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Resolvin D1 Stimulates Alveolar Fluid Clearance through Alveolar Epithelial Sodium Channel, Na,K-ATPase via ALX/cAMP/PI3K Pathway in Lipopolysaccharide-Induced Acute Lung Injury

Qian Wang,*1 Xia Zheng,*1 Yang Cheng,* Yi-Lan Zhang,* Hai-Xu Wen,* Zhen Tao,* Hui Li,* Yu Hao,* Ye Gao,* Liang-Min Yang,* Fang Gao Smith,* Chang-Jiang Huang,† and Sheng-Wei Jin*

Resolvin D1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid) (RvD1), generated from ω-3 fatty docosahexaenoic acids, is believed to exert anti-inflammatory properties including inhibition of neutrophil activation and regulating inflammatory cytokines. In this study, we sought to investigate the effect of RvD1 in modulating alveolar fluid clearance (AFC) on LPS-induced acute lung injury. In vivo, RvD1 was injected i.v. (5 μg/kg) 8 h after LPS (20 mg/kg) administration, which markedly stimulated AFC in LPS-induced lung injury, with the outcome of decreased pulmonary edema. In addition, rat lung tissue protein was isolated after intervention and we found RvD1 improved epithelial sodium channel (ENaC) α, γ, Na,K-adenosine triphosphatase (ATPase) α1, β1 subunit protein expression and Na,K-ATPase activity. In primary rat alveolar type II epithelial cells stimulated with LPS, RvD1 not only upregulated ENaC α, γ and Na,K-ATPase α1 subunits protein expression, but also increased Na+ currents and Na,K-ATPase activity. Finally, protein kinase A and cGMP were not responsible for RvD1’s function because a protein kinase A inhibitor (H89) and cGMP inhibitor (Rp-cGMP) did not reduce RvD1’s effects. However, the RvD1 receptor (formyl-peptide receptor type 2 [FPR2], also called ALX [the lipoxin A4 receptor]) inhibitor (BOC-2), cAMP inhibitor (Rp-cAMP), and PI3K inhibitor (LY294002) not only blocked RvD1’s effects on the expression of ENaC α in vitro, but also inhibited the AFC in vivo. In summary, RvD1 stimulates AFC through a mechanism partly dependent on alveolar epithelial ENaC and Na,K-ATPase activation via the ALX/cAMP/PI3K signaling pathway. The Journal of Immunology, 2014, 192: 3765–3777.

A

cute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are devastating clinical syndromes that frequently lead to acute respiratory failure characterized by alveolar epithelial injury, flooding of protein-rich fluid in the alveolar spaces, and the expression of proinflammatory mediators (1, 2). Currently, supportive care with lung-protective ventilation and a fluid-conservative strategy is the mainstay of clinical management with no effective pharmacological therapies. Despite improvements, morbidity and mortality rates of ARDS remain as high as 30–40% (3, 4). Timely and effective removal of excessive alveolar edema fluid is critical for minimizing damage to the surrounding tissue and for the resolution of inflammation (5).

Alveolar fluid clearance (AFC) is accomplished by active ion transport, predominantly by the alveolar epithelium. As a matter of fact, the alveolar epithelium is not only a tight epithelial barrier that resists the movement of edema fluid into the alveoli, but it is also actively involved in the transport of ions and solutes, a process that is essential for edema fluid clearance. Alveolar type II epithelial (ATII) cells play a primary role in the process of edema fluid clearance (6). Considerable experimental evidence indicates that active Na+ transport is the dominant ion transport mechanism involved in AFC in the normal lung (7, 8). This solute transport drives osmotic water transport and, accordingly, AFC (9). Na+ ions enter ATII cells at the apical surface primarily through amiloride-sensitive sodium channels, such as the epithelial sodium channel (ENaC), and are pumped out on the basolateral surface by Na,K-adenosine triphosphatase (ATPase). Thus, both ENaC and Na,K-ATPase are accredited with key roles in the resolution of pulmonary edema (10). Several recent in vivo studies have demonstrated vasopressin-2 receptor antagonist (11), GABA receptor (12), β-adrenergic (13), triiodo-l-thyronine (14), and leukotriene D4 (15) could markedly upregulate AFC in experimental animal models. We previously reported that treatment with lipoxin A4, an anti-inflammatory and proresorption mediator,
and lipoxin receptor agonist (BML-111) significantly stimulated AFC in oleic acid–induced lung injury and decreased pulmonary edema (16). We have also found that i.v. β-agonists (salbutamol) reduced extravascular lung water and improved lung injury in ARDS patients (17–19). However, in a multicenter, randomized, controlled clinical trial, i.v. infusion of salbutamol given to patients with early ARDS significantly increased 28-d mortality as treatment was poorly tolerated because of tachycardia, arrhythmias, and lactic acidosis (20).

Resolvins are ω-3 docosahexaenoic acid–derived metabolites biosynthesized during the resolution phase of inflammatory response that elicit distinct anti-inflammatory and proresolving bioactions, including halting transendothelial migration of human neutrophils (21–23), upregulation of monocyte ingestion of apoptotic neutrophils, and enhanced macrophage phagocytosis of zymosan and apoptotic polymorphonuclear neutrophils (PMNs) (24). Previous studies have shown that resolvin D1 (7S,8R,17S-trihydroxy-4Z,8E,10Z,12E,14E, 19Z-docosahexaenoic acid) (RvD1) exerts potent anti-inflammatory and proresolving actions in several animal models of sepsis, peritonitis, taraxin, and ALI (25, 26). Furthermore, a recent study showed that RvD1 improved survival rate and attenuated ALI induced by LPS (27). Darre et al. (28) have reported that goblet cell mucous secretion is an important component of ocular allergy and early dry eye, and stimulated secretion is terminated by RvD1 via active resolution of inflammation. Another study showed that RvD1 accelerated the airway mucous metaplasia in the resolution of established allergic airway responses (29). However, no studies to date have addressed the effect of RvD1 on pulmonary edema (16). We have also found that i.v. β2–AR agonists have been shown to enhance AFC transport via a cAMP-dependent mechanism under physiological conditions (30) and in experimental models of lung injury (31), as well as in one prospective study of extravascular lung water in patients with ALI (19). In contrast, PI3K has been identified as integral for regulation of ENaC-mediated AFC by insulin (32).

