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Yanghe Pingchuan granules mitigates oxidative stress and inflammation in a bronchial asthma rat model: role of the IKK/IκB/NF-κB signalling pathway

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Background: Bronchial asthma (BA) is a chronic inflammatory airway disease. Previous research has shown that Yanghe Pingchuan granules (YPG), among the granules formulated by the First Affiliated Hospital of the Anhui University of Chinese Medicine, exerts a precise therapeutic effect on BA. We previously showed that YPG improves airway inflammation in BA rats. Other studies have shown that the inhibitor of kappa-B kinase (IKK)/inhibitor of NF-κB (IκB)/nuclear factor kappa-B (NF-κB) signalling pathway plays a key role in inflammation mediation. Therefore, this study explored whether YPG could intervene in BA through the IKK/IκB/NF-κB signalling pathway.

Methods: Ovalbumin-induced method was used to established BA rat model. After successful modelling, the authors used YPG to intervene the rats in BA rats. Hematoxylin-eosin (HE) staining was used to detect the bronchial pathological changes in BA rats, enzyme-linked immunosorbent assay (ELISA) was used to detect the changes of inflammatory factors (IL-1β and IL-6) and oxidative stress indexes malondialdehyde (MDA), superoxide dismutase (SOD) and nitrogen monoxide (NO), Quantitative real-time polymerase chain reactionCR and western blot were used to detect the expression of IKK/IκB/NF-κB signalling pathway.

Results: In BA model rats, YPG significantly improved the inflammatory response in bronchial tissues, reduced inflammatory factors IL-1β and IL-6, alleviated oxidative stress, reduced MDA and NO, and increased SOD. Quantitative real-time polymerase chain reaction and western blot results showed that YPG could block the IKK/IκB/NF-κB signalling pathway.

Conclusion: These findings showed that YPG had a definite therapeutic effect on BA, which may be related to blocking the IKK/IκB/NF-κB signalling pathway and improving inflammation and oxidative stress.

Keywords: bronchial asthma, Yanghe Pingchuan granules, IKK/IκB/NF-κB signalling pathway, inflammation, oxidative stress

Introduction

Bronchial asthma (BA) is a chronic respiratory syndrome characterized by cough, breathlessness and wheezing symptoms[1]. Current global prevalence is 4.3% and global incidence is expected to reach 400 million by 2025[2]. BA is characterized by continuous airway inflammation, airway hyperresponsiveness and airway remodelling[3]. Airway inflammation is considered to be an important factor in causing BA. Therefore, controlling airway inflammation is important to treating BA[4]. Research has suggested that airway smooth muscle (ASM) responses play an important role in BA and that ASM contraction (ASMC) leads to bronchoconstriction[5]. Proinflammatory mediators secreted by ASMCs also perpetuate airway inflammation[6]. Understanding the mechanisms involved in airway damage, especially inflammation, is therefore important for discovering effective therapeutic approaches for BA.

The nuclear factor kappa-B (NF-κB) signalling pathway is important to the inflammatory response, antigen presentation of immune cells and stimulation of humoral and cellular immunity, among which the most important is activation of the IKK/IκB/NF-κB pathway. The NF-κB family consists of: NF-κB1 (p105/p50); NF-κB2 (100/p52); RelA (p65); Relb and cRel[7]. Activation of the trimeric IκB kinase (IKK) complex is an important step in the canonical NF-κB activation pathway[8]. The IKK complex, composed of NF-κB essential modulator and the IKKα and IKKβ subunits, phosphorylates the inhibitory IκB proteins, including IκB-α, leading to their proteasomal degradation[9]. NF-κB,

HIGHLIGHTS

- Traditional Chinese medicine Yanghe Pingchuan Granule (YPG) can regulate inflammation and oxidative stress to improve asthma symptoms.
- The mechanism of YPG regulating inflammation and oxidative stress may be related to IKK/IκB/NF-κB signalling pathway.

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a potent inflammatory mediator, enhances cytokines in several disorders, including BA[10]. IκB phosphorylation activates the NF-κB p65 subunit (NF-κB-p65), which is responsible for increased cytokine production and inflammatory cascade promotion[11]. Inflammatory mediators interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are commonly associated with respiratory diseases, and mark dendritic cells maturation regulation and neutrophil accumulation promotion[12,13]. Among nitric oxide (NO)-regulated mediators, NF-κB is a key airway inflammatory response mediator. NO may inhibit NF-κB activation via a feedback mechanism. MDA expression can be measured to reflect the degree of lipid peroxidation, and is often combined with superoxide dismutase (SOD) measurement. Oxygen free radical scavenging can be indirectly measured by SOD activity.

