Laboratory Investigation (2015) 95, 1258–1268

Lipoxin A_4 activates alveolar epithelial sodium channel gamma via the microRNA-21/PTEN/AKT pathway in lipopolysaccharide-induced inflammatory lung injury

Wei Qi[1,6], Hui Li[1,6], Xiao-Hong Cai[2], Jia-Qi Gu[1], Jin Meng[1], Hai-Qing Xie[1], Jun-Li Zhang[1], Jie Chen[1], Xian-Guan Jin[1], Qian Tang[1], Yu Hao[1], Ye Gao[1], Ai-Qing Wen[3], Xiang-Yang Xue[4], Fang Gao Smith[5] and Sheng-Wei Jin[1]

Lipoxin A_4 (LXA_4), as an endogenously produced lipid mediator, promotes the resolution of inflammation. Previously, we demonstrated that LXA_4 stimulated alveolar fluid clearance through alveolar epithelial sodium channel gamma (ENaC-γ). In this study, we sought to investigate the mechanisms of LXA_4 in modulation of ENaC-γ in lipopolysaccharide (LPS)-induced inflammatory lung injury. miR-21 was upregulated during an LPS challenge and downregulated by LXA_4 administration *in vivo* and *in vitro*. Serum miR-21 concentration was also elevated in acute respiratory distress syndrome patients as compared with healthy volunteers. LPS increased miR-21 expression by activation of activator protein 1 (AP-1). In A549 cells, miR-21 upregulated phosphorylation of AKT activation via inhibition of phosphatase and tensin homolog (PTEN), and therefore reduced the expression of ENaC-γ. In contrast, LXA_4 reversed LPS-inhibited ENaC-γ expression through inhibition of AP-1 and activation of PTEN. In addition, an miR-21 inhibitor mimicked the effects of LXA_4; overexpression of miR-21 abolished the protective effects of LXA_4. Finally, both AKT and ERK inhibitors (LY294002 and UO126) blocked effects of LPS on the depression of ENaC-γ. However, LXA_4 only inhibited LPS-induced phosphorylation of AKT. In summary, LXA_4 activates ENaC-γ in part via the miR-21/PTEN/AKT signaling pathway.
Laboratory Investigation (2015) **95,** 1258–1268; doi:10.1038/labinvest.2015.109; published online 24 August 2015

Acute respiratory distress syndrome (ARDS) is a devastating syndrome characterized by dysregulated inflammation and alveolar barrier disruption that impairs pulmonary gas exchange, leading to refractory arterial hypoxemia and respiratory failure.[1,2] Bacterial or viral pneumonia and sepsis are the most common causes of acute lung injury (ALI) and ARDS,[1] wherein Gram-negative bacteria are a prominent cause. Lipopolysaccharide (LPS), the outer membrane of Gram-negative bacteria, is one of the mainly pro-inflammatory reaction factors in ALI, leading to neutrophil recruitment and pulmonary edema.[3] After decades of efforts, the mortality of ARDS has declined, perhaps secondary to more widespread use of lung-protective ventilation, reductions in nosocomial infections and supportive care.[2,4]

Recently, the critical importance of timely and effective removal of excessive alveolar edema, namely alveolar fluid clearance (AFC), is better recognized in ARDS.[5,6] It is now well established that the mechanism of AFC is the alveolar fluid cleared by active Na^+ transport[7] across the alveolar epithelium via an apical alveolar epithelial sodium channel (ENaC)[8–10] and through basolateral Na^+-K^+-ATPases.[9,11] The water then moves passively from the air spaces to the alveolar intersititium as the result of the transepithelial osmotic gradient caused by active Na^+ transport. Hence, both ENaC and Na^+-K^+-ATPases are pivotal in timely and effective removal of excessive alveolar edema fluid.[12]

There is a growing appreciation that there are short-term regulatory and long-term regulatory mechanisms in active Na^+ transport. Short-term regulatory mechanisms mainly involve dopamine and β-adrenergic agonists; transcriptional and posttranscriptional mechanisms are key to long-term regulation.[11] We previously reported that an intravenous

[1]Department of Anesthesia and Critical Care, Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Zhejiang, China; [2]Department of Pediatrics, Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Zhejiang, China; [3]State Key Laboratory of Trauma, Burns and Combined Injury, Institute of Surgery Research, Daping Hospital, Third Military Medical University, Chongqing, China; [4]Department of Microbiology, Wenzhou Medical University, Zhejiang, China and [5]Academic Department of Anesthesia, Critical Care, Pain and Resuscitation, Birmingham Heartlands Hospital, Heart of England NHS Foundation Trust, Birmingham, UK

Correspondence: Professor S-W Jin, MD, PhD or Professor FG Smith, MD, PhD, Department of Anesthesia and Critical Care, Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, 109 Xueyuan Road, Zhejiang 325027, China.
E-mail: jinshengwei69@163.com or f.gaosmith@bham.ac.uk
[6]These authors contributed equally to this work.

Received 7 February 2015; revised 20 April 2015; accepted 29 April 2015

β-agonist (salbutamol) reduced extravascular lung water in ARDS patients.[13–16] However, we found salbutamol significantly increased the 28-day mortality rate because of its side effects, such as tachycardia, arrhythmia, and lactic acidosis, in a multicenter, randomized, controlled clinical trial.[17] Therefore, new therapeutic agents need to be identified.

Lipoxins are eicosanoids that are formed during inflammation via transcellular biosynthetic routes that possess distinct anti-inflammatory and pro-resolution properties, including the repression of pro-inflammatory cytokine production, inhibition of leukocyte-mediated injury, and stimulation of macrophage clearance of apoptotic neutrophils.[18,19] Lipoxin A_4 (LXA_4) has emerged as a founding member of the first class of lipid mediators that can function as 'braking signals' in inflammation, and are switched on in the resolution phase of an inflammatory response. We previously described distinct pro-resolution and anti-edema properties of LXA_4 and aspirin-triggered LXA_4 in a rat ALI model.[10,20,21] We found that LXA_4 significantly stimulated AFC through upregulation of ENaC-γ protein expression,[10] which is a regulatory subunit of ENaC.[22] Thus, LXA_4 is a useful research tool and might be developed as a novel therapeutic intervention.

MicroRNAs (miRNAs) are a class of gene products that are implicated in several lung diseases[23–25] and have emerged as novel biomarkers[26] and possible therapeutic strategies.[27] Recent studies revealed that miR-21 was dynamically regulated in LPS-induced ALI.[28,29] Also, studies have showed that miR-16 upregulated ENaC-β,[30] miR-101 and miR-144 targeted cystic fibrosis transmembrane conductance regulator 3′ UTR,[31] miR-96 and miR-330 bound to the 3' UTR of aquaporin 5.[32] Previous studies demonstrated that each specialized pro-resolving mediator regulated a distinct panel of miRNAs.[33–35] More recently, LXA_4 exhibited antifibrotic properties by upregulation of let-7c expression in renal fibrosis.[36] However, whether LXA_4 augments AFC through a particular miRNA in LPS-induced ALI, and if so, what the underlying mechanisms are remain unclear.