This study was designed to investigate whether RvD1 could exert protective effects on AFC in LPS-induced ALI in vivo. In addition, we investigated the effect of RvD1 on the protein expression of ENaC and Na,K-ATPase, and the activity of Na,K-ATPase in vivo and in vitro. Finally, to better understand the mechanisms of action of RvD1, we used ALX receptor (BOC-2), cAMP inhibitor (Rp-cAMP), cGMP inhibitor (Rp-cGMP), PI3K inhibitor (LY294002), and PKA inhibitor (H89) in vivo and in vitro to investigate how this signaling pathway regulates ENaC and AFC.

Materials and Methods

**Materials**

RvD1, LY294002 (PI3K inhibitor), and H89 (PKA inhibitor) were from Cayman Chemical Company (Ann Arbor, MI). LPS (Escherichia coli serotype 055:B5) and ouabain were purchased from Sigma (St. Louis, MO). Myeloperoxidase (MPO), cAMP, and cGMP ELISA kits were from R&D Systems (Minneapolis, MN). BOC-2 (ALK inhibitor), Rp-cAMP (cAMP inhibitor), and Rp-cGMP (cGMP inhibitor) were obtained from Biomol-Enzo Life Sciences (Farmingdale, NY). Anti-ENaC α, β, and γ, and anti-Na,K-ATPase α1 and β1 were purchased from Abcam (Cambridge, MA).

**Animal preparation**

Experiments were performed on adult male Sprague–Dawley rats (250–300 g; Shanghai Experimental Animal Center of China). Rats were provided water and food ad libitum. The use of animals in this study was approved by the Animal Studies Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University.

Rats were randomized into five groups (n = 8): control group, LPS group, LPS + Alcohol group, LPS + RvD1 group, and RvD1 group. The LPS-induced lung injury model was produced by 20 mg/kg LPS injected via caudal vein. In the RvD1 group, rats received RvD1 (5 μg/kg) i.v. via caudal vein. In other groups, rats received RvD1 or alcohol or equivalent volume of saline via caudal vein 8 h after LPS exposure. Animals were anesthetized with an i.p. injection of 5% chloral hydrate (7 ml/kg), after which a tracheotomy tube was placed. Rats were sacrificed after 60 min of mechanical ventilation and lungs were harvested.

**Pathological studies**

The right lower lung lobes were harvested and fixed with 10% neutral-buffered formalin for 24 h, then embedded in paraffin and stained with H&E for light microscope analysis. A semiquantitative scoring system was adopted to evaluate the lung injury including alveolar congestion, alveolar hemorrhage, infiltration or aggregation of neutrophils in the airspace or vessel wall, and thickness of alveolar wall/hyaline membrane formation and inflammatory cell infiltration. The grading scale to score the pathologic findings was as follows: 0 = no injury; 1 = slight injury (25%); 2 = moderate injury (50%); 3 = severe injury (75%); and 4 = very severe injury (almost 100%). The results were graded from 0 to 4 for each item, as described previously (33, 34). The four variables were summed to represent the lung injury score (total score: 0–16).

**Measurement of AFC in live rats**

AFC was measured in living rats as previously described (14, 35, 36) with some modifications. Clearance is expressed as a percentage of total instilled volume cleared after 60 min. AFC was determined by Evans blue–tagged albumin concentration changes, which has been clearly characterized by our laboratory (16).

For preparation of the alveolar instillate, a 5% albumin instillate solution was prepared by dissolving 50 mg/ml BSA in modified lactated Ringer’s solution: 137 mM NaCl, 4.67 mM KCl, 1.82 mM CaCl2·2H2O, 1.25 mM MgSO4·7H2O, 5.55 mM dextrose, and 12 mM HEPES. The pH was adjusted to 7.4 at 37°C. The albumin solution was labeled with 0.15 mg/ml Evans blue. In brief, after anesthesia with 5% anaesthesia, 1 ml of 7% chloral hydrate (7 ml/kg), a polystyrene endotracheal tube was inserted through a tracheotomy. Rats were ventilated with a constant volume ventilator (model HX-300 Animal ventilators; Tai- meng Company of Chengdu, China) with an inspired oxygen fraction of 100%, a respiratory rate of 45–50 breaths/min and 2.8 ± 0.2 ml tidal volumes, positive end expiratory pressure was kept at 2–3 cm H2O during the baseline period. After tracheotomy, the rats were allowed to stabilize for 10 min. The animals were then placed in the left lateral decubitus position, and instillation tubing (16G Epidural catheter) was gently passed through the tracheotomy tube into the left lung. A total of 1.5 ml (5 ml/kg) of the instillate solution was instilled at a rate of 0.08 ml/min using a syringe pump. After instillation was complete, 0.2 ml air was injected to clear the instillation catheter. The concentrations of Evans blue–labeled albumin in the instilled and aspirated solutions were measured by a spectrophotometer at a wavelength of 621 nm. AFC was calculated using the following equation: 

$$AFC = \frac{C_0 - C_1}{C_0} \times 100\%$$

where C0 is the protein concentration of the instillate before instillation, and C1 is the concentration of albumin in the instillate after being aspirated (35).

