Protective effects of metformin on lipopolysaccharide-induced airway epithelial cell injury via NF-κB signaling inhibition

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Abstract. Asthma is a heterogeneous disease characterized by chronic airway inflammation. It has been demonstrated that metformin, an extensively used drug for the treatment of type 2 diabetes, improves airway inflammation and remodeling. However, the mechanism by which this occurs remains poorly understood. The present study investigated the protective effects of metformin in lipopolysaccharide (LPS)-induced human bronchial epithelial (16HBE) cells injury and the associated mechanisms. 16HBE cells were preincubated with metformin for 1 h and subsequently exposed to LPS for 12 h. A lactate dehydrogenase (LDH) leakage assay was used to determine the extent of injury to 16HBE cells. The expression of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) was measured by ELISA. The protein expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), as well as proteins associated with nuclear factor (NF)-κB signaling, was measured by western blotting. Immunofluorescence assays confirmed the nuclear translocation of NF-κB p65. The LDH leakage assays suggested that metformin significantly reduced LPS-induced 16HBE cell injury. Furthermore, it was confirmed that metformin suppressed the LPS-induced secretion of TNF-α, IL-6, ICAM-1 and VCAM-1. The mechanism occurred at least partially via inhibition of NF-κB signaling. The results demonstrated that metformin inhibited NF-κB mRNA expression and the nuclear translocation of NF-κB p65. To the best of our knowledge, the present study was the first to demonstrate that metformin ameliorated LPS-induced bronchial epithelial cell injury via NF-κB signaling suppression.

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Introduction

Asthma is a significant public health problem with increasing prevalence, affecting 1-18% of the population in different countries (1). Although certain pathogenic mechanisms in asthma have been well established, chronic inflammatory responses are a main factor contributing to disease progression, which are less well understood (2). Therefore, identifying the mechanisms and factors that are involved in airway inflammation is critical for the discovery of novel strategies to control respiratory diseases, including asthma.

Airway epithelial cells, the core of the stable mucosal surface microenvironment, are key in the respiratory defense barrier. Once the airway epithelial structure is damaged and the natural protection is compromised, airway inflammation or infection may occur (3). Lipopolysaccharide (LPS) is an endotoxin that induces inflammatory reactions in immune and non-immune cells, including the airway epithelium, resulting in pro-inflammatory cytokines and chemokine production (4).

Increasing amounts of research have determined that NF-κB, through diverse signaling cascades, serves a primary role in inflammatory and apoptotic processes (5-7). The expression of several cytokines, including TNF-α and IL-6 is regulated by NF-κB; furthermore, NF-κB activation evokes further activation of NF-κB, therefore enlarging the inflammatory reaction and epithelial injury (8). Metformin, a widely used drug for the treatment of type 2 diabetes, has been demonstrated to improve airway inflammation and remodeling (9). However, the mechanisms underlying these anti-inflammatory effects have not yet been reported.

In the present study, the protective effects of metformin against LPS-induced human bronchial epithelial cells

inflammation were investigated and the potential mechanisms of action involving the NF-κB signaling pathways were discussed.

Materials and methods

Materials. Metformin, LPS and NF-κB activation-nuclear translocation assay kits were purchased from Peprotech, Inc. (Rocky Hill, NJ, USA). Phospho-NF-κBp65 (Ser536) antibody, NF-κB p65 antibody, ammonium pyrrolidinedithiocarbamate (PDTC) and lysis buffer were all acquired from BD Biosciences (Franklin Lakes, NJ, USA). The LDH activity assay kit was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). VCAM-1 and ICAM-1 antibodies were purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). Bicinchoninic acid (BCA) protein assay kit and NF-κB p65 Transcription Factor assay kits were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

Cell culture. 16HBE cells were donated by the State Key Laboratory of Respiratory Diseases, Guangzhou Medical University (Guangzhou, China) and cultured in minimum essential medium (National Veterinary Institute, Uppsala, Sweden) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin sulfate. 16HBE cells were incubated in a humidified environment of 5% CO_2/95% air at 37˚C; culture medium was replenished every 48 h according to the manufacturer's protocol. 16HBE cells at passages 3-5 were collected for subsequent experimentation.

