

RCTR1 promotes alveolar fluid clearance by activating alveolar epithelial sodium channels and Na, K-ATPase in LPS-induced acute lung injury

Qian Yang^a, Hao-ran Xu^a, Shu-yang Xiang^a, Chen Zhang^b, Yang Ye^a, Chen-xi Shen^a, Hong-xia Mei^a, Pu-hong Zhang^a, Hong-yu Ma^a, Sheng-xing Zheng^a, Fang-gao Smith^{a,c}, Sheng-wei Jin^a, Qian Wang^a

^aDepartment of Anesthesia and Critical Care, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Zhejiang 325027, China

^bWenzhou Medical University

^cInstitute of Inflammation and Aging, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK

Running title: RCTR1 promotes alveolar fluid clearance

Corresponding Author: Sheng-Wei Jin, MD, PhD E-mail: jsw@wmu.edu.cn

Qian Wang, MD, PhD Email: wqian84@163.com

Address: Department of Anesthesia and Critical Care, the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, 109 Xueyuan Road, Wenzhou, Zhejiang Province, P. R. China 325027.

Telephone: 0086-577-88002806 Fax number: 0577-88832693

Number of manuscript pages: 18

Number of figures: 7

Number of tables: 0

Number of references: 44

Number of words in Abstract: 221

Number of words in Introduction: 385

Number of words in Discussion: 1050

Abbreviations:

AEC II: type II alveolar epithelial cells

AFC: alveolar fluid clearance

ALI: acute lung injury

ARDS: acute respiratory distress syndrome

ENaC: epithelial sodium channels

LPS: lipopolysaccharide

RCTR1: Resolvin conjugates in tissue regeneration 1

ABSTRACT

Acute respiratory distress syndrome (ARDS), a common and fatal clinical condition, is characterized by the destruction of epithelium and augmented permeability of the alveolar-capillary barrier. Resolvin conjugates in tissue regeneration (RCTR1) is an endogenous lipid mediator derived from DHA, exerting pro-resolution effects in the process of inflammation. In our research, we evaluated the role of RCTR1 in alveolar fluid clearance (AFC) in Lipopolysaccharide-induced ARDS/ALI rat model. Rats were injected with RCTR1 (5 µg/kg) via caudal veins 8h after LPS (14 mg/kg) treatment, then AFC was estimated after 1h of ventilation. Primary type II alveolar epithelial cells (AEC II) were incubated with LPS (1 ug/ml) with or without RCTR1 (10 nM) for 8 h. Our results showed that RCTR1 significantly enhanced the survival rate, promoted the AFC, and alleviated LPS-induced ARDS/ALI in vivo. Furthermore, RCTR1 remarkably elevated the protein expression of sodium channels and Na, K-ATPase, and the activity of Na, K-ATPase in vivo and in vitro. Additionally, RCTR1 also decreased Nedd4-2 level via up-regulating P-Akt expression. Besides, inhibitors of ALX, cAMP, and PI3K (BOC-2, KH-7, and LY294002) notably inhibited the effects of RCTR1 on AFC. In summary, RCTR1 enhances the protein levels of sodium channels and Na, K-ATPase, and the Na, K-ATPase activity to improve AFC in ALI through ALX/cAMP/PI3K/Nedd4-2 pathway, suggesting that RCTR1 may become a therapeutic drug for ARDS/ALI.

Keywords: ARDS, RCTR1, Alveolar fluid clearance, ENaC, Na, K-ATPase, Nedd4-2

Significance Statement

RCTR1, an endogenous lipid mediator, enhanced the rate of AFC to accelerate the resolution of inflammation in the LPS-induced murine lung injury model. RCTR1 up-regulates the expression of ENaC and Na, K-ATPase in vivo and in vitro to accelerate the AFC. The efficacy of RCTR1 on the ENaC and Na, K-ATPase level was in an ALX/cAMP/PI3K/Nedd4-2-dependent manner.

INTRODUCTION

Acute respiratory distress syndrome (ARDS), a common and potentially fatal clinical condition, is featured by augmented permeability of the alveolar-capillary barrier and alveolar edema, leading to respiratory failure with a high mortality rate (Matthay MA et al., 2019; Sweeney and McAuley, 2016). Despite improvements in supportive treatments for ARDS, effective pharmacotherapies remain scarce (Meyer and Calfee, 2017). Most ARDS patients show the cripple ability of alveolar fluid clearance (AFC), thus the efficient clearance of superfluous alveolar edema fluid at an early phase of the syndrome is imperative for effective treatment (Sznajder, 2001; Ware and Matthay, 2001).

The primary mechanism responsible for AFC involves the vectorial transport of Na⁺ across the alveolar epithelium through the apical ENaC and basolateral Na, K-ATPases (Huppert and Matthay, 2017). Then, an osmotic gradient is created by the active sodium transport, which actuates the reabsorption of edema fluid from alveoli into pulmonary interstitium (Matthay MA et al., 2002). Hence, both sodium channels and Na, K-ATPase play pivotal roles in the removal of pulmonary edema (Matthay MA 2014). Nedd4-2, a ubiquitin ligase, can negatively regulate ENaC by promoting its internalization and degradation, while phosphorylation mediated by Akt can inhibit its activity (Lee et al., 2007; Manning and Kumar, 2018). Furthermore, a previous study revealed that the cAMP-PI3K pathway contributed to the transport of sodium and regulated the distribution of sodium channels and Na, K-ATPase (Niu et al., 2019; Thomas et al., 2004).

Resolvin conjugates in tissue regeneration (RCTRs) are a new series of DHA-derived lipid mediators that exert potent properties facilitating the resolution of inflammation and tissue repair (Krishnamoorthy N. et al., 2018). Macrophages and apoptotic neutrophils produce RCTR1 (8R-glutathionyl-7S,17S-dihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahex-aenoic acid) which has been identified in the spleen, blood, and in resolving infectious exudates from sepsis patients (Serhan et al., 2018). Previous studies demonstrated that RCTR1 (1 nmol/l) could promote the phagocytosis and efferocytosis of human macrophages (de la Rosa et al., 2018). However, the effect

of RCTR1 on the resolution of pulmonary edema and AFC remains unknown.

In the present study, we hypothesized that RCTR1 treatment would alleviate lipopolysaccharide (LPS)-induced ARDS/ALI and pulmonary edema through accelerating alveolar fluid clearance. Additionally, we investigated the effects of RCTR1 on sodium channels and Na, K-ATPase expression, and the Na, K-ATPase activity in vivo and in vitro. Moreover, inhibitors (ALX, cAMP, and PI3K) were used to explore the underlying mechanisms of RCTR1 treatment.

MATERIALS AND METHODS

Materials

RCTR1 (Item No.24896) was purchased from Cayman Chemical Company. LPS (*Escherichia coli* serotype 055:B5) was obtained from Sigma. ELISA kits for cAMP, MPO, TNF- α , IL-1 β , and IL-10 were from R&D Systems. KH7 (cAMP antagonist) and LY294002 (PI3K antagonist) were obtained from MedChemExpress. MK-2206 (Akt inhibitor) was from Target Molecule Corp. BOC-2 (ALX inhibitor) was from Biomol-Enzo Life Sciences. Anti-Nedd4-2, T-Akt, and P-Akt antibodies were from Cell Signaling Technology. Anti-ENaC subunits (α , β , or γ) antibodies were purchased from Affinity. Anti-Na, K-ATPase subunits (α 1 or β 1) antibodies were obtained from Abcam.

Animal preparation

Male Sprague-Dawley rats (250–300 g) were purchased from the Shanghai Laboratory Animal Center. Rats were housed in a pathogen-free room maintaining a light-dark cycle for 12 h with air temperature (22 to 26 °C) and relative humidity (60%–65%) with free access to water and food (MD17111, Medicience Ltd, China). The research was authorized by the Animal Studies Ethics Committee of Wenzhou Medical University and was implemented according to the Guide for the Care and Use of Laboratory Animals (Institution of Laboratory Animal Research, The National Academies Press, Eighth edition, 2011).