In the present study, we investigated the central role of miR-21 in LPS-dependent inflammatory lung injury. Based on *in silico* bioinformatic analysis and real-time PCR, we focused on miR-21 and its potential target ENaC-γ mRNA. We also evaluated ARDS patients' serum miR-21 levels. In addition, we investigated the effect of LXA_4 on the protein expression of ENaC-γ and phosphatase and tensin homolog (PTEN), and the phosphorylation of AKT and ERK *in vitro*. Finally, to better understand the mechanisms of action of LXA_4, we used a miR-21 mimic, an miR-21 inhibitor, an AP-1 inhibitor (SR11302), an AKT inhibitor (LY294002), and an ERK inhibitor (UO126) to investigate how this signaling pathway regulates ENaC-γ protein expression.

MATERIALS AND METHODS

Reagents

LPS, LY294002 (AKT inhibitor), and U0126 (ERK inhibitor) were obtained from Sigma-Aldrich (St Louis, MO, USA). LXA_4 was from Cayman Chemical Company (Ann Arbor, MI, USA). SR11302 (AP-1 inhibitor) was purchased from Tocris Bioscience (Bristol, UK). RPMI 1640, fetal bovine serum, trypsin, and enzyme-free cell dissociation buffer were purchased from Gibco (Grand Island, NY, USA). Penicillin and streptomycin in saline citrate buffer were from Invitrogen (Carlsbad, CA, USA).

Animals

Specific pathogen-free adult male SD rats, weighing 250–300 g, obtained from Slac Laboratory Animal (Shanghai, China), were housed under controlled temperature and humidity in a day–night cycle, with free access to standard laboratory food and water. The study was approved by the Animal Studies Ethics Committee of Wenzhou Medical University.

Cell Culture

A549 Cells were cultured in RPMI 1640 containing 10% fetal bovine serum with 100 U/ml penicillin, and 100 μg/ml streptomycin at $1 \times 10^6/cm^2$, and incubated in a humidified atmosphere with 5% CO_2 at 37 °C. Before stimulation or transfection, cells were cultured overnight in 1% FBS.

Study Participants and Sample Processing

All patients and healthy volunteers provided written informed consent. This study complied with the Declaration of Helsinki and was approved by the Committee on Ethics of the Second Affiliated Hospital of Wenzhou Medical University. Study enrollment occurred between January and June 2014. ARDS was defined and classified according to the Berlin definition.[37] According to the Berlin definition, diagnostic criteria for ARDS rely on four categories: (1) timing: within 1 week of a known clinical insult or new or worsening respiratory symptoms; (2) radiography: bilateral opacities—not fully explained by effusion, lobar/lung collapse or nodule; (3) origin of lung edema: respiratory failure not fully explained by cardiac failure or fluid overload, and (4) oxygenation impairment: subdivided into three categories according to the degree of hypoxemia severity (mild, moderate, and severe). Eligible patients were at least 18 years of age and diagnosed within 48 h with a PaO_2/FiO_2 ratio of <300. Exclusion criteria included pre-existing severe disease of any major organs, pregnancy, pulmonary hypertension, malignant disease, HIV infection, or if informed consent could not be obtained. Volunteers were not taking any medications for 2 weeks before commencement of the study.

Blood from ARDS patients and healthy volunteers were put into EDTA-containing tubes (BD Biosciences, NJ, USA). Tubes were rotated end-over-end at room temperature for 30 min and centrifuged (795 *g*, 20 min, 4 °C) by Beckman JB-6 (Beckman Coulter, Danvers, MA, USA). The serum samples were aliquoted into 1.5-ml RNase-free Eppendorf tubes (Ambion, Carlsbad, CA, USA).

Bioinformatics Analysis and miRNA Prediction

The microRNA databases and target prediction tool TargetScan 6.2 (Cambridge, MA, USA; http://www.targetscan.org/index.html)[38] and miRWalk (Mannheim, Germany; http://mirwalk.uni-hd.de/)[39] were used to identify potential miRNA targets.

Transfections and Reporter Assays

Cells were transfected with an miR-21 mimic, inhibitor, or negative controls (RiboBio, Guangzhou, China) according to the manufacturer's protocol.

Human SCNN1G (ENaC-γ mRNA) coding sequence (CDS; 828–835 bp) containing the putative binding sites of miR-21 were amplified by PCR, inserted into the firefly luciferase reporter vector pmiR-RB-REPORT^{TM} (RiboBio) between the restrictive sites *Xho*I and *Not*I, and validated by sequencing. Their mutant constructs with a mutation of the miR-21 seed sequence were generated with the mutagenic oligonucleotide primers, according to the manual of Gene-Tailor Site-Directed Mutagenesis System (Invitrogen). Cells were plated at 1×10^5 cells/well on 24-well plates, and transfected with pmiR-RB-SCNN1G CDS (30 ng) or their mutant constructs. Twenty-four hours post transfection, firefly and Renilla luciferase activities were consecutively measured, according to the dual-luciferase assay manual (Promega, Madison, WI, USA). The Renilla luciferase signal was normalized to the firefly luciferase signal for each individual analysis.

Reverse Transcription and Real-Time PCR

Total RNA was extracted using the Trizol reagent (Invitrogen), according to the manufacturer's instructions. Pri-miR-21, ENaC-γ, and PTEN mRNA were quantified by real-time PCR assay using the SYBR Green real-time PCR Master Mix regents (Toyobo, Osaka, Japan) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the normalization control. For determination of miRNAs expression, total RNA (2 μg) was reversely transcribed with Bulge-Loop miRNA-specific reverse transcription-primers (RiboBio). Real-time PCR reactions were done with SYBR Green real-time PCR Master Mix regents (Toyobo) and Bulge-Loop primers (RiboBio) on the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with small nuclear RNA U6 as the normalization control. For determination of serum miRNA concentration, specific TaqMan assays for miRs and the TaqMan Micro-RNA Reverse Transcription Kit were used, followed by real-time PCR using the Universal PCR Master Mix (Ambion) according to the manufacturer's protocol with cel-miR-39 as the normalization control.

Western Blot

Western blot analysis from A549 cells lysis solution was performed as described previously.[10] The protein extracts were separated in SDS-polyacrylamide gels and transferred to PVDF membranes. The primary antibodies used included the following: β-actin (1:2000; Santa, Santa Cruz, CA, USA), GAPDH (1:5000; Bioworld, St Louis, MN, USA), ENaC-γ (1:1000; Abcam, Cambridge, MA, USA), PTEN (1:500; Abcam), phospho-AKT (1:1000; Cell Signaling Technology, Boston, USA), AKT (1:1000; Cell Signaling Technology), phospho-ERK (1:1000; Cell Signaling Technology), and ERK (1:1000; Cell Signaling Technology). And after primary antibodies incubated overnight at 4 °C, the secondary horseradish peroxidase-conjugated IgG, which were either goat anti-mouse or goat anti-rabbit, were used at 1:3000 dilution and imaged with the Image Quant LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Statistical Analysis

Data are presented as means ± s.e.m. All data were analyzed by the Student's *t*-test or by one-way ANOVA followed by Tukey's *post-hoc* test for multiple comparisons. Statistical analysis and graphs were done with GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). Results with $P<0.05$ were considered statistically significant.

