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**CRedit authorship contribution statement**

**Hong Jiang:** Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing - review and editing. **Ziyu Bai:** Investigation, Formal analysis, Writing - original draft. **Yongyu Ou:** Formal analysis, Writing – original draft. **Huiling Liu:** Investigation. **Zilin Si:** Investigation. **Yafang Liu:** Formal analysis. **Xiaoqiong Liu:** Formal analysis. **Xiaoqing Liu:** Conceptualization. **Zhihao Zhang:** Conceptualization, Writing - review and editing, Supervision. **Ninghua Tan:** Conceptualization, Resources, Writing - review and editing, Supervision, Project administration.

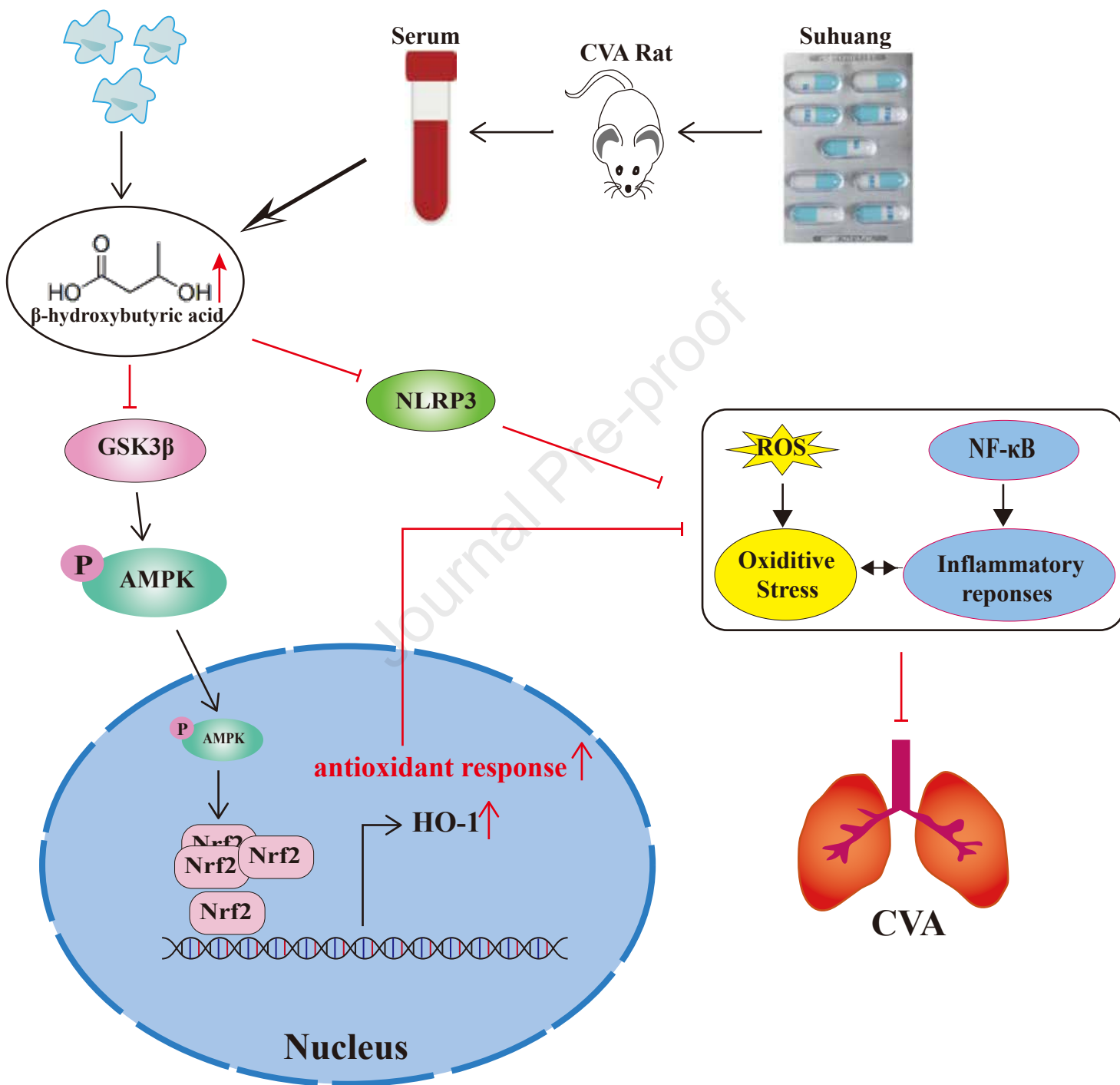
LPS-induced  
BMDM cells

Journal Pre-proof

Serum

CVA Rat

Suhuung



**$\beta$ -Hydroxybutyric acid upregulated by Suhuang antitussive capsule ameliorates cough  
variant asthma through GSK3 $\beta$ /AMPK-Nrf2 signal axis**

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**ABSTRACT**

*Ethnopharmacological relevance:* Cough variant asthma (CVA) is a chronic inflammatory disease characterized by cough as the main symptom. Suhuang antitussive capsule (Suhuang), one of traditional Chinese patent medicines, mainly treats CVA clinically. Previous studies have shown that Suhuang significantly improved CVA, post-infectious cough (PIC), sputum obstruction and airway remodeling. However, the effect of Suhuang on ovalbumin-induced (OVA-induced) metabolic abnormalities in CVA is unknown.

*Aim of the study:* This study aimed to identify potential metabolites associated with efficacy of Suhuang in the treatment of CVA, and determined how Suhuang regulates metabolites, and differential metabolites reduce inflammation and oxidative stress.

