Biochemistry and Biophysics Reports 7 (2016) 367–373

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Repeated lipopolysaccharide exposure causes corticosteroid insensitive airway inflammation via activation of phosphoinositide-3-kinase δ pathway

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ARTICLE INFO

Article history:
Received 24 April 2016
Received in revised form
18 July 2016
Accepted 19 July 2016
Available online 28 July 2016

Keywords:
Airway inflammation
Corticosteroid-resistance
COPD
HDAC2
PI3K Nrf2

ABSTRACT

Corticosteroid resistance is one of major barriers to effective management of chronic inflammatory respiratory diseases, such as chronic obstructive pulmonary disease (COPD) and severe asthma. These patients often experience exacerbations with viral and/or bacterial infection, which may cause continuous corticosteroid insensitive inflammation. In this study, we observed that repeated exposure of lipopolysaccharide (LPS) intranasally attenuated the anti-inflammatory effects of the corticosteroid fluticasone propionate (FP) on neutrophils and CXCL1 levels in bronchoalveolar lavage (BAL) fluid in an *in vivo* murine model. Histone deacetylase-2 (HDAC2) and NF-E2 related factor 2 (Nrf2) levels in lungs after LPS administration for 3 consecutive days were significantly decreased to $38.9 \pm 6.3\%$ (mean ± SEM) and $77.5 \pm 2.7\%$ of the levels seen after only one day of LPS exposure, respectively. In addition, 3 days LPS exposure resulted in an increase of Akt phosphorylation, indicating activation of the phosphoinositide-3-kinase (PI3K) pathway by 4-fold in lungs compared with 1 day of exposure. Furthermore, combination treatment with theophylline and FP significantly decreased the neutrophil accumulation and CXCL1 concentrations in BAL fluid from $22.5 \pm 1.8 \times 10^4$ cells/mL and 214.6 ± 20.6 pg/mL to $7.9 \pm 0.5 \times 10^4$ cells/mL and 61.9 ± 13.3 pg/mL, respectively. Combination treatment with IC87114, a selective PI3Kδ inhibitor, and FP also significantly decreased neutrophils and CXCL1 levels from $16.8 \pm 0.7 \times 10^4$ cells/mL and 182.4 ± 4.6 pg/mL to $5.9 \pm 0.3 \times 10^4$ cells/mL and 71.4 ± 2.7 pg/mL, respectively. Taken together, repeated exposure of LPS causes corticosteroid-insensitive airway inflammation *in vivo*, and the corticosteroid-resistance induced by LPS is at least partly mediated through the activation of PI3Kδ, resulting in decreased levels of HDAC2 and Nrf2. These findings provide a potentially new therapeutic approach to COPD and severe asthma.

1. Introduction

Reduced responsiveness to the anti-inflammatory effects of corticosteroids is one of the important clinical problems in chronic respiratory inflammatory diseases, including COPD and severe asthma [1–4]. Corticosteroids are one of the most effective therapeutic drugs for asthma. However in COPD and some patients with asthma, corticosteroids fail to suppress chronic inflammation, even though some reports suggested that high doses of systemic corticosteroids treatment could attenuate chronic inflammation in COPD patients [1,4–6].

Corticosteroids suppress inflammatory gene expression by recruiting HDAC2 to the activated inflammatory transcriptional complex, resulting in the deacetylation of histones and thus inflammatory gene suppression [7]. In *in vivo* and *in vitro* models, oxidative stress activates phosphoinositide-3-kinase-δ (PI3Kδ) and reduces HDAC2 deacetylase activity [8,9]. In addition, cigarette smoke extracts or oxidative stress induces HDAC2 phosphorylation, ubiquitination, and nitration, resulting in HDAC2 degradation [10–12]. The decreased activity and expression of HDAC2 induces glucocorticoid receptor (GR) acetylation, resulting in loss of anti-inflammatory effects of corticosteroids and also a reduction in anti-oxidative defenses due to a reduction in the key transcriptional regulator of anti-oxidant genes, NF-E2 related factor 2 (Nrf2) [7,13–15].

COPD patients are prone to exacerbations, as a result of upper respiratory tract virus infections such as rhinovirus, and/or

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http://dx.doi.org/10.1016/j.bbrep.2016.07.020

bacterial infections, such as *Streptococcus pneumoniae* and *Haemophilus influenzae* [16–20]. In addition, lungs of COPD patients are also chronically colonized by bacteria such as *H. influenzae*, resulting in chronic exposure to endotoxins [21–24]. Therefore, continuous inflammatory stimuli induced by viruses and/or bacteria contribute to the pathogenesis of COPD. In fact, we previously reported that repeated stimulation by poly(I:C), an RNA virus infection mimic, attenuated anti-inflammatory effects of corticosteroids [25]. However, there are few studies investigating the effects of repeated bacterial stimuli on corticosteroid sensitivity in airways.