The rat model of acute lung injury was established by the injection of LPS (14 mg/kg) via the tail vein. For exploring the optimal RCTR1 dose in vivo, rats were received

RCTR1 of 1, 5, or 10 µg/kg intravenously 8 h after LPS administration. Then, rats were randomly divided into eight groups (n=4-6), including control, LPS, LPS+RCTR1, RCTR1, LPS+RCTR1+BOC-2, LPS+RCTR1+KH-7, LPS+RCTR1+LY294002, and LPS+RCTR1+MK-2206 groups. RCTR1 (5 µg/kg) was administrated intravenously 8 h after LPS injection. In the LPS+RCTR1+BOC-2 or KH-7 or MK-2206 or LY294002 groups, we treated the rats with BOC-2 (0.6 µg/kg), KH-7 (1 mg/kg), MK-2206 (0.3 mg/kg) or LY294002 (3 mg/kg), respectively, though the tail vein 30 min before RCTR1 treatment (Zhang PH et al., 2020; Zhang Z. et al., 2017; Zhuo et al., 2018). After anesthetization with 30 mg/kg of 2% pentobarbital sodium intraperitoneally, the experimental rats were mechanically ventilated for 1 h. After measuring AFC and obtaining a lung tissue sample, the experiment was terminated when the animal was euthanized. For surviving curve, rats in LPS group and LPS+RCTR1 group (n=12) were observed until day 5 without receiving the AFC measurement, and finally, the surviving rats were euthanized with 200 mg/kg 2% pentobarbital sodium.

Measurement of AFC

The measurement method of alveolar fluid clearance (AFC) on live rats was described previously (Wang et al., 2013). After anesthesia, the perfusion solution including 5% albumin and Evans blue (150 µg/ml) was perfused into each rat's left lung through a tracheostomy tube. After 1 h of mechanical ventilation, the alveolar perfusion solution was collected. Both the original and final protein concentrations of the perfusion solution were detected using spectrophotometry at 621 nm wavelength. AFC was calculated using this formula: $AFC = (1 - C_0/C_1)$, in which C_0 and C_1 represented the initial and final concentrations of the Evans blue-labeled albumin in alveolar perfusion solution, respectively.

Pathological studies

After being fixed in 4% paraformaldehyde and embedded in paraffin, the lung tissue blocks were cut into 5-µm sections and then stained with H&E for microscopic observation. The lung injury scores were evaluated based on alveolar edema, alveolar collapse, inflammatory cells infiltration, and the thickness of the alveolar wall

(Matute-Bello et al., 2011). The degree of the lung damage was scored from 0 (no damage) to 16 (severe damage).

ELISA

The concentrations of IL-10, MPO, TNF- α , IL-1 β , and cAMP in the homogenates of lung tissue were detected using their respective ELISA kits following the protocol provided by the manufacturer.

Na, K-ATPase activity measurement

Lung tissue homogenates were harvested for measuring the Na, K-ATPase activity using a minimal ATP enzyme test kit (Jiancheng Company).

Transmission electron microscopy

Lung tissue samples were fixed 24 h in 2.5% glutaraldehyde, then postfixed 60 min in 1% osmium tetroxide. After rinsing with PBS three times, samples were sequentially dehydrated in gradient acetone and then embedded in epoxy resin. After staining with uranyl acetate and cut into 60–100 nm slices, subsequently, the ultrathin sections were observed and photographed by Zeiss EM 10C transmission electron microscope (HITACHI, H-7500) operated with an accelerating voltage of 60 kV.

Immunohistochemistry

After dewaxed and hydrated, lung sections were repaired with citrate solution and were treated with 3% H₂O₂ to restrain the activity of endogenous peroxidase. Subsequently, the sections were blocked with 10% donkey serum, incubated with primary antibodies overnight, and then incubated with HRP-labeled secondary antibodies for 1 h. After stained with DAB and hematoxylin, the lung sections were observed and photographed by microscope.

Extraction and preparation of primary rat type II alveolar epithelial cells

The lung tissues were digested by elastase for 20 min to extract the primary rat AEC II. Then the cell suspension was loaded onto IgG-coated plates for the removal of fibroblasts and alveolar macrophages as described previously (Dobbs et al., 1988). The primary AEC II were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum in 5% CO₂ and 95% air atmosphere. After serum-derived for 24 h, AEC II was cultured with LPS (1 mg/ml) with or without

RCTR1 (10 nM). BOC-2 (10 μ M), KH-7 (10 μ M), and LY294002 (10 μ M) were added to the medium 1 h before LPS and/or RCTR1 administration (Niu et al., 2019; Zhang PH et al., 2020).

Western blotting

After extraction, proteins from the lung tissue samples and primary epithelial cells were resolved by gel electrophoresis and transferred onto PVDF membranes. After incubating in primary and secondary antibodies, the protein bands were visualized by an Odyssey CLx imager and evaluated using ImageJ.

Confocal imaging

The primary AEC II was fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked with 10% donkey serum. Cells were immunostained with primary antibodies for 16 h at 4 °C and then incubated with relevant secondary antibodies at room temperature for 1 h. A confocal laser-scanning microscope (Leica) was used for capturing the cell images.

Statistical analysis

The data are presented as mean \pm SD. One-way ANOVA was performed to compare multiple individual datasets. Statistical analyses were processed using GraphPad Prism software (version 8.0.1), with significance accepted at P values < 0.05.

RESULTS

RCTR1 increased the survival rate and AFC in Lipopolysaccharide-induced acute lung injury

First, we investigated whether RCTR1 could improve the survival rate of the LPS-induced ALI rat model (Figure 1A). The mortality of the LPS+RCTR1 group was less than that of LPS group (Figure 1B). After administration of 14 mg/kg LPS, different doses of RCTR1 (1 μ g/kg, 5 μ g/kg, and 10 μ g/kg) were injected into the rats intravenously to evaluate the effect of RCTR1 on AFC which was detected after 1 h of sustained ventilation. (Figure 1C). However, compared to the LPS group, RCTR1 at 5 μ g/kg remarkably improved AFC (Figure 1C). There was no statistical difference between the RCTR1 5 μ g/kg and 10 μ g/kg doses (Figure 1C). Consequently, RCTR1

at a concentration of 5 ug/kg was used in our study.

RCTR1 mitigated Lipopolysaccharide-induced ALI in vivo

Next, we investigated the effect of RCTR1 on LPS-induced lung injury. Compared with the control group, there was significant damage presented with the LPS group, manifested by an increased lung injury scores determined from pulmonary histology with alveolar edema, neutrophils infiltration, hemorrhage, and thickening of the alveolar wall (Figure 2A, B). Treatment with RCTR1 markedly alleviated the LPS-induced morphologic changes, indicated by significantly decreased lung injury scores (Figure 2A, B). However, the control and RCTR1 groups showed no statistical difference (Figure 2B). Compared with the control group, the lung tissue homogenates in the LPS group revealed elevated levels of inflammatory factors, including TNF- α , MPO, IL-1 β , and IL-10 (Figure 2C-F). Of note is the observation that these inflammatory factors were obviously reduced after RCTR1 treatment (Figure 2C-F). Additionally, the ultrastructure of lung tissues observed with a transmission electron microscope in the LPS group showed severe vacuolation of lamellar bodies and destruction of the air-blood barrier featuring damaged epithelial bridges and capillary walls (Figure 2G, H). Treatment with RCTR1 produced obvious recovery from these pathological changes (Figure 2G, H).

RCTR1 up-regulated LPS-inhibited sodium channels and Na, K-ATPase expression in vivo

Subsequently, our study assessed the mechanism by which RCTR1 augments the AFC that is mediated by sodium channels and Na, K-ATPase. Administration of LPS (14 mg/kg) for 8 h resulted in significant down-regulation of the protein levels of ENaC subunits (α , β , and γ) and Na, K-ATPase subunits (α 1 and β 1) in lung tissue homogenates, which was reversed by RCTR1 treatment (Figure 3A-F). Immunohistochemical analysis demonstrated that ENaC α subunit was located in the apical surfaces of the alveolar epithelium. The decrease in protein expression of ENaC α subunit was recovered in the LPS+RCTR1 group, which agreed with the western blot results (Figure 3H). Furthermore, LPS-suppressed Na, K-ATPase activity was enhanced by the RCTR1 treatment in vivo (Figure 3G).