*Materials and methods:* Rats were given 1 mg OVA/100 mg aluminum hydroxide in the 1st and 7th days by intraperitoneal injection and challenged by atomizing inhalation of 1% OVA saline solution after two weeks to establish the CVA model. Rats were intragastrically (i.g.) administrated with Suhuang at 1.4 g/kg and  $\beta$ -hydroxybutyric acid ( $\beta$ -HB) were given with different concentrations (87.5 and 175 mg/kg/day) by intraperitoneal injection for 2 weeks. After 26 days, GC-MS-based metabolomic approach was applied to observe metabolic changes and search differential metabolites. The number of coughs, coughs latencies, enzyme-linked immunosorbent assay (ELISA), histological analysis and quantitative-polymerase chain reaction (Q-PCR) were used to investigate the effects of Suhuang. Then  $\beta$ -HB on CVA rats, NLRP3 inflammasome and GSK3 $\beta$ /AMPK/Nrf2 signalling pathway were detected by western blotting.

*Results:* The results showed that Suhuang treatment significantly enhanced the serum level of  $\beta$ -HB. Interestingly, exposure to exogenous  $\beta$ -HB was also protective against OVA-induced CVA.  $\beta$ -HB significantly reduced the number of coughs and lengthened coughs latencies, improved lung injury, reduced the secretion of various cytokines, and directly inhibited the NLRP3 inflammasome. In addition,  $\beta$ -HB increased the nuclear accumulation of Nrf2 by activating the GSK3 $\beta$ /AMPK signaling axis, and then inactivating the NF- $\kappa$ B signaling pathway, effectively protecting OVA-induced CVA from oxidative stress and inflammation.

*Conclusions:* The results of this study shows that  $\beta$ -HB can reduce inflammation and oxidative stress, the increased production of  $\beta$ -HB in serum might be the crucial factor for Suhuang to

exert its effect in the treatment of CVA.

**Key words:** Suhuang antitussive capsule; Pulmonary dysfunction; Cough variant asthma; Metabolomics;  $\beta$ -Hydroxybutyric acid; GSK3 $\beta$ /AMPK/Nrf2

**Chemical compounds studied in this article:** Dexamethasone (PubChem CID: 5743); Lipopolysaccharide (PubChem CID: 11970143); Ovalbumin (PubChem CID: 56832351);  $\beta$ -Hydroxybutyric acid (PubChem CID: 92135 ); Compound C (PubChem CID: 11524144); Trigonelline (PubChem CID: 5570); Sodium nitroprusside (PubChem CID:11953895).

