

Cathelicidin LL-37 restoring glucocorticoid function in smoking and lipopolysaccharide-induced airway inflammation in rats

Jian-Zhen Weng, Yan Wang, Tie-Ying Sun

Department of Respiratory and Critical Care Medicine, Beijing Hospital, National Center of Gerontology, Beijing 100730, China.

Abstract

Background Glucocorticoids have been widely used to treat patients with chronic obstructive pulmonary disease (COPD). Nevertheless, corticosteroid insensitivity is a major barrier to the effective treatment of COPD and its mechanism remains unclear. This study aimed to evaluate the effect of cathelicidin LL-37 on corticosteroid insensitivity in COPD rat model, and to explore the involved mechanisms.

Methods COPD model was established by exposing male Wistar rats to cigarette smoke combined with intratracheal instillation of lipopolysaccharide (LPS). Inhaled budesonide and LL-37 were consequently applied to COPD models separately or collectively to confirm the effects on inflammatory cytokines (tumor necrosis factor [TNF]- α and transforming growth factor [TGF]- β) by enzyme-linked immunosorbent assay (ELISA) and lung tissue histopathological morphology. Expression of histone deacetylase-2 (*HDAC2*) and phosphorylation of Akt (p-AKT) in lung were also measured.

Results Briefly, COPD model rats showed an increased basal release of inflammatory cytokines (lung TNF- α : 45.7 ± 6.1 vs. 20.1 ± 3.8 pg/mL, $P < 0.01$; serum TNF- α : 8.9 ± 1.2 vs. 6.7 ± 0.5 pg/mL, $P = 0.01$; lung TGF- β : 122.4 ± 20.8 vs. 81.9 ± 10.8 pg/mL, $P < 0.01$; serum TGF- β : 38.9 ± 8.5 vs. 20.6 ± 2.3 pg/mL, $P < 0.01$) and COPD related lung tissue histopathological changes, as well as corticosteroid resistance molecular profile characterized by an increase in phosphoinositide 3-kinase (PI3K)/Akt (0.5 ± 0.1 fold of control vs. 0.2 ± 0.1 fold of control, $P = 0.04$) and a decrease in *HDAC2* expression and activity (expression: 13.1 ± 0.4 μ mol/ μ g vs. 17.4 ± 1.1 μ mol/ μ g, $P < 0.01$; activity: 1.1 ± 0.1 unit vs. 1.4 ± 0.1 unit, $P < 0.01$), compared with control group. In addition, LL-37 enhanced the anti-inflammatory effect of budesonide in an additive manner. Treatment with combination of inhaled corticosteroids (ICS) and LL-37 led to a significant increase of *HDAC2* expression and activity (expression: 15.7 ± 0.4 μ mol/ μ g vs. 14.1 ± 0.9 μ mol/ μ g, $P < 0.01$; activity: 1.3 ± 0.1 unit vs. 1.0 ± 0.1 unit, $P < 0.01$), along with decrease of p-AKT compared to budesonide monotherapy (0.1 ± 0.0 fold of control vs. 0.3 ± 0.1 fold of control, $P < 0.01$).

Conclusions This study suggested that LL-37 could improve the anti-inflammatory activity of budesonide in cigarette smoke and LPS-induced COPD rat model by enhancing the expression and activity of *HDAC2*. The mechanism of this function of LL-37 might involve the inhibition of PI3K/Akt pathway.

Keywords: Chronic obstructive pulmonary disease; Glucocorticoid insensitivity; Histone deacetylase-2; Phosphoinositide 3-kinase/Akt pathway

Introduction

Chronic obstructive pulmonary disease (COPD) is a common and heterogeneous chronic lung disorder and a leading cause of morbidity and mortality worldwide.^[1-4] The pathogenesis of COPD is associated with abnormal inflammation mainly affecting the lung parenchyma and peripheral airways. Glucocorticoids are the most effective anti-inflammatory drugs for many chronic inflammatory and immune diseases but are relatively ineffective against COPD.^[5] Many studies have evaluated the anti-inflammatory effects of glucocorticoids on COPD,

reporting inconsistent results regarding the inflammatory levels in relation to number of inflammatory cells and cytokines,^[6-8] regarding the crucial clinical outcomes such as mortality,^[9-11] quality of life,^[9,12,13] and lung function.^[9,14,15] The relatively low responsiveness of COPD to glucocorticoids, namely the glucocorticoid resistance/insensitivity, may be one of the underlying reasons accounting for these inconsistent results.^[16]