RESULTS

Specific miRNAs are Upregulated by LPS Challenge and Downregulated with LXA_4 Administration

In silico bioinformatic analysis (TargetScan and miRWalk) revealed that miR-181a/b, miR-340, miR-137 were likely targeting the Na,K-ATPase β1 3′-UTR, miR-29a/b/c were likely targeting the Na,K-ATPase α1 5′-UTR, and miR-21 was able to potentially regulate the expression of ENaC-γ mRNA by binding to its CDS (Figure 1a). To investigate whether these miRNAs had roles in Na,K-ATPase α1, Na,K-ATPase β1, and ENaC-γ mRNA post-transcriptional regulations, we next sought to determine whether LPS regulates the expression of these miRNAs and the effects of LXA_4. Hierarchical clustering grouped these miRNAs into distinct clusters based on their relative abundance at the different treatment, *in vivo* and *in vitro* (Figure 1b and c). In particular, among the few miRNAs highly expressed in the present of LPS, miR-21, miR-29b, and miR-340 displayed >2.5-fold increases compared with control group and attenuated in the LXA_4 treatment. Conversely, a large group of miRNAs showed less of an increase (<0.4-fold changes). Among these, we focused on miR-21 because it showed consistent upregulation from LPS stimulation and downregulation due to LXA_4 treatment *in vivo* and *in vitro*. Therefore, we next determined the expression levels of miR-21 in a previously described LPS-induced rat ALI model[40] using real-time PCR. MiR-21 was upregulated by LPS treatment and downregulated by LXA_4 treatment (Figure 2a). Consistent with the results from the *in vivo* studies, miR-21 was significantly upregulated by stimulation with LPS when compared with the control group, and attenuated in the LXA_4 treatment in A549 cells (Figure 2b).

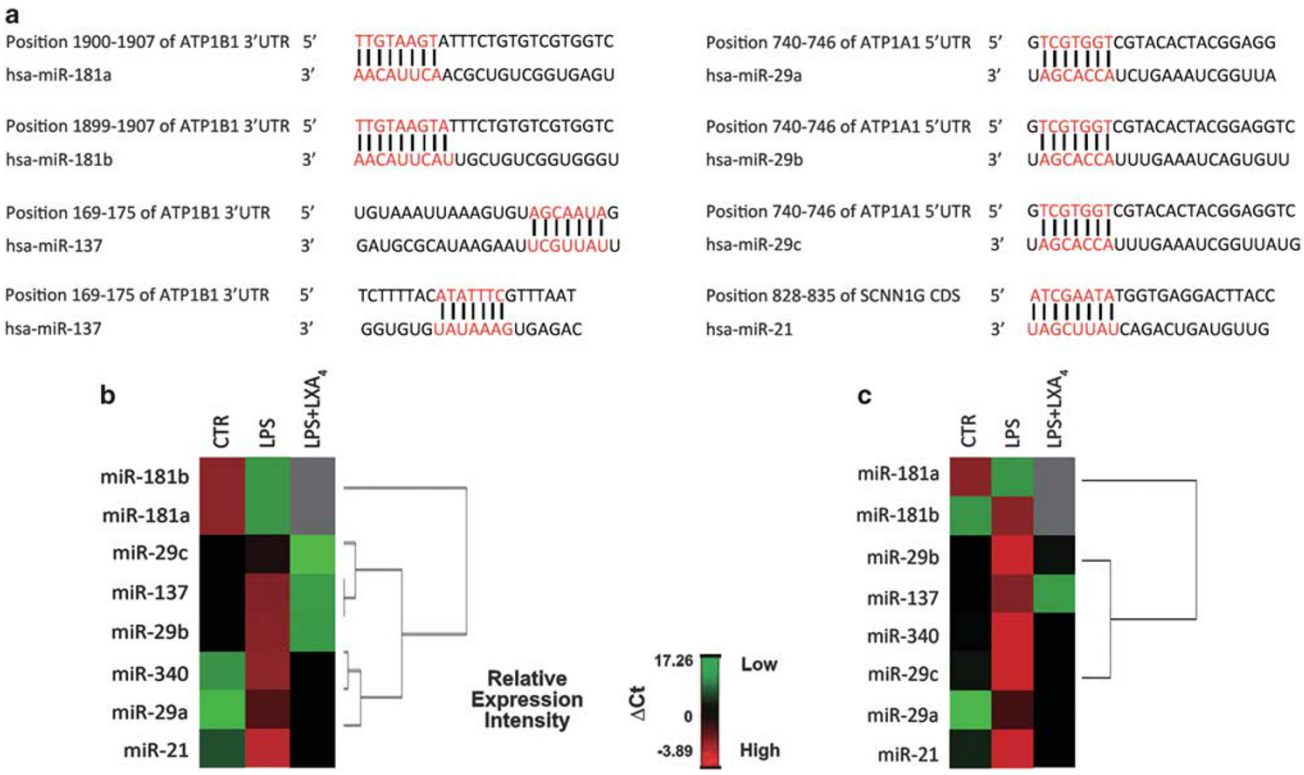


Figure 1 LXA_4-regulated miRNAs target genes with specific roles in alveolar fluid clearance. (**a**) The putative miRNAs binding sites located in Na,K-ATPase β1, α1, or ENaC-γ mRNA. (**b**) SD rats were injected with LXA_4 (2 μg/kg) or vehicle (1 ml saline) 8 h after LPS (20 mg/kg) stimulation through caudal vein, lung tissue homogenate samples are harvested 1 h later ($n=3$ per group), and miRNA fractions were isolated. (**c**) A549 cells treated with LPS (1 μg/ml) and/or LXA_4 (100 nM) for 8 h ($n=4$). Heatmap cluster represents relative expression of 8 miRNAs determined with real-time PCR after normalization with U6. Relative expression intensities are indicated in a green–red color code based on ΔC_t values. ENaC-γ, epithelial sodium channel gamma; LPS, lipopolysaccharide; LXA_4, lipoxin A_4.

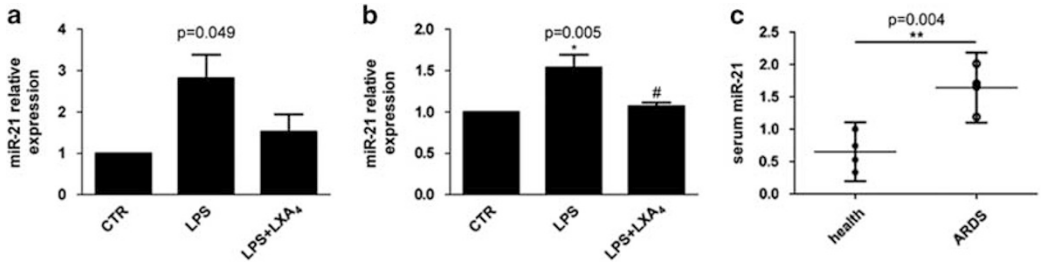


Figure 2 miR-21 is increased in inflammatory lung injury. (**a**) SYBR Green real-time PCR validation of miR-21 expression levels in a rat ALI model. SD rats were injected with LXA_4 (2 μg/kg) or vehicle (1 ml Saline) 8 h after LPS (20 mg/kg) stimulation through caudal vein, lung tissue homogenate samples are harvested 1 h later ($n=3$ per group). (**b**) miR-21 expression measured by SYBR Green real-time PCR in A549 cells treated with LPS (1 μg/ml) and/or LXA_4 (100 nM) for 8 h ($n=4$). (**c**) TaqMan real-time PCR detection of serum miR-21 level of ARDS patients at admission to ICU revealed ($P=0.03$, *t*-test) elevation of miR-21 in ARDS patients ($n=4$) as compared with healthy controls ($n=4$). Plots are displayed, where the vertical line indicates the median per group, and horizontal lines show 95% confidence interval. $^*P<0.05$ *versus* control group, $^{\#}P<0.05$ *versus* LPS group, $^{**}P<0.01$. ALI, acute lung injury; ARDS, acute respiratory distress syndrome; LPS, lipopolysaccharide; LXA_4, lipoxin A_4.