RCTR1 improved AFC partly through stimulating the ALX/cAMP/PI3K/Nedd4-2 pathway in vivo

Then, to explore the signaling pathway involved in the improvement in AFC following RCTR1 treatment, we determined the concentration of cAMP in lung tissue homogenates by ELISA. The cAMP level in the LPS group was significantly reduced compared with the control group, and RCTR1 treatment notably enhanced the cAMP level in LPS-challenged rats (Figure 4A). However, the use of BOC-2 (ALX inhibitor, 0.6 µg/kg) suppressed the up-regulating effect of RCTR1 on cAMP, indicating that RCTR1 activates the ALX/cAMP pathway (Figure 4A).

Next, the co-administration of BOC-2 (ALX antagonist, 0.6 µg/kg), KH-7 (cAMP antagonist, 1 mg/kg), and LY294002 (PI3K antagonist, 3 mg/kg) with RCTR1 was investigated to determine whether its efficacy on improving AFC was mediated by the ALX/cAMP/PI3K pathway. We found that AFC in the LPS+RCTR1+BOC-2 or KH-7 or LY294002 groups was lower than that in the LPS+RCTR1 group (Figure 4B), illustrating that BOC-2, KH-7, and LY294002 partly inhibited the improving effect of RCTR1 on AFC. Consequently, RCTR1 increases AFC partly depending on the ALX/cAMP/PI3K pathway.

As a ubiquitin ligase, Nedd4-2 is a crucial negative regulator of ENaC, which can be phosphorylated and inactivated by Akt. Therefore, the protein expression of Ser⁴⁷³-phosphorylated-Akt (P-Akt) was detected. Our results revealed that the ratio of P-Akt/T-Akt in LPS group was lower than that in the control group, whereas RCTR1 treatment markedly enhanced the ratio (Figure 4C). By contrast, the ratio of P-Akt/T-Akt was lower in the LPS+RCTR1+BOC-2 or KH-7 or LY294002 groups compared with the LPS+RCTR1 group, demonstrating that BOC-2, KH-7, and LY294002 could partly block the enhancement of P-Akt by RCTR1 (Figure 4C). Furthermore, we evaluated the Nedd4-2 expression in lung tissue using western blot. Compared with the control group, LPS treatment up-regulated the Nedd4-2 expression, which was down-regulated by RCTR1 (Figure 4D). However, the groups of LPS+RCTR1+BOC-2 or KH-7 or LY294002 or MK-2206 showed higher protein expression of Nedd4-2 than the LPS+RCTR1 group, indicating that the

down-regulating effect of RCTR1 on Nedd4-2 expression could be partly suppressed by the inhibitors (BOC-2, KH-7, LY294002, and MK-2206) (Figure 4D, E). Therefore, RCTR1 activates the ALX/cAMP/PI3K/Nedd4-2 pathway.

The regulation of RCTR1 on the expression of Na, K-ATPase α 1 subunits in primary AEC II

The primary AEC II were incubated in LPS (1 μ g/ml) and different doses of RCTR1 (1 nM, 10 nM, 50 nM, 100 nM). The protein levels of Na, K-ATPase α 1 subunits decreased obviously in the LPS-treated cells and increased obviously with RCTR1 (10 nM, 50 nM, 100 nM) (Figure 5A). There were no statistical differences among the 10 nM, 50 nM, and 100 nM doses (Figure 5A). Next, we cultured the primary AEC II with 1 μ g/ml LPS and 10 nM RCTR1 for different durations (4 h, 8 h, 12 h). Then, our results showed that the upregulation of protein levels of Na, K-ATPase α 1 was significant at 8 h and 12 h, and there were no statistical differences between 8 h and 12 h (Figure 5B). Therefore, RCTR1 (10 nM) was used for 8 h in the subsequent experiments in vitro.

RCTR1 enhanced the protein expression of sodium channels and Na, K-ATPase in primary rat AEC II via the ALX/cAMP/PI3K/Nedd4-2 pathway

Primary AEC II were stimulated with 1 μ g/ml LPS with or without RCTR1 (10 nM) treatment for 8 h. A considerable increase in the expression of ENaC α subunit and Na, K-ATPase α 1 subunit was observed in the RCTR1-treated AEC II (Figure 6A). The protein levels of ENaC subunits (α , β , and γ) and Na, K-ATPase subunits (α 1 and β 1) were obviously diminished in LPS-challenged cells and promoted by RCTR1 treatment (Figure 6B-G). Moreover, RCTR1 up-regulated the LPS-induced decrease in P-Akt, and the up-regulating effect was partly blocked by BOC-2 (10 μ M), KH-7 (10 μ M), and LY294002 (10 μ M) (Figure 6H). Additionally, RCTR1 inhibited the LPS-induced elevation in the Nedd4-2 expression, while BOC-2, KH-7, and LY294002 reversed this effect (Figure 6I). In conclusion, RCTR1 promoted the expression of sodium channels and Na, K-ATPase through the ALX/cAMP/PI3K/Nedd4-2 pathway in vitro.

DISCUSSION

Our data reveal an important therapeutic role for RCTR1 in LPS-induced ARDS/ALI. Our data show that RCTR1 effectively enhanced the rate of AFC, improved survival rates, accelerated the lung inflammation resolution, and alleviated pulmonary damage in the Lipopolysaccharide-induced ALI model. Furthermore, our data demonstrated that treatment with RCTR1 up-regulates the ENaC and Na, K-ATPase protein expression in vivo and in vitro, and the Na, K-ATPase activity in vivo. Moreover, RCTR1 enhanced the expression of P-Akt and decreased the Nedd4-2 levels in vivo as well as in vitro. The efficacy of RCTR1 on AFC was partially inhibited by BOC-2, KH-7, and LY294002, demonstrating that RCTR1 improved AFC and the ENaC level via the ALX/cAMP/PI3K/Nedd4-2 signaling pathway (Figure 7).

The disrupted alveolar epithelium and endothelium, which leads to the increased alveolar-capillary permeability, is the primary characteristic of ARDS/ALI (Matthay M. A. et al., 2019; Thompson et al., 2017). Impaired AFC is closely related to the progression and severity of ARDS (Jabaudon et al., 2015; Ware and Matthay, 2001). Thus, recovery from ARDS necessitates the removal of alveolar edema fluid. Our results indicated that RCTR1 significantly enhanced AFC and survival rates in LPS-induced ALI. Additionally, interstitial edema, increased neutrophil infiltration, alveolar wall thickening, damaged air-blood barrier, and vacuolar lamellar bodies in AEC II, which were the specific pathological changes observed in the LPS-challenged lung, were reversed by RCTR1 treatment. The increased concentration of a series of inflammatory cytokines in the LPS-challenged lung tissue was suppressed by RCTR1. Our experimental data imply that RCTR1 protects lung tissue and facilitates the resolution of inflammation and alveolar edema in LPS-induced ALI.

As is well-known, vectorial transport of Na⁺ is requisite for AFC (Matthay MA et al., 2002). Na⁺ transport from the alveoli into epithelial cells depends on sodium channels located on the apical membrane of alveolar epithelium (Eaton et al., 2009). Subsequently, intracellular sodium ions are pumped into the interstitium via the Na, K-ATPase on the basolateral surfaces of alveolar epithelium. Then, an osmotic gradient is established inside and outside the alveoli, which propels the removal of

fluid from alveoli into lung interstitium via aquaporins (Sartori and Matthay, 2002). Then, oedematous fluid in the interstitium can be cleared by the microcirculatory and lymphatic systems (Matthay M. A. et al., 2012; Matthay M. A. et al., 2019). ENaC, composed of α , β , and γ subunits, is the main driving force of AFC (Guidot et al., 2006; Hummler E, 2010). ENaC α -deficient mice lost the ability to clear edema fluid in alveoli and died within 40 h after birth (Hummler et al., 1996). The most important subunits of Na, K-ATPase in the lung are $\alpha 1$ and $\beta 1$, acting synergistically with apical ENaC to regulate AFC (Vadász et al., 2007). The impaired function of Na, K-ATPase serves as an indicator of lung injury (Guazzi et al., 2015). It has been reported that elevated expression of sodium channels and Na, K-ATPase could promote sodium transport, resulting in improved clearance of oedematous fluid in alveoli (Morty RE, 2007; Sartori and Matthay, 2002; Wang et al., 2013). Our study showed that RCTR1 increased the expression of sodium channels and Na, K-ATPase, and the Na, K-ATPase activity in LPS-induced ALI rats as well as in primary AEC II stimulated with LPS. These findings imply that RCTR1 improves Na⁺ transport across the cytomembranes from the alveoli into the pulmonary interstitium.