We hypothesized that repeated stimulation with LPS causes corticosteroid-resistant inflammation in lungs. We examined the impact of repeated intranasal LPS exposure on 3 consecutive days on the anti-inflammatory effects of the corticosteroids fluticasone propionate (FP) in mice *in vivo*. We also investigated the role of PI3K signaling in chronic LPS exposure.

2. Materials and methods

2.1. Experimental animals

All animal studies were approved by the guidelines of the Nihon University Animal Care and Use Committee. Specific pathogen-free male A/J mice aged 5 weeks were obtained from Sankyo Labo Service Co Inc. (Tokyo, Japan). Mice were housed without environmental enrichment in a climate-controlled room on a 12 h light-dark cycle (24 ± 1 °C and 55 ± 5% humidity). Mice consumed water and standard chow *ad libitum*.

2.2. Drug and chemicals

Fluticasone propionate (FP), lipopolysaccharide from *Escherichia coli* (LPS), and propidium iodide were purchased from Sigma-Aldrich (St. Louis, MO, USA). IC87114, an inhibitor of PI3Kδ, was purchased from Chemdea (Ridgewood, NJ, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-neutrophils (7/4) antibody was purchased from Acris Antibodies GmbH (Herford, Germany). Anti-Nrf2 (ab31163) and anti-HDAC2 (ab12169) antibodies were purchased from Abcam plc (Cambridge, UK). Anti-Akt antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-phospho-Akt (Ser473), clone SK703, was purchased from Merck Millipore Co. (Darmstadt, Germany). Anti-β-actin (AC-15) antibody was purchased from Sigma-Aldrich Co, LLC (St. Louis, MO, USA). HRP-conjugated anti-rabbit IgG and anti-mouse IgG and ECL Plus were purchased from GE Healthcare UK Ltd. (Buckingham, UK).

2.3. Drug treatment and LPS instillation

Mice were anesthetized with 2% isoflurane, then, LPS (0.1 mg/mL, 40 μL/animal, twice daily) was administrated intranasally (i.n.). The i.n. applications of LPS were repeated on the second and third days. Mice were sacrificed 24 h after the i.n. LPS administration on day 3 and the lungs were isolated and subjected to further experiments. The 35 μL/animal of saline, FP (0.05 mg/mL in saline), theophylline (2.0 mg/mL in saline), IC87114 (4.0 mg/mL in saline) were administered i.n. 2 h before each LPS administration. In a pilot study, we confirmed that 4.0 mg/mL of IC87114 almost completely inhibited PI3Kδ activity in lung of mouse (data not shown).

2.4. Bronchial alveolar lavage (BAL) fluid

At designated time points, BAL fluid were collected after anesthetizing the mice, and then, placing a cannula into the trachea through which total 100 mL/kg was flushed three times. The BAL fluid cells were collected by centrifugation (500 × *g*, 4 °C, 10 min) and hemolysis of contaminating erythrocytes induced by re-suspending in 0.2% NaCl. After isotonization, by addition of the same volume of 1.6% NaCl, the total number of BAL cells was counted and aliquoted for flow cytometry analysis.

2.5. Flow cytometry analysis

A BAL cell suspension was incubated with FITC-conjugated anti-neutrophil antibody (clone 7/4, 2 μg/mL) and propidium iodide (2 μg/mL). The cells were subsequently analyzed using a flow cytometer (ALTRA II; Beckman Coulter, Tokyo, Japan) [25].

2.6. Immunoblotting and Enzyme-Linked Immunosorbent Assay (ELISA)

For the detection of HDAC2, Nrf2, and β-actin, lung tissue was homogenized in a lysis buffer containing 10 mM of Tris-HCl, 150 mM of NaCl, 0.5% of sodium deoxycholate, 1 mM of DTT, 0.5% of Nonidet P-40, and a tablet of protease inhibitor cocktail (cOmplete Mini®, Roche Diagnostic GmbH, Mannheim, Germany), adjusted to pH7.4. For the detection of Akt/p-Akt, lung tissue was homogenized in a lysis buffer containing 50 mM of Tris-HCl, 150 mM of NaCl, 1% of Nonidet P-40, 0.25% of sodium deoxycholate, 1 mM of PMSF, a tablet of cOmplete Mini, and Pierce™ Phosphatase Inhibitor Mini Tablets® (Thermo Fisher Scientific, Rockford, IL, USA), adjusted to pH7.4. The lysate containing 40 μg of protein was separated by electrophoresis on 10% sodium dodecyl sulfate (SDS) polyacrylamide gel, and then, transferred to nitrocellulose membrane. The membrane was blocked with blocking buffer (5% skim milk TBS/T (20 mM) for the detection of HDAC2, Nrf2, and β-actin, or 1% BSA in TBS/T (20 mM) for the detection of p-Akt/Akt) for 1hr at room temperature and incubated with primary antibody (1:1000) in blocking buffer overnight at 4 °C. The membrane was washed with TBS/T and incubated with secondary antibody for 2 h. After three additional washes, the immunoreactive bands were visualized by ECL Plus on X-ray film.