**Primary rats ATII cells isolation, culture, and treatment**

Primary rats ATII cells were isolated from Sprague–Dawley rats (200–250 g) by collagenase digestion of lung tissue and then diffusively adhered on IgG-coated plates as described by Dobbs et al. (37). The purity of ATII cells was assessed by modified Papanicolaou stain based on the presence of dark blue inclusions. Cell viability was assessed by trypan blue exclusion (>95%). ATII cells were seeded onto plastic culture dishes at 1 × 10^5/cm² and cultured in a 5% CO2, 95% air atmosphere in DMEM containing 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin after isolation. For all experiments, cells were subcultured into six-well plates and maintained until subconfluence (80%), and cells were serum deprived for 24 h after the addition of LPS (1 μg/ml) in the presence or absence of RvD1 (50 nM).

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Western blotting for ENaC, Na,K-ATPase in rat lung tissues and primary alveolar epithelial cells

Proteins were obtained with RIPA lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, leupeptin) and PMSF. Samples were ultrasonicated 3 times, for 5 s, and then spun at 12,000 x g/min for 30 min. Protein concentrations of the supernatants were determined by using a BCA protein assay kit (Thermo Scientific, Rockford, IL). Proteins were separated by 10% SDS polyacrylamide gels and transferred to PVDF membranes. After blocking with 5% nonfat dried milk in TBS containing 0.05% Tween 20, the membranes were incubated with primary Abs ENaC α, β, γ (1:500), 1:1000, 1:3000), Na,K-ATPase α, β1, β1 (1:1000), and β-actin (internal control, 1:500) overnight at 4˚C, and then reacted with HRP-conjugated secondary Ab (1:1000; Santa Cruz Company) at room temperature for 1.5 h. The protein bands were detected by ECL and visualized by UVP Gel imaging system (Upland, CA). The band intensity was analyzed by AlphaEaseFC (version 4.0).

Electrophysiology

Patch-clamp recording was measured as previously reported (38). Immediately before each experiment, a coverslip bearing primary ATII cells was removed from the culture plate into a recording chamber, which was mounted on the stage of an inverted fluorescent microscope (Leica DM IRB). For whole-cell mode of patch-clamp recording, cells were perfused continuously with extracellular fluid containing (in mM): 140 NaCl, 4.5 KCl, 3.6 CaCl2, 2.4 MgCl2, 10 glucose, and 10 HEPES (pH 7.4). Pipettes were backfilled with internal solution with the following ionic composition (in mM): 135 K-gluconate, 2 MgCl2, 10 NaCl, 4 Na2ATP, and 10 HEPES. Pipettes were made from capillary glass electrode with a P-87 micropipette puller (Sutter Instrument Company), and resistance varied from 3 to 5 MΩ.

Immunohistochemistry

The paraffin was dewaxed with xylene, hydrated with ethanol, and repaired with citrate buffer solution (pH 6.0), and then treated with 3% H2O2 to inhibit endogenous peroxidase activity for 10 min and rinsed with phosphate buffer solution (pH 7.6). It was blocked with BSA for 30 min and incubated with primary Abs at 4˚C for 24 h. Then, biotinylated anti-rabbit IgG (Santa Cruz Company) were reacted for 1 h in an incubator at 37˚C. After washing with phosphate buffer solution for three times, it was reacted with avidin–peroxidase complex (Sigma) for 30 min and then stained with 3,3’-diaminobenzidine (DAB; Sigma) a coloring agent, for 5 min. For control staining, it was also reacted with hematoxylin for 30 s. PBS was a substitute for the primary Abs in the earlier process as a negative control staining, it was also reacted with hematoxylin for 30 s. PBS and primary alveolar epithelial cells

The hydrolytic activity of Na,K-ATPase was measured as ouabain-sensitive ATP hydrolysis under maximal velocity conditions by measuring the release of inorganic phosphate from ATP, as previously described (39, 40). In brief, the rat lung tissues and treated alveolar epithelial cells were digested, subjected to centrifugal sedimentation, lyzed, and homogenized. The minimal ATP enzyme test kit (Jiangcheng Company, Nanjing, China) was used to assay Na,K-ATPase activity following manufacturer’s instructions.

RNA isolation, RT-PCR

Total RNA was isolated from primary ATII cells using a TRizol reagent (Invitrogen, Carlsbad, CA) followed by phenol-chloroform extraction and ethanol precipitation (Fisher Scientific, Houston, TX). RNA purity was checked by spectrophotometry, and RNA integrity was confirmed by visualization of 28S and 18S bands on 1.5% agarose gel. RNA samples were reverse-transcribed into cDNA using an RT-PCR kit, according to the manufacturer’s instructions. Each PCR product was run on a 2.0% agarose gel containing ethidium bromide, and semiquantitative analysis was performed using UVG gel densitometry.

Measurement of cAMP and cGMP concentration

Primary ATII cells were harvested and sonicated. cAMP and cGMP concentration in cell lysates was measured via ELISA kits according to the manufacturer’s instructions.

Confocal imaging

ATII cells were respectively treated with saline, LPS (1 μg/ml), LPS+Alcohol (the same volume of RvD1), LPS+RvD1 (50 nM), and RvD1 for 12 h after fixing in 4% paraformaldehyde and blocked with PBS containing 10% donkey serum for 30 min. The cells were then incubated in a 1:20 dilution of monoclonal mouse anti-Na,K-ATPase, and goat anti-ENaC α at 4˚C for 48 h, followed by Alexa Fluor donkey anti-goat and donkey anti-mouse IgG incubation (1:100 and 1:300; Jackson) at room temperature for 2 h. Cell images were acquired with confocal laser-scanning microscope (Leica) and analyzed by Image Pro plus 6.3 software (Media Cybernetics, Crofton, MA).