## Abbreviations

CVA: cough variant asthma; BMDM: Bone-marrow-derived macrophage; Dex: dexamethasone; BALF: broncho alveolar lavage fluid; *i.g.*: intragastrically; *i.p.*: intraperitoneally; LPS: lipopolysaccharide; OVA: ovalbumin; ELISA: enzyme-linked immunosorbent assay; CC: compound c; Trig: trigonelline; SNP: sodium nitroprusside; NLRP3: NOD-like receptor family pyrin domain containing-3; Nrf2: nuclear factor erythroid 2-related factor 2, HO-1: heme oxygenase 1; AMPK: adenosine 5'-monophosphate activated protein kinase; GSK3 $\beta$ : glycogen synthase kinase 3 beta.

## 1. Introduction

Asthma is one of the predisposing chronic diseases, affecting 339 million people globally (Johnson et al., 2022). It is characterized as a chronic pulmonary disease with mucus hypersecretion, airway hyperresponsiveness and inflammation (Xu et al., 2021). Cough variant asthma (CVA) is considered to be a precursor of classic asthma (Saito et al., 2015), which is characterized by eosinophilic inflammation of the whole lower airways (Niimi, 2021). Lung inflammation could lead to pulmonary dysfunction. CVA in traditional Chinese medicine disease differentiation and syndrome differentiation can belong to the category of "wind cough", the treatment is based on the principle of wind drainage, spasmolysis and cough relief (Bu et al., 2016).

Suhuang antitussive capsule (Suhuang) is a widely used traditional Chinese patent

medicine for treating CVA and post-cold cough (PIC) in China, which was formed based on the theory of "wind cough" (Lai et al., 2018). It contains nine traditional Chinese medical herbs (Qin et al., 2021). As shown in Table S1, these original plant species were authenticated by Professor Ninghua Tan (Department of TCMs Pharmaceuticals, China Pharmaceutical University, Nanjing, China). Suhuang has been shown multiple pharmacological effects such as immunomodulatory property, and attenuation airway inflammation/hyperresponsiveness/remodeling (Wang et al., 2018; Bu et al., 2016). Furthermore, in our previous studies, the main components and quality control fingerprint of Suhuang were studied (Li et al., 2017; Liu et al., 2019; Wu et al., 2020). Suhuang inhibited NLRP3 inflammasome activation and endoplasmic reticulum stress to protect CVA (Qin et al., 2019). Suhuang preserved mitochondrial homeostasis to ameliorate the unresolved inflammatory response by inactivating NF- $\kappa$ B signal and NLRP3 inflammasome (Qin et al., 2021). Simultaneously, Suhuang treated sputum obstruction by EGFR-ERK signaling and up-regulating hepatocyte growth factor (HGF) secretion by colon-dependent means (Tong et al., 2019). Suhuang attenuated airway remodeling via inhibiting JAK2/STAT3 signaling pathway (Guo et al., 2021). Besides, Suhuang ameliorated PIC via AhR-Nrf2 pathway (Liang et al., 2022). However, the metabolomic change of Suhuang in the treatment of CVA remains unclear.

Metabolomics has the potential to elucidate potential metabolic pathways and discover key metabolites and their associations with disease. Previous studies showed that fatty acid, amino acid and phosphatidylcholine metabolism were altered in ovalbumin-induced (OVA-induced) allergic asthma (Hsu et al., 2021; Shou et al., 2019).

In this study, in order to identify potential metabolites associated with efficacy of Suhuang in the treatment of OVA-induced CVA rats, we used GC-MS-based metabolomic approach to profile serum metabolites. We found that the serum level of  $\beta$ -hydroxybutyric acid ( $\beta$ -HB) was remarkably decreased in OVA-induced mice, while Suhuang treatment significantly increased the level of  $\beta$ -HB. It has been reported that  $\beta$ -HB, an endogenous metabolite, exhibits protective effects in a variety of diseases, such as acute kidney injury (Tajima et al., 2019), liver injury (Miyauchi et al., 2019), cancer (Feng et al., 2019), aging and senescence processes (Fang et al., 2021) and diabetes-related complications (Trotta et al., 2019). In addition,  $\beta$ -HB possesses multiple pharmacological activities, such as anti-inflammatory, antioxidant and

immunomodulatory property (Shimazu et al., 2013; Youm et al., 2015). However, the role of  $\beta$ -HB on CVA has barely been clarified.