Cell viability assay. Cultured 16HBE cells ($1x10^4$ cells/well) were plated in 96-well plates and grown until 90% confluence was reached. Cells were incubated with increasing concentrations of LPS (0.5, 2.5 and 12.5 μg/ml) for 12 h, or pre-incubated with metformin (50, 100, 150 or 250 μg/ml) for 1 h. For the controls, 16HBE cells were incubated with medium only for the same duration. At the end of treatment, the supernatant was removed and 20 μl MTT (5 mg/ml) was added with 80 μl medium for 4 h at 37˚C, following which the product was dissolved with 100 μl dimethyl sulfoxide. Absorbance (490 nm) was measured using a microplate reader (Spectramax Plus 384; Molecular Devices, LLC, Sunnyvale, CA, USA).

Lactate dehydrogenase (LDH) leakage. LDH activity, a biomarker cell injury, is an indicator of cell membrane permeability. Following treatment, culture medium was collected and centrifuged for 10 min at 1,000 x g at room temperature, and LDH activity in the supernatant was determined. LDH activity was measured with a spectrophotometer (Shanghai Instrument Factory, Shanghai, China) according to the manufacturer's protocol.

ELISA. Following treatment with metformin, the culture medium supernatant was collected to measure the concentration of TNF-α (PT512; Beyotime Institute of Biotechnology) and IL-6 (PI326; Beyotime Institute of Biotechnology) with commercial ELISA kits (TNF-α, 550610; IL-6, 550799; BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. Absorbance at 450 nm was detected using a microplate reader.

NF-κB localization. NF-κB nuclear translocation was determined according to the protocol in the NF-κB activation-nuclear translocation assay kit. Following treatment, 16HBE cells were washed in PBS and then fixed in 4% paraformaldehyde for 15 min at room temperature, washed again and blocked in bovine serum albumin (BSA; Beyotime Institute of Biotechnology, Shanghai, China) for 1.5 h in the same conditions. Following incubation with NF-κB p65 primary antibodies (1:50; cat. no. SN368-4; Beyotime Institute of Biotechnology) overnight at 4˚C, washed thrice with PBS and incubated with the Cy3-conjugated secondary antibody (cat. no. SN368-5; Beyotime Institute of Biotechnology) for 2 h at room temperature. Next, 16HBE cells were stained with DAPI for 5 min at room temperature and visualized with a fluorescence microscope (Nikon Corporation, Tokyo, Japan).

Protein extraction and western blotting. 16HBE cells were collected and the protein was extracted by lysis buffer. Lysates were centrifuged at 4˚C for 20 min at 13,000 x g. The protein concentration in the supernatant was evaluated by BCA protein assay kit. Equal amounts of protein (25 μg) were fractionated by 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes, which were blocked in 5% BSA for 1 h. Cells were subsequently incubated at 4˚C for 12 h with phospho-NF-κB p65 (1:600; cat. no. 558377), NF-κB p65 (1:1,000; cat. no. 610868), ICAM-1 (1:1,000; cat. no. sc-107), VCAM-1 (1:600; cat. no. sc-73252) and GAPDH (1:1,000; cat. no. sc-293335) antibodies. The membranes were then incubated with goat anti-rabbit IgG (1:5,000; Beyotime Institute of Biotechnology; cat. no. A0208) secondary antibodies for 1 h at room temperature and washed with Tris-buffered saline/0.1% Tween-20. The blots were analyzed with Syngene software (CFX3.0; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

RNA isolation, cDNA synthesis and polymerase chain reaction (PCR). Total RNA was isolated from 16HBE cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). The purity and concentration of the isolated RNA was measured in a DU650 spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA). RNA (1 μg) was reverse transcribed using a Revert Aid Premium First Strand cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan). The condition of reverse transcription was 37˚C for 15 min and 85˚C for 5 sec. For quantitative (q)PCR, a Taqman probe (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used and 0.8 μl cDNA was amplified in a 16 μl reaction system. The primer sequences were as follows: NF-κB p65 sense, 5'-GTGCAGCCTCTT CGTCCTC-3' and antisense, 5'-GTGCACTACAGACGA GCCATT-3'; GAPDH sense, 5'-CGTATCGGACGCCTG GTT-3' and antisense, 5'-CGTGGGTAGAGTCATACTGGA AC-3'. The thermo cycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Each sample was measured in duplicate. 18 s rRNA was applied for each sample as an internal control in order to normalize gene expression levels. The results were expressed as GAPDH using qPCR and the $2^{-\Delta\Delta Cq}$ method (10).

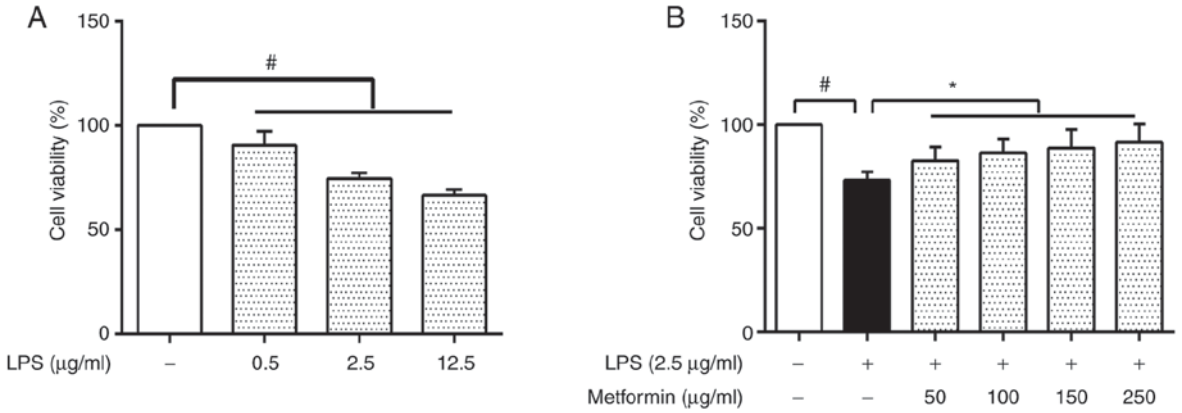

Figure 1. Metformin exerts protective effects in 16HBE cells injured with LPS. (A) 16HBE cells were incubated with different concentrations of LPS for 12 h. Cell viability was assessed using a MTT assay (n=6). (B) Protective effects of metformin on LPS-induced 16HBE cells injury. 16HBE cells were incubated with different concentrations of metformin for 1 h, then exposed to LPS (2.5 μmol/l) for 12 h. Cell viability was assessed via an MTT assay (n=6). $^{\#}$P<0.01 vs. the control; *P<0.01 vs. the LPS group. LPS, lipopolysaccharide.

Statistical analysis. Data were obtained from a minimum of three to five independent experiments and expressed as the mean ± standard deviation. The Student's t-test was used to compare two groups and one-way analysis of variance followed by Scheffe's test was used for multiple comparisons. SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Metformin reduces LPS-induced 16HBE cell injury. 16HBE cell viability was measured with an MTT assay. Treatment of 16HBE cells with LPS (0.5-12.5 μg/ml) significantly reduced the viability in a dose-dependent manner (P<0.01; Fig. 1A). Furthermore, exposure to 02.5 μg/ml LPS for 12 h significantly decreased cell viability to 72.2±4.3% (P<0.01; Fig. 1A). Therefore, on the basis of previous experiments (11), 2.5 μg/ml LPS for 12 h was used as the model condition. It was confirmed that the protective effect of metformin on LPS-induced 16HBE cells injury was dose-dependent (Fig. 1B).

Metformin concentrations of 50 and 250 mg/ml were selected for further experiments. LDH leakage, as a biomarker of cell membrane damage, was significantly increased in 16HBE cells exposed to 2.5 μg/ml LPS (P<0.01); preincubation with metformin for 1 h significantly reduced LDH leakage (P<0.01; Fig. 2).

Metformin reduces inflammatory cytokine secretion in 16HBE cells. To investigate whether metformin inhibited inflammatory cytokines secretion in 16HBE cells treated with 2.5 μg/ml LPS, the expression of TNF-α and IL-6 was measured by ELISAs. TNF-α and IL-6 (Fig. 3) expression in LPS-induced 16HBE cells was significantly reduced by metformin in a dose-dependent manner (P<0.01).

Metformin decreases the expression of ICAM-1 and VCAM-1 in injured 16HBE cells. PDTC is an inhibitor of NF-κB, which was used to treat 16HBE cells prior to the detection of ICAM-1 and VCAM-1 expression in LPS-induced cells. The western blot analysis demonstrated that VCAM-1 and ICAM-1 expression increased in LPS-treated 16HBE cells. In addition, the effects of LPS on ICAM-1 and VCAM-1 expression were significantly inhibited by PDTC (P<0.01; Fig. 4). These results suggested that NF-κB signaling was involved in the LPS-induced expression of ICAM-1 and VCAM-1 in 16HBE cells. Furthermore, 16HBE cells were treated with metformin at 50 and 250 μg/ml, and ICAM-1 and VCAM-1 expression was significantly inhibited, compared with the LPS only group.

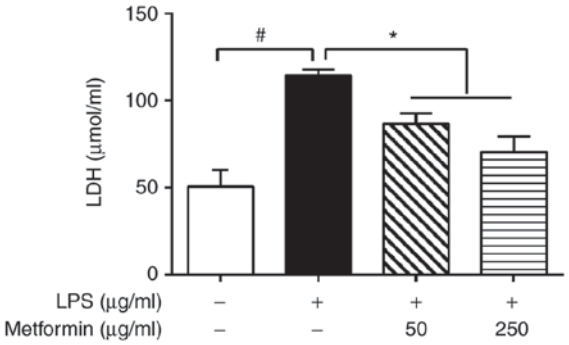

Figure 2. Metformin decreases LDH leakage in injured 16HBE cells. An LDH leakage assay was performed using a commercial LDH activity assay kit (n=6). Data are presented as the mean ± standard deviation. $^{\#}$P<0.01 vs. the control; *P<0.01 vs. the LPS group. LDH, lactate dehydrogenase; LPS, lipopolysaccharide.

Metformin suppresses LPS-induced NF-κB activation. NF-κB p65 phosphorylation promotes nuclear translocation, triggering inflammatory cytokine transcription. LPS induces the NF-κB signaling pathway via p65 phosphorylation and subsequent nuclear translocation. In the present study, p65 phosphorylation was inhibited by metformin in a dose-dependent manner. In addition, LPS markedly reduced p65 expression in the cytoplasm; this decrease was inhibited by metformin pretreatment (Fig. 5A). The decrease in cytosolic NF-κB expression induced by LPS may have been mediated by an increase in nuclear NF-κB p65 expression, which was also prevented by metformin pretreatment (Fig. 5B). Therefore, these results demonstrated that LPS-mediated NF-κB activation was inhibited by metformin.

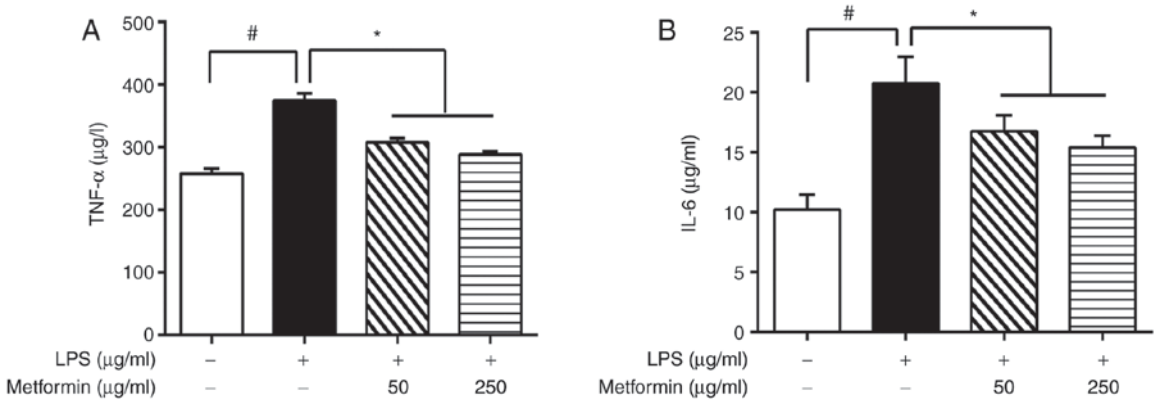

Figure 3. Metformin reduces the LPS-induced secretion of TNF-α and IL-6. (A) 16HBE cells were incubated with metformin (50 and 250 μg/ml) for 1 h, then exposed to LPS (2.5 μmol/l) for 12 h. TNF-α and (B) IL-6 were detected in the culture supernatants using commercial ELISA kits (n=6). Data are presented as the mean ± standard deviation. #P<0.01 vs. the control; *P<0.01 vs. the LPS group. IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide.

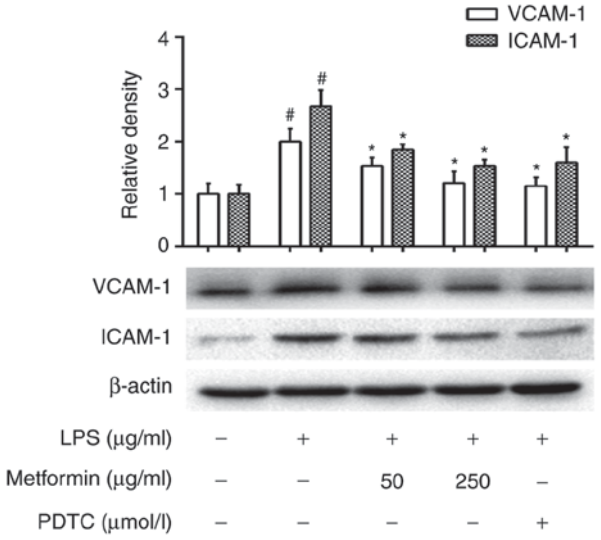

Figure 4. Metformin reduces LPS-induced ICAM-1 and VCAM-1 expression. 16HBE cells were incubated with metformin (50 and 250 μg/ml) or PDTC (20 μM) for 1 h, then exposed to LPS (2.5 μmol/l) for 12 h. The expression of ICAM-1 and VCAM-1 was analyzed by western blotting (n=3). Data are presented as the mean ± standard deviation. #P<0.01 vs. the control; *P<0.01 vs. the LPS group. LPS, lipopolysaccharide; ICAM, intercellular adhesion molecule; VCAM, vascular cellular adhesion molecule; PDTC, pyrrolidinedithiocarbamate.

NF-κB p65 mRNA expression is decreased by metformin in 16HBE cells treated with LPS. qPCR results revealed that compared with the control group, NF-κB mRNA expression in the LPS group was significantly increased (P<0.01). Pretreatment with different concentrations of metformin for 1 h resulted in a significant decrease in NF-κB p65 mRNA expression (P<0.01; Fig. 6).

Discussion

Asthma is a chronic inflammatory disease of the airways, characterized by the infiltration of numerous inflammatory cells into the airway mucosa and airway smooth muscle (12). Bronchial epithelial cells are the specific targets for airway inflammation and their injury results in further airway inflammation, mucosal edema and contraction, which underpins the pathological basis of airway hyperactivity (13). Therefore, structural integrity dysfunction in the airway epithelium promotes asthma. It has been well established that asthma predominantly involves Th2 lymphocytes, as well as the production of cytokines, including IL-6 and TNF-α (14).

NF-κB is a nuclear transcription factor that is critical in the regulation of apoptosis and inflammation, as well as various autoimmune diseases. NF-κB activation is thought to be activated by a variety of stimuli, including growth factors, cytokines and lymphokines (15). The NF-κB protein family includes five members, of which the P50/p65 dimer is the most widely expressed (16,17). Iκb is a protein that binds and inhibits NF-κB, and is frequently combined with P50/p65 to form a trimer (18). The classical activation pathway of NF-κB is stimulated by inflammatory factors, which results in Iκb phosphorylation and NF-κB nuclear translocation to bind the specific gene sequence (19). Certain studies demonstrated that LPS stimulated macrophages and increased the production of inflammatory factors, including TNF-α, which serves an important role in severe refractory asthma (20,21). The over-expression of inflammatory agents is produced in vascular smooth muscle cells via the relevant NF-κB pathway. Additionally, LPS induced NF-kB expression and phosphorylation (22). Therefore, it was hypothesized that the pathogenesis of asthma would be further clarified by investigating the LPS-NF-κB-TNF-α pathway.

Adhesion molecules are membrane glycoproteins that affect the cells surface and regulate cell-cell and extracellular matrix-cell interactions. Through mediating epithelial cell and leukocyte activation, ICAM-1 and VCAM-1 trigger a proinflammatory response (23). The NF-κB signaling pathway not only participates in the inflammatory process, but also induces ICAM-1 and VCAM-1 expression. Therefore, selectively blocking the NF-κB activation pathway is key to inhibiting the inflammatory response. Metformin is widely used to treat patients with type 2 diabetes. Previously, certain studies have demonstrated that metformin reduces inflammatory cell migration and serves an anti-inflammatory role by reducing the expression of adhesion molecules and inflammatory factors (24,25). Calixto *et al* demonstrated that metformin inhibits the exudate of inflammatory cells in obese asthma

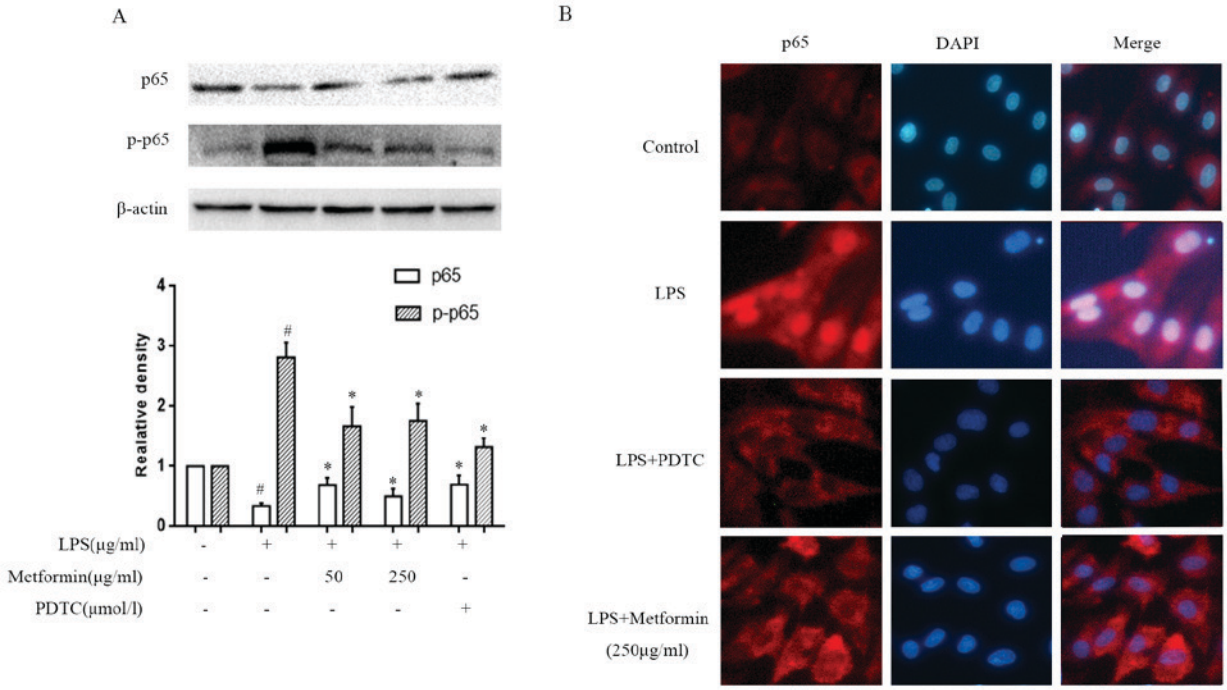

Figure 5. Metformin-mediated blockade of LPS-induced NF-κB activation. (A) 16HBE cells were incubated with metformin (50 and 250 μg/ml) or PDTC (20 μM) for 1 h, then exposed to LPS (2.5 μmol/l) for 12 h. NF-κB p65, p65 phosphorylation were examined by western blotting (n=4). Data are presented as the mean ± standard deviation. $^{\#}$P<0.01 vs. the control; *P<0.01 vs. the LPS group. (B) Metformin attenuated the nuclear translocation of NF-κB p65. 16HBE cells were incubated with metformin (50 and 250 μg/ml) or PDTC (20 μM) for 1 h, then exposed to LPS (2.5 μmol/l) for 12 h. Nuclear translocation of NF-κB p65 was detected using an immunofluorescence assay (x200 magnification; n=5). Images were obtained using a Nikon fluorescent microscope (nuclei with DAPI staining indicated by blue; NF-κB p65 with Cy3 staining indicated in red). In untreated 16HBE cells NF-κB p65 was localized predominantly in the cytoplasm (control group). Cells exposed to LPS only exhibited significant translocation of p65 to the cell nucleus. In the cells pretreated with metformin or PDTC (20 μM) for 1 h then exposed to LPS (2.5 μmol/l) for 12 h, NF-κB p65 was significantly retained in the cytoplasm. LPS, lipopolysaccharide; NF, nuclear factor; PDTC, pyrrolidinedithiocarbamate; p, phosphorylated.

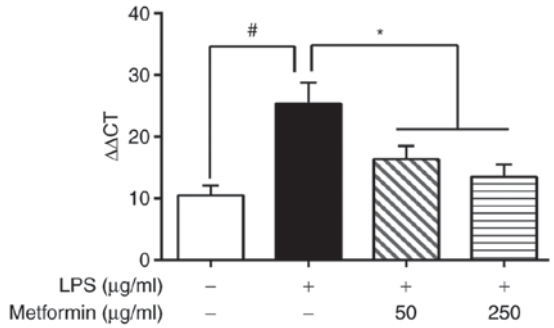

Figure 6. The effect of metformin on NF-κB p65 mRNA in 16HBE cells. Compared with the control group, pretreatment of different doses of metformin for 1 h resulted in NF-κB p65 mRNA expression being decreased. A total of three independent experiments for one 16HBE cells culture. Data were presented as the mean ± standard deviation. $^{\#}$P<0.01 vs. control; *P<0.01 vs. the LPS group. LPS, lipopolysaccharide.

mice with high lipid-induced obesity. Metformin also reduces the production of eosinophil chemoattractant and TNF-α in the bronchoalveolar lavage fluid of obese mice (26). Furthermore, it may also inhibit airway smooth muscle cell proliferation via an AMP-activated protein kinase-dependent pathway (27). Therefore, there is evidence that metformin may be useful in the treatment of asthma. Notably, previous studies have been demonstrated as an important clinical benefit of metformin therapy in asthmatic and diabetic patients (28,29). In the present study, whether metformin inhibited bronchial epithelial inflammation through the inhibition of NF-κB activation was investigated. The concentration of metformin used reflected the therapeutic dose of metformin administered to diabetic patients and therefore may reach similar plasma concentrations.

In the present study, the results of the LDH leakage experiments demonstrated that pretreatment with metformin significantly reduced LPS-induced 16HBE cells injury. Additionally, metformin significantly reduced the secretion of proinflammatory cytokines TNF-α and IL-6, therefore diminishing the inflammatory phenotype of 16HBE cells. These results suggested that metformin may effectively inhibit cytokine production. It was also demonstrated that metformin inhibited the expression of ICAM-1 and VCAM-1 in 16HBE cells injured with LPS, suggesting that metformin inhibited airway inflammation to a certain extent. These anti-inflammatory properties may be associated with the suppression of transcription factor activation. NF-κB translocating into the nucleus activates the transcription of proinflammatory cytokines, chemokines, adhesion molecules and growth factors, therefore participating in immune defense and the inflammatory response (30). Therefore, inhibiting NF-κB activity may be crucial for controlling airway inflammation, as well as delaying the progression of bronchial epithelial cell inflammation in asthma. In the present study, different concentrations of metformin were used to pretreat 16HBE cells prior to LPS treatment. Actually, it is difficult to calculate the drug *in vitro* concentration according to the clinical drug concentration due to the multiple influencing factors, including

drug absorption and bioavailability, hepatic elimination and binding to the vector protein. However, if the pharmacokinetic data were available, it would be useful to calculate the drug *in vitro* concentration. In the present study, two doses of metformin (50 and 250 μg/ml) were used according to the formula: Drug *in vitro* concentration (μg/ml)=60x clinical drug concentration (mg/kg/d)x2x10^3÷5,000. The results demonstrated that metformin inhibited NF-κB activation in LPS-induced 16HBE cells in a dose-dependent manner.

In the present study, to the best of our knowledge, it was demonstrated for the time that metformin alleviated LPS-induced airway epithelial inflammation via inhibition of NF-κB activation. These results indicated that metformin alleviated LPS-induced 16HBE cells inflammation and was a modulator of NF-κB signaling. Therefore, metformin may have the potential to treat inflammatory airway diseases, including asthma. Indeed, there are a number of pathophysiological mechanisms that occur during the development and progression of asthma, and it is impossible to explain by several inflammatory cytokines or single pathways alone, but numerous fundamental studies may be the way to investigate the truth and their use should be considered.

Therefore, in future studies, the authors will focus on other inflammatory immune cells and signaling pathways that participate in asthma development, potentially leading to clinical research to confirm the relevance of metformin in asthma therapy.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JS performed the experiments, analyzed data, interpreted results, and wrote the manuscript. NH performed experiments and analyzed data. HZ conducted the research. KL and WM participated in the study design and coordination and helped review the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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