NF-κB activation occurs in important sites (e.g., airway epithelial cells, submucosal cells and smooth muscle cells) in patients with asthma. NF-κB pathway activation can produce large numbers of cytokines, inflammatory mediators and enzymes (including TGF-β1, TNF-α, MMP-9 and IL-4/5/13) involved in the pathological mechanism of asthma, which can lead to inflammatory responses, inducing acute BA exacerbation. Therefore, the IKK/I-κB/NF-κB signalling pathway plays an important role in BA pathogenesis[14,15].

Yanghe Pingchuan granules (YPG), a medication prepared at the First Affiliated Hospital of the Anhui University of Chinese Medicine[16], is composed of Ephedra sinica Stapf., Rehmannia glutinosa (Gaertn.) DC, Inula japonica Thunb., Morinda officinalis F.C.How, Schisandra chinensis (Turcz.) Baill., Sinapis alba L., Draba nemorosa L., Angelica sinensis (Oliv.) Diels and Platycodon grandiflorus (Jacq.) A.DC. YPG are known to invigorate the kidney, activate the blood, resolve phlegm and relieve cough and asthma[17,18], and have been used in clinical BA treatment for many years, with clear curative effects.

We previously confirmed[19] that YPG exerts a definite therapeutic effect on airway remodelling and airway inflammation in a rat BA model, and have described quality control experiments for these granules. However, it remains unclear whether YPG can reduce airway inflammation by regulating the IKK/IκB/NF-κB signalling pathway. Therefore, we applied a series of detection methods to investigate the IKK/IκB/NF-κB signalling pathway mechanisms underlying the effects of YPG in BA.

Materials and methods

Chemicals and instruments

The YPG was prepared by the First Affiliated Hospital of the Anhui University of Chinese Medicine, batch number: 20201011[19]. Guilong Kechuanning capsules were provided by Guilong Pharmaceuticals (Anhui Co. Ltd., Ma Anshan). NF-κB and ovalbumin (OVA) were provided by Sigma-Aldrich (MO, USA). The RNA extraction kit and fluorescence quantitative polymerase chain reaction (PCR) kit were provided by Sangong Bioengineering (Shanghai Co., Ltd.). We also used a real-time PCR instrument (Roche, Switzerland, 480II) and an ultraviolet spectrophotometer (Nanodrop2000; Thermo, USA).

Animal model

Specific pathogen-free male Sprague Dawley rats, weighing 220 ± 30 g, were provided by Anhui Medical University (Production license: SCXK (Anhui) 2017-001). The rats were randomly divided into six groups: normal group (N), model group (M), YPG low dose group (YPG L), YPG middle dose group (YPG M), YPG high dose group (YPG H) and contrast group (Aminophylline tablets). The BA model was established according to previous reports[20,21] using 10% OVA (100 mg OVA + 100 mg aluminium hydroxide + 1 ml saline) once every 7 days from days 1–14. Every 2 days during days 15–30, rats in the non-saline groups were stimulated with 1% atomized OVA for 30 minutes. Rats in the normal group received saline. Rats in the OVA group were orally gavaged with YPG L (3.69 g/kg), YPG M (7.38 g/kg), YPG H (14.76 g/kg), or Aminophylline (50 mg/kg), respectively, 30 min before OVA stimulation (normal group rats again received saline). Rats were sacrificed and intraperitoneally injected with 2% pentobarbital sodium on day 36.

All animal experiments were approved by the Animal Experiment Ethics Committee of Anhui University of Chinese Medicine (license number: AHUCM-rats-2022093).

Histopathology

Lung tissue from the same part of the right lung was fixed in 4% paraformaldehyde. The sample was rinsed under running water, dehydrated in gradient ethanol solution, and then transferred to xylene solution. Sections were then cut at 4 μm and stained with hematoxylin and eosin for routine histological examination. The slices were dewaxed in xylene, then transferred to gradient ethanol solution for hydration, hematoxylin staining solution for 5–10 minutes, and 0.5–1% hydrochloric acid alcohol (70% alcohol preparation) for colour separation. After running water rinse, transfer to 0.1–0.5% eosin dye for 1–5 min, and gradient ethanol dehydration[22]. The pathological characteristics of the synovium in each group were observed under a microscope.