In this study, our results showed protective effect of  $\beta$ -HB on OVA-induced rats was relevant to the regulation of the GSK3 $\beta$ /AMPK-Nrf2 axis and NLRP3 inflammasome function.  $\beta$ -HB could directly inactivated the NLRP3 inflammasome, and simultaneously activated AMPK and inhibited GSK3 $\beta$  to up-regulate the Nrf2 pathway and inactivated the NF- $\kappa$ B signaling pathway for anti-inflammatory and antioxidant effects.

## 2. Materials and methods

### 2.1 Reagents and chemicals

Suhuang antitussive capsule (Suhuang) (China Standard of National Drug, YBZ00172008) was provided by Beijing Haiyan Pharmaceutical Co., Ltd., Yangtze River Pharmaceutical Group (Beijing, China).  $\beta$ -HB (Cat.# 300-85-6), purity > 95%, was purchased by the J&K Scientific (Beijing, China). Dexamethasone (Dex, Cat.# 190703) was purchased by Zhejiang Xianju Pharmaceutical Co., Ltd (Hangzhou, China). Lipopolysaccharide (LPS, Cat.# 297-473-0) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Compound C (Cat.# 866405-64-3) and trigonelline (Cat.# 535-83-1) (CC and Trig, specific inhibitors of AMPK and Nrf2, respectively) and sodium nitroprusside (SNP, Cat.# 14402-89-2) were offered by MedChemExpress (St. Newark, NJ, USA). OVA (Cat.# 9006-59-1) was purchased from Sigma-Aldrich (St. Louis, MO, USA). ELISA kits for rat COX-2, iNOS and IL-6 were purchased from Cusabio Biotech (Wuhan, China). Antibodies for Nrf2, HO-1, NLRP3, casapase-1 (P20), IL-1 $\beta$ , Lamin B and GAPDH were supplied by Proteintech (Chicago, IL, USA) or Abcam (Cambridge, MA, USA). Antibodies for p65, p-p65, IKB $\alpha$ , p-IKB $\alpha$ , AMPK, p-AMPK, GSK3 $\beta$ , p-GSK3 $\beta$  were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.).

### 2.2 Animals and experimental protocols

Male Wistar rats (220–260 g, 8-10 weeks) were offered by the Laboratory Animal Center of Nanjing Qinglongshan (Nanjing, Jiangsu, China). All animals were given free access to water

and food. CVA rat model was established according to our previous study (Qin et al., 2019). Briefly, Rats were sensitized by 1 mg OVA/100 mg aluminum hydroxide on days 1 and 7. From the 14th day, rats were kept by stimulating with 1% OVA saline solution for 14 days. For the first animal experiment, the rats were randomly divided into 4 groups and treated as follows: control group (n = 10), CVA group (n=10), CVA + Suhuang group (i. g., 7 g/kg, n = 10), CVA + Dex group (i. g., 0.5 mg/kg, n = 10). For the second animal experiment, the rats were randomly grouped into 6 groups and treated as follows: control group (n = 6), CVA group (n =6), CVA +  $\beta$ -HB-L group (i. p., 87.5 mg/kg , n = 6), CVA +  $\beta$ -HB-H group (i. p., 175 mg/kg , n = 6), CVA +  $\beta$ -HB-H +Trig group (i. p., 20 mg/kg , n = 6), CVA + Dex group (i. g., 0.5 mg/kg, n = 6). The optimal dosage of  $\beta$ -HB was explored in the pre-experiment. Challenge with OVA aerosol and drugs administration were done as a single daily dose from day 14 to day 26. The scheme of establishing the CVA rat model was shown in Fig. 1. All experimental animals used in this study were handled in accordance with the National Institutes of Health Guide for the Care and approved by the Ethics Committee of China Pharmaceutical University (Ethical clearance number: 2022-09-005).