Measurement of Na,K-ATPase activity in rat lung tissues and primary ATII cells

ATII cells were respectively treated with saline, LPS (1 μg/ml), LPS+Alcohol (the same volume of RvD1), LPS+RvD1 (50 nM), and RvD1 for 12 h after fixing in 4% paraformaldehyde and blocked with PBS containing 10% donkey serum for 30 min. The cells were then incubated in a 1:20 dilution of monoclonal mouse anti-Na,K-ATPase, and goat anti-ENaC α at 4˚C for 48 h, followed by Alexa Fluor donkey anti-goat and donkey anti-mouse IgG incubation (1:100 and 1:300; Jackson) at room temperature for 2 h. Cell images were acquired with confocal laser-scanning microscope (Leica) and analyzed by Image Pro plus 6.3 software (Media Cybernetics, Crofton, MA).

FIGURE 1. RvD1 protected lung tissues in LPS-induced ALI. RvD1 (5 μg/kg) was administered to Sprague-Dawley rats 8 h after LPS (20 mg/kg) stimulation through caudal vein, ventilating for 60 min, and the effect of RvD1 was assessed (A) by histology in H&E-stained sections (original magnification ×400). Lung injury scores (B) were recorded from 0 (no damage) to 16 (maximum damage) according to the criteria described in Materials and Methods. Lung tissues MPO (C) was also measured to quantitatively define the resolution of infiltrated cells. Data are presented as mean ± SEM. n = 8. Alcohol is resolvent. *p < 0.05, **p < 0.01 versus control group. *p < 0.05, **p < 0.01 versus LPS group. &p < 0.05, &&p < 0.01 versus LPS+Alcohol group.
Statistical analysis

Data are represented as mean ± SEM. All data were analyzed by the Student t test or by one-way ANOVA followed by Tukey’s post hoc test for multiple comparisons. Significance was determined at the p < 0.05 level. Statistical analyses were performed using Prism 5.0 software (GraphPad Software, San Diego, CA).

Results

RvD1 protected lung tissues from LPS-induced ALI—histopathologic evaluation

First, we evaluated the effect of RvD1 on LPS-induced ALI. The control group revealed normal pulmonary histology (Fig. 1A). In contrast, lung tissues in the LPS group were significantly damaged, with interstitial edema, hemorrhage, thickening of the alveolar wall, and infiltration of inflammatory cells into the interstitium and alveolar spaces, as evidenced by an increase in lung injury score (p < 0.01). All the morphologic changes were less pronounced in the LPS+RvD1 group. RvD1 significantly attenuated LPS-induced pathologic changes by the evidence of a decrease in lung injury score (p < 0.01). There was no significant difference between the control and RvD1 groups (p > 0.05) (Fig. 1B). The lung tissues homogenate MPO concentration was significantly increased in the LPS group compared with the control group (p < 0.01), but greatly attenuated in the RvD1 treatment group compared with the LPS group (p < 0.05) (Fig. 1C).

RvD1 upregulated AFC in LPS-induced ALI in vivo

Next, we examined the effect of RvD1 on AFC in LPS-induced ALI in vivo. RvD1 at a concentration of 5 µg/kg or vehicle alone was injected through caudal vein 8 h after LPS (20 mg/kg) administration, and AFC was determined after 60 min. AFC in the LPS group was as expected, reduced compared with the control group (p < 0.01), whereas RvD1 increased AFC after LPS-induced ALI (p < 0.05). However, there was no significant difference between the control and RvD1 groups (p > 0.05) (Fig. 2).

RvD1 enhanced ENaC and Na,K-ATPase in LPS-induced ALI in vivo

To clarify whether RvD1 effects on AFC are mediated by ENaC and Na,K-ATPase, the protein was isolated from rat lungs and then measured by Western blotting and immunohistochemistry. In addition, the activity of Na,K-ATPase was measured.

In vivo, the protein expression of ENaC α and γ subunits in rat lung tissue homogenates was increased in the LPS+RvD1 group compared with the LPS group (p < 0.05). However, no significant change in protein expression of ENaC β subunit was observed after treatment with RvD1 (p > 0.05) (Fig. 3A).

Immunohistochemical analysis was used to determine the distribution of ENaC α, β, and γ subunits in rat lungs after drug intervention. Positive immunostained cells appeared brown. The expression of ENaC α, β, and γ subunits were specifically localized to the apical and basal membrane of the alveolar epithelium. The number of positive cells expressing ENaC α, γ subunits were significantly decreased in the LPS group, but increased in the LPS+RvD1 group compared with the LPS group (Fig. 3B, 3D), whereas ENaC β subunit showed no difference between the LPS and LPS+RvD1 groups (Fig. 3C).

In vivo, the protein expression of Na,K-ATPase α1 and β1 subunits in rat lung tissue homogenates were increased in the LPS+RvD1 group compared with the LPS group (p < 0.05; Fig. 4A). Immunohistochemical analysis was used to determine the distribution of Na,K-ATPase α1 and β1 subunits in rat lungs after drug intervention. Positive immunostained cells appeared brown. The expression of α1 and β1 subunits were specifically localized to the apical and basal membrane of the alveolar epithelium. The number of positive cells expressing Na,K-ATPase α1 and β1 subunits were significantly decreased in the LPS group, but increased in the LPS+RvD1 group compared with the LPS group (Fig. 4B, 4C).

In addition, the activity of Na,K-ATPase was measured. LPS markedly decreased the Na,K-ATPase activity compared with the control group (p < 0.01), whereas RvD1 augmented Na,K-ATPase activity 8 h after LPS-induced ALI (p < 0.05) (Fig. 4D).

Dose and time dependency of LPS regulated ENaC expression in primary ATII cells

The dose–response and temporal expression patterns relationship of LPS regulating ENaC expression were determined in primary ATII cells by Western blotting. Cells were incubated with varying concentrations of LPS including 0.5, 1, 2, 5, and 10 µg/ml. In all responses, the ENaC α subunit protein expression was decreased dose-dependently with a concentration of 1 µg/ml producing a maximal effect. In subsequent experiments, the ENaC and Na,K-ATPase expression in ATII cells was assessed using 1 µg/ml LPS (Fig. 5A).

