cells

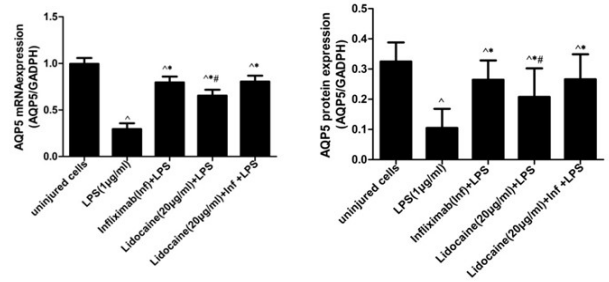

FIG 5A

FIG5C

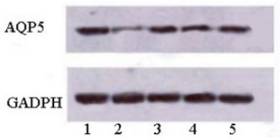

FIG 5B

Fig. 5: Effect of Infliximab and/or lidocaine on AQP5 expression in uninjured cells and LPS injured cells. Levels of AQP-5 proteins were measured in duplicate via western blot (B, C), Threshold levels of mRNA expression ($^{\Delta\Delta}$Cq) were normalized to housekeeping genes (A). Values are reported as means ± standard deviations. ^ P < 0.05, versus uninjured cells; *P<0.05, versus LPS (1μg/ml); #P<0.05, Lidocaine (20μg/ml) +LPS versus Lidocaine (20μg/ml) +Infliximab+LPS.

The viability, production and purity of isolated primary AEC II cells from rats

The Taipan blue assay showed that the cell viability was about 93.2+1.6% after isolation from rat tissue. The pAEC II cell numbers were approximately (8.0±0.3) ×10^7.

After removing the remaining macrophages, the number of cells was estimated at (5.8±0.5)×107, the purity was 89±3.1% as detected by ALP and electron microscopy. These are common methods of evaluating primary AEC II cells.

Effect of lidocaine pretreatment on AQP-5 expression in primary AEC II cells injured by LPS

The expression of AQP-5 in primary AEC II cells was down-regulated after LPS exposure for 4h, compared to controls. Lidocaine pretreation restored the mRNA and protein expressions ofAQP-5 in AEC II cells (fig. 3 A-C). The dose-dependent effect of lidocaine pretreatment was observed from 2μg/ml to 200μg/ml in LPS injured AEC II cells, however, AQP5 expression after treatment different dose of lidocaine were significantly inhibited compared to uninjured cells (fig. 3 A-C).

Effect of lidocaine pretreatment on TNF-α release in primary AEC II cells injured by LPS

Four hours after inducing LPS injuring in AEC II cells, TNF-α levels were increased in the supernatants, as compared to ethyl alcohol or lidocaine controls, which cells were only exposed to ethyl alcohol or lidocaine without LPS, and there was no difference observed between the controls. TNF-α release was significantly attenuated by pretreatment with lidocaine in the LPS injured AEC II cells, the effects were dose dependent, increasing from 2μg/ml to 200μg/ml, however, TNF-α concentration were increased compared to uninjured cells(fig 4).

Effect of TNF-α release on the regulation of AQP5 expression in mRNA and protein

Infliximab, an anti-TNF-α neutralizing antibody, was used to assess the effects of TNF-α release on the down-regulation of AQP5 expression in terms of mRNA and protein. The first, AQP5 expression was evaluated in these groups, uninjured cells group cultured in normal condition, lidocanine or/and Infliximab uninjured cells groups cultured with lidocaine or/and Infliximab, the difference in AQP5 expression was not observed in these groups (data not shown), it was shown AQP5 expression in unjured cells was not changed by lidocaine or/and Infliximab. Then, the effect of Infliximab on AQP5 expression was assessed in LPS injured cell, its expression restored significantly in cells that were pretreated with Infliximab, compared to cells that underwent LPS injury only, however, its expression was inhibited compare with uninjured cells. To investigate whether lidocaine up-regulates AQP5 expression through inhibition TNF-α release in LPS injured ALI model, cells were pretreated with lidocaine 20μg/ml and/or Infliximab at 1μg/ml, then injured with LPS for 4 hrs, there were no difference in AQP5 expression between groups Infliximab and lidocaine combine with Infliximab, the less expression of AQP-5 was seen when cells pretreated with lidocaine (20μg/ml) compared to lidocaine (20μg/ml) combined with Infliximab or Infliximab (fig. 5A-C).