The exact molecular mechanism of glucocorticoid resistance has not yet been fully elucidated. Recent studies have shown that reduced histone deacetylase-2 (*HDAC2*)

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Correspondence to: Prof. Tie-Ying Sun, Department of Respiratory and Critical Care Medicine, Beijing Hospital, National Center of Gerontology, No.1 Dahua Road, Dongchen District, Beijing 100730, China
E-Mail: suntieying@126.com

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activity, which is critical for glucocorticoid-dependent anti-inflammatory action, is induced by oxidant stress and abnormal inflammation and may be involved in the development of glucocorticoid insensitivity.^[17-20] In addition, altered activity of signaling pathways kinase, such as phosphoinositol-3-kinase δ (PI3K δ), could inflict HDAC2 and suppress the function of glucocorticoid receptor- α indirectly.^[21] Moreover, cigarette exposure is considered a major risk factor for COPD. Oxidant stress induced by cigarette smoke has shown to promote COPD glucocorticoid resistance model, which was correlated with reduced HDAC2 activity.^[22-24]

LL-37, the only peptide of the cathelicidin family found in the human body, is an important molecule of host innate immunity against invading microbes. Apart from direct antibacterial effects, LL-37 has the ability to decrease infection-induced inflammatory effects by inhibiting the activation of *c-Jun N-terminal kinase* (JNK) and the Akt signal pathways while decreasing the pro-inflammatory cytokine levels.^[25,26] Accordingly, we hypothesized that LL-37 could restore corticosteroid sensitivity by enhancing HDAC2 expression through inhibition of the PI3K/Akt pathway.

In this study, we investigated the role and effect of LL-37 on glucocorticoid resistance using the rat model induced by cigarette smoke exposure and lipopolysaccharide (LPS). Furthermore, we examined the role of HDAC2 and phosphorylation of Akt (p-AKT) to explore the related mechanism.

Methods

Ethical approval

All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Beijing Hospital institutional animal care and conducted according to the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the Institutional Animal Care and Use Committee (IACUC) guidelines.

COPD rat model

The 10-week-old male Wistar rats weighing 252 ± 7 g were obtained from Xingrong experimental animals company (Beijing, China). Rats in control group ($n=5$) was maintained in specific-pathogen-free (SPF) laboratory with temperature of $24.0 \pm 0.5^\circ\text{C}$ and humidity of 50% to 60% for 6 weeks. COPD rats ($n=20$) were exposed to cigarette smoke for 28 days (1 cigarette/rat, 1 h for every treatment, twice a day) and intratracheal instillation of LPS for 2 days (Sigma Company, USA; $200 \mu\text{L}/\text{rat}$, 1 g/L) as previously described.^[27] From the 29th day, the COPD rats were randomized in additional groups ($n=5/\text{group}$): Bud group, rats receiving aerosol inhalation of budesonide (AstraZencca Company, Sweden; 2 mg/20 mL per rat); LL37 group, rats receiving intratracheal instillation of LL-37 (LLGDFFRKSK EKIGKEFKRIVQRIKDFLRNLPRTES-COOH, synthesized using F-moc chemistry at Saibaisheng Biotechnology, China; 1.5 mg/kg); Bud+LL-37 group, rats receiving a combination of budesonide and intratracheal installation for 2 weeks; and COPD group: received no treatment.

Enzyme-linked immunosorbent assay

The levels of tumor necrosis factor- α (TNF- α) and transforming growth factor β (TGF- β) in serum and lung tissue homogenate were measured using Quantikine ELISA kit (R&D Systems, Germany).

HDAC2 expression and activity

HDAC2 expression level and activity of lung were measured by HDAC2 assay kit (Epigentek, USA) and P-4002 HDAC activity assay kit (Epigentek, USA) respectively according to the manufacturer's instructions.

Detection of p-AKT by western blotting

To determine the protein content in lungs tissue, cytoplasmic proteins were prepared according to the manufacturer's instructions. The p-AKT level was detected by sodium dodecyl sulfate-polyacrylamide gelelectrophoresis/Western blotting with antibody (Abcam, UK). Equal loading of sample was confirmed by immunoblotting of β -actin.