Masson's staining

We used 4% paraformaldehyde to fix tissues (55°C, 1 h). Masson's trichrome (1%) staining was used to determine the extent of bronchial fibrosis (5–10 min, 55°C)[23]. Then, we evaluated the extent of fibrosis in the lungs and bronchial tissue with an optical microscope and performed pathological grading.

Enzyme-linked immunosorbent assay

Elisa kits (ELISA LAB, China) were used to measure IL-6, IL-1β, NO, SOD and malondialdehyde (MDA) levels in rat serum, according to the manufacturer's instructions[24]. OD values were read at 450 nm, and IL-6, IL-1β, NO, SOD and MDA levels were determined based on standard curves.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was applied to measure IKK-β, IκB- α and NF-κB-p65 mRNA levels. Total RNA samples were from the right lung tissues, seeded in a six-well culture plate at a cell density of 1×10^6 cells/well for 24 h of incubation and exposed to lysis buffer (Science Biotech, China). Then, the OD 260/280 nm value of total RNA was determined by UV spectrophotometer to determine the mRNA concentration. mRNA

Table 1
Primer sequences

Fragment	Primer sequence
IKK-β	Forward, 5′ -ACCAGAATCCGGGAAGACACAG-3′
	Reverse, 5′-AGACGAGATCCATGTCCAGTGTG-3′
IκB-α	Forward, 5′ -TGGCCAGTGTAGCAGTCTTGAC-3′
	Reverse, 5′ -ATCAGCACCCAAAGTCACCAAG-3′
NF-κB-p65	Forward, 5′ -CACAGATACCACTAAGACGCACC-3′
	Reverse, 5′ -AGTCCTTCCCCACAAGTTCATG-3′
β-actin	Forward, 5′ -GACGTTGACATCCGTAAAGACC-3′
	Reverse, 5′ -CTAGGAGCCAGGGCAGTAATCT-3′

was reverse-transcribed into cDNA with the cDNA reverse transcription kit, according to the manufacturer's instructions. qRT-PCR was performed according to the LightCycler 480 II system (Roche Diagnostics GmbH, Germany) using 2 × S6 Universal SYBR qPCR Mix (EnzyArtisan, China). The thermal cycling conditions included: pre-denaturation at 95°C for 30 sec; denaturation at 95°C for 10 sec; annealing and extension at 60°C for 30 sec for 45 cycles; and extension at 72°C for 5 min. The amplification target genes were IKK-β, IκB-α and NF-κB-p65, with β-actin as the internal reference gene. The $2^{-\Delta\Delta Ct}$ method was applied to normalize the relative mRNA level. All primer sequences were devised and synthesized by EnzyArtisan (Shanghai, China). The primers sequences are shown in Table 1.

Western blot

Western blot was used to analyze the expressions of IKK-β, IκB-α and NF-κB-p65 proteins and phosphorylated proteins. Total protein from right lung tissue were seeded in a six-well culture plate at a cell density of 1 × 106 cells/well for 24 h of incubation, exposed to RIPA lysis buffer and quantified using the BCA protein assay kit (Leagene, Beijing, China). Protein samples were electrophoresed on 10% polyacrylamide gels at 120 V for 60–90 min, then transferred to a PVDF membrane at 100 mA for 2 h. The membrane was blocked with 5% nonfat milk in TBST for 2 h and incubated with primary antibodies. The protein band was then transferred to secondary antibodies and incubated at room temperature for 1.5 h. Target protein levels were normalized to β-actin and quantitative protein expression analysis was carried out using Image-J software.

Statistical analysis

All analyses were processed with SPSS 23.0 statistical software. Experimental values are expressed as mean ± standard deviation (SD), and one-way ANOVA was used to test for significant between-group differences. Differences were considered significant at P less than 0.05 or P less than 0.01.

Results

Pathological changes

YPG-induced changes in BA model bronchial pathology are shown in Figure 1. There were no obvious pathological changes in the bronchial tissue of the normal group. Compared with the normal group, the BA model rats had large inflammatory cell infiltrations around the bronchial wall, along with a series of pathological changes, including microvascular leakage, proliferations of goblet cells and airway epithelial cells, smooth muscle layer thickening and bronchial mucosa thickening and elongation. After YPG treatment, inflammatory cells like eosinophil and lymphocyte infiltrations were reduced significantly, the tube wall and smooth muscle layer thicknesses were close to those of the normal group, and the bronchial mucosa structure was largely improved. The therapeutic effect was related to YPG dose. Likewise, the positive control group showed significant improvement.

