Resolvin Conjugates in Tissue Regeneration 1 Promote Alveolar Fluid Clearance by Activating Alveolar Epithelial Sodium Channels and Na, K-ATPase in Lipopolysaccharide-Induced Acute Lung Injury

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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 1 (RCTR1) is an endogenous lipid mediator derived from docosahexaenoic acid, exerting prosesolution effects in the process of inflammation. In our research, we evaluated the role of RCTR1 in alveolar fluid clearance (AFC) in lipopolysaccharide-induced ARDS/acute lung injury (ALI) rat model. Rats were injected with RCTR1 (5 μg/kg) via caudal veins 8 hours after lipopolysaccharide (LPS) (14 mg/kg) treatment, and then AFC was estimated after 1 hour of ventilation. Primary type II alveolar epithelial cells were incubated with LPS (1 μg/ml) with or without RCTR1 (10 nM) for 8 hours. 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 vitro. Additionally, RCTR1 also decreased neural precursor cell expressed developmentally downregulated 4-2 (Nedd4-2) level via upregulating Ser473-phosphorylated-Akt expression. Besides this, inhibitors of receptor for lipoxin A4 (ALX), cAMP, and phosphatidylinositol 3-kinase (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.

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 upregulates the expression of epithelial sodium channels (ENaCs) 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 (Sweeney and McAuley, 2016; Matthay et al., 2019). Despite improvements in supportive treatments for ARDS, effective pharmacotherapies remain scarce (Meyer and Calfee, 2017). Most patients with ARDS 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 epithelial sodium channels (ENaCs) and

ABBREVIATIONS: AEC II, type II alveolar epithelial cells; AFC, alveolar fluid clearance; ALI, acute lung injury; ALX, receptor for lipoxin A4; ARDS, acute respiratory distress syndrome; ENaC, epithelial sodium channel; IL, interleukin; LPS, lipopolysaccharide; MPO, myeloperoxidase; Nedd4-2, neural precursor cell expressed developmentally downregulated 4–2; P-Akt, Ser473-phosphorylated-Akt; PI3K, phosphatidylinositol 3-kinase; RCTR1, resolvin conjugates in tissue regeneration 1; T-Akt, total protein kinase B; TNF-α, tumor necrosis factor α.
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 (Matthey et al., 2002). Hence, both sodium channels and Na, K-ATPase play pivotal roles in the removal of pulmonary edema (Matthey, 2014). Nedd4-2, a ubiquitin ligase, can negatively regulate ENaC by promoting its internalization and degradation, whereas phosphorylation mediated by Akt can inhibit its activity (Lee et al., 2007; Manning and Kumar, 2018). Furthermore, a previous study revealed that the cAMP–phosphatidylinositol 3-kinase (PI3K) pathway contributed to the activity (Lee et al., 2007; Manning and Kumar, 2018). Furthermore, the effect 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 24896) was purchased from Cayman Chemical Company (Ann Arbor, MI). LPS (Escherichia coli serotype 055:B5) was obtained from Sigma (St. Louis, MO). ELISA kits for cAMP, myeloperoxidase (MPO), tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), and IL-1β were from R&D Systems (Minneapolis, MN). KH-7 (1 mg/kg), MK-2206 (0.3 mg/kg), or LY294002 (3 μg/kg) was administered intravenously 8 hours after LPS injection. In the 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 hours after LPS injection. In the LPS+RCTR1 + BOC-2, LPS+RCTR1 + KH-7, LPS+RCTR1 + MK-2206, or LPS+RCTR1 + 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 μg/kg), respectively, through the tail vein 30 minutes before RCTR1 treatment (Zhang et al., 2017; Zhuo et al., 2018; Zhang et al., 2020). After anesthesia with 30 mg/kg of 2% pentobarbital sodium intraperitoneally, the experimental rats were mechanically ventilated for 1 hour. 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 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 hour 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 – C0/C1), in which C0 and C1 represented the initial and final concentrations of the Evans blue–labeled albumin in alveolar perfusion solution, respectively.

Pathologic 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 cell 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 (interleukin) IL-10, MPO, TNF-α, IL-1β, 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). Lung tissue samples were fixed 24 hours in 2.5% glutaraldehyde and then postfixied 60 minutes in 1% osmium tetroxide. After being rinsed with PBS three times, samples were sequentially dehydrated in gradient acetone and then embedded in epoxy resin. After being stained 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 being 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 horseradish peroxidase–labeled secondary antibodies for 1 hour. After being stained with diaminobenzidine 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 minutes to extract the primary rat type II alveolar epithelial cells (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 containing 10% fetal bovine serum in 5% CO$_2$ and 95% air atmosphere. After being serum-derived for 24 hours, AEC II were cultured with LPS (1 mg/ml) with or without RCTR1 (10 nM). BOC-2 (10 μM), KH-7 (10 μM), and LY294002 (10 μM) were added to the medium 1 hour before LPS and RCTR1 administration (Niu et al., 2019; Zhang 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 poly(vinylidene) fluoride 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 were 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 hours at 4°C and then incubated with relevant secondary antibodies at room temperature for 1 hour. A confocal laser-scanning microscope (Leica) was used for capturing the cell images.