Morphology

Lung tissues were cut into sections and stained with hematoxylin and eosin (H&E). Then Olympus PM-10 AD optical microscope and photographic system (Olympus, Tokyo, Japan) were used to observe the morphology.

Statistical analysis

Data were expressed as means \pm standard deviation (SD). All statistical analysis was performed using SPSS version 20.0 (IBM, USA). Levene test was used to assess the equality of variances of groups. Data were evaluated by one-way analysis of variance (ANOVA) with least-significant difference (LSD) or Games-Howell *post hoc* test for comparisons between groups. Statistical significance was also assessed using Student's *t*-test. A $P < 0.05$ was considered statistically significant.

Results

Cigarette smoke and LPS inducing COPD related manifestations in rats

Local and systemic inflammations are characteristics of COPD. In order to determine the inflammatory level of COPD, we analyzed the TNF- α and TGF- β levels of lung homogenate tissue and serum in COPD model rats. Briefly, the significant increases in inflammatory cytokines from lung homogenate tissue or serum were found in the COPD group, compared with control group (lung TNF- α : 45.7 ± 6.1 pg/mL *vs.* 20.1 ± 3.8 pg/mL, $t=8.0$, $P < 0.01$; serum TNF- α : 8.9 ± 1.2 pg/mL *vs.* 6.7 ± 0.5 pg/mL, $t=3.7$, $P=0.01$; lung TGF- β : 122.4 ± 20.8 pg/mL *vs.* 81.9 ± 10.8 pg/mL, $t=3.9$, $P < 0.01$; serum TGF- β : 38.9 ± 8.5 pg/mL *vs.* 20.6 ± 2.3 pg/mL, $t=4.6$, $P < 0.01$; Figure 1). In addition, bronchial smooth muscle thickening and significant inflammatory cells infiltration with evidence of alveolar wall rupture fused to the bulla were observed in the lung tissue of COPD group; those morphological changes