Bronchial fibrosis and collagen deposition

Masson's trichrome staining results are shown in Figure 2. Normal group lung tissue was normal, with little increase in

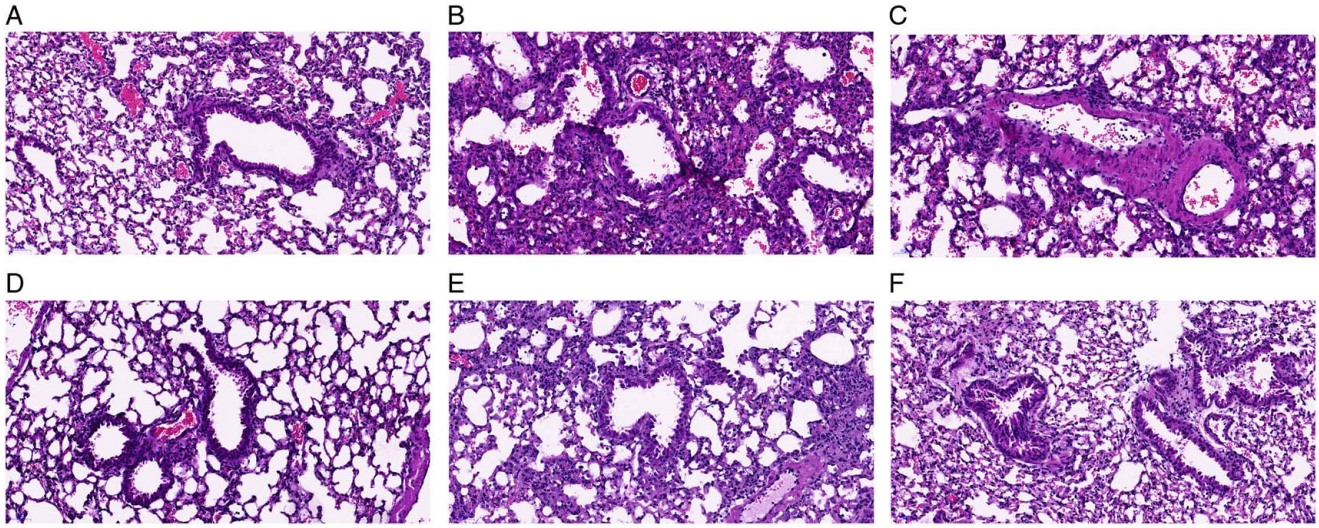

Figure 1. Effect of Yanghe Pingchuan granules (YPG) on histopathologic changes in lungs of an ovalbumin -induced asthma rat model. HE staining was used to detect the specific performance of lung tissue in each group. Magnification, × 400; scale bars, 50 μm. Normal group (A), model group (B), YPG L group (C), YPG M group (D), YPG H group (E), Aminophylline group (F).