To determine the dynamic expression of ENaC in primary ATII cells, we incubated cells with 1 µg/ml LPS for 6, 12, 24, and 48 h. The expression of ENaC α and γ subunits protein was significantly decreased initially at 6 h with recovery occurring at 48 h and 1 µg/ml LPS treatment for 12 h producing a maximal effect (Fig. 5B).

RvD1 increased ENaC expression and Na+ currents in primary rat ATII cells

To clarify whether the effect of RvD1 on AFC is mediated by ENaC, the protein was isolated from primary ATII cells and then measured by Western blotting and confocal laser-scanning microscopy. In addition, the Na+ current in primary rat ATII cells was measured.

In vitro, rat primary ATII alveolar epithelial cells were incubated with RvD1 at a concentration of 50 nM in the presence or absence of LPS (1 µg/ml) for 12 h at 37°C. The protein expression of ENaC α and γ subunits was increased in the LPS+RvD1 group compared with the LPS group (p < 0.05). However, no significant change in protein expression of ENaC β subunit was observed after treatment with RvD1 (p > 0.05) (Fig. 6A).

The effect of RvD1 on the subcellular distribution of the ENaC α subunit was further examined by confocal laser-scanning microscopy. RvD1 increased ENaC α subunit abundance in the plasma.
membrane, which was best seen at the junctions between adjacent cells (Fig. 6B). These microscopic images thus corroborate the results obtained by immunohistochemistry of rat lung tissues.

Average current–voltage relationships from whole-cell recordings of primary rat ATII cells treated with RvD1 (50 nM) in the presence or absence of LPS (1 μg/ml) for 12 h were measured in this study (Fig. 6C). Data were presented as pA/pF. The curve showed the Na+ currents in the LPS group were lower from −100 mV to +100 mV than the control group, and the current in the RvD1 treatment group was higher than the LPS group. Statistical analysis showed that cell exposed to LPS had significantly lower Na+ currents than those of the control cells (p < 0.05). However, the Na+ currents in the RvD1 treatment group were higher than the LPS group (p > 0.05).

RvD1 promoted Na,K-ATPase expression and Na,K-ATPase activity in primary rat ATII cells

The protein expression of Na,K-ATPase in primary ATII cells was measured by Western blotting and confocal laser-scanning microscopy. In addition, the activity of Na,K-ATPase was measured.

In vitro, the protein expression of the Na,K-ATPase α1 subunit was increased in the LPS+RvD1 group compared with the LPS group (p < 0.05). However, no significant change in protein expression of the Na,K-ATPase β1 subunit was observed after treatment with RvD1 (p > 0.05) (Fig. 7A).

The effect of RvD1 on the subcellular distribution of the Na,K-ATPase α1 subunit was further examined by confocal laser-scanning microscopy. RvD1 increased Na,K-ATPase α1 subunit abundance in the plasma membrane, which was best seen at the junctions between adjacent cells (Fig. 7B). These microscopic images thus corroborate the results obtained by immunohistochemistry of rat lung tissues.

Furthermore, the role of RvD1 on the Na,K-ATPase activity in primary rat ATII epithelial cells stimulated with LPS was assessed. Primary ATII cells after LPS (1 μg/ml) exposure with RvD1 (50 nM) treatment for 12 h were digested, subjected to centrifugal sedimentation, lysed, and homogenized to analyze the activity of Na,K-ATPase with kits; Na,K-ATPase activity was significantly increased in the LPS+RvD1 group compared with the LPS group (p < 0.05; Fig. 7C).

RvD1 increased ENaC expression regulated by ALX in primary ATII cells stimulated with LPS

To monitor the effect of RvD1 receptors on the ENaC expression in ATII cells, we used RT-PCR to measure the mRNA expression of GPR32 and ALX (RvD1 receptors). The inhibitory effect of LPS on ALX mRNA expression was abrogated by treatment with RvD1. However, the GPR32 mRNA was not expressed in rat primary ATII cells. As a positive control, GPR32 mRNA was expressed in A549

FIGURE 3. RvD1 enhanced ENaC expression in LPS-induced ALI in vivo. RvD1 (5 μg/kg) was administrated to Sprague–Dawley rats 8 h after LPS (20 mg/kg) stimulation through caudal vein, ventilating for 60 min, and the right lung tissue was harvested to measure the protein expression of ENaC α, β, and γ subunits by Western blotting (A) and immunohistochemistry (B–D) (original magnification ×400); brown cells represented positive cells. DAB was used to stain the cells. Data are presented as mean ± SEM, n = 5. Alcohol is resolvent. *p < 0.05, **p < 0.01 versus control group. *p < 0.05, **p < 0.01 versus LPS group. *p < 0.05, **p < 0.01 versus LPS+Alcohol group.
human epithelial adenocarcinoma cells, and the inhibitory effect of LPS on GPR32 mRNA expression was abolished by treatment with RvD1 (Fig. 8A). In addition, BOC-2 (10 μM) (41) was used to measure the ENaC protein expression in LPS-stimulated ATII cells by Western blotting. ENaC α and γ subunit protein expression were increased in the LPS+RvD1 group compared with the LPS group, but this function was abolished by BOC-2 (Fig. 8B).

RvD1-induced cAMP elevation in the primary ATII cells stimulated with LPS was dependent on PI3K

To test whether RvD1 (50 nM) has an impact on cAMP and cGMP levels in ATII cells, we measured cAMP and cGMP concentration in primary ATII cells stimulated with LPS (1 μg/ml) for 12 h by ELISA kits. We found cAMP concentration was decreased in the LPS group compared with the control group (p < 0.05), and RvD1 treatment abrogated the increased cAMP concentration compared with the LPS group (p < 0.05). In addition, LY294002 (10 μM) or H89 (10 μM) was used to treat primary ATII cells in the presence of RvD1 and LPS for 12 h. cAMP concentration was decreased in the LPS+RvD1+LY294002 group compared with the LPS+RvD1 group (p < 0.05), but not decreased in the LPS+RvD1+H89 group (Fig. 9A). However, we could not detect a significant change of cGMP level in these groups (Fig. 9B).