miR-21 Serum Concentration Is Elevated in ARDS

We next investigated miR-21 serum concentration in ARDS patients (see Supplementary Table 1 for details). The miR-21 serum concentrations were elevated in ARDS patients (at admission to the ICU and before therapeutic intervention) compared with healthy volunteers ($n=4$; Figure 2c).

AP-1 Mediated LPS-Induced miR-21 Expression

To clarify the regulatory mechanism by which LPS and/or LXA_4 affect miR-21 expression, we analyzed primary transcripts of the miR-21 gene (pri-miR-21) in A549 cells. LPS increased the quantities of pri-miR-21, whereas LXA_4 abolished the LPS-induced pri-miR-21 expression (Figure 3a), consistent

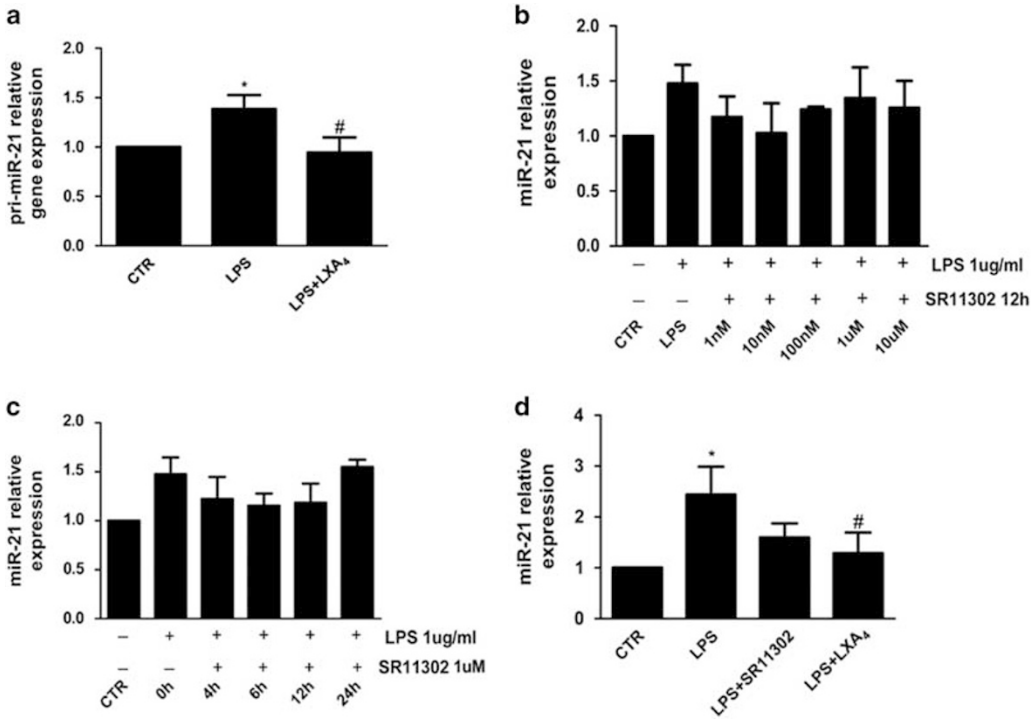


Figure 3 LPS-simulated miR-21 expression depends on AP-1. (**a**) SYBR Green real-time PCR measurement of pri-miR-21 in A549 cells treated with LPS (1 μg/ml) and/or LXA_4 (100 nM) for 8 h. Expression was normalized to GAPDH ($n=4$). (**b**) SYBR Green real-time PCR detection of dose-dependent miR-21 expression in A549 cells stimulated with LPS and SR11302. A549 cells are pre-incubated with different concentrations of SR11302 for 30 min including 1, 10, 100, 1000, 10 000 nM before LPS (1 μg/ml 12 h) treatment to measure miR-21 expression. (**c**) SYBR Green real-time PCR detection of temporal-dependent miR-21 expression in A549 cells stimulated with LPS and SR11302. A549 cells are pre-treated with SR11302 (1 μM) for 30 min then stimulated with LPS (1 μg/ml) for 4, 6, 12, 24 h to detect miR-21 expression ($n=3$). (**d**) miR-21 expression measured by SYBR Green real-time PCR in A549 cells incubated with LPS (1 μg/ml; 8 h) after pre-treated with SR11302 (10 nM; AP-1 inhibitor) or LXA_4 (100 nM) for 30 min ($n=5$). U6 expression is selected as endogenous control for normalization. Relative expression is calculated using the $\Delta\Delta C_t$ method of analysis. $^*P<0.05$ *versus* control group, $^{\#}P<0.05$ *versus* LPS group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide; LXA_4, lipoxin A_4.

with the mature miR-21, indicating that LPS and LXA_4 regulate miR-21 biosynthesis at the transcriptional level.

Because AP-1 is one of the conserved enhancer elements in the miR-21 promoter region,[41,42] we next explored whether AP-1 was involved in LPS- and/or LXA_4-mediated miR-21 biosynthesis. Indeed, pretreatment of the A549 cells with the selective AP-1 inhibitor retinoid SR11302[43] reduced the production of LPS-induced miR-21, in a dose- and time-dependent manner. In all cases, miR-21 expression decreased in a dose-dependent manner with a concentration of 10 nM producing a maximal effect (Figure 3b). The dynamic expression of miR-21 decreased initially at 4 h with recovery occurring at 24 h and 6–12 h producing a maximal effect (Figure 3c). In a subsequent experiment, miR-21 expression decreased 35% by treatment with 10 nM SR11302 for 12 h, whereas LXA_4 significantly decreased LPS-induced miR-21 expression (Figure 3d).