The CXCL1 concentrations in BAL fluid supernatant were analyzed by using Quantikine mouse CXCL1/KC enzyme-linked immunosorbent assay kit (R&D systems Inc., Minneapolis, MN, USA).

2.7. Statistical analysis

Results are expressed as means ± SEM. Multiple comparisons were performed by analysis of variance followed by the Dunnett multiple comparison test performed using the PRISM 6 software program (GraphPad Software Inc., CA, USA). The comparison between two groups was performed by unpaired *t*-test with Welch correction or Mann-Whitney test. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Repeated dosing with i.n. LPS caused corticosteroid-insensitive lung inflammation

To determine whether repeated i.n. administration of LPS induces airway inflammation and reduces the anti-inflammatory effects of FP, we examined the effects of FP in mice after intranasal instillation of LPS on days 0, 1 and 2. Twenty four hours after the last administration of LPS, the numbers of neutrophils in BAL fluid were analyzed by flow cytometry. After i.n. LPS administration, there was marked neutrophilia in BAL fluid collected 24 h after 1, 2 or 3 consecutive days of challenge, and the number of

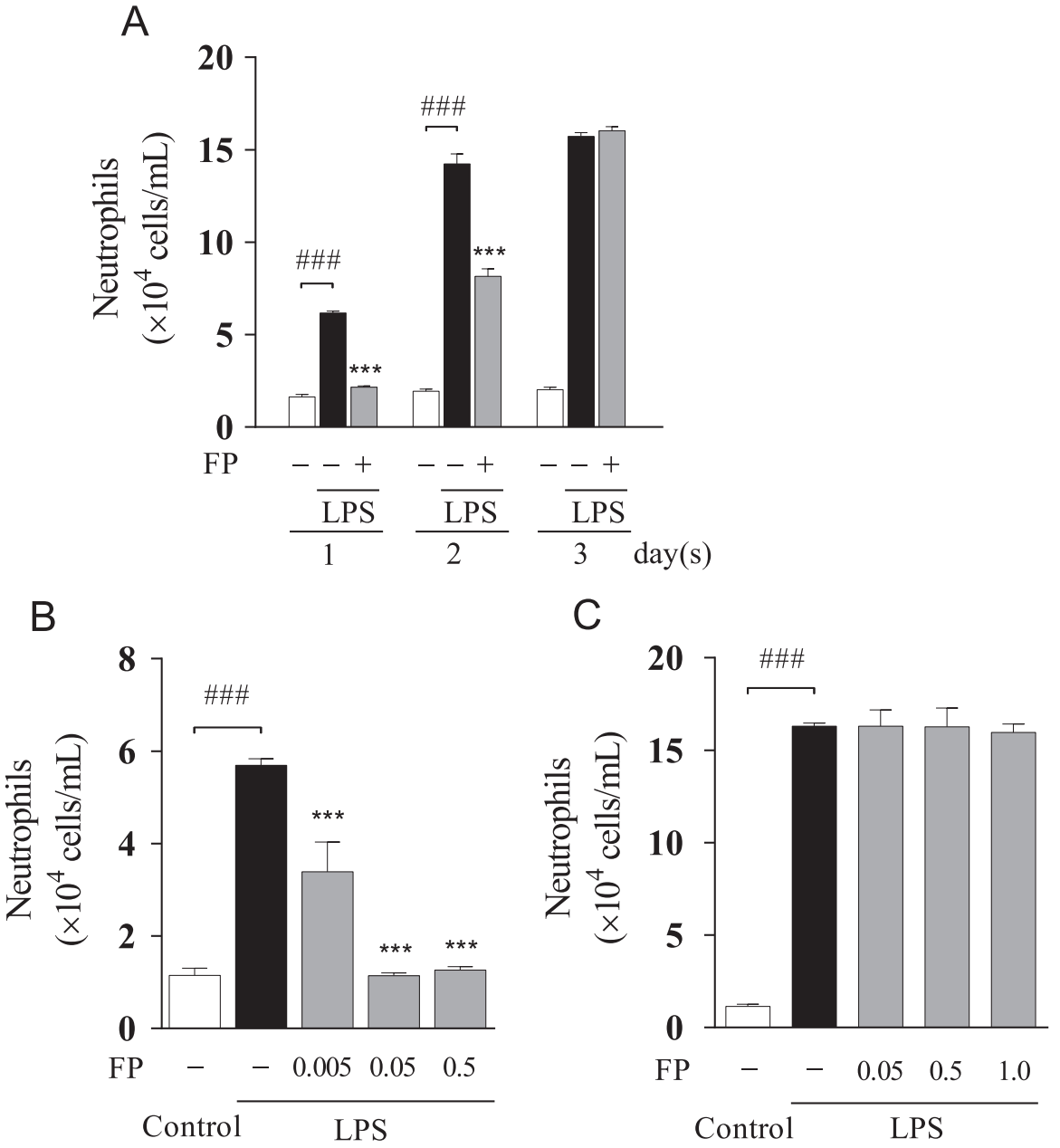

Fig. 1. Repeated exposure of LPS caused corticosteroid-insensitive airway neutrophilia in BAL fluid. Mice were intranasally administered with LPS and FP. LPS and 0.05 mg/mL of FP were administered for 1–3 days (A). LPS and indicated dose of FP were treated for 1 (B) or 3 days (C), respectively. Twenty four hours after the last exposure of LPS, mice were sacrificed. The neutrophil numbers in BAL fluid were analyzed by flow cytometry. Results represent mean ± SEM (n=3, $^{###}P < 0.001$, compared with control and vehicle group, $^{***}P < 0.001$, compared with vehicle group and dosing groups).