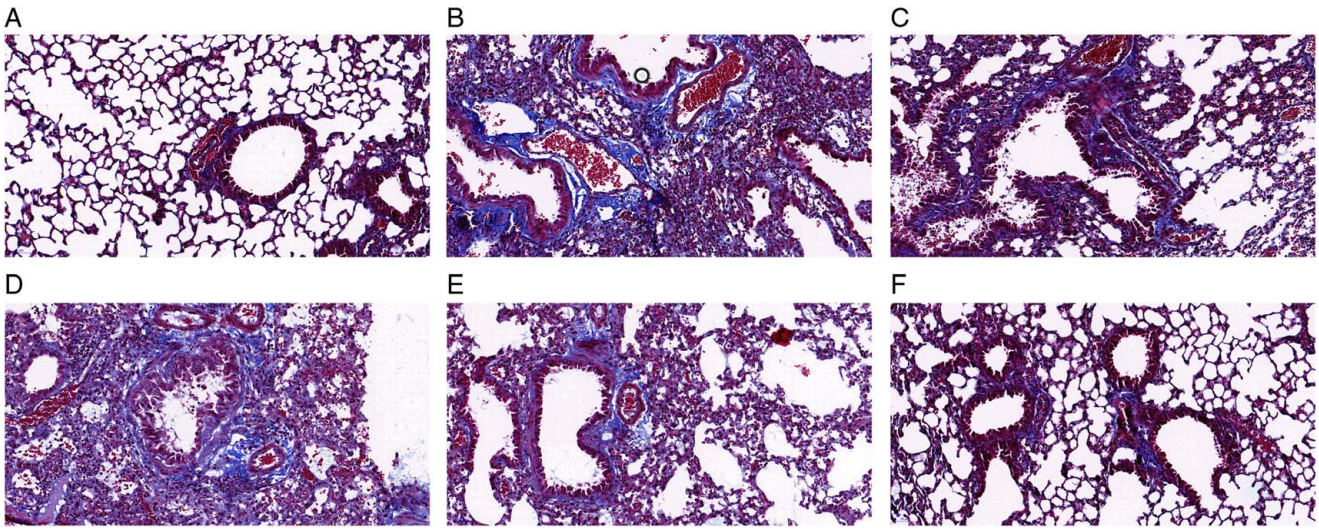

Figure 2. Effect of Yanghe Pingchuan granules (YPG) on bronchial fibrosis in an ovalbumin -induced asthma rat model. Masson staining was used to detect pulmonary fibrosis in each group. Magnification, ×400; scale bars, 50 μm. Normal group (A), model group (B), YPG L group (C), YPG M group (D), YPG H group (E), Aminophylline group (F).

precipitation of collagen fibres (blue) or muscle fibres. Compared with the normal group, fibrosis in model group lung tissue, and expressions of red and blue precipitates, were significantly increased. After drug treatment, lung tissue fibrosis in each administration group was significantly improved, and the therapeutic effect was correlated with YPG dose; the positive drug group had a better therapeutic effect.

YPG effects on inflammatory factors

Expressions of inflammatory factors IL-1β and IL-6 in BA rat model before and after YPG intervention are shown in Figure 3. Compared with the normal group, IL-1β and IL-6 expression in the model group was significantly increased ($P < 0.01$). After YPG treatment, IL-1β and IL-6 expression in each administration group was significantly downregulated ($P < 0.01$). YPG decreased IL-1β and IL-6 expression in a dose-dependent manner. Aminophylline in the positive control group had better therapeutic effect.

YPG effects on oxidative stress

Results of NO, MDA and SOD ELISA analyses are shown in Figure 4, reflecting YPG effects on oxidative stress in BA model rats. Compared with the normal group, NO and MDA expressions in the model group were significantly increased, and antioxidant SOD expression was significantly decreased, indicating severe oxidative stress. After YPG treatment, NO and MDA contents were significantly downregulated, while SOD content increased, and the regulatory effects were positively correlated with YPG dose. These results indicate that YPG may control oxidative stress in a BA rat model.

YPG effects on the IKK/IκB/NF-κB signalling pathway

qRT-PCR was used to detect IKK-β, IκB-α and NF-κB-p65 mRNA levels. Western blot was used to detect protein and phosphorylated protein expressions. As shown in Figure 5, compared with the normal group, IKK-β and NF-κB-p65 mRNA expression levels in the model group were significantly increased, and IκB-α was decreased. After YPG and aminophylline

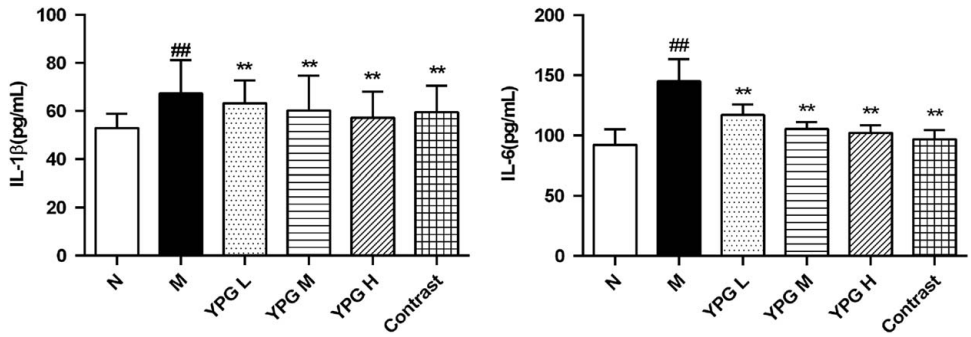

Figure 3. Effect of Yanghe Pingchuan granules (YPG) on pulmonary inflammation in bronchial asthma (BA) rats. ELISA was used to detected the expression of IL-6 and IL-1β in serum of rats in each group. Normal group (N), Model group (M), YPG L group (YPG L), YPG M group (YPG M), YPG H group (YPG H), Aminophylline group (Contrast). n = 8. $^{\#\#}P < 0.01$ vs normal group (N). $^{**}P < 0.01$ vs model group (M).