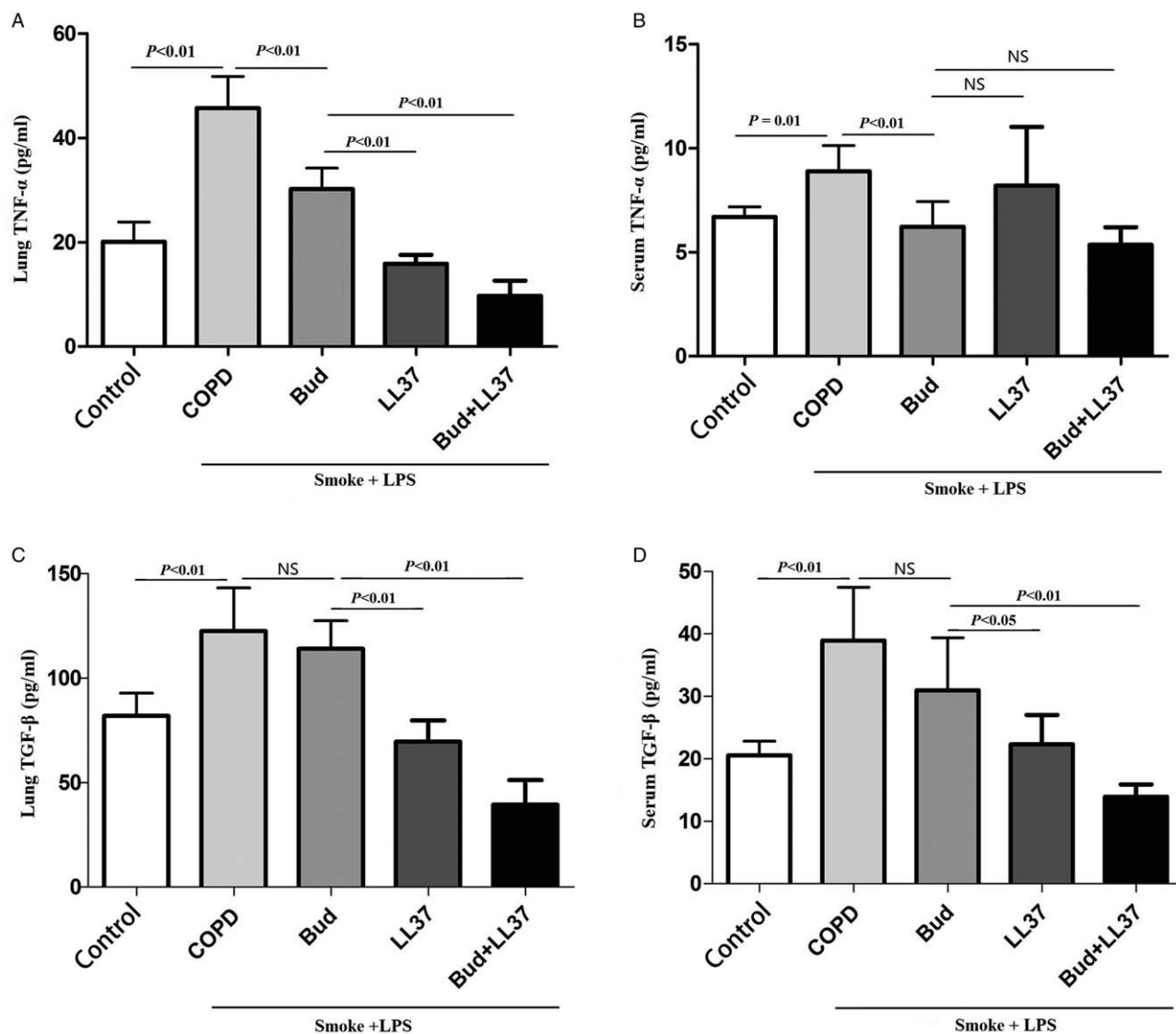


Figure 1: Concentration of inflammatory cytokines TNF- α (A and B) and TGF- β (C and D) in lung tissue homogenate and serum of rats. COPD: Chronic obstructive pulmonary disease; LPS: Lipopolysaccharide; NS: No significance; TNF: Tumor necrosis factor; TGF: Transforming growth factor.

closely resemble the manifestations from lung tissue of COPD group [Figure 2].

Corticosteroid insensitivity and HDAC2 activity and expression in COPD model rats

Compared with COPD group, no significant differences in lung and serum TGF- β levels were found in Bud group after inhaled corticosteroids (ICS) treatment (lung TGF- β : Bud group 114.0 ± 13.4 pg/mL, $t=0.8$, $P=0.47$; serum TGF- β : Bud group 30.9 ± 8.4 pg/mL, $t=1.5$, $P=0.18$). Contrary, significantly decreased lung and serum TNF- α levels were found in Bud group compared to COPD group (lung TNF- α : Bud group 30.2 ± 4.0 pg/mL, $t=4.8$, $P < 0.01$; serum TNF- α : Bud group 6.2 ± 1.2 pg/mL, $t=3.5$, $P < 0.01$; Figure 1). Moreover, the evidence of inflammatory cells infiltration and alveolar wall rupture was slightly but significantly decreased in Bud group [Figure 2].

Furthermore, compared with control group (expression: 17.4 ± 1.1 $\mu\text{mol}/\mu\text{g}$; and activity: 1.4 ± 0.1 unit), both expression and activity of HDAC2 in the lung decreased significantly in the COPD group (expression: 13.1 ± 0.4 $\mu\text{mol}/\mu\text{g}$, $t=8.6$, $P < 0.01$; activity: 1.1 ± 0.1 unit, $t=6.4$, $P < 0.01$) and Bud group (expression: 14.1 ± 0.9 $\mu\text{mol}/\mu\text{g}$, $t=5.2$, $P < 0.01$; activity: 1.0 ± 0.1 unit, $t=5.8$, $P < 0.01$; Figure 3A and 3B). In addition, the level of p-AKT was significantly higher in COPD group (0.5 ± 0.1 fold of control, $t=3.1$, $P=0.04$), but not in Bud group (0.3 ± 0.1 fold of control, $t=0.6$, $P=0.58$), compared to control group (0.2 ± 0.1 fold of control) [Figure 4].