neutrophils in LPS administration for 3 days induced a 2.6-fold increase compared with LPS administration for 1 day. In mice with exposure of LPS for one day(twice daily), i.n. treatment with 0.05 mg/mL of FP completely inhibited an increase in neutrophils in BAL fluid, but FP did not show any beneficial effects on the level of neutrophils in mice which received i.n. LPS in 3 consecutive days (Fig. 1A). Further studies with FP (0.05, 0.5, and 1 mg/mL, i.n.) confirmed that the neutrophilia was not affected even by the highest dose of FP, although FP significantly reduced the neutrophilia after the LPS administration for 1 day, in a dose-dependent manner (Fig. 1B and C).

CXCL1 concentrations increased significantly in BAL fluid from mice which received LPS for 1, 2, and 3 days compared with control, and LPS exposure on 3 consecutive days induced 1.7-fold increase compared with exposure of LPS for 1 day. Although treatment with 0.05 mg/mL of FP markedly suppressed the increase of CXCL1 at 24 h after LPS exposure for 1 or 2 days (Fig. 2A) in the model of 3 consecutive days LPS exposure, FP (0.05, 0.5, and 1 mg/mL) had no significant effect on CXCL1 levels (Fig. 2B).

3.2. Repeated LPS induced phosphorylation of Akt, and decreased HDAC2 and Nrf2 expression

We examined the effects of i.n. LPS exposure on HDAC2 and Nrf2 protein levels by western blotting. In our analysis, the molecular weight of HDAC2, Nrf2, Akt/p-Akt and β-actin in lung homogenates matched with predicted band size of 55, 68, 60, and 42 kDa, respectively (Fig. 3A). When mouse lung was collected at 24 h after LPS exposure for 1 day, the level of HDAC2 was not altered, but the expression of Nrf2 was significantly increased. In contrast, after LPS exposure on 3 consecutive days the expression