LXA_4 Attenuates LPS-Inhibited ENaC-γ Expression through Inhibition of miR-21, but miR-21 Does Not Target ENaC-γ mRNA Directly

We previously demonstrated that LPS decreased ENaC-γ protein expression, and that LXA_4 reversed the suppression in primary rat alveolar type II epithelial cells.[10] Therefore, we hypothesized that LXA_4-attenuated expression of LPS-induced miR-21 might underlie the protective mechanism of LXA_4. First, we observed that LPS stimulated a significant reduction in ENaC-γ mRNA and protein expression. However, treatment with LXA_4 and SR11302 increased ENaC-γ expression (Figure 4a and b). We therefore proposed that miR-21 may be a pivotal mediator of LPS-induced ENaC-γ expression. Second, to test this hypothesis, we transfected miR-21 mimic or inhibitor in A549 cells resulting in a 1000-fold induction or 2.2-fold repression in miR-21 expression levels, respectively (see Supplementary Figure 1 for details). However, there was no significant change in ENaC-γ protein expression (Figure 4c and d). Third, to further address whether miR-21 is involved in LPS-inhibited ENaC-γ expression, A549 cells that had been stimulated with LPS (1 μg/ml) for 8 h after transfection of miR-21 mimic caused a further decrease in ENaC-γ protein expression, and miR-21 inhibitor diminished the effect (Figure 4e and f). These data suggest that upregulation of miR-21 by LPS was necessary for subsequent downregulation of ENaC-γ expression. Furthermore, miR-21 mimic abolished the protective effect of LXA_4 in attenuating LPS-mediated ENaC-γ depression (Figure 4g

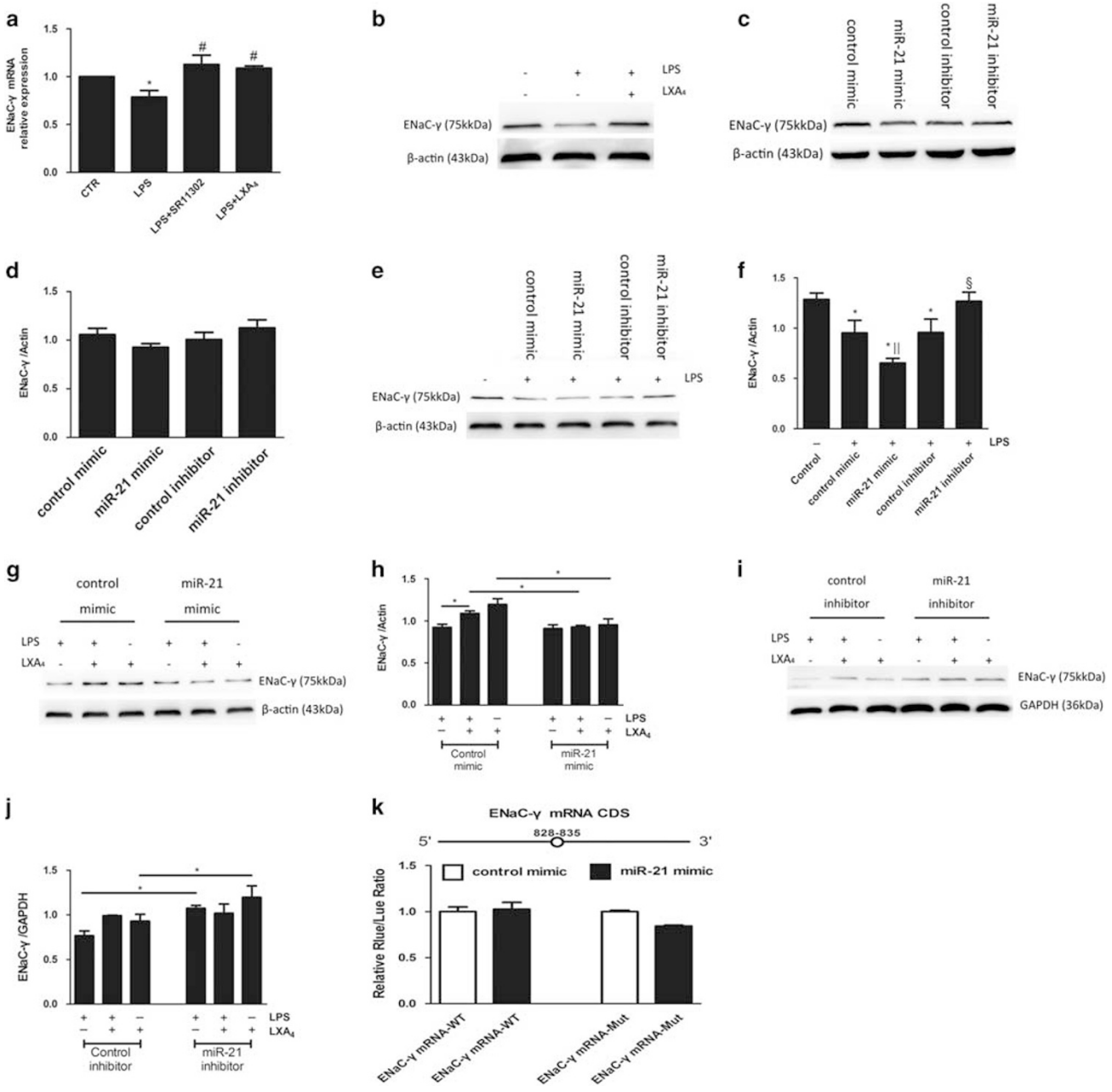
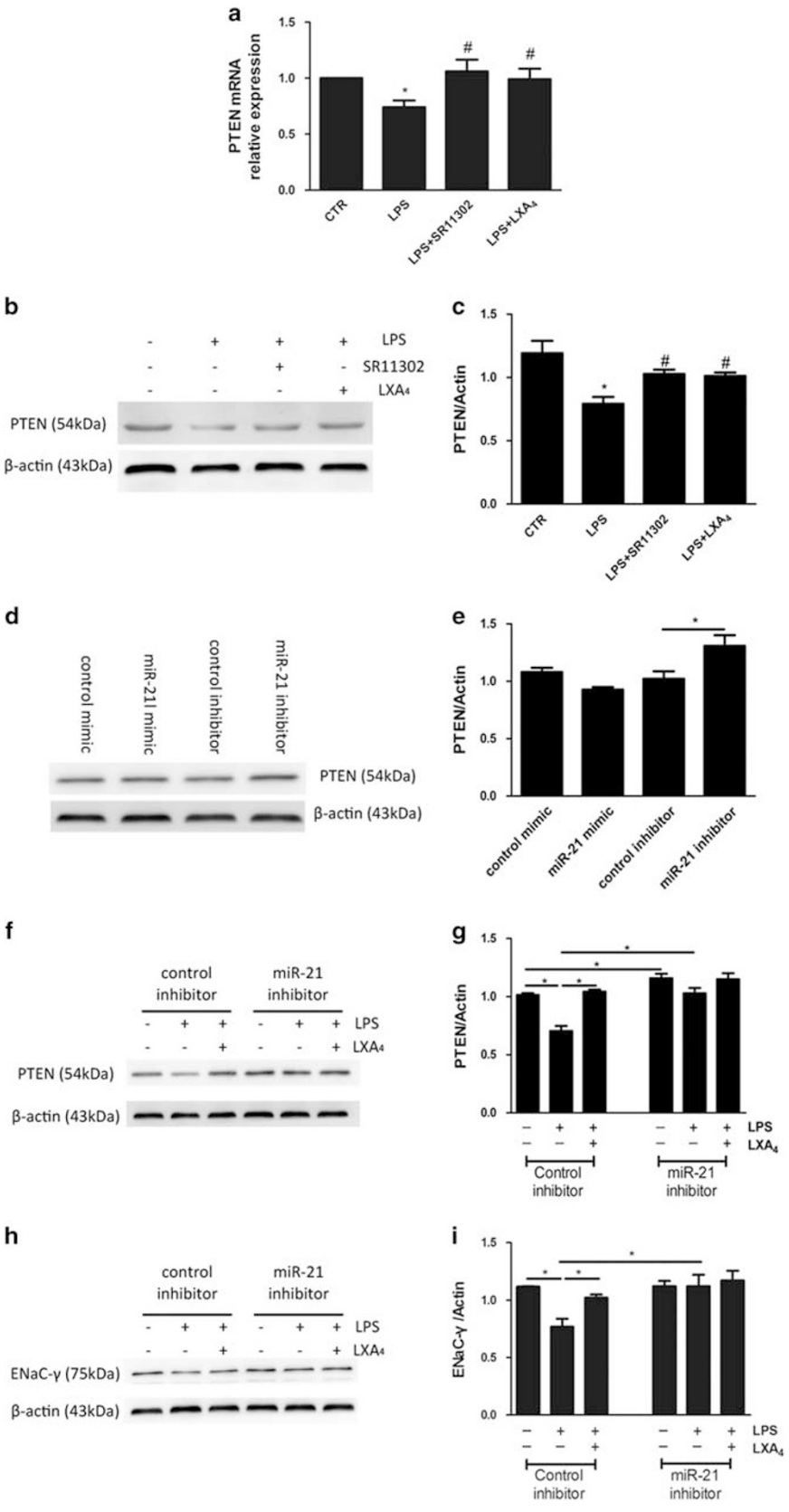