LL-37 treatment improving corticosteroid sensitivity in COPD model rats

LL-37 showed additive and synergistic inhibition with budesonide on lung and serum TGF- β levels (lung TNF- α :

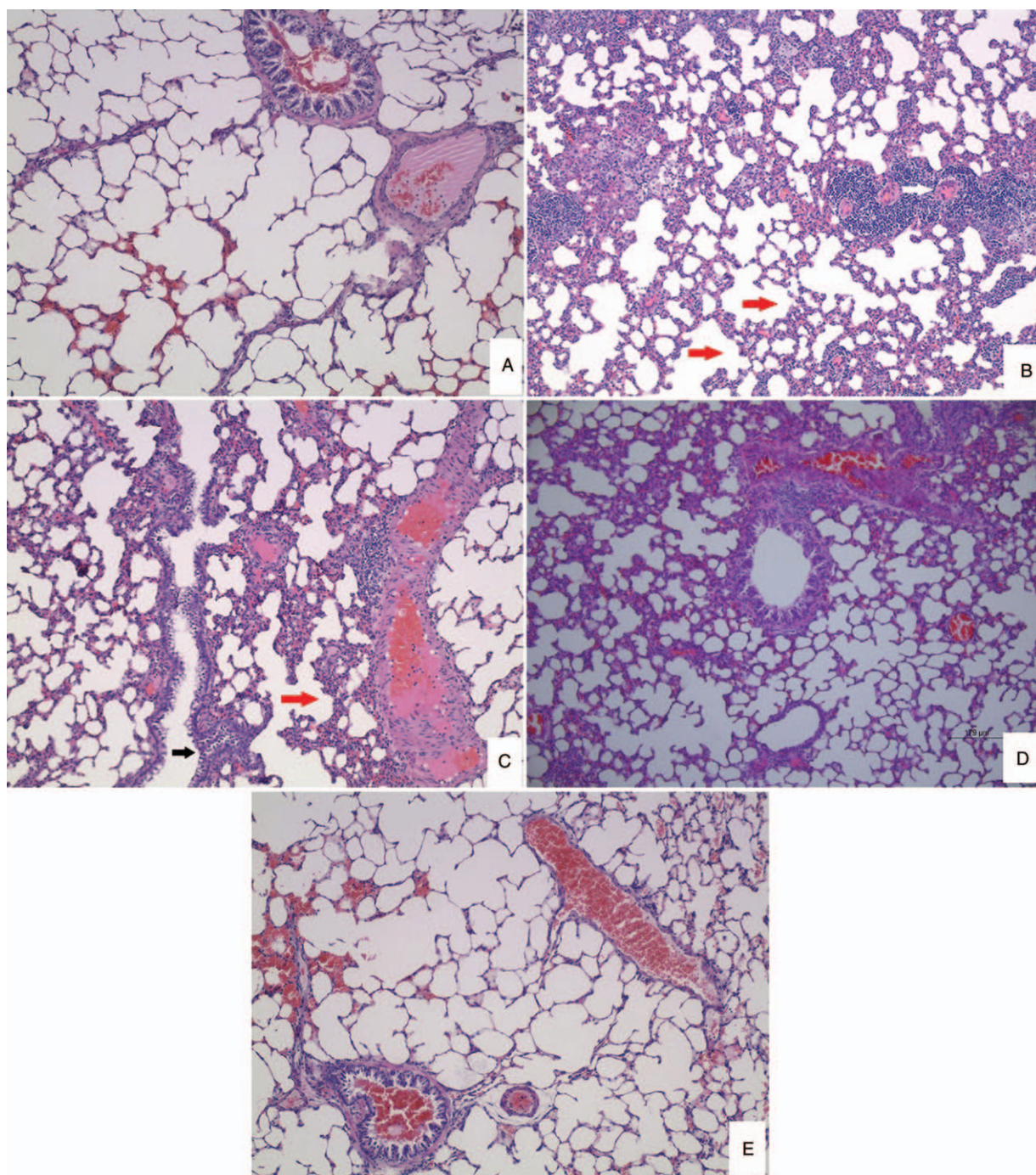


Figure 2: Morphological manifestations of lung tissue of rats (HE staining; original magnification, $\times 100$). (A) Control group: structures of airway and alveoli were normal. Bronchial cilia were well arranged. No obvious inflammation infiltration. (B) COPD group: numerous lymphocytes and neutrophils infiltrated surrounding bronchi and vessels (white arrow). Alveolar walls ruptured into bulla (red arrow) and some alveolar septa widened. (C) Bud group: infiltration of inflammatory cells and destruction of alveolar walls (red arrow) also can be seen with a slightly improvement compare to COPD group. Cilia of bronchial epithelia distributed poorly (black arrow). (D) LL37 group: the severity of infiltration of inflammatory cells and destruction of alveolar walls was similar with Bud group. (E) Bud+LL37 group: the degree of inflammation and emphysema was mild. Morphological manifestations were very close to control group. COPD: chronic obstructive pulmonary disease.