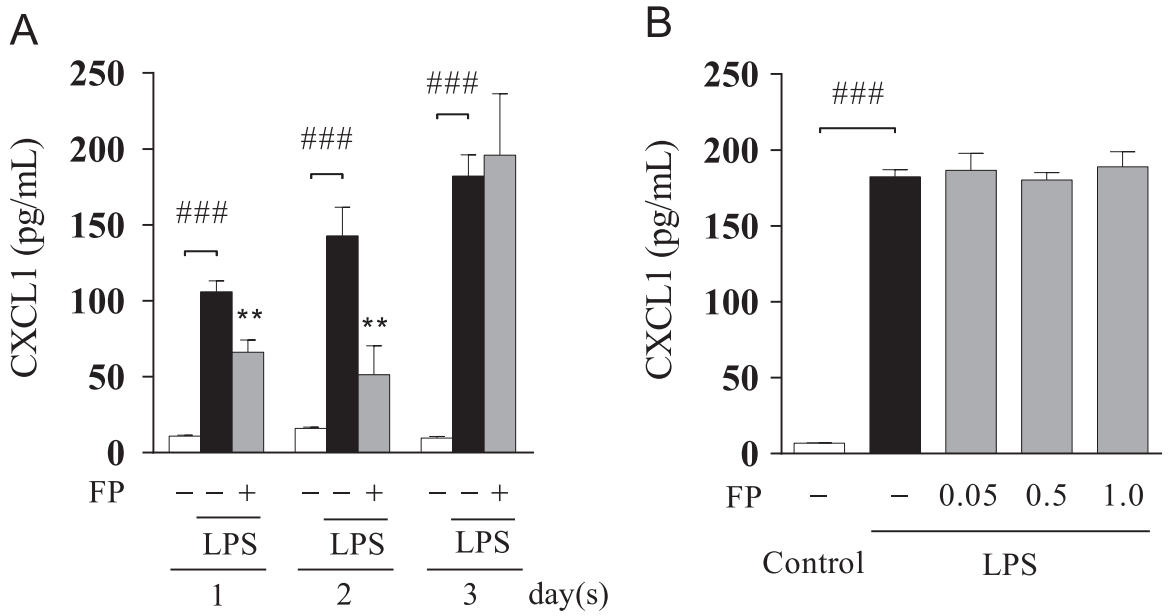

Fig. 2. Repeated exposure of LPS reduced the effect of FP on chemokine expression in BAL fluid. Mice were intranasally administered with LPS and 0.05 mg/kg of FP for 1–3 days (A). Mice were intranasally treated with LPS and indicated dose of FP for 3 days (B), respectively. Twenty four hours after the last exposure of LPS, mice were sacrificed. The CXCL1 levels in BAL fluid were measured by ELISA. Results represent mean ± SEM (n=3, $^{###}P<0.001$, compared with control and vehicle group, $^{**}P<0.01$, compared with vehicle group and dosing groups).

of HDAC2 and Nrf2 were decreased by 27.9 ± 7.5% and 68.4 ± 3.8% respectively, when compared with non-treatment controls. After 3 days exposure to LPS, HDAC2 and Nrf2 expression were decreased by 38.9 ± 6.3% and 77.5 ± 2.7% respectively, when compared with LPS exposure for 1 d (Fig. 3A and B).

We also examined the effect of LPS exposure on Akt phosphorylation at Ser-473 as a marker of the activation of PI3K signaling pathway. In mouse lung collected at 24 h after 1, 2 or 3 consecutive days exposure of LPS, there was an increase in p-Akt/Akt ratios by 2.5-, 7.6- and 10.3-fold compared with that in