RvD1 activated the cAMP/PI3K pathway in vitro

To further investigate whether RvD1 regulated ENaC expression via cAMP, PKA, and PI3K pathway, we used Rp-cAMP (cAMP inhibitor, 10 μM), H89 (PKA inhibitor, 10 μM), and LY294002 (PI3K inhibitor, 10 μM) to measure the ENaC protein expression by Western blotting in primary ATII cells stimulated with LPS. The protein level of ENaC α and γ subunit protein expression was markedly decreased in the LPS+RvD1+RP-cAMP group compared with the LPS+RvD1 group (p < 0.05), but Rp-cAMP alone did not obviously change ENaC α and γ subunits expression (Fig. 10). ENaC α protein expression was reduced by LY294002 treatment compared with the RvD1 treatment group, but there was no significant difference between the LPS+RvD1 and LPS+RvD1+H89 groups (Fig. 11A). There was no significant difference in ENaC γ protein expression between the LPS+RvD1, LPS+RvD1+LY294002, and LPS+RvD1+H89 groups (Fig. 11B).

RvD1 promoted AFC through activating the ALX/cAMP/PI3K pathway in vivo

To further investigate ALX/cAMP/PI3K-dependent actions of RvD1 in vivo, we coadministered RvD1 (5 μg/kg) and BOC-2 (600 ng/kg), Rp-cAMP (5 mg/kg), Rp-cGMP (5.5 mg/kg), LY294002 (3 mg/kg) or H89 (10 mg/kg) to Sprague–Dawley rats through caudal vein 8 h after LPS (20 mg/kg) administration, and AFC was determined after 60 min. AFC in the LPS+RvD1+BOC-2, LPS+RvD1+Rp-cAMP, and LPS+RvD1+LY294002 groups was reduced compared with LPS+RvD1 group (p < 0.05), whereas there was no significant changes in the LPS+RvD1+Rp-cGMP and LPS+RvD1+H89 groups (p > 0.05) (Fig. 12).

Discussion

We have provided evidence for the proresolution actions of RvD1 in ARDS. Treatment with RvD1 improved AFC and decreased pulmonary edema in LPS-induced ALI in rats. RvD1 most potently regulated AFC with upregulating the protein expression of ENaC α, γ and Na,K-ATPase α1, increasing the activity of Na,K-ATPase
RvD1 increased Na⁺ currents in primary ATII cells. RvD1 enhanced the subcellular distribution of ENaC and Na,K-ATPase, specifically localized to the apical and basal membrane of the alveolar epithelium. Moreover, BOC-2, LY294002, and Rp-cAMP blocked the increased AFC and ENaC expression response to RvD1 in vivo and in vitro, indicating RvD1 increased ENaC expression to promote AFC via the ALX/PI3K/cAMP signaling pathway.

Pulmonary edema is a hallmark of LPS-induced ALI and a life-threatening condition, consisting of various degrees of water and proteins, resulting from an imbalance between forces driving fluid into the airspaces and biological mechanisms for its removal (27, 42). It is widely accepted that resolution of alveolar edema is the key step to patient survival (5). Previously, clinical studies have shown that impaired alveolar fluid transport mechanisms contribute to the development, severity, and outcome of pulmonary edema in humans (43). The AFC process is crucial to efficient gas exchange in the lung (9), and patients with ALI who have intact AFC have lower morbidity and mortality than those with compromised AFC (44). Our data clearly demonstrate that RvD1 has no effect on AFC in healthy, perfused intact rat lungs; however, it enhances the rate of AFC 8 h after LPS challenge with the outcome of decreased pulmonary edema, suggesting that RvD1 plays a role in the resolution of inflammation.

It is well accepted that Na⁺ reabsorption from the alveolar spaces via ENaC is of particular importance in the process to remove edema fluid from the alveolar spaces in ALI/ARDS. Several observations confirm that transepithelial sodium transport plays a major role in the clearance of fluid from the airspace not only under normal conditions, but also during experimental lung injury (6, 7). Apical located ENaC is composed of three homologous subunits, α, β, and γ, which are expressed in a number of epithelial tissues including alveolar epithelial cells. Unable to clear alveolar edema fluid, ENaC γ gene knockout mice died within 40 h after birth (45). In our study, RvD1 not only enhanced lung tissues homogenate ENaC α and γ subunits protein expression in LPS-induced ALI, but also increased ENaC α, γ subunit protein expression and Na⁺ currents in primary ATII cells stimulated with LPS. Furthermore, immunohistochemical analysis of rat lung tissues and confocal
In primary ATII cells, laser-scanning microscopy results draw identical outcomes. Consistent with our findings, similar results have shown that upregulation of ENaC increased pulmonary edema fluid reabsorption (46) and reduced ENaC expression delayed reab-

**FIGURE 7.** RvD1 promoted Na,K-ATPase expression and Na,K-ATPase activity in primary rat ATII cells stimulated with LPS. Rat primary ATII cells were treated with RvD1 (50 nM) in the presence of LPS (1 μg/ml) for 12 h. After incubation, the cells were harvested and sonicated. Na,K-ATPase α1 and β1 subunits protein expression in the cell lysates was detected by Western blotting (A) and confocal laser-scanning microscopy (B) using a specific Ab (original magnification ×400). Alexa Fluor second Ab was used to stain the cells. In addition, Na,K-ATPase activity was measured by kits (C). Data are presented as mean ± SEM. n = 8. Alcohol is resolvent. *p < 0.05 versus control group. #p < 0.05, ##p < 0.01 versus LPS group. &&p < 0.05, &&&p < 0.01 versus LPS+Alcohol group.