15.9 ± 1.7 pg/mL in LL37 group, and 9.7 ± 2.9 pg/mL in Bud+LL37 group; serum TNF- α : 8.2 ± 2.8 pg/mL in LL37 group, and 5.4 ± 0.8 pg/mL in Bud+LL37 group; Lung TGF- β : 69.6 ± 10.0 pg/mL in LL37 group, and 39.4 ± 11.8 pg/mL in Bud+LL37 group; serum TGF- β : 22.3 ± 4.7 pg/mL in LL37 group, and 13.9 ± 2.0 pg/mL in Bud+LL37 group; Figure 1). Furthermore, the combination of LL37 and budesonide could significantly improve the

destruction of lung tissue induced by smoke and LPS [Figure 2].

In addition, lung *HDAC2* expression and activity were both significantly increased in Bud+LL37 group (expression: 15.7 ± 0.4 $\mu\text{mol}/\mu\text{g}$, $t=3.5$, $P < 0.01$; activity: 1.3 ± 0.1 unit, $t=4.2$, $P < 0.01$), but not in LL37 group (expression: 13.1 ± 0.4 $\mu\text{mol}/\mu\text{g}$, $t=2.1$, $P=0.07$; activity:

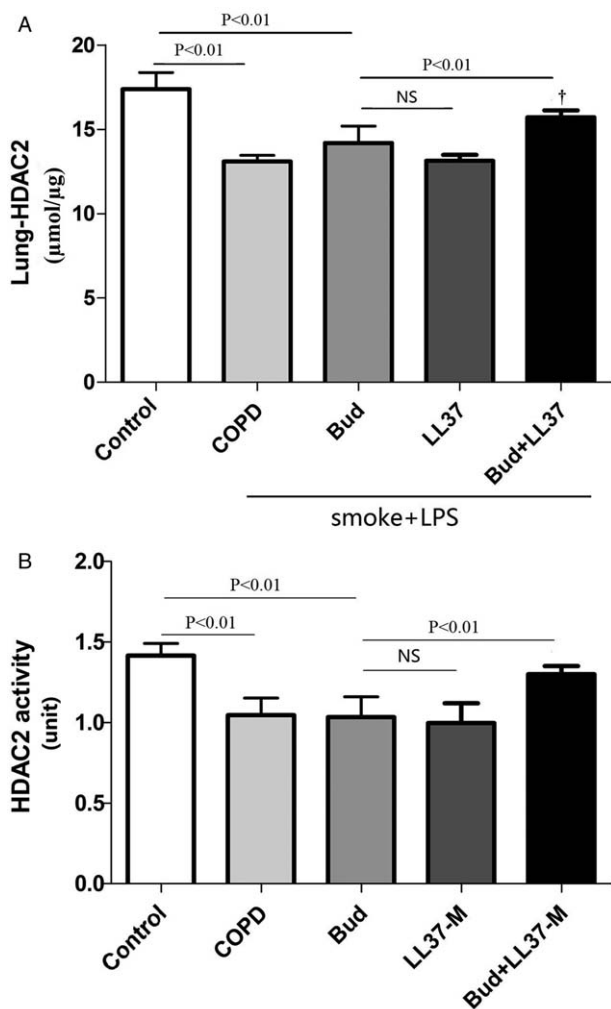


Figure 3: Expression of HDAC2 (A) and activity of HDAC2 (B) in lung tissues. COPD: chronic obstructive pulmonary disease; HDAC2: histone deacetylase-2; LPS: lipopolysaccharide; NS: no significance.