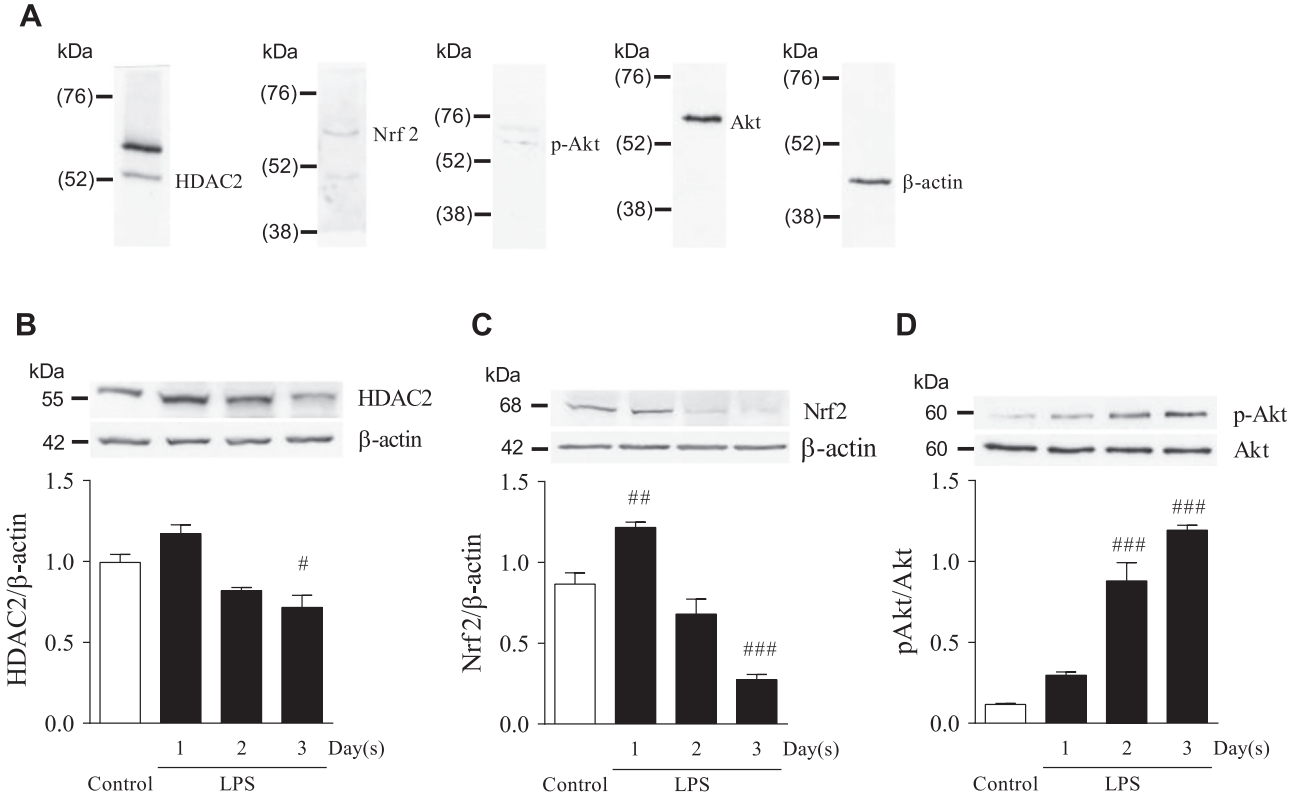

Fig. 3. LPS exposure for 3 days reduced HDAC2, Nrf2 levels, and increased Akt phosphorylation. Mice were administered with LPS for 1–3 days, and one day after the last administration, sacrificed. Lung homogenate was separated on SDS-PAGE, and the HDAC2, Nrf2, Akt, p-Akt, and β-actin protein levels were determined by immunoblotting. HDAC2, Nrf2, Akt/p-Akt and β-actin were preliminary detected with predicted band size (A). Upper panel shows a typical immunoblot of HDAC2 (B), Nrf2 (C), Akt, p-Akt (D) and β-actin in the lung homogenates. Lower panel shows the ratios of HDAC2/β-actin (B), Nrf2/β-actin (C) or p-Akt/Akt (D), calculated by measuring band density. Results represent mean ± SEM (n=3, $^{#}P<0.05$, $^{##}P<0.01$, $^{###}P<0.001$, compared with control and dosing groups).