### 2.3 Cells and culture conditions

Bone-marrow-derived macrophage cells (BMDMs) were isolated from Male C57BL/6 mice (18-20 g, 6-8 weeks) supplied by the Laboratory Animal Center of Nanjing Qinglongshan (Nanjing, Jiangsu, China) and cultured as described previously. BMDMs were obtained from the femurs and tibias, and resuspended in differentiation medium (DMEM, 10% FBS, 20% L929 conditional medium), maintained and cultured at 37 °C in a 5% CO<sub>2</sub> incubator for 7 days to harvest differentiated adherent cells (Heap et al., 2021).

### 2.4 Cell viability assay

BMDM cells viability in response to  $\beta$ -HB treatment were analyzed using the MTT assay following the manufacturer's protocol. The absorbance was detected with a microplate reader (Biotek Epoch, Winooski, VT, USA) at 490 nm.

### 2.5 H&E staining and immunohistochemistry

Lung tissues were embedded in paraffin and sectioned. H&E and Masson staining were used for the histological evaluation (Chen et al., 2021). Immunohistochemistry was performed for the tissue level of Nrf2, HO-1 and p65 (Oh et al., 2021). The images were captured by an optical microscope (Leica DMI8, Wetzlar, Heessen, Germany).

## 2.6 Immunofluorescence

BMDM cells were fixed with 4% paraformaldehyde, permeated with 0.3% Triton X-100 and then blocked with 5% BSA (Qiu et al., 2018). BMDM were further incubated with anti-Nrf2, anti-p65 antibodies at 4 °C overnight. After incubation with fluorescent secondary antibody, nuclei were stained with DAPI (Beyotime). The images were captured with a microscope (Leica DMI8).

## 2.7 Real-time quantitative PCR analysis

Total RNAs was extracted from the tissue homogenates and BMDM cells using Trizol reagent (Takara, Otsu, Japan) as directed by the specification. RNAs were reverse transcribed to cDNAs for quantitative PCR (Zhou et al., 2021). The primers sequences were listed in Table S2.

## 2.8 ELISA assay

BMDM cells culture supernatant and lung tissue homogenates were used to determine the protein content of COX-2, IL-6 and iNOS according to the ELISA kit requirements. The absorbance was measured with a microplate reader (Biotek Epoch, Winooski, VT, USA) at 450 nm.

## 2.9 Analysis of BALF

The rats were anesthetized, the right lung was ligated, and the left lung was lavaged three times with tracheal cannula with 2 ml PBS (Hu et al., 2019). The supernatant and precipitate of extracted BLAF were centrifuged at 1000rpm for 5min at 4°C for further detection (Jiang et al., 2016).

## 2.10 Western blotting analysis

The total proteins from the lung tissues or BMDM cells were exposed using RIPA buffer with phenylmethanesulfonyl fluoride (PMSF). The nuclear proteins from the lung tissues or BMDM cells were extracted using a detection kit (Beyotime, Shanghai, China). Equal amounts of the extracted proteins were subjected to SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Ji et al., 2016). The membranes were blocked in 5% non-fat milk in TBST for 2 h and continued to be incubated with primary antibody at 4 °C overnight. Next, the corresponding secondary antibodies were reacted with membranes at room temperature for 2 h, then the membranes were detected by a Tanon 5200 Multi chemiluminescent substrate system (Tanon, Shanghai, China).

## 2.11 RNA interference (RNAi) of Nrf2

Small interfering RNA (siRNA) was designed to target Nrf2 to reduce Nrf2 expression. Nrf2 siRNA was transfected into BMDM cells by using siRNA transfection reagent (22Y0302M2, Polyplus Transfection, France) (Liao et al., 2020). After 6 h of transfection, BMDM cells were pretreated with  $\beta$ -HB for 2 h, then treated with LPS (1  $\mu$ g/mL) for the appropriate time periods. The siRNA sequences of Nrf2 were listed in Table S3.

## 2.12 Intracellular ROS measurement

BMDM cells were seeded into confocal dish for 12 h incubation. After pretreating with  $\beta$ -HB for 1 h, cells stimulated with LPS (1  $\mu$ g/mL) for 6 h were incubated with DCFH-DA (10  $\mu$ M) for 30 min at 37 °C in the dark. Subsequently, the fluorescence intensity was measured with a confocal microscope.