**FIGURE 8.** RvD1 increased ENaC expression regulated by ALX in primary ATII cells stimulated with LPS. Rat primary ATII cells were treated with RvD1 (50 nM) in the presence of LPS (1 μg/ml) for 12 h. After incubation, the cells were harvested and sonicated. Expression of GPR32 and ALX mRNA was determined by RT-PCR; the GPR32 mRNA expression in A549 human epithelial adenocarcinoma cells was also measured for a positive control (A). Next, BOC-2 (10 μM) and RvD1 were coincubated in primary ATII cells stimulated with LPS for 12 h; the ENaC α and γ subunits protein expression in the cell lysates was detected by Western blotting (B). Data are presented as mean ± SEM. *p < 0.05 versus control group, #p < 0.05 versus LPS group, &p < 0.05 versus LPS+RvD1 group.

n = 4.
sorption of fluid during pulmonary edema after thiourea-induced lung injury (47). These findings, therefore, suggest that RvD1 promotes AFC through upregulation of ENaC α and γ subunits protein expression.

The Na,K-ATPase is known for its function in maintaining a transepithelial osmotic gradient, secondary to the establishment of sodium transport, and its role in the clearance of lung edema fluid (48). It works in concert with apical ENaC, produces an osmotic gradient, which constitutes the primary driving force of alveolar fluid reabsorption across the alveolar epithelium (49). Na,K-ATPase is composed of an α subunit that hydrolyzes ATP and exchanges intracellular Na⁺ for extracellular K⁺, and a β subunit that controls enzyme assembly and insertion into the plasma membrane (15, 50). Early studies showed AFC was increased by upregulation of Na,K-ATPase activity and facilitates the expression of Na,K-ATPase in alveolar cells (10, 51). Analogously, our study demonstrates that RvD1 not only increases Na,K-ATPase α1 expression in rat lung tissues and primary ATII cells after LPS challenge by Western blotting, immunohistochemistry, and confocal laser-scanning microscopy measurement, but also upregulation of Na,K-ATPase activity in vivo and in vitro. Together, our data from the lung tissues and cell culture indicate that RvD1 promotes AFC through both of the essential mechanisms of transepithelial active sodium transport proteins in the lung mediated by ENaC and Na,K-ATPase.

The anti-inflammatory and proresolving autacoids mediate their bioactions via specific GPCRs. Recently, two GPCRs for RvD1 were identified and validated using a GPCR/β-arrestin–coupled system, namely, orphan receptor GPR32 and ALX. Expression levels of GPR32 and ALX were increased after monocyte exposure.

**FIGURE 9.** PI3K was involved in the control of cAMP concentration after treatment of RvD1. Primary ATII cells were incubated with LY294002 (10 μM) or H89 (10 μM) in the presence of RvD1 (50 nM) and LPS (1 μg/ml) for 12 h. After incubation, the cells were harvested and sonicated. cAMP (A) and cGMP (B) concentration in the cell lysates was detected by ELISA kits. Data are expressed as mean ± SEM for each group. *p < 0.05 versus control group, **p < 0.05 versus LPS group, &p < 0.05 versus LPS+RvD1 group. n = 4.

**FIGURE 10.** RvD1 increased ENaC expression dependent on cAMP in the primary ATII cells stimulated with LPS. Rat primary ATII cells were treated with Rp-cAMP (cAMP inhibitor, 10 μM) in the presence of RvD1 (50 nM) and LPS (1 μg/ml) for 12 h. After incubation, the cells were harvested and sonicated. ENaC α and γ subunits protein expression in the cell lysates was detected by Western blotting using a specific Ab against (A, B). Data are expressed as mean ± SEM for each group. **p < 0.01 versus control group, *p < 0.05 versus LPS group, &p < 0.05 versus LPS+RvD1 group. n = 4.
to GM-CSF or zymosan A for 24–48 h. Indeed, human monocyte-derived macrophages treated with RvD1 display enhanced zymosan phagocytosis, a response further increased when these cells were coadministered with either GPR32 or ALX. Moreover, RvD1 potently regulates human PMN recruitment and lipid mediator biosynthesis, as well as enhances clearance of zymosan via binding GPR32 and ALX (24). Another study showed, with human macrophages, RvD1 stimulated phagocytosis of E. coli in a GPR32-dependent manner (52). However, interestingly, GPR32 was not present in rat primary ATII cells in this study, which is consistent with a previous report, where an ortholog for human GPR32 was identified in the chimpanzee but is absent in the dog genome and is a pseudogene in the rat and mouse (53). In this study, RvD1 increased the ALX mRNA expression in ATII cells stimulated with LPS. In addition, RvD1 increased ENaC α and β subunits protein expression, but the beneficial effects were abrogated by ALX antagonist (BOC-2) in vitro, indicating that the effect of RvD1 increasing ENaC expression was through binding ALX. Along these lines, BOC-2, recently identified via a medicinal chemistry screen with anti-inflammatory actions in vivo (16), also abolished the beneficial effects of RvD1 on AFC in vivo. This is consistent with a previous report where RvD1 improved survival rate and attenuated ALI in mice induced by LPS with protective mechanisms that might be related to selective reaction with ALX (27). These results, taken together, suggest that the RvD1 response is ALX dependent.