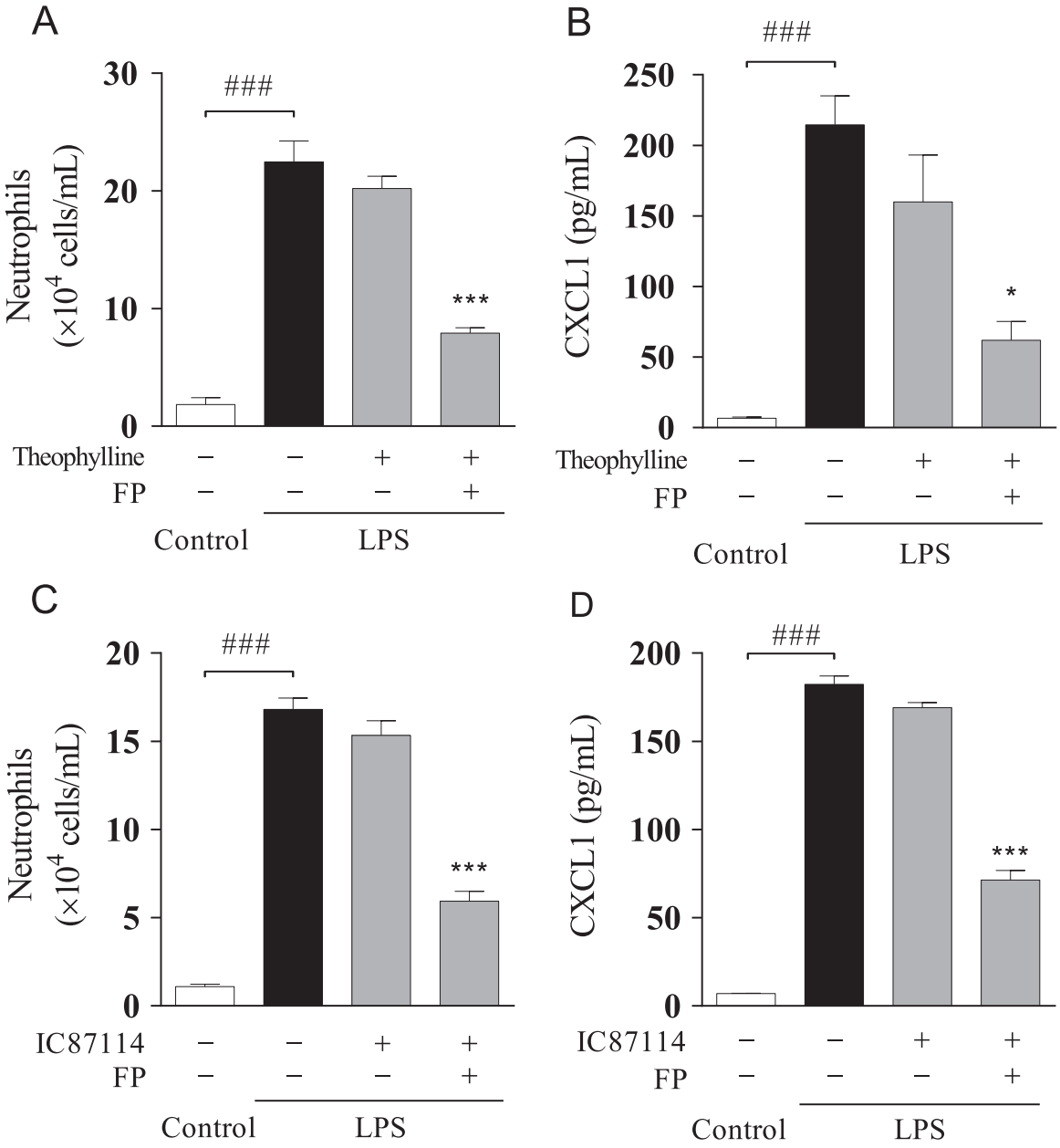

Fig. 4. PI3K inhibition restored the corticosteroid responsiveness in mice exposed with LPS. Mice were administered with LPS, and theophylline (A, B) or IC87114 (C, D) for 3 days, respectively. Twenty four hours after the last exposure of LPS, mice were sacrificed. The neutrophil numbers in BAL fluid were analyzed by flow cytometry (A, C). The CXCL1 levels in BAL fluid were measured by ELISA (B, D). Results represent mean ± SEM (n=3, $^{###}P<0.001$, compared with control and vehicle group, $^{*}P<0.05$, $^{***}P<0.001$, compared with vehicle group and dosing groups).

control, respectively (Fig. 3D). LPS had no effect on basal Akt levels, suggesting the increase in p-Akt/Akt ratio is due to an increase of phosphorylation levels.

3.3. *Effect of PI3Kδ inhibitor on LPS-induced corticosteroid-insensitive airway inflammation*

After an i.n. exposure of LPS on 3 consecutive days, theophylline (2.0 mg/mL, i.n.), an allosteric PI3Kδ inhibitor, or IC87114 (4.0 mg/mL, i.n.), a selective PI3Kδ specific small molecule inhibitor, were administered twice daily before daily LPS administration. Theophylline alone did not suppress the increase in neutrophils or CXCL1 levels in BAL fluid from mice with repeated LPS exposure for 3 days and FP alone did not have any effects, either, as previously presented (Fig. 4A and B). In contrast, a combination of theophylline and FP significantly suppressed LPS-induced airway neutrophilia in BAL fluid from $22.5 \pm 1.8 \times 10^4$ cells/mL to $7.9 \pm 0.5 \times 10^4$ cells/mL and also elevation of CXCL1 levels in BAL fluid from 214.6 ± 20.6 pg/mL to 61.9 ± 13.3 pg/mL. Combination treatment with IC87114 and FP also significantly suppressed LPS-induced accumulation of neutrophils from $16.8 \pm 0.7 \times 10^4$ cells/mL to $5.9 \pm 0.3 \times 10^4$ cells/mL and CXCL1 from 182.4 ± 4.6 pg/mL to 71.4 ± 2.7 pg/mL, although IC87114 alone did not affect neutrophils and CXCL1 (Fig. 4C and D). These data suggest that PI3Kδ activation contributes to the corticosteroid insensitivity in mice induced by 3 days exposure of LPS.