**Statistical Analysis.** The data are presented as mean ± 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 (Fig. 1A). The mortality of the LPS+RCTR1 group was less than that of the LPS group (Fig. 1B). After administration of 14 mg/kg LPS, different doses of RCTR1 (1 μg/kg, 5 μg/kg, and 10 μg/kg) were injected into the rats intravenously to evaluate the effect of RCTR1 on AFC, which was detected after 1 hour of sustained ventilation (Fig. 1C). However, compared with the LPS group, RCTR1 at 5 μg/kg remarkably improved AFC (Fig. 1C). There was no statistical difference between the RCTR1 5 μg/kg and 10 μg/kg doses (Fig. 1C). Consequently, RCTR1 at a concentration of 5 μg/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 increased lung injury scores determined from pulmonary histology with alveolar edema, neutrophil infiltration, hemorrhage, and thickening of the alveolar wall (Fig. 2, A and B). Treatment with RCTR1 markedly alleviated the LPS-induced morphologic changes, as indicated by significantly decreased lung injury scores (Fig. 2, A and B). However, the control and RCTR1 groups showed no statistical difference (Fig. 2B). Compared with the control group, the lung tissue homogenates in the LPS group revealed elevated levels of inflammatory factors, including TNF-$\alpha$, MPO, IL-1β, and IL-10 (Fig. 2, C–F). Of note is the observation that these inflammatory factors were obviously reduced after RCTR1 treatment (Fig. 2, C–F). Additionally, the ultrastructure of lung tissues observed with a transmission electron microscope in the LPS group showed severe vacuolization of lamellar bodies and destruction of the air-blood barrier featuring damaged epithelial bridges and capillary walls (Fig. 2, G and H). Treatment with RCTR1 produced obvious recovery from these pathologic changes (Fig. 2, G and H).

**RCTR1 Upregulated 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 hours resulted in significant downregulation of the protein levels of ENaC subunits ($\alpha$, $\beta$, and $\gamma$) and Na, K-ATPase subunits ($\alpha1$ and $\beta1$) in lung tissue homogenates, which was reversed by RCTR1 treatment (Fig. 3, A–F). Immunohistochemical analysis demonstrated that ENaC $\alpha$ subunit was located in the apical surfaces of the alveolar epithelium. The decrease in protein expression of ENaC $\alpha$ subunit was located in the apical surfaces of the alveolar epithelium. The decrease in protein expression of ENaC $\alpha$ subunit was recovered in the LPS+RCTR1 group, which agreed with the Western blot results (Fig. 3H). Furthermore, LPS-suppressed Na, K-ATPase activity was enhanced by the RCTR1 treatment in vivo (Fig. 3G).

![Fig. 1](https://example.com/fig1.png)

**Fig. 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 hours, rats were treated with RCTR1 (5 μg/kg), and then AFC was measured in vivo after 1 hour 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, $n = 6$. 

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158 Yang et al.
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 after 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 (Fig. 4A). However, the use of BOC-2 (ALX inhibitor, 0.6 μg/kg) suppressed the upregulating effect of RCTR1 on cAMP, indicating that RCTR1 activates the ALX/cAMP pathway (Fig. 4A).

Next, the coadministration 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, LPS + RCTR1 + KH-7, or LPS +
RCTR1 + LY294002 groups was lower than that in the LPS + RCTR1 group (Fig. 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 P-Akt was detected. Our results revealed that the ratio of P-Akt/T-Akt in the LPS group was lower than that in the control group, whereas RCTR1 treatment markedly enhanced the ratio (Fig. 4C). By contrast, the ratio of P-Akt/T-Akt was lower in the LPS + RCTR1 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 (Fig. 4C). Furthermore, we evaluated the Nedd4-2 expression in lung tissue using Western blot. Compared with the control group, LPS treatment upregulated the Nedd4-2 expression, which was downregulated by RCTR1 (Fig. 4D). However, the groups of LPS + RCTR1 + BOC-2, LPS + RCTR1 + KH-7, LPS + RCTR1 + 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 (Fig. 4C). Therefore, RCTR1 activates the ALX/cAMP/PI3K/Nedd4-2 pathway.