## 2.13 Metabolomics methods for serum samples

Separations were performed on an Agilent 7890 chromatograph coupled with a 5977B MS system (Agilent Technologies, Santa Clara, CA, USA). Serum sample preparation and GC/MS analysis are described in detail in the supplementary material.

## 2.14 Statistical analysis

Data are presented as mean  $\pm$  SEM (GraphPad Prism 9.0). Data were analyzed by Student's t-test and one-way ANOVA. Statistical significance was analyzed by  $p$  values  $< 0.05$ . The raw GC-MS data were transformed for multivariate analysis (Zhang et al., 2018). OPLS-DA was used to visualize the clustering and trends, the differential metabolites were identified by variable importance in the projection (VIP  $> 1.0$ ),  $P$  values less than 0.05 and a library search (NIST and Fiehn).

### 3. Results

#### 3.1 *Su Huang relieved OVA-induced pulmonary dysfunction and inflammatory responses in rats*

In order to control the quality of Su Huang, LC-MS or GC-MS analysis were applied to determine and identify some main active ingredients in Su Huang in the previous studies (Li et al., 2017; Liu et al., 2019; Tong et al., 2019). The chemical components of Su Huang include ephedrine, arctiin, arctigenin, schisandrin, and schisandrol B, which provide scientific reference for Su Huang as a stable pharmacological experimental object in this study.

To determine the role of Su Huang on pulmonary damage, we examined number of coughs and coughs latencies (Fig. S1A). Su Huang significantly reduced the number of coughs and extended coughs latencies. OVA-induced rats displayed excessive infiltration of inflammatory cells in bronchoalveolar lavage fluid, Su Huang treatment led to reduction in the number of total cells and differential inflammatory cells including lymphocytes, neutrophils and eosinophils (Fig. S1B). H&E examination showed that the bronchial airways and around blood vessels were infiltrated by a large number of inflammatory cells and the exudates in the bronchial lumen were increased after challenged by OVA. However, Su Huang effectively alleviated these lung injuries. Masson trichrome staining indicated that the collagen deposition is reduced after Su Huang treatment (Fig. S1C-D). Moreover, to analyze the effects of Su Huang on OVA-induced inflammatory responses, mRNA and protein levels of COX-2 and IL-6 in the lung tissues were examined. Our results suggested that Su Huang treatment effectively reduced mRNA and protein expression of COX-2 and IL-6 (Fig. S1E-F). These data indicated that pulmonary damage could be substantially attenuated by Su Huang treatment, which was in line with our previous study (Qin et al., 2019).

### 3.2 Regulation of endogenous metabolites in serum by Suhuang

GC-MS based serum metabolomics was used to analyze the differential metabolites of Suhuang treatment. As shown in Fig. 2A, OPLS-DA was used to analyze the metabolic differences of the control, CVA model and Suhuang groups in the serum samples. The OPLS-DA plots showed that there was a obvious separation from the normal and model groups, and the metabolic state of the Suhuang group is closer to that of the normal group. Twelve serum differential metabolites are listed in Table S4, and we performed a heatmap to show the relative changes of these differential metabolites in each group in Fig. 2B. As shown in Fig. 2C, metabolic pathway enrichment analysis was carried out using the differential metabolites, we found that Suhuang affected a variety of metabolic pathways such as lactose degradation, galactose metabolism and sphingolipid metabolism. Among these metabolites, previous studies have shown that  $\beta$ -HB is closely associated with inflammatory diseases (Shimazu et al., 2013; Youm et al., 2015), and  $\beta$ -HB in the Suhuang-administered group was significantly increased (Fig. 2D), so we speculated that  $\beta$ -HB was a key biomarker for Suhuang treatment of CVA. It was previously reported that peroxisome proliferator-activated receptor alpha (PPAR- $\alpha$ ) was an upstream regulatory protein that affected  $\beta$ -HB production (Grabacka et al., 2016). We found that Suhuang could activate PPAR $\alpha$ , which might be responsive for the elevated level of  $\beta$ -HB in the Suhuang group (Fig. 2E). Cumulatively, these results demonstrated that Suhuang could upregulate the serum level of  $\beta$ -HB.