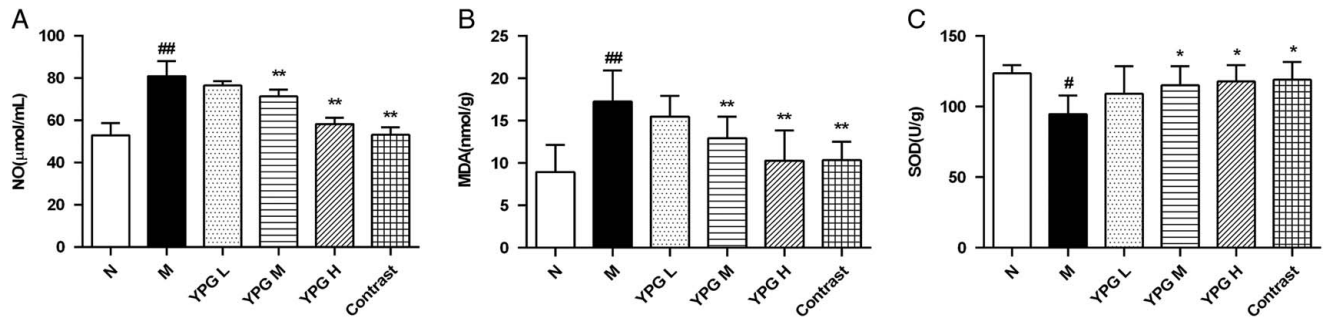

Figure 4. Regulation of pulmonary oxidative stress by Yanghe Pingchuan granules (YPG) in bronchial asthma (BA) rats. ELISA was used to detected the expression of NO, MDA and SOD in serum of rats in each group. (A) The level change of NO. (B)The level change of MDA. (C)The level change of SOD. Normal group (N), Model group (M), YPG L group (YPG L), YPG M group (YPG M), YPG H group (YPG H), Aminophylline group (Contrast).n = 8. $^{\#\#}P < 0.01$ vs normal group (N), $^{**}P < 0.01$ vs model group (M), $^{*}P < 0.05$ vs model group (M).

treatment, compared with the model group, each administration group showed significantly decreased IKK-β and NF-κB-p65 expressions and increased IκB-α level, and the regulatory effect was positively correlated with dose. Protein expression results were consistent with those of mRNA expression (Fig. 6).

Discussion

BA is a chronic airway inflammatory disease involving a variety of inflammatory cells and inflammatory mediators. Asthma onset can cause airway stenosis and obstruction, which lead to corresponding symptoms like shortness of breath, chest tightness and coughing[25]. During asthma pathogenesis, cytokines and other inflammatory mediators and inflammatory cells form a complex inflammatory system[25]. Therefore, reducing the release of inflammatory factors and controlling airway inflammation is an effective BA treatment method.

BA pathogenesis is also characterized by inflammatory phenomena, and the involvement of the NF-κB signalling pathway in various inflammatory diseases has been confirmed. NF-κB is a well-studied transcription factor that plays an important role in the airway inflammation process, including amplification of the cascade reaction[26]. NF-κB binds to IκB in the cytoplasm in an inactive state. When IκB is phosphorylated by the IKK complex, NF-κB is released and translocated into the nucleus, triggering multiple intracellular inflammatory signalling pathways[27,28]. The IKK complex consists of two kinases, IKKα and IKK-β, which are essential for NF-κB transcription factor activation. Between them, IKK-β is the major in vivo IKK, which is essential for activating nuclear translocation of canonical NF-κB heterodimers[29]. NF-κB activation can promote inflammatory factor expressions, including NO, COX-2 and IL-6, and Th2 cytokine (IL-4, IL-5 and IL-13) secretions in allergic airway inflammation[30].