Figure 4 LXA$_4$ attenuates LPS-inhibited ENaC-γ protein expression through miR-21, but miR-21 does not target ENaC-γ mRNA. (**a**) SYBR Green real-time PCR and (**b**) western blot measurement of ENaC-γ mRNA and protein expression in A549 cells stimulated with LPS (1 μg/ml) and/or LXA$_4$ (100 nM) for 8 h ($n=4$). *$P<0.05$ *versus* control group, $^{\#}P<0.05$ *versus* LPS group. (**c** and **d**) Western blot and corresponding densitometry analysis of ENaC-γ protein expression in A549 cells transfected with control mimic, miR-21 mimic, control inhibitor, and miR-21 inhibitor ($n=3$). (**e** and **f**) Western blot and corresponding densitometry analysis of ENaC-γ protein expression in A549 cells transfected with control mimic, miR-21 mimic, control inhibitor, and miR-21 inhibitor with stimulation of LPS (1 μg/ml; 8 h) ($n=4$). *$P<0.05$ *versus* control group, $^{\|}P<0.05$ *versus* control mimic, $^{\S}P<0.05$ *versus* control inhibitor. (**g** and **h**) Western blot and corresponding densitometry analysis of ENaC-γ protein expression in A549 cells transfected with control mimic or miR-21 mimic and stimulated with LPS (1 μg/ml) and/or LXA$_4$ (100 nM) for 8 h ($n=4$). *$P<0.05$. (**i** and **j**) Western blot and corresponding densitometry analysis of ENaC-γ protein expression in A549 cells transfected with control inhibitor or miR-21 inhibitor and stimulated with LPS (1 μg/ml) and/or LXA$_4$ (100 nM) for 8 h ($n=3$). *$P<0.05$. (**k**) Luciferase/Renilla ratio results for 293T cells co-transfected with miR-21 or control miRNA mimic together with pmiR-RB-SCNN1G CDS (wild type or mutant) for 24 h. CDS, coding sequence; ENaC-γ, epithelial sodium channel gamma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide; LXA$_4$, lipoxin A$_4$.

and h). Conversely, ENaC-γ protein expression was induced upon miR-21 inhibitor transfection after LPS stimulation (Figure 4i and j). Together, these findings indicated that LXA$_4$ has protective effect on ENaC-γ protein expression through downregulation of miR-21 expression. Finally, luciferase reporter assays containing wild-type and mutant seed region


a
PTEN mRNA relative expression
1.5
1.0
0.5
0.0
CTR
LPS
LPS+SR11302
LPS+LXA4
b
LPS
SR11302
LXA4
PTEN (54kDa)
β-actin (43kDa)
c
PTEN/Actin
d
control mimic
miR-21l mimic
control inhibitor
miR-21 inhibitor
PTEN (54kDa)
β-actin (43kDa)
e
PTEN/Actin
control mimic
miR-21 mimic
control inhibitor
miR-21 inhibitor
f
control inhibitor
miR-21 inhibitor
LPS
LXA4
PTEN (54kDa)
β-actin (43kDa)
g
PTEN/Actin
Control inhibitor
miR-21 inhibitor
h
ENaC-γ (75kDa)
β-actin (43kDa)
i
ENaC-γ /Actin

miR-21-binding site of the ENaC-γ mRNA CDS confirmed that there was no interaction between miR-21 mimic and this site (Figure 4k).

LXA_4 Attenuates LPS Deduced ENaC-γ Expression via miR-21/PTEN Pathway

Because of previous evidence that PTEN is downregulated in the LPS-induced rat ALI model,[44] and that miR-21 is a direct target of PTEN,[45] we investigated PTEN expression after an LPS challenge *in vitro*. A549 cells stimulated with LPS revealed significant downregulation of PTEN mRNA and protein. Conversely, LXA_4 alleviated this effect, whereas SR11302 showed an analogous result (Figure 5a–c). Moreover, transfection with an miR-21 inhibitor significantly increased PTEN protein expression (Figure 5d and e). Furthermore, in A549 cells transfected with miR-21 inhibitor, miR-21 silencing prevented LPS-mediated depression of PTEN and ENaC-γ protein expression (Figure 5f–i). Together, these results indicated that miR-21/PTEN was the key signaling pathway underling the LXA_4 attenuated LPS-inhibited ENaC-γ protein expression.

LPS Decreased ENaC-γ Protein Expression in a miR-21/PTEN AKT- and ERK-Dependent Manner

AKT and ERK are regulated by the miR-21/PTEN pathway.[46] Therefore, we examined whether these signaling pathways were involved in the regulation of ENaC-γ protein expression after LPS treatment. First, we examined the phosphorylation of AKT and ERK in LPS-stimulated A549 cells. We observed significant phosphorylation of AKT and ERK with 30 min LPS stimulation in A549 cells (Figure 6a–d). Pretreatment of A549 cells with AP-1inhibitor (SR11302), AKT inhibitor (LY294002), or ERK inhibitor (U0126) abrogated LPS-decreased ENaC-γ protein expression (Figure 6e and f).

LXA_4 Augments ENaC-γ Protein Expression through Attenuation of LPS-Induced Phosphorylation of AKT

The addition of SR11302, LY294002, and LXA_4 abolished LPS-induced upregulation of phosphorylation of AKT (Figure 6a and b). However, only U0126 reversed LPS-induced upregulation of phosphorylation of ERK (Figure 6c and d). After pretreatment of LXA_4, ENaC-γ protein expression was significantly elevated compared with LPS group (Figure 6e and f). Our data indicate that LXA_4 attenuated LPS-inhibited ENaC-γ protein expression in part through miR-21/PTEN/AKT pathway.