4. Discussion

Corticosteroid resistance is one of major barriers of effective management of chronic inflammatory respiratory diseases such as

COPD and severe asthma [1–4]. In addition, respiratory RNA viruses, such as human rhinovirus, respiratory syncytial virus, influenza virus, are common causes of COPD and asthma exacerbations [16–20]. We have previously reported that repeated doses of poly(I: C), which mimics RNA virus infection, attenuated the anti-inflammatory effect of corticosteroids in mice [25]. Bacterial infections, such as *Haemophilus influenzae* and *Streptococcus pneumoniae,* also cause exacerbations in patients with COPD. In the present study, we examined whether repeated stimulation with LPS induced corticosteroid resistance in airway inflammation in mice. The data clearly indicated that repeated exposure of LPS on 3 consecutive days induced corticosteroid insensitive neutrophil accumulation and CXCL1 induction in BAL fluid although airway inflammation after LPS exposure for 1 day remained corticosteroid sensitive (Figs. 1 and 2). Therefore, repeated inflammatory stimulation by LPS may result in the attenuation of anti-inflammatory effects of corticosteroids. Some investigators have reported that LPS induces internalization of Toll-like receptor (TLR)–4, leading down-regulation of cell surface TLR4 and tolerance of LPS in peritoneal macrophages from C57BL/6 mice or human peripheral blood monocytes [26–28]. In current study, however, repeated exposure of LPS amplified inflammatory responses in A/J mice (Figs. 1 and 2). In addition, other groups also reported that low dose LPS augmented pro-inflammatory cytokine expression in murine bone marrow-derived macrophages and a human macrophage cell line [29,30]. Thus, further studies on why repeated LPS stimuli induce corticosteroid-insensitive inflammation rather than the LPS tolerance in our model are needed.

We also demonstrated that intranasal administration of LPS for 3 days resulted in a decrease in HDAC2 (Fig. 3B). There is also a marked reduction of HDAC2 activity and expression in lungs from patients with COPD [31]. In HDAC2 knock-out mice, corticosteroids have less effect on LPS induced airway inflammation [32]. HDAC2 has also been shown to be a prerequisite molecule of corticosteroid action as activated GR recruits HDAC2 molecule and inhibits NF-κB-dependent pro-inflammatory gene expression by deacetylation of histones in epithelial and mononuclear culture cells *in vitro* [33–37]. Thus, HDAC2 reduction observed after LPS treatment on 3 consecutive days likely explains the molecular mechanism of corticosteroid-insensitivity in repeated LPS-induced airway inflammation. In this study, Nrf2 levels were also decreased after repeat doses of LPS (Fig. 3C). Nrf2 knockout mice also showed an impaired response to corticosteroids on airway inflammation induced by LPS [32]. As HDAC2 is also known as a protein deacetylase, HDAC2 down-regulation causes Nrf2 acetylation, resulting in decreased stability and activity of Nrf2 in macrophages [13], which causes reduction of anti-oxidant capacity in cells and amplification of oxidative stress. Amplified oxidative stress might decrease HDAC2 further as oxidative stress such as H_2O_2 and cigarette smoke conditioned media are reported to decrease HDAC2 protein expression via protein modifications, such as phosphorylation, ubiquitination and nitration [11,38,39]. Notably, viral or bacterial infection or LPS exposure are reported to induce oxidative stress in cells [40,41].

Furthermore, we also demonstrated that the level of phosphorylation of Akt was increased after repeated exposure to LPS, which indicates the activation of PI3K signaling pathway (Fig. 3D). Thus, our study suggests that time-dependent decrease in HDAC2 and Nrf2 are inversely-related to the p-Akt phosphorylation levels, and the activation of PI3K signaling pathway causes corticosteroid insensitivity through a decrease in HDAC2 and Nrf2 levels. PI3Kδ is activated by oxidative stress, such as cigarette smoke *in vivo* and cigarette smoke extract *in vitro*, and negatively regulates the HDAC2 activity via Akt phosphorylation, which contributes to the attenuation of anti-inflammatory effects of corticosteroids [8,42]. Our results showed that both theophylline and IC87114, which are allosteric and a δ isoform-selective PI3K inhibitors, respectively, restored the effects of FP on repeated LPS exposure-induced airway neutrophilia and elevation of CXCL1 in BAL fluid (Fig. 4). This is in agreement with the fact that these PI3K inhibitors restored corticosteroid sensitivity in cigarette smoke exposed mice [8]. Another report also suggests that PI3Kδ and Akt activation induced by oxidative stress cause corticosteroid insensitivity via the activation of mammalian target of rapamycin independently in the HDAC2 activity [43]. Thus, in the present corticosteroid-refractory murine model, repeated dosed LPS induced PI3Kδ-dependent decrease in HDAC2 and Nrf2 may, at least in part, contributes to the corticosteroid insensitive airway inflammation.

In conclusion, we report that repeated LPS exposure induced corticosteroid refractory airway inflammation potentially via a decrease in HDAC2 and Nrf2 expression by PI3Kδ activation. Understanding the molecular mechanisms of corticosteroid resistance may result in the development of new therapeutic strategies to treat diseases characterized by corticosteroid resistant inflammation. The mouse model we describe with repeated LPS exposure may prove to be a useful *in vivo* model to assess the effects of novel therapies.

Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.07.020.

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