### 3.3 $\beta$ -HB relieved OVA-induced pulmonary damage and inflammatory responses in rats

To assess the protective effects of  $\beta$ -HB on pulmonary damage, we determined number of coughs and coughs latencies.  $\beta$ -HB significantly decreased the number of coughs and increased the coughs latencies, indicating that it could ameliorate lung injury (Fig. 3A). H&E staining showed that  $\beta$ -HB reduced inflammation in bronchial airways and around blood vessels, and reduced the exudate in the bronchial lumen. Masson trichrome staining showed that the collagen deposition was reduced after  $\beta$ -HB treatment (Fig. 3B-C). Moreover, ELISA kits were

used to detect protein levels of COX-2, IL-6 and iNOS, the results suggested that the expression of COX-2, IL-6 and iNOS decreased after treatment with  $\beta$ -HB in the lung tissues (Fig. 3D). Furthermore,  $\beta$ -HB treatment ameliorated OVA-induced excessive infiltration of inflammatory cells in bronchoalveolar lavage fluid (Fig. 3E). Altogether, our results showed that  $\beta$ -HB can protect lung tissue against OVA-induced injury.

#### *3.4 $\beta$ -HB treatment suppressed OVA-activated NLRP3 inflammasome and NF- $\kappa$ B signaling pathways in rats*

Given that  $\beta$ -HB could effectively inhibited inflammatory responses, we further verified whether  $\beta$ -HB could block NLRP3 inflammasome-mediated inflammation. The results showed  $\beta$ -HB inactivated NLRP3 inflammasome, as a proof of decreasing caspase-1 cleavage and IL-1 $\beta$  production (Fig. 4A-B). NF- $\kappa$ B signal pathway have been seen as mechanisms by which  $\beta$ -HB plays a role in inflammation. As presented in Fig. 4C-F,  $\beta$ -HB treatment obviously suppressed phosphorylation of NF- $\kappa$ B and I $\kappa$ B $\alpha$  in OVA-induced rats. These results strongly suggested that  $\beta$ -HB treatment inhibited the activation of NLRP3 inflammasome and NF- $\kappa$ B signaling induced by OVA exposure *in vivo*.

#### *3.5 $\beta$ -HB treatment induced phosphorylation of GSK3 $\beta$ and AMPK as well as upregulation of Nrf2 in OVA-induced rats*

Nuclear factor erythroid 2-related factor 2 (Nrf2) is responsible for improvement of asthma. We found that the protein level of Nrf2 in lung tissues was enhanced by  $\beta$ -HB. Reportedly, Nrf2 nuclear retention could be regulated by GSK3 $\beta$ /AMPK (Lv et al., 2017). Thus, we explored the effect of  $\beta$ -HB on GSK3 $\beta$  and AMPK phosphorylation and the antioxidant and anti-inflammatory activities of  $\beta$ -HB. Our results showed that  $\beta$ -HB treatment enhanced AMPK and GSK3 $\beta$  phosphorylation and promoted the protein expression of HO-1 and Nrf2 (Fig. 5A-D). The data collectively revealed that the positive effects of  $\beta$ -HB treatment might be crucial to the GSK3 $\beta$ /AMPK/Nrf2 axis.

### 3.6 $\beta$ -HB treatment blocked LPS-induced inflammatory responses in BMDM cells

We next investigated the effect of  $\beta$ -HB on LPS-induced inflammation in BMDM cells. Firstly, As shown in Fig. 6A, MTT assay showed  $\beta$ -HB at the concentration of 5-80 mM exerted no cytotoxicity to BMDM cells. Therefore, the concentrations of  $\beta$ -HB for exposure to BMDM cells were finally chosen to be 10, 20 and 40 mM. Then we found that  $\beta$ -HB decreased the mRNA levels of LPS-induced inflammatory mediators including COX-2, IL-6 and iNOS in a dose-dependent manner (Fig. 6B). In addition, we investigated the effect of  $\beta$ -HB treatment on LPS-induced NF- $\kappa$ B activation.  $\beta$ -HB pretreatment effectively reduced p