DISCUSSION

In the present study, we have identified a unique role for LXA_4 on miR-21 expression in LPS-induced inflammatory lung injury. Our data show that miR-21 was upregulated by LPS challenge and downregulated with LXA4 administration *in vivo* and *in vitro*. Furthermore, serum miR-21 concentrations were elevated in ARDS patients. MiR-21 upregulated phosphorylation of AKT via inhibition of PTEN and therefore reduced the expression of ENaC-γ. However, LXA_4 reversed LPS-inhibited ENaC-γ expression through inhibition of AP-1 and activation of PTEN, indicating that LXA_4 activated ENaC-γ via the miR-21/PTEN/AKT signaling pathway (summarized in Figure 7).

We previously reported the protective effects and anti-edema properties of LXA_4 in oleic acid (OA) and LPS-induced rat ALI model.[10,20,21] The mechanisms associated with LXA_4 in the resolution of AFC are of broad interest. In this study, we examined miRNA expression profiling after treatment with LXA_4 in LPS-induced inflammatory lung injury. Our results confirm that LPS induced sustained expression of miR-21 *in vivo* and *in vitro*. However, LXA_4 inhibited miR-21 expression. Moschos *et al*,[29] using a mouse aerosolized LPS model, reported that miR-21 was upregulated and showed time-dependent increases, consistent with our *in vivo* result. Lee *et al* reported that miR-21 was also upregulated throughout the 24 h following OA challenge, with a time-course analysis of miRNA expression in rat OA-induced ALI.[47] These *in vitro* and *in vivo* results suggest the importance of miR-21 in ALI. Coincidently, we also found an increase in miR-21 serum concentrations in ARDS patients. Therefore, miR-21 may be a novel biomarker for prediction of the severity and outcome of ARDS.

Pri-miR-21 was also elevated by LPS stimulation and was attenuated by LXA_4 treatment, which implies a transcriptional regulatory mechanism. AP-1, whose binding site is located in miR-21 promoter region, has proven to be one of the conserved enhancer elements of miR-21.[41] Previous studies demonstrated that LXA_4 and aspirin-triggered LXA_4 reduced AP-1 activity in the presence of LPS.[48] Here we used the AP-1 inhibitor SR11302 and abolished LPS-induced miR-21 expression in a dose- and time-dependent manner. Moreover, LXA_4 mimicked the effect of SR11302, through inhibition of

Figure 5 miR-21/PTEN pathway and the LPS and LXA_4 regulation of ENaC-γ. (**a**) SYBR Green real-time PCR and (**b**) western blot measurement of PTEN mRNA and protein expression in A549 cells incubated with LPS (1 μg/ml; 8 h) after pre-treated with SR11302 (10 nM; AP-1 inhibitor) or LXA_4 (100 nM) for 30 min ($n=4$). (**c**) Densitometry analysis of PTEN protein expression in **b** ($n=3$). $^{*}P<0.05$ *versus* control group, $^{\#}P<0.05$ *versus* LPS group. (**d** and **e**) Western blot and corresponding densitometry analysis of PTEN protein expression in A549 cells transfected with control mimic, miR-21 mimic, control inhibitor, and miR-21 inhibitor ($n=3$). Western blot and corresponding densitometry analysis of PTEN (**f** and **g**) and ENaC-γ (**h** and **i**) protein expression in A549 cells transfected with control inhibitor or miR-21 inhibitor and stimulated with LPS (1 μg/ml) and/or LXA_4 (100 nM) for 8 h ($n=3$). $^{*}P<0.05$. ENaC-γ, epithelial sodium channel gamma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide; LXA_4, lipoxin A_4; PTEN, phosphatase and tensin homolog.

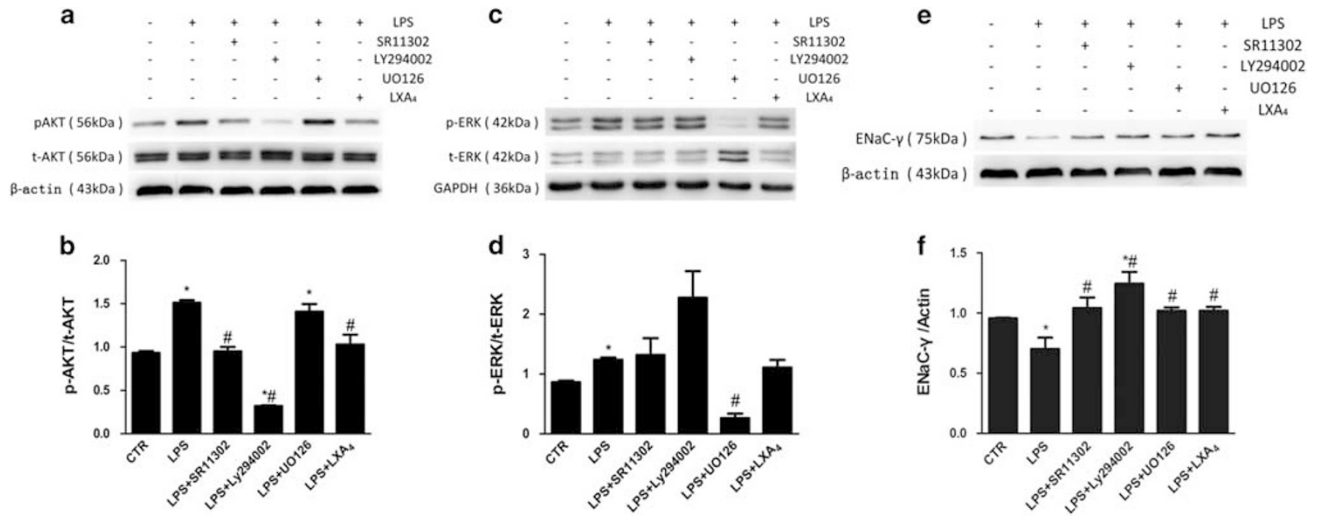


Figure 6 LXA_4 augmented LPS-reduced ENaC-γ protein expression via miR-21/PTEN/AKT pathway. Western blot and corresponding densitometry analysis of p-AKT (**a** and **b**), p-ERK (**c** and **d**), and ENaC-γ (**e** and **f**) protein expression in A549 cells stimulated with LPS (1 μg/ml; 12 h) after pre-incubated with SR11302 (10 nM; AP-1 inhibitor), LY294002 (10 μM, PI3K/Akt inhibitor), U0126 (20 μM, ERK inhibitor), or LXA_4 (100 nM) for 30 min ($n=3$). $^*P<0.05$ *versus* control group, $^{\#}P<0.05$ *versus* LPS group. ENaC-γ, epithelial sodium channel gamma; LPS, lipopolysaccharide; LXA_4, lipoxin A_4; PTEN, phosphatase and tensin homolog.