**The Regulation of RCTR1 on the Expression of Na, K-ATPase a1 Subunits in Primary AEC II.** The primary AEC II were incubated in LPS (1 μg/ml) and different doses of RCTR1 (1, 10, 50, and 100 nM). The protein levels of Na, K-ATPase a1 subunits decreased obviously in the LPS-treated cells and increased obviously with RCTR1 (10, 50, and 100 nM) (Fig. 5A). There were no statistical differences among the 10, 50, and 100 nM doses (Fig. 5A). Next, we cultured the primary AEC II with 1 μg/ml LPS and 10 nM RCTR1 for different durations (4, 8, and 12 hours). Then, our results showed that the upregulation of protein levels of Na, K-ATPase a1 was significant at 8 and 12 hours, and there were no statistical differences between 8 and 12 hours (Fig. 5B). Therefore, RCTR1 (10 nM) was used for 8 hours 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 μg/ml LPS with or without RCTR1 (10 nM) treatment of 8 hours. A considerable increase in the expression of ENaC α subunit and Na, K-ATPase α1 subunit was observed in the RCTR1-treated AEC II (Fig. 6A). The protein levels of ENaC subunits (α, β, and γ) and Na, K-ATPase...
Fig. 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 received BOC-2 (0.6 ug/kg), KH-7 (1 mg/kg), or LY294002 (3 mg/kg), respectively, 0.5 hours 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 ± SD, n = 4–6.
subunits (α1 and β1) were obviously diminished in LPS-challenged cells and promoted by RCTR1 treatment (Fig. 6, B–G). Moreover, RCTR1 upregulated the LPS-induced decrease in P-Akt, and the upregulating effect was partly blocked by BOC-2 (10 μM), KH-7 (10 μM), and LY294002 (10 μM) (Fig. 6H). Additionally, RCTR1 inhibited the LPS-induced elevation in the Nedd4-2 expression, whereas BOC-2, KH-7, and LY294002 reversed this effect (Fig. 6I). In conclusion, RCTR1 promoted Nedd4-2 expression, whereas BOC-2, KH-7, and LY294002, demonstrating that RCTR1 inhibited the LPS-induced elevation in the Nedd4-2 expression, whereas BOC-2, KH-7, and LY294002 reversed this effect (Fig. 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 demonstrate that treatment with RCTR1 upregulates 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 (Fig. 7).

ARDS/ALI is characterized by damage to alveolar epithelial cells and endothelial cells, which in turn leads to increased alveolar capillary permeability. (Thompson et al., 2017; Matthay et al., 2019). Impaired AFC is closely related to the progression and severity of ARDS (Ware and Matthay, 2001; Jabaudon et al., 2015). 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 pathologic 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 a requisite for AFC (Matthay 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, edematous fluid in the interstitium can be cleared by the microcirculatory and lymphatic systems (Matthay et al., 2012, 2019). ENaC, which is composed of α, β, and γ subunits, is the main driving force of AFC (Guidot et al., 2006; Hummler and Planès, 2010). ENaC α-deficient mice lost the ability to clear edema fluid in alveoli and died within 40 hours after birth (Hummler et al., 1996). The most important subunits of Na, K-ATPase in the lung are α1 and β1, which act 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 edematous fluid in alveoli (Sartori and Matthay, 2002; Morty et al., 2007; 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.
Fig. 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 μg/ml) with or without RCTR1 (10 nM) for 8 hours. The distribution of ENaC α subunit and Na, K-ATPase α1 subunit in primary AEC II was determined by immunofluorescent images, the fluorescent dye 4'-6-Diamidino-2-phenylindole (DAPI) is used in fluorescence microscopy as a nuclear stain. (B–G) The protein levels of ENaC subunits (α, β, and γ) and Na, K-ATPase subunits (α1 and β1) in primary AEC II. (H and I) Protein levels of P-Akt and Nedd4-2 in AEC II. Results are presented as mean ± SD, n = 6.
Belonging to specialized proresolving mediators, docosahexaenoic acid–derived RCTR1 plays a key role in orchestrating the inflammation resolution (Basil and Levy, 2016; Krishnamoorthy et al., 2018). Although the specific receptor for RCTR1 remains to be determined, ALX/FPR2 has been identified as the receptor for other members of the specialized proresolving mediators, such as lipoxin a4 and resolvin D1 (Krishnamoorthy et al., 2010, 2012). The ALX receptor, a 7-transmembrane G-protein–coupled receptor, transmits cell-type–specific signaling pathways (Chiang et al., 2006; Serhan et al., 2008). Our results demonstrate that the stimulative efficacy of RCTR1 on AFC was partially repressed by the 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 the 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 (Thomas et al., 2004; Lecouona et al., 2009). 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 (Thomas et al., 2004; Soundararajan et al., 2009; Deng et al., 2012). 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 physiologic processes (Lee et al., 2007; Hoxhaj and Manning, 2020). 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 shows that RCTR1 could reverse the LPS-increased Nedd4-2 expression, whereas 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 Laboratory of Anesthesiology, The Second Affiliated Hospital, Wenzhou Medical University.

Authorship Contributions

Participated in research design: Yang, Xu, Jin, Wang.
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