cAMP and cGMP are important second messengers by which cells transduce extracellular signals into intracellular responses. ALX was the first receptor cloned and identified as a GPCR for lipoxin and resolvin with demonstrated cell-type–specific signaling pathways (54). Extracellular signals interact with GPCRs to activate the AC and increase the intracellular cAMP levels. A previous study showed that LPS-induced immune response leads to a decrease of intracellular cAMP (55). Another study showed that LPS activates Gs, thereby inhibiting AC and formation of cAMP (56). Stimulation with cAMP not only increases Na+ transport within 5 min, but also promotes Na,K-ATPase recruitment to the plasma membrane (57, 58). It is consistent with a model where the early effect of cAMP is to increase trafficking of ENaC to the apical cell surface whereas the sustained effect requires the synthesis of ENaC (57). Extracellular signals also interact with GPCRs to activate the guanylyl cyclases and increase the intracellular cGMP levels. In this context, there is evidence for substantial compartmentalization of two signaling pathways on regulating AFC, cAMP, and cGMP. In accordance with previously, the intracellular cAMP level was decreased after LPS stimulation, and RvD1 abrogated the decrease observed in the LPS group in vitro. More importantly, the effect of RvD1 on ENaC is abolished by Rp-cAMP, an inhibitor of cAMP. Of interest, there was no significant difference of intracellular cGMP level between the RvD1 treatment and LPS group. Finally, as further proof, Rp-cAMP and Rp-cGMP were used in vivo, and we found that the Rp-cAMP, not Rp-cGMP, reduced AFC in LPS-induced ALI, indicating that RvD1 promoted AFC by activating cAMP via ALX, but not cGMP.

It is well-known that PI3K signals are implicated in regulating ENaC trafficking and activity (59). The PI3K has been identified as integral for regulation of ENaC-mediated AFC by insulin (32). Previous studies showed that RvD1 treatment in Par-Cro cells activates the AC and increases ENaC expression of ENaC and the beneficial effects were abrogated by ALX antagonist (BOC-2) in vitro, indicating that the effect of RvD1 increasing ENaC expression was through binding ALX. Along these lines, BOC-2, recently identified via a medicinal chemistry screen with anti-inflammatory actions in vivo (16), also abolished the beneficial effects of RvD1 on AFC in vivo. This is consistent with a previous report where RvD1 improved survival rate and attenuated ALI in mice induced by LPS with protective mechanisms that might be related to selective reaction with ALX (27). These results, taken together, suggest that the RvD1 response is ALX dependent.

FIGURE 11. RvD1-induced cAMP elevation in the primary ATII cells stimulated with LPS was dependent on PI3K. Rat primary ATII cells were treated with H89 (PKA inhibitor, 10 μM) and LY294002 (PI3K inhibitor, 10 μM) in the presence of RvD1 (50 nM) and LPS (1 μg/ml) for 12 h. After incubation, the cells were harvested and sonicated. ENaC α (A) and γ (B) subunits protein expression in the cell lysates was detected by Western blotting using a specific Ab. Data are expressed as mean ± SEM for each group. *p < 0.05 versus control group, #p < 0.05 versus LPS group, ##p < 0.01 versus LPS+RvD1 group. n = 4.

FIGURE 12. RvD1 improves AFC through activating the ALX/cAMP/PI3K pathway in vivo. RvD1 (5 μg/kg) and BOC-2 (600 ng/kg), Rp-cAMP (5 mg/kg), Rp-cGMP (5.5 mg/kg), LY294002 (3 mg/kg), or H89 (10 mg/kg) were coadministered to Sprague-Dawley rats through caudal vein 8 h after a tracheostomy to the left lung; AFC was measured over 60 min in ventilated animals. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01 versus control group. #p < 0.01 versus LPS group. ##p < 0.05 versus LPS+RvD1 group. n = 8.
but not the ENaC γ. Indeed, assembly and expression of functional active sodium channels was strictly dependent on ENaC α (45). The typical result of the production of cAMP was activation of PKA (61). However, previous studies have shown that ENaC was not activated by PKA in the oocyte and the bilayer systems (62, 63). Consistently, we also found the cAMP effects were not mediated by PKA, because coincubation with H89, a PKA inhibitor, did not block RvD1-induced increase in cAMP concentration and expressions of ENaC α, γ in ATII cells stimulated with LPS. Moreover, LY294002 and H89 were used in vivo, and we found LY294002, not H89, reduced AFC in LPS-induced ALI. Moreover, LY294002 and H89 were used in vivo, and we found LY294002, not H89, reduced AFC in LPS-induced ALI. The implication for our work is that RvD1 promoting AFC is largely dependent on PI3K. The key role for RvD1 during LPS-induced ALI in vivo and in vitro is summarized in Fig. 13.

In this study, we have shown that RvD1 reduced pulmonary edema primarily by its actions on the alveolar ENaC and Na,K-ATPase. However, its potential actions on other inflammatory mechanisms may be contributing as an anti-inflammatory and proresolution mediator. First, RvD1 has been shown to improve vascular permeability changes and enhance both epithelial and endothelial barrier integrity to decrease alveolar edema in acid-initiated ALI (64). Second, a previous study showed that the number of PMNs in the BALF was statistically increased in the LPS-induced lung injury group compared with saline-instilled controls at 3 h and reached maximum numbers at 24 h (65). Therefore, there is still a substantial ongoing increase in lung PMNs 8 h after LPS such that the effect of RvD1 treatment could be to attenuate PMN influx into the lung. Finally, RvD1 increased the apoptosis of neutrophils to enhance their clearance (66), and treatment for 30 min with RvD1 upregulated macrophages engulfing apoptotic neutrophils (67).

In conclusion, these data demonstrate that RvD1 alleviated pulmonary edema, enhanced AFC, and attenuated lung injury partially through stimulation of ENaC and Na,K-ATPase via activation of the ALX/cAMP/P13K pathway in LPS-induced ALI without affecting normal lung. Thus, treatment with RvD1 in critically ill patients with ALI has the potential to augment lung edema clearance. Our findings reveal a novel mechanism for pulmonary edema fluid reabsorption and RvD1 may provide a new therapy for the resolution of ALI/ARDS.

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Disclosures

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