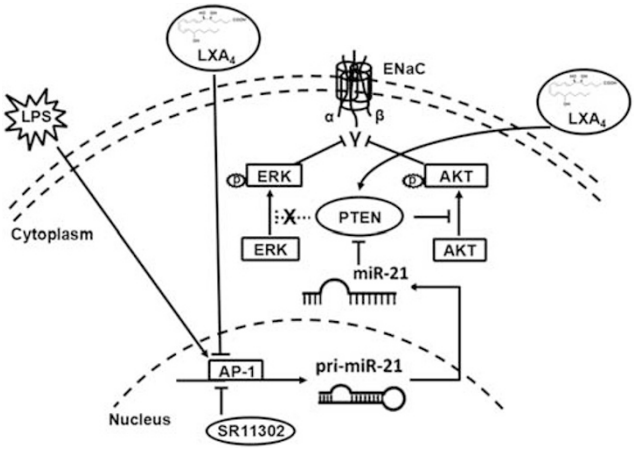


Figure 7 Schematic representation of miR-21/PTEN/AKT pathway in LXA_4 regulation of LPS-inhibited ENaC-γ protein expression. Through inhibition of PTEN, miR-21 upregulated phosphorylation of AKT activation therefore reduced the expression of ENaC-γ. However, via inhibiting AP-1 and activating PTEN, LXA_4 reversed LPS-inhibited ENaC-γ expression, unveiling a novel therapeutic entry point wherein LXA_4 activated ENaC-γ through downregulation of miR-21 expression. ENaC-γ, epithelial sodium channel gamma; LPS, lipopolysaccharide; LXA_4, lipoxin A_4; PTEN, phosphatase and tensin homolog.

the LPS-mediated AP-1 pro-inflammatory signal, which as a consequence may lead to depression of miR-21.

ENaC-γ is a regulatory subunit of ENaC promotes the resolution of AFC.[9] Our results on miRNA expression patterns and *in silico* predictions led us to propose that miR-21 mediated the effects of LXA_4 in promoting ENaC-γ expression in inflammatory lung injury responses. This hypothesis was supported by our findings transfecting the miR-21 mimic and inhibitor into A549 cells. Overexpression of miR-21 abolished the protective effects of LXA_4 in LPS-reduced ENaC-γ expression; however, miR-21 inhibitor mimicked the effects of LXA_4. Our data clearly demonstrate that miR-21 had no effect on ENaC-γ expression in physiological conditions. However, transfection with miR-21 mimic or inhibitor exacerbated LPS-inhibited ENaC-γ expression. Vaporidi *et al*[49] demonstrated that ectopic expression of miR-21 reduced lung compliance and increased alveolar-arterial oxygen differences and protein levels in bronchoalveolar lavage in a ventilator-induced mouse ALI model.[49] Here, we observed that treatment with the miR-21 mimic abolished the protective effect of LXA_4 on LPS-reduced ENaC-γ expression. In addition, both SR11302 and LXA_4 reversed LPS-decreased ENaC-γ protein expression. Our data demonstrate that downregulation of miR-21 is a necessary step in LXA_4 diminishing LPS-reduced ENaC-γ expression.

Luciferase reporter assays demonstrated that miR-21 did not target ENaC-γ mRNA directly. However, we observed the depression of PTEN expression under LPS stimulation, and prevented by LXA_4 and SR11302 pretreatment. Consistent with previous studies that upregulated miR-21 depressed PTEN mRNA and protein expression in ventilator-induced and OA-stimulated rat ALI model.[47,49] In addition, PTEN contains miR-21-binging site within 3′ UTR.[45] Hence, we provide evidence linking LPS-mediated upregulation of miR-21 with downregulation of PTEN mRNA and protein in alveolar epithelia. Furthermore, transfection of an miR-21 inhibitor increased PTEN protein expression. Consequently, loss of miR-21, LPS-inhibited PTEN, and ENaC-γ were attenuated, demonstrating the importance of this miR-21/PTEN pathway in the mechanism of LPS action on PTEN and ENaC-γ expression. This observation unveils a novel pathway

wherein miR-21/PTEN is responsible for LXA_4 in augmenting ENaC-γ protein expression in LPS-stimulated inflammatory lung injury.

Both AKT and ERK are downstream regulators of the miR-21/PTEN pathway.[46] It is also known that ENaC activity is regulated by a complex network of signaling pathways in addition to the AKT and ERK pathways.[50] We previously demonstrated that the AKT pathway mediated the LPS-induced decrease of cystic fibrosis transmembrane conductance regulator protein expression in primary ATII cells.[20] LPS resulted in a rapid phosphorylation of AKT and ERK, which peaked at 30 min.[20] In the current study, the addition of LY294002 abrogated the LPS-induced phosphorylation of AKT, whereas pretreatment of A549 cells with SR11302 and LXA_4 decreased phosphorylation of AKT to normal levels 30 min after LPS stimulation. Interestingly, pretreatment with SR11302 and LXA_4 failed to depress LPS-induced phosphorylation of ERK. It has been reported that aspirin-triggered LXA_4 inhibited myeloperoxidase suppression of neutrophil apoptosis via downregulation of AKT and ERK.[51] In contrast, Prieto *et al.* found that pretreatment with LXA_4 promoted a rapid activation of ERK after staurosporine challenge in RAW 264.7 cells.[52] These differences were probably a consequence of signaling convergence and cross-talk between the AKT and ERK cascades.

Although multiple studies exist regarding the regulation of ion channels by miRNAs,[30–32] our results demonstrate that miR-21/PTEN/AKT pathway involves LXA_4 and LPS regulation of ENaC-γ protein expression. However, there are three aspects that need to be further addressed. First, to verify the relationship between miR-21 and ARDS there needs to recruit more patients. Second, ENaC-γ activity and trafficking such as ion and fluid transport need to be evaluated.[53] Finally, the mechanism we demonstrated based on our *in vitro* study; it would be of interest to further substantiate our findings that miR-21/PTEN/AKT pathway contributes to the lung anti-edema effects of LXA_4 *in vivo*.

In this study, we have shown that downregulation of miR-21 controlled ENaC-γ expression via the PTEN/AKT signaling pathway after LXA_4 treatment in LPS-dependent inflammatory lung injury. However, miR-21 may also affect other signaling pathways in lung injury. MiR-21 has been shown to affect ERK–MAP kinase signaling in cardiac fibroblasts, which in turn positively regulates cardiac fibroblast survival, leading to fibrosis, hypertrophy, and cardiac dysfunction.[54] A recent study demonstrated that miR-21 was activated and involved in the pathophysiologic processes of ischemia/reperfusion-induced acute kidney injury. Therefore, miR-21 can serve as potential targets for modulation by specific miR-21 antagomiRs[27] to achieve protective effects.[55] Furthermore, miR-21 has been demonstrated as a multi-faceted miRNA[42] and deregulated in almost all types of cancers, and therefore was classified as an oncomiR,[56] especially in cardiovascular and pulmonary diseases. Hence, pharmacological downregulation of miR-21, with miR-21 antagomiR or agents like LXA_4, may be a new therapies in the future.

In conclusion, our study demonstrates that LXA_4 alleviated LPS-inhibited ENaC-γ protein expression via miR-21/PTEN/AKT signaling pathway. Thus, treatment with LXA_4 for downregulation of miR-21 could represent a novel target for the treatment of the critically ill patients with inflammatory lung injury. Our findings reveal a new mechanism of LXA_4 in reversing LPS-reduced ENaC-γ protein expression and LXA_4 may provide a new therapy for the resolution of inflammatory lung injury.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

ACKNOWLEDGMENTS

We thank Hongxia Mei and Qingquang Lian for their support and assistances. This work was sponsored by the grants from the National Natural Science Foundation of China (no. 30772088, no. 30930089, no. 81270132, no. 81070061), and by the Natural Science Foundation of Zhejiang Province (no. LY14H150007, no. LY14H150011).

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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