1.0 ± 0.1 unit, $t=0.59$, $P=0.56$; Figure 3A and 3B), along with suppression of Akt pathway (LL-37 group: 0.2 ± 0.1 fold of control, $t=1.1$, $P=0.28$; Bud+LL37 group: 0.1 ± 0 fold of control, $t=3.0$, $P<0.01$), compared with Bud group [Figure 4].

Discussion

COPD is a chronic inflammatory disorder, in which innate immune responses are not a relevant component.^[28] Cigarette smoking is the most commonly encountered risk factor for COPD. Animal models of cigarette smoke-induced COPD reliably reflect the inflammatory and pathogenic mechanisms of the disease. Using a guinea pig or murine, it usually takes 6-month exposure period to establish the COPD model.^[29] Combining induction agents could shorten the modeling period and induce severer stage. In present study, COPD model was established by exposing male Wistar rats to cigarette smoke combined with intratracheal instillation of LPS. Consequently, elevated inflammatory cytokines levels and destruction of lung structure were observed confirming the

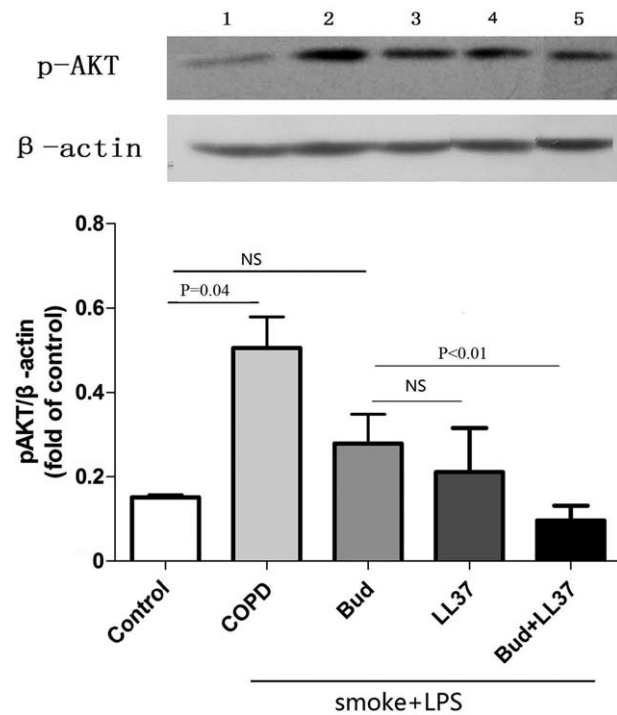


Figure 4: Expression of p-AKT in lung tissues. COPD: chronic obstructive pulmonary disease; NS: no significance; p-AKT: phosphorylation of Akt; LPS: lipopolysaccharide.

presence of COPD-like inflammation in the exposed animals. However, COPD is a complex disease and no such animal model has completely replicated the inflammatory response of COPD to date. Our models, with the shortcomings of short modeling time, could only partially reflect the characteristics of COPD.

LL-37 is the only peptide of the cathelicidin family found in the human body, that acts as an effector molecule of the innate immune system.^[30] Apart from broad antibacterial effects, it also serves as a potent immunoregulator having a delicate role in inflammatory/anti-inflammatory balance in infectious and inflammatory diseases.^[31] Significantly higher levels of LL-37 have been observed in sputum, airway epithelium and BALF samples in COPD patients compared to healthy individuals.^[32-36] In addition, LL-37 expression in airway epithelium has shown to be positively correlated with airway wall thickness and collagen deposition.^[37] Moreover, high sputum hCAP18/LL-37 levels were associated with increased risk of exacerbation, non-typeable Haemophilus influenzae colonization, higher age and higher levels of inflammatory markers.^[36] Overall, these studies have suggested that LL-37 was involved in the pathogenesis of COPD, which was somewhat contradictory with our results. Yet, compared to healthy individuals, significantly higher LL-37 levels were found in bronchoalveolar lavage fluid (BALF) in patients with early stages of COPD (GOLD I-II), and significantly lower LL-37 levels in patients with advanced COPD (GOLD III-IV).^[35] Although there were also some contradictory reports,^[33,38] these data suggested that the role of LL-37 and its regulation in COPD was a complicated process, especially in patients with the advanced stage of COPD.