IL-6, TNF-α and IL-1β are common inflammatory mediators associated with lung disease; these markers regulate dendritic cell maturation and promote neutrophil accumulation at inflammation sites. NF-κB can regulate proinflammatory cytokine release, including IL-6 and TNF-α[31,32]. NO is an endogenous gaseous signal mainly generated by NO synthase[33], which regulates bronchial inflammation and vascular smooth muscle relaxation. Studies have shown that NO expression levels in lung diseases like asthma, chronic obstructive pulmonary disease, pulmonary fibrosis and cancer differ significantly from normal. NO is involved in regulating various signalling pathways related to transcription factor activation and expression, as well as post-translational regulation of various inflammatory mediator activities. Among NO-regulated mediators, the NF-κB signalling pathway is a key airway inflammatory response mediator[34]. An extensive literature demonstrates that oxidative stress imbalance is closely related to the pathological process of neutrophilic asthma. Excessive reactive oxygen species can reduce the efficacy of glucocorticoids in neutrophilic asthma and stimulate histamine release, resulting in

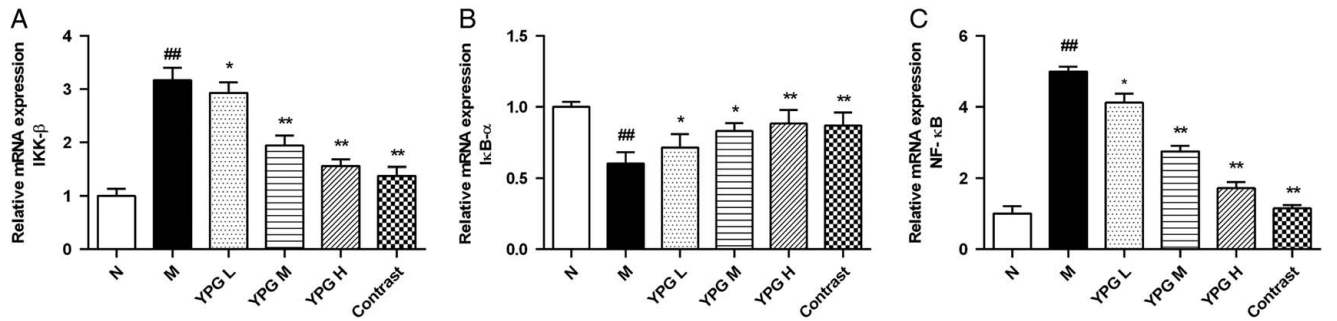

Figure 5. Yanghe Pingchuan granules (YPG) effect on the IKK/IκB/NF-κB signalling pathway in bronchial asthma (BA) rat model. qRT-PCR was used to detected the mRNA levels of IKK, IκB and NF-κB in BA rats. (A) The level change of IKK, (B)The level change of IκB-α, (C)The level change of NF-κB. Normal group (N), Model group (M), YPG L group (YPG L), YPG M group (YPG M), YPG H group (YPG H), Aminophylline group (Contrast). $^{\#\#}P < 0.01$ vs normal group (N), $^{**}P < 0.01$ vs model group (M), $^{*}P < 0.05$ vs model group (M).