Belonging to specialized pro-resolving mediators (SPMs), DHA-derived RCTR1 plays a key role in orchestrating the inflammation resolution (Basil and Levy, 2016; Krishnamoorthy N. et al., 2018). While the specific receptor for RCTR1 remains to be determined, ALX/FPR2 has been identified as the receptor for other members of the SPMs, such as LXA4 and RvD1 (Krishnamoorthy S. et al., 2012; Krishnamoorthy S. et al., 2010). The ALX receptor, a 7-transmembrane GPCR, transmits cell-type-specific signaling pathways (Chiang et al., 2006; Serhan et al., 2008). Our results demonstrated that the stimulative efficacy of RCTR1 on AFC was partially repressed by ALX inhibitor (BOC-2), implying that the effect of RCTR1 partly depends on the ALX receptor. As a primary second messenger, cAMP is responsible for signal transduction inside and outside the cell to trigger physiological changes (Nachury and Mick, 2019). The binding of extracellular ligands to ALX receptor can activate intracellular adenylate cyclase and then influence cAMP levels (Wang et al., 2013). The activation of cAMP promotes Na, K-ATPase transfer to plasma membranes to

boost Na⁺ transport and mediates AFC in the removal of alveolar edema (Lecuona et al., 2009; Thomas et al., 2004). In the current study, RCTR1 elevated intracellular cAMP levels (which decreased with LPS treatment), an effect that BOC-2 inhibited. Furthermore, the cAMP antagonist (KH-7) partly abrogated the efficacy of RCTR1 on AFC, indicating that RCTR1 improves AFC through the ALX/cAMP pathway.

It is generally accepted that PI3K can be activated by cAMP signals and that it regulates ENaC distribution and activity (Deng et al., 2012; Soundararajan et al., 2009; Thomas et al., 2004). Our results showed that LY294002 (PI3K inhibitor) significantly suppressed RCTR1-induced improvements in AFC, demonstrating that the efficacy of RCTR1 is dependent on PI3K signals. Akt, the serine/threonine protein kinase, is an important signaling molecule downstream of PI3K signal pathway, controlling various physiological processes (Hoxhaj and Manning, 2020; Lee et al., 2007). Our studies have revealed that RCTR1 increased LPS-reduced P-Akt levels, whereas BOC-2, KH-7, and LY294002 partly inhibited the effect of RCTR1 on P-Akt. It has been identified that Nedd4-2, as an E3 ubiquitin ligase, can ubiquitinate ENaC resulting in the internalization and degradation of the sodium channels, whereas P-Akt can phosphorylate Nedd4-2 to prevent its interaction with ENaC (Kim et al., 2018; Manning and Kumar, 2018). Consistently, our research also showed that RCTR1 could reverse the LPS-increased Nedd4-2 expression, while the inhibitory effect of RCTR1 was partly suppressed by BOC-2, KH-7, and LY294002. These data illustrate that RCTR1 decreases Nedd4-2 levels thereby diminishing the internalization and degradation of ENaC. In conclusion, RCTR1 promotes AFC by increasing the ENaC levels by stimulating the ALX/PI3K/P-Akt/Nedd4-2 signaling pathway.

In summary, our results show that RCTR1 promotes the clearance of alveolar edema fluid through enhancing the protein levels of sodium channels and Na, K-ATPase, and improving the Na, K-ATPase activity, which is mediated by the ALX/cAMP/PI3K/Nedd4-2 pathway. Thus, RCTR1 exhibits potent effects in alleviating ALI and facilitating the resolution of pulmonary edema. Overall, our research indicates that RCTR1 may become a therapeutic drug for ARDS/ALI.

Acknowledgments

This work was performed in the Zhejiang Province Key Lab of Anesthesiology, the Second Affiliated Hospital, Wenzhou Medical University

Conflict of interest

The authors confirm that there are no conflicts of interest.

Authorship Contributions

Participated in research design: Qian Yang, Hao-ran Xu, Sheng-wei Jin, Qian Wang.

Conducted experiments: Qian Yang, Hao-ran Xu, Shu-yang Xiang, Chen Zhang, Yang Ye, Chen-xi Shen. Performed data analysis: Qian Yang, Hong-xia Mei, Pu-hong Zhang, Hong-yu Ma, Sheng-xing Zheng. Wrote or contributed to the writing of the manuscript: Qian Yang, Fang-gao Smith, Qian Wang.

REFERENCES

- Basil MCLevy BD (2016) Specialized pro-resolving mediators: endogenous regulators of infection and inflammation. *Nat Rev Immunol* 16:51-67
- Chiang N, Serhan CN, Dahlén SE, Drazen JM, Hay DW, Rovati GE, Shimizu T, Yokomizo TBrink C (2006) The lipoxin receptor ALX: potent ligand-specific and stereoselective actions in vivo. *Pharmacological reviews* 58:463-87
- de la Rosa X, Norris PC, Chiang N, Rodriguez AR, Spur BWSerhan CN (2018) Identification and Complete Stereochemical Assignments of the New Resolvin Conjugates in Tissue Regeneration in Human Tissues that Stimulate Proresolving Phagocyte Functions and Tissue Regeneration. *The American journal of pathology* 188:950-966
- Deng W, Li CY, Tong J, Zhang WWang DX (2012) Regulation of ENaC-mediated alveolar fluid clearance by insulin via PI3K/Akt pathway in LPS-induced acute lung injury. *Respiratory research* 13:29
- Dobbs L, Williams MGonzalez R (1988) Monoclonal antibodies specific to apical surfaces of rat alveolar type I cells bind to surfaces of cultured, but not freshly isolated, type II cells. *Biochimica et biophysica acta* 970:146-56
- Eaton DC, Helms MN, Koval M, Bao HFJain L (2009) The contribution of epithelial sodium channels to alveolar function in health and disease. *Annu Rev Physiol* 71:403-23
- Guazzi M, Phillips SA, Arena RLavie CJ (2015) Endothelial dysfunction and lung capillary injury in cardiovascular diseases. *Progress in cardiovascular diseases* 57:454-62
- Guidot DM, Folkesson HG, Jain L, Sznajder JI, Pittet JFMatthay MA (2006) Integrating acute lung injury and regulation of alveolar fluid clearance. *American journal of physiology. Lung cellular and molecular physiology* 291:L301-6
- Hoxhaj GManning BD (2020) The PI3K-AKT network at the interface of oncogenic signalling and cancer metabolism. *Nature reviews. Cancer* 20:74-88
- Hummler E, Barker P, Gatzky J, Beermann F, Verdumo C, Schmidt A, Boucher RRossier BC (1996) Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice. *Nature genetics* 12:325-8
- Hummler E PC (2010) Importance of ENaC-mediated sodium transport in alveolar fluid clearance using genetically-engineered mice. *Cell. Physiol. Biochem.*
- Huppert LAMatthay MA (2017) Alveolar Fluid Clearance in Pathologically Relevant Conditions: In Vitro and In Vivo Models of Acute Respiratory Distress Syndrome. *Frontiers in immunology* 8:371
- Jabaudon M, Blondonnet R, Roszyk L, Bouvier D, Audard J, Clairefond G, Fournier M, Marceau G, Déchelotte P, Pereira B, Sapin VConstantin JM (2015) Soluble Receptor for Advanced Glycation End-Products Predicts Impaired Alveolar Fluid Clearance in Acute Respiratory Distress Syndrome. *American journal of respiratory and critical care medicine* 192:191-9
- Kim Y, Yoo H, Hong A, Kwon M, Kang S, Kim KSong YJFjopotFoASfEB (2018) NEDD4L limits cAMP signaling through ubiquitination of CREB-regulated transcription coactivator 3. *32:4053-4062*
- Krishnamoorthy N, Abdalnour RE, Walker KH, Engstrom BDLevy BD (2018)

Specialized Proresolving Mediators in Innate and Adaptive Immune Responses in Airway Diseases. *Physiol Rev* 98:1335-1370

Krishnamoorthy S, Recchiuti A, Chiang N, Fredman G, Serhan CN (2012) Resolvin D1 receptor stereoselectivity and regulation of inflammation and proresolving microRNAs. *The American journal of pathology* 180:2018-27

Krishnamoorthy S, Recchiuti A, Chiang N, Yacoubian S, Lee CH, Yang R, Petasis N, Serhan CN (2010) Resolvin D1 binds human phagocytes with evidence for proresolving receptors. *Proceedings of the National Academy of Sciences of the United States of America* 107:1660-5

Lecuona E, Minin A, Trejo HE, Chen J, Comellas AP, Sun H, Grillo D, Nekrasova OE, Welch LC, Szleifer I, Gelfand V, Snajder JJ (2009) Myosin-Va restrains the trafficking of Na⁺/K⁺-ATPase-containing vesicles in alveolar epithelial cells. *Journal of cell science* 122:3915-22

Lee IH, Dinudom A, Sanchez-Perez A, Kumar S, Cook DI (2007) Akt mediates the effect of insulin on epithelial sodium channels by inhibiting Nedd4-2. *The Journal of biological chemistry* 282:29866-73

Manning JA, Kumar S (2018) Physiological Functions of Nedd4-2: Lessons from Knockout Mouse Models. *Trends in biochemical sciences* 43:635-647

Matthay M (2014) Resolution of pulmonary edema. Thirty years of progress. *American journal of respiratory and critical care medicine* 189:1301-8

Matthay M, Folkesson H, Clerici C (2002) Lung epithelial fluid transport and the resolution of pulmonary edema. *Physiological reviews* 82:569-600

Matthay M, Zemans R, Zimmerman G, Arabi Y, Beitler J, Mercat A, Herridge M, Randolph A, Calfee C (2019) Acute respiratory distress syndrome. *Nat Rev Dis Primers* 5:18

Matthay MA, Ware LB, Zimmerman GA (2012) The acute respiratory distress syndrome. *The Journal of clinical investigation* 122:2731-40

Matthay MA, Zemans RL, Zimmerman GA, Arabi YM, Beitler JR, Mercat A, Herridge M, Randolph AG, Calfee CS (2019) Acute respiratory distress syndrome. *Nat Rev Dis Primers* 5:18

Matute-Bello G, Downey G, Moore B, Groshong S, Matthay M, Slutsky A, Kuebler W (2011) An official American Thoracic Society workshop report: features and measurements of experimental acute lung injury in animals. *American journal of respiratory cell molecular biology* 44:725-38

Meyer N, Calfee C (2017) Novel translational approaches to the search for precision therapies for acute respiratory distress syndrome. *Lancet Respir Med* 5:512-523

Morty RE, EO, Seeger W (2007) Alveolar fluid clearance in acute lung injury: what have we learned from animal models and clinical studies? *Intensive Care Med* 33:1241-1242

Nachury MV, Mick DU (2019) Establishing and regulating the composition of cilia for signal transduction. *Nat Rev Mol Cell Biol* 20:389-405

Niu F, Xu X, Zhang R, Sun L, Gan N, Wang A (2019) Ursodeoxycholic acid stimulates alveolar fluid clearance in LPS-induced pulmonary edema via ALX/cAMP/PI3K pathway. *Journal of cellular physiology* 234:20057-20065

- Sartori CMatthay MA (2002) Alveolar epithelial fluid transport in acute lung injury: new insights. *The European respiratory journal* 20:1299-313
- Serhan CN, Chiang NDalli J (2018) New pro-resolving n-3 mediators bridge resolution of infectious inflammation to tissue regeneration. *Molecular aspects of medicine* 64:1-17
- Serhan CN, Chiang NVan Dyke TE (2008) Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol* 8:349-61
- Soundararajan R, Melters D, Shih IC, Wang JPearce D (2009) Epithelial sodium channel regulated by differential composition of a signaling complex. *Proceedings of the National Academy of Sciences of the United States of America* 106:7804-9
- Sweeney RMcAuley D (2016) Acute respiratory distress syndrome. *Lancet* 388:2416-2430
- Sznajder J (2001) Alveolar edema must be cleared for the acute respiratory distress syndrome patient to survive. *American journal of respiratory and critical care medicine* 163:1293-4
- Thomas CP, Campbell JR, Wright PJHusted RF (2004) cAMP-stimulated Na⁺ transport in H441 distal lung epithelial cells: role of PKA, phosphatidylinositol 3-kinase, and sgk1. *Am J Physiol Lung Cell Mol Physiol* 287:L843-51
- Thompson BT, Chambers RCLiu KD (2017) Acute Respiratory Distress Syndrome. *N Engl J Med* 377:562-572
- Vadász I, Raviv SSznajder JI (2007) Alveolar epithelium and Na,K-ATPase in acute lung injury. *Intensive Care Med* 33:1243-1251
- Wang Q, Lian Q, Li R, Ying B, He Q, Chen F, Zheng X, Yang Y, Wu D, Zheng S, Huang C, Smith FJin S (2013) Lipoxin A(4) activates alveolar epithelial sodium channel, Na,K-ATPase, and increases alveolar fluid clearance. *American journal of respiratory cell and molecular biology* 48:610-8
- Ware LMatthay M (2001) Alveolar fluid clearance is impaired in the majority of patients with acute lung injury and the acute respiratory distress syndrome. *American journal of respiratory and critical care medicine* 163:1376-83
- Zhang P, Han J, Cao F, Liu Y, Tian C, Wu C, Smith F, Hao YJin SJJocp (2020) PCTR1 improves pulmonary edema fluid clearance through activating the sodium channel and lymphatic drainage in lipopolysaccharide-induced ARDS. 235:9510-9523
- Zhang Z, Fang P, Guo L, He B, Shi M, Zhu YBo P (2017) Akt2-Dependent Beneficial Effect of Galanin on Insulin-Induced Glucose Uptake in Adipocytes of Diabetic Rats. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 41:1777-1787
- Zhuo XJ, Hao Y, Cao F, Yan SF, Li H, Wang Q, Cheng BH, Ying BY, Smith FGJin SW (2018) Protectin DX increases alveolar fluid clearance in rats with lipopolysaccharide-induced acute lung injury. *Exp Mol Med* 50:49

Footnotes

This work was sponsored by grants from the National Natural Science Foundation of China (no. 81570076) and by the Natural Science Foundation of Zhejiang Province

(LY18H010005), Provincial Medical and health science and technology project (2021455293) and Research Fund for Lin He's Academician Workstation of New Medicine and Clinical Translation (19331102).

Figure Legends

Figure 1: RCTR1 enhanced the survival rate and AFC in LPS-induced ALI. A.

Rats were injected with LPS (14 mg/kg) via caudal veins to establish ALI model. After 8 h, rats were treated with RCTR1 (5 µg/kg), and then AFC was measured in vivo after 1 h of ventilation. Finally, rats were euthanized for tissue collection. Furthermore, rats in LPS and LPS+RCTR1 groups received the observation of the survival rate for 5 days. **B.** Kaplan-Meier survival curves were recorded in the LPS group and LPS+RCTR1 group and were compared by a Log-rank test. n=12. **C.** After administration of RCTR1 with different doses, AFC was measured. Results are presented as mean ± SD, n=6.

Figure 2: The effect of RCTR1 on the histology, ultrastructure of lung tissues, and inflammation factors levels in LPS-induced ALI. A.

Rats were received RCTR1 (5 µg/kg) via the tail vein 8 h after LPS administration. After ventilation for 1 h, lung tissues were harvested. Lung tissue sections stained with H&E were used to evaluate the effects of RCTR1 on lung histology. Original magnification ×200, ×400. **B.** Acute lung injury scores were determined based on the standards mentioned in the Methods. **C-F:** Concentrations of IL-10, MPO, TNF-α, and IL-1β were detected in lung tissue homogenates by ELISA. **G-H:** Lamellar bodies and air-blood barriers were observed through TEM among the groups. Magnification x20000. lb, lamellar body; ac, air capillary; bc, blood capillary; ecm, extracellular matrix of the capillary wall; en, endothelial cell; ep, epithelial bridge. Results are presented as mean ± SD, n=6.

Figure 3: RCTR1 elevated ENaC and Na, K-ATPase expression and Na, K-ATPase activity. A-F: The protein expression of ENaC subunits (α , β , γ) and Na, K-ATPase subunits ($\alpha 1$, $\beta 1$) in lung tissue homogenates among different groups. **G.** The Na, K-ATPase activity was detected by an ELISA kit. **H.** The distribution of ENaC α in lung tissues was evaluated by immunohistochemical images. Results are presented as mean \pm SD, n=6.

Figure 4: The efficacy of RCTR1 on AFC was partly mediated by the ALX/cAMP/PI3K/Nedd4-2 pathway. A. Levels of the cAMP in lung tissue homogenates were detected by ELISA. **B.** After LPS administration, rats were received BOC-2 (0.6 ug/kg), KH-7 (1 mg/kg), or LY294002 (3 mg/kg) respectively 0.5 h before RCTR1 treatment. The ratio of AFC among groups was subsequently measured in vivo. **C-E.** The protein expressions of P-Akt and Nedd4-2 were determined by western blotting analysis. Results are presented as mean \pm SD, n=4-6.

Figure 5: The effect of RCTR1 on the expression of Na, K-ATPase $\alpha 1$ subunit in primary AEC II. A. The primary AEC II were cultured with LPS (1 ug/ml) and RCTR1 with different concentrations. The protein levels of Na, K-ATPase $\alpha 1$ were evaluated. **B.** The primary AEC II were incubated with LPS (1 ug/ml) and RCTR1 (10 nM) for different hours. Then, the protein expression of Na, K-ATPase $\alpha 1$ was evaluated. Results are presented as mean \pm SD, n=6.

Figure 6: RCTR1 improved the expression of ENaC and Na, K-ATPase through the ALX/cAMP/PI3K/Nedd4-2 pathway in vitro. A. The primary AEC II were cultured with LPS (1 μ g/ml) with or without RCTR1 (10 nM) for 8 h. The distribution of ENaC α subunit and Na, K-ATPase $\alpha 1$ subunit in primary AEC II was determined by immunofluorescent images. **B-G:** The protein levels of ENaC subunits (α , β , γ) and Na, K-ATPase subunits ($\alpha 1$, $\beta 1$) in primary AEC II. **H-I.** Protein levels of P-Akt and Nedd4-2 in AEC II. Results are presented as mean \pm SD, n=6.

Figure 7: RCTR1 promotes AFC through activating the expression of ENaC and

**Na, K-ATPase in LPS-induced ARDS/ALI through the
ALX/cAMP/PI3K/Nedd4-2 pathway.**

Figure 1

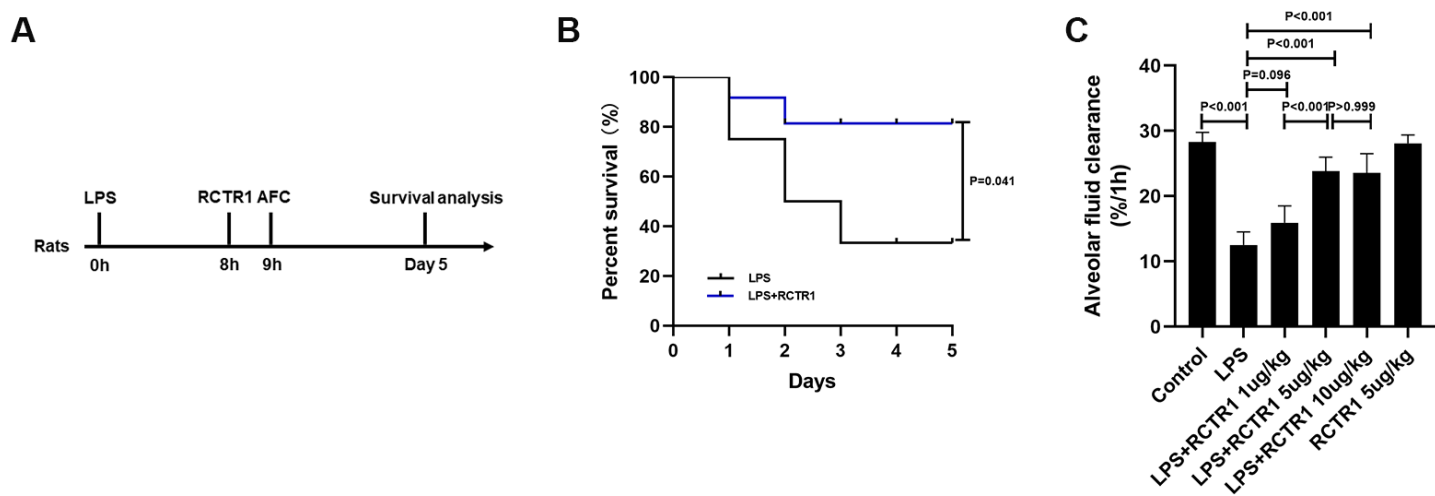


Figure 2

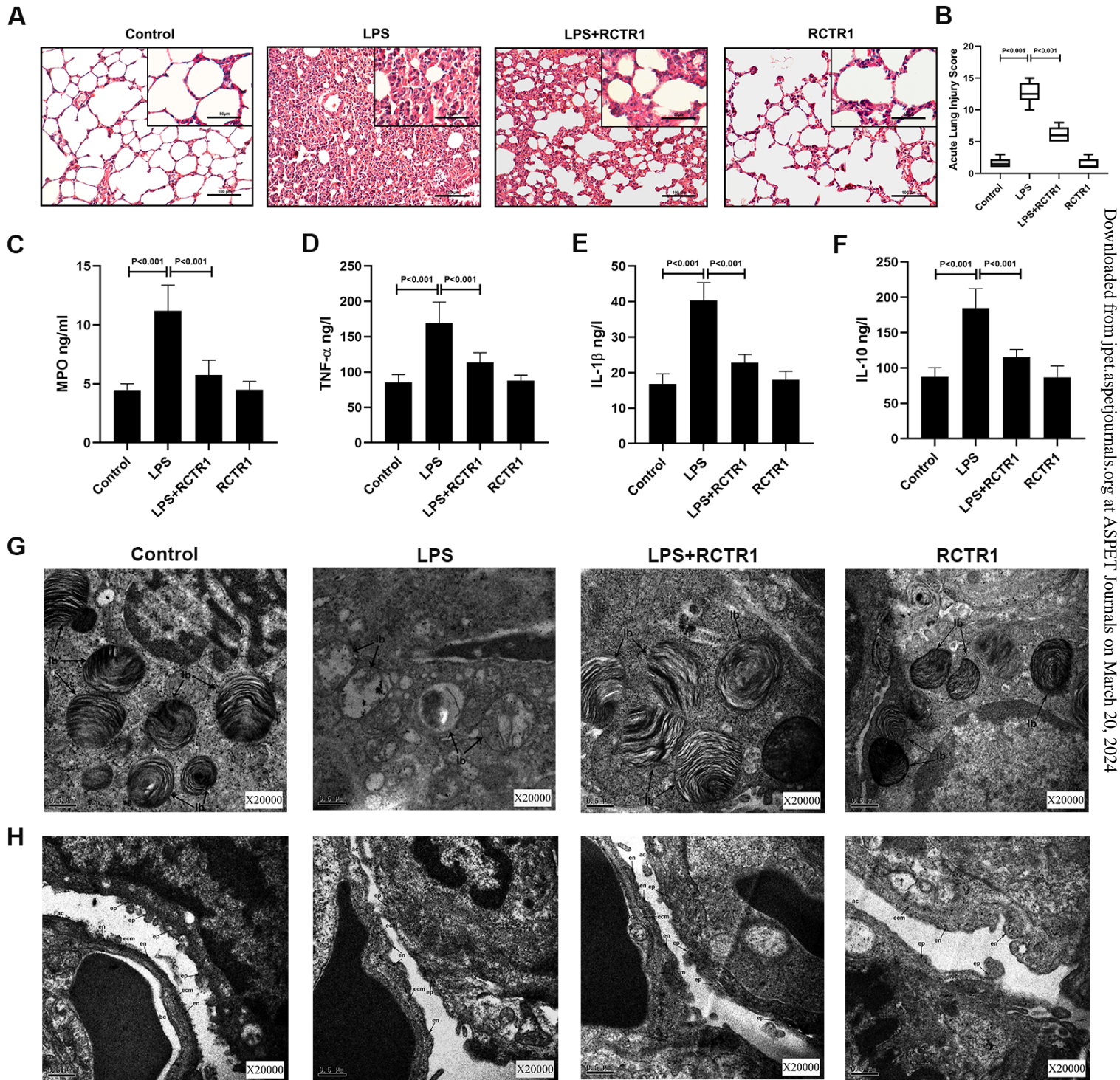


Figure 3

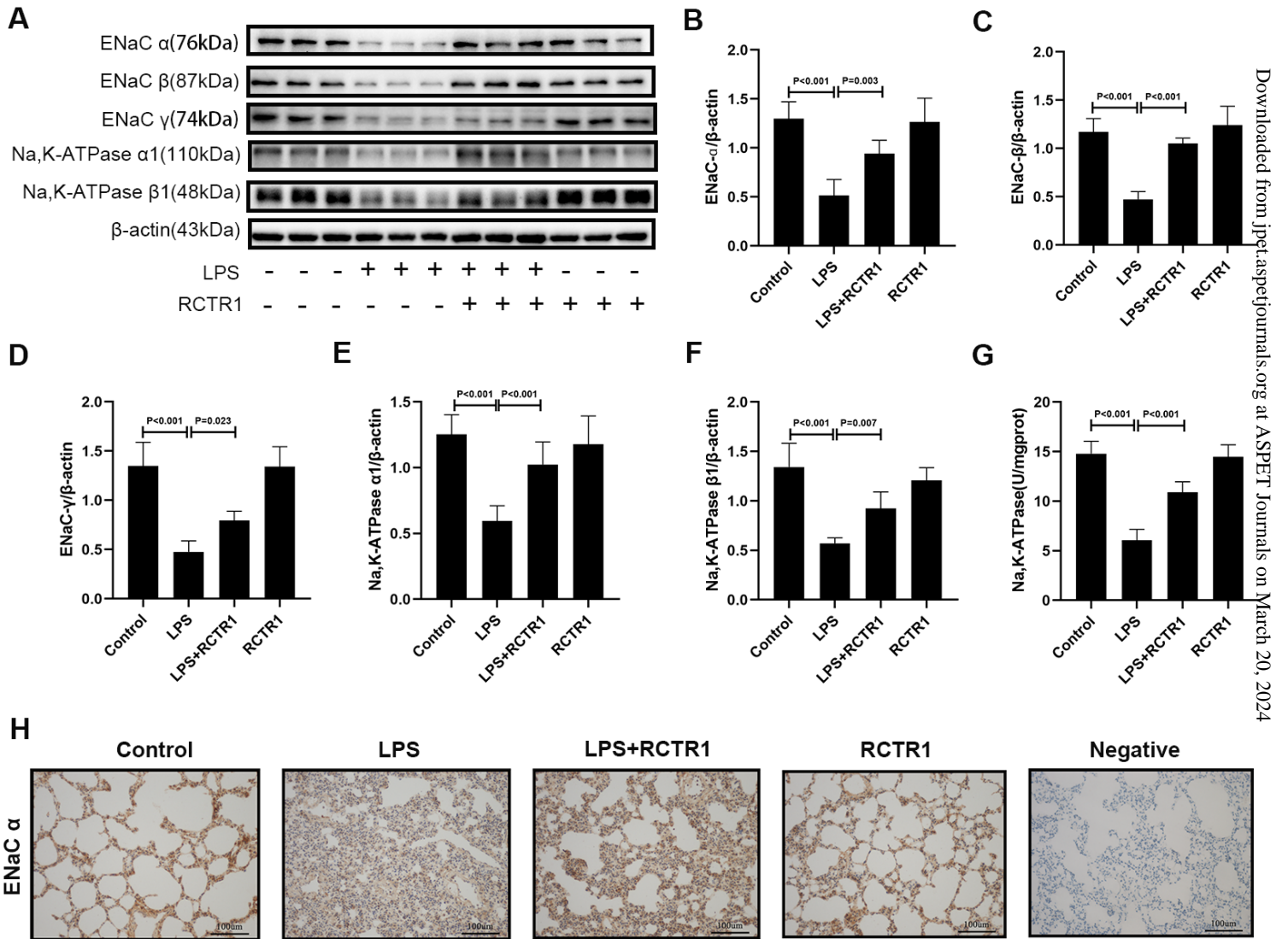


Figure4

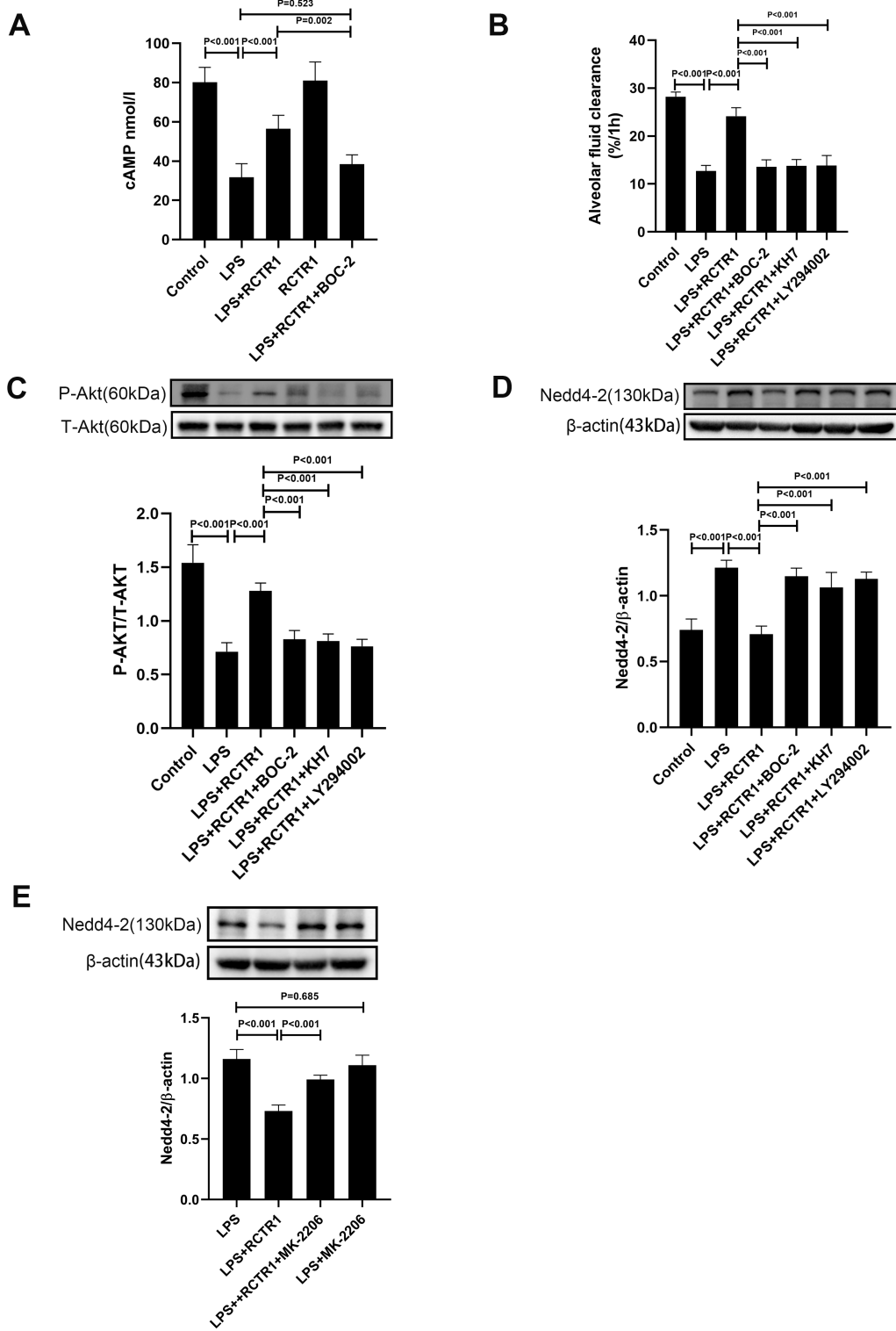


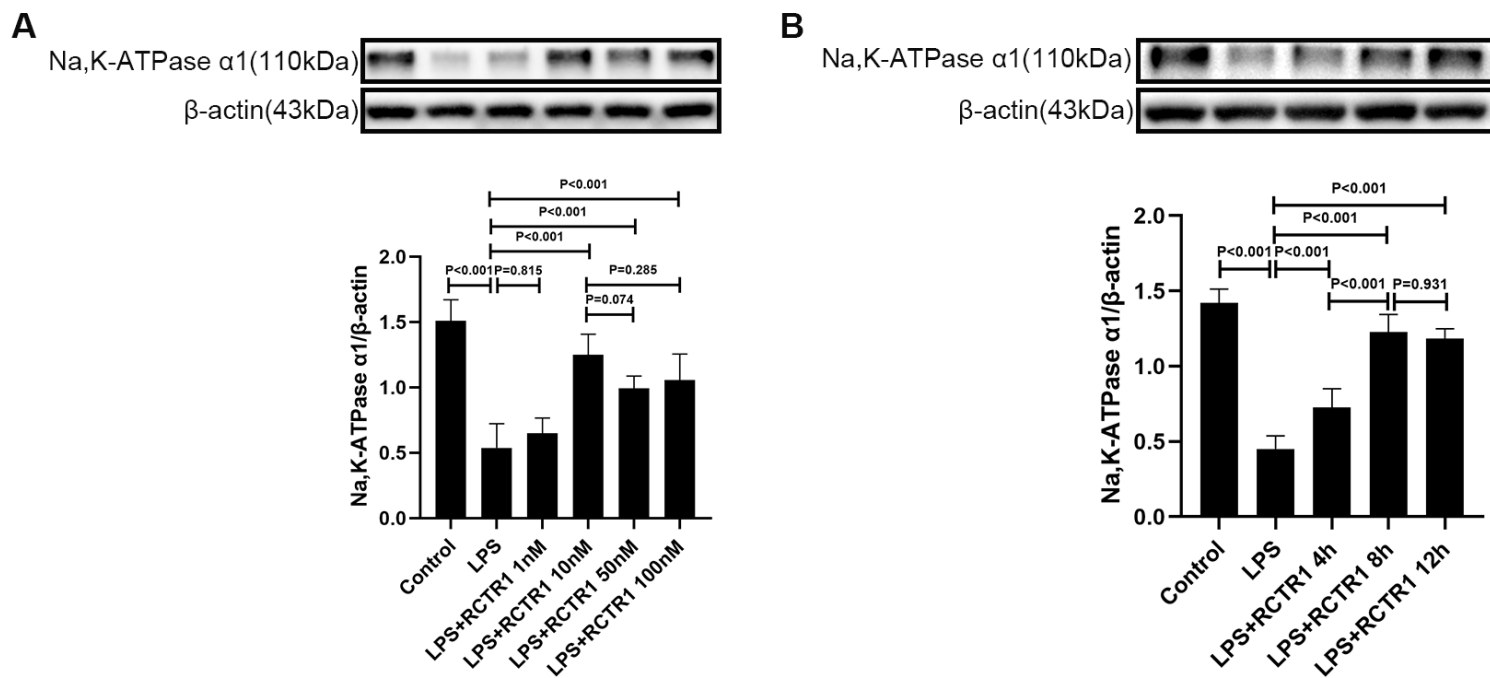
Figure 5

Figure 6

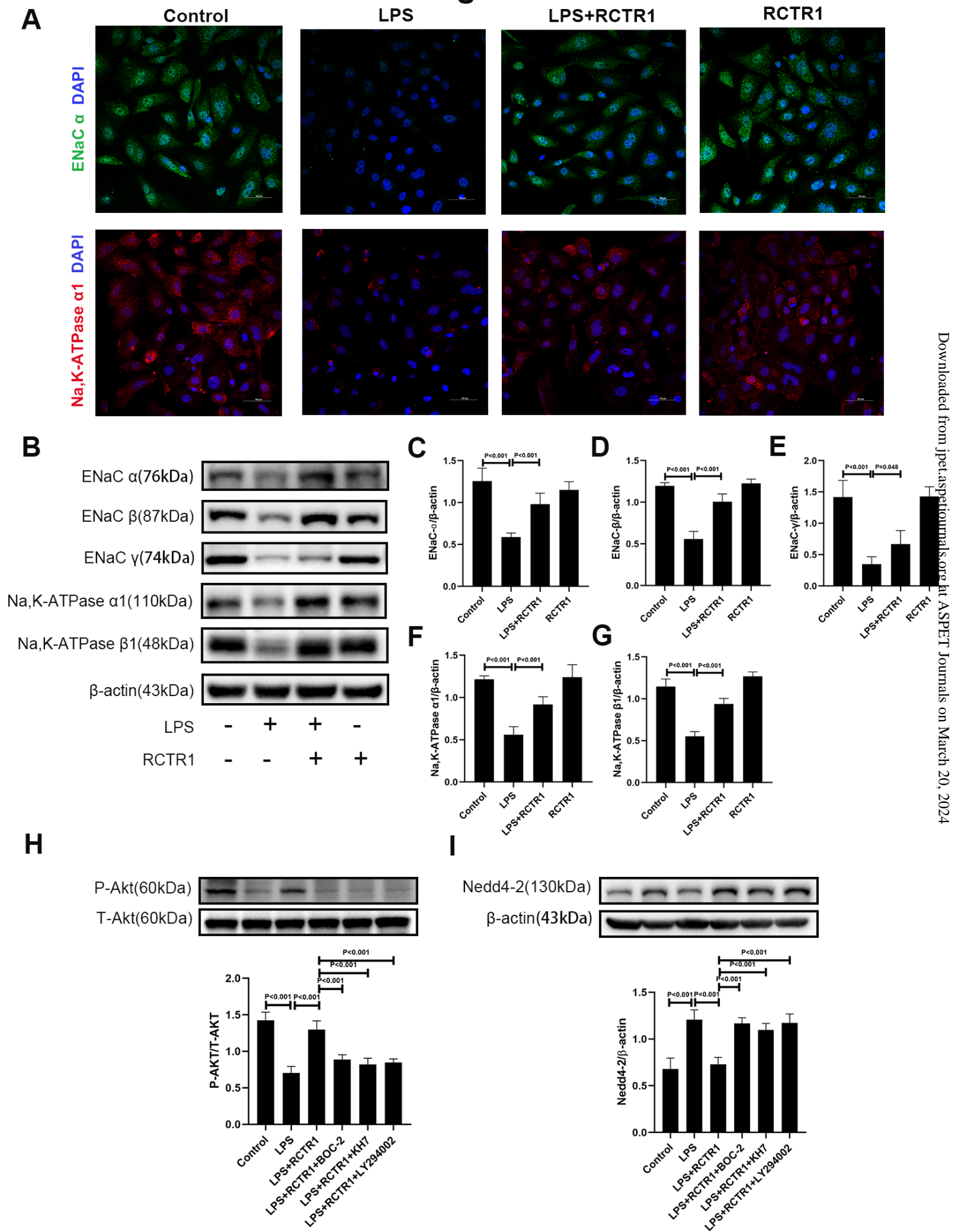


Figure 7