Moreover, protein structures are closely related to their functions. An increased presence of peptidylarginine deiminase (PADIs) and citrullinated proteins have been found in the lungs of smokers and COPD patients, which led to post-translational modification of proteins like LL-37 by converting cationic peptidylarginine residues to neutral peptidylcitrulline.^[39,40] Previous study has reported that citrullinated LL-37 was less efficient at neutralizing LPS, and more prone to degradation by proteases.^[39] Cell death events were crucial for balancing inflammatory reactions. LL-37 facilitated clearance of apoptotic neutrophils (ie, non-functional cells with intact membranes) from the system by surrounding macrophages in an immunologically silent manner; this process in turn resulted in a massive secretion of anti-inflammatory mediators.^[41,42] The secondary necrosis of apoptotic neutrophils represents an important immunomodulatory function of LL-37, that was abolished by citrullination,^[39] which could potentially explain the contradictory reports concerning the role of LL-37 in COPD. Immunomodulatory function of LL-37 relies on normal structure, which is altered in COPD and smokers. Our study proved that exogenous treatment of naïve LL-37 could regulate the abnormal inflammation, thus supporting this point of view.

Glucocorticoid resistance is a main barrier of ICS implication in COPD. A body of evidence has verified the important role of *HDAC2* in the induction of steroid resistance. The levels of *HDAC2* were decreased in lung parenchyma, bronchial biopsies and alveolar macrophages in patients with COPD and in smokers,^[43] as well as in macrophages and lungs of mice exposed to cigarette smoke. They were also correlated with disease severity and exacerbation.^[44] In patients with very severe COPD, the expression of *HDAC-2* was less than 5% of the expression observed in normal lung.^[45] In our study, *HDAC2* expression was significantly decreased in COPD model rats, along with unsatisfactory response to ICS, which was consistent with previous reports.^[27,46]

Considering these data, over the last decades *HDAC2* has become one of the most attractive targets for restoration of glucocorticoid sensitivity. It has been reported that the glucocorticoid resistance of COPD bronchoalveolar macrophages could be completely reversed by overexpressing *HDAC2*.^[47] So far, several potential therapeutic avenues for restoring *HDAC2* function to improve steroid efficacy in COPD have emerged. A low dose of theophylline has shown to improve the anti-inflammatory action of corticosteroid in COPD.^[48,49] Although the exact molecular targets of theophylline remain unresolved, the downstream mechanisms involve the restoration of *HDAC2* activity.^[21] Similar to theophylline, some other agents such as the curcumin,^[50] nortriptyline,^[51] and erythromycin^[17,50,52,53] have been proposed to mediate restoration of corticosteroid function through a protection of *HDAC-2* expression and activity. In our study, LL-37 treatment enhanced the expression and activity of *HDAC2* in lung of COPD model rats. Combining LL-37 with ICS suppressed the inflammatory cytokines more effectively compared to monotherapy showing notable improvements and changes in lung tissue.

PI3K δ /Akt activity was increased, and *HDAC2* reduced in COPD patients.^[21,54] This suggested that activation of PI3K δ was responsible for the dysfunction of *HDAC2*. Indeed, the activation of PI3K δ could reduce *HDAC2* activity in smoking-induced inflammation.^[21,24,51] Most of the above mentioned medicines appeared to restore *HDAC2* via selective inhibition of PI3K δ ,^[17,50-53] and selective PI3K δ inhibitors were also effective.^[21,54] In our study, the significantly increased *HDAC2* expression and activity, as well as inhibition of Akt expression, were observed in the group receiving combination therapy compared to Bud group. Still, the differences in p-AKT expression between control group and Bud group were not significantly different, while *HDAC2* expression and activity was lower in Bud group. PI3K activation was not completely related to the expression of *HDAC2* and corticosteroid response as it has been the case in the other studies. This discrepancy might be due to the limited number of experimental animals in our study and complexity of mechanisms and pathways that regulate *HDAC2* expression and activity.

In conclusion, this study indicated that LL-37 improved the anti-inflammatory activity of budesonide in cigarette smoke and LPS-induced COPD model rats by enhancing the expression and activity of *HDAC2*. The mechanism of this function of LL-37 may involve the inhibition of PI3K/Akt pathway.

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Conflicts of interest

None.

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