DISCUSSION

During fetal lung development or after lung injury, the damaged epithelium can be repaired through AEC II converted to AEC I, these cells can also synthesize and secrete active substances on the lung surface, involving lung water transport, enhancing local immunity, and other important physiological functions (Hasan, 2014) Damage of AEC II is believed to play a key role in ALI/ARDS. AQPs down-regulation is involved in the edema process seen in ALI, which accompanies inflammation in the lung tissue (Hong-Min, *et al*., 2016; Sun, *et al*., 2015).

Primary isolation and culture of AEC II became one of the main approaches and as a critical step to study its biological functions. The lamellar body as one of specific markers for AEC II, it can be clearly observed in the cytoplasm through transmission electron microscopy, as the gold standard currently for identification AEC II. SP-A specially expresses in AEC II cells, it is the most abundant and easy to test, the specificity sensitivity is high. ALP is a specific marker for the differentiation of AEC II cells, also is a marker of membrane enzyme, can be observed by light microscopy. In the study, we performed lung perfusion, elastase digestion and rat IgG panning to isolate primary AEC II cells from rats, ALP and SP-A were used as markers to identification AEC II cells. Our results demonstrated that these steps were efficient to isolate AEC II cells, the purity was 89±3.1% detected by ALP, SP-A and electron microscopy, which can confirm our further tests.

LPS plays a vital role in the pathogenesis of LPS induced lung injury. LPS-induced AEC II cells act as a direct injury *in vitro* lung model. In our study LPS caused an increase in the release of the inflammatory factor TNF-α and the down-regulation of the expression of AQP5, which has been previously confirmed in other reports (Hasan, *et al*., 2014; Hong-Min, *et al*., 2016; Fang, *et al*., 2019). Corticosteroids, heparin or pulmonary surfactant replacement therapy, etc, have been used in ALI patients, nevertheless, the protective effect is limited in clinic (Sun, *et al.*, 2015; Malaque, *et al*., 2015; Xu*et al*., 2016; Chen, *et al*., 2018), other therapeutic agents are required. In this study, the effects of lidocaine pretreatment on LPS-induced AEC II cell injury was investigated. We founded that TNF-α release from AEC II was significantly inhibited when AEC II were pretreated with lidocaine prior to LPS-induced injury, however, TNF-α release from lidocaine pretreated injured cells were much increased compared to uninjured cells, it is reasonable TNF-α releasing was significantly inhibited by lidocaine pretreatment in LPS injured AEC II cells. Additionally, APQ5 expression was preserved in the AEC II cells in a

dose dependent fashion with lidocaine pretreatment, however, pretreatment with a TNF-α neutralizing antibody (Infliximab) on LPS injured cells caused AQP5 expression to be preserved to some degree, however, the expression was reduced compared to controls, supporting the concept that TNF-α plays a critical role in down-regulation of AQP5 expression in LPS injured AEC II cells. When cells were pretreated with both lidocaine and Infliximab, AQP5 expression were restored, the less expression of AQP-5 was seen when cells pretreated with lidocaine compared to lidocaine combined with Infliximab. It was reasonable lidocaine was restored AQP5 expression in LPS-injured cells through inhibition of TNF-α releasing, attenuate the inflammation response. It is consistent with the report lidocaine could ameliorate the LPS-induced lung injury and excessive inflammatory responses *in vivo*. The further will be needed to explore the signaling of lidocaine restore AQP5 expression via TNF-α. In addition, lidocaine is a sodium channel inhibitor, and how such a drug affects the production of a pro-inflammatory cytokine such as TNF-α is a topic of great interest. Besides, impaired alveolar fluid clearance (AFC) is a major feature of ALI/ARDS that contributes to mortality (Elizabeth, *et al*., 2010; John, *et al*., 2017; Yasmeen, *et al*., 2016). The primary mechanism involved in alveolar edema is ion transport acrossthe alveolar epithelium, primarily through $Na^{+}K$-ATPase, epithelial sodium channels (ENaC), and aquaporin (AQP)-5 channels, the further mechanistic experimentsin which lidocaine is administered after LPS challenge on the improvement of alveolar fluid clearance, regulation of alveolar epithelial channels and the mechanism of how lidocaine inhibits TNF-α secretion in lung cells should be performed.

CONCLUSION

In conclusion, the use of lidocaine pretreatment alleviated lung injury, attenuated TNF-α releasing, then restored lung AQP-5 expression *in vitro* established by LPS-induced primary AT II cells, which is providing a potential for becoming the anti-inflammatory agent against lung injury.

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