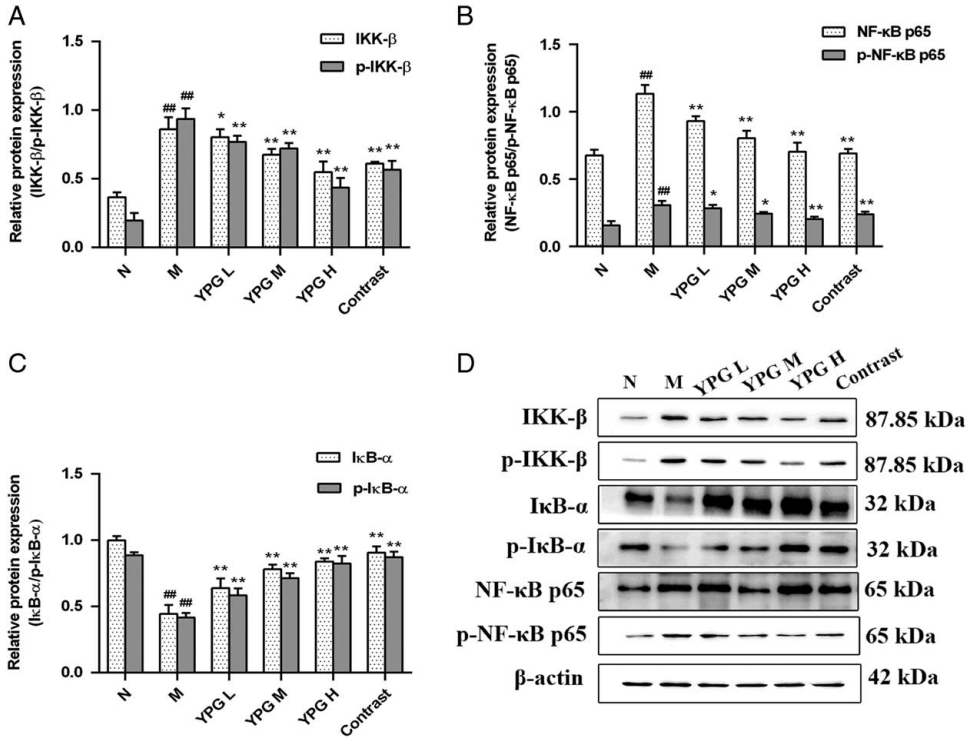

Figure 6. Yanghe Pingchuan granules (YPG) effect on IKK/IκB/NF-κB signalling pathway expression in (BA) rat model. WB was used to detected the protein levels of IKK, IκB and NF-κB in BA rats. Normal group (N), Model group (M), YPG L group (YPG L), YPG M group (YPG M), YPG H group (YPG H), Aminophylline group (Contrast). $^{\#\#}P < 0.01$ vs normal group (N), $^{**}P < 0.01$ vs model group (M), $^{*}P < 0.05$ vs model group (M).

endothelial cell damage and shedding, disrupting the function of β-adrenergic receptors, and thus aggravating airway remodelling, increasing respiratory resistance and aggravating asthma. MDA expression reflects the degree of systemic lipid peroxidation, and is often combined with SOD measurement. SOD, an important antioxidant enzyme and oxidative stress marker, plays an important role in scavenging free radicals, preventing cell damage and promoting oxidation and antioxidant balance.

Our previous YPG experiment series included quality control and basic pharmacodynamics. We determined that YPG exerts a specific therapeutic effect on BA and that its mechanism of action may be related to PI3K/PKB signal pathway regulation. Interestingly, previous studies also showed that YPG has a regulatory effect on the BA rat model inflammatory response. The next step was to clarify the mechanism by which YPG regulates the inflammatory response and oxidative stress in asthma treatment. Thus, we focused herein on the IKK/IκB/NF-κB signalling pathway.

These experiments showed that YPG significantly improves inflammation and oxidative stress BA rat model bronchus, indicating that YPG exerts a BA therapeutic effect by inhibiting the inflammatory response. YPG also significantly inhibits expressions of key genes and proteins, including IKK-β, IκB-α and NF-κB-p65 in the IKK/IκB/NF-κB signalling pathway, indicating that YPG can inhibit the inflammatory response and oxidative stress in BA model rats, possibly by regulating the IKK/IκB/NF-κB signalling pathway.

This study provides a new idea for the treatment of asthma by traditional Chinese medicine. Based on the advantages of multi-components and multi-targets of traditional Chinese medicine, the research group can optimize the prescription composition of YPD by using bioinformatics technology such as network pharmacology in the later stage in order to improve the therapeutic effect of YPD on asthma and reduce side effects.

There are some limitations to this experiment. Although the expression changes of NO, MDA and SOD, which are indicators of oxidative stress, were detected, the underlying mechanism of YPG regulating oxidative stress through IKK/IκB/NF-κB signalling pathway still needs to be further studied.

Conclusion

Results showed that the regulation of inflammatory response and oxidative stress by YPG may be closely related to blocking the IKK/IκB/NF-κB signalling pathway. The anti-inflammatory effect of YPG may bring new inspiration to the treatment of asthma with traditional Chinese medicine.

Ethical approval

Inapplicability.

Consent

The present study followed Anhui university of traditional chinese medicine guidelines for humane animal treatment and complied with relevant legislation;

Source of funding

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Author contribution

Y.H., Y.W.: study concept or design. X.D., L.P.: data collection. C.G., Y.C.: data analysis or interpretation. Y.J., Y.S.: animal experiment. B.H.: writing the paper.

Conflicts of interest disclosure

The authors declare that they have no competing interests.

Research registration unique identifying number (UIN)

No human studies were involved in this study.

Guarantor

None.

Data availability statement

The data used to support the findings of this study are included within the article.

Provenance and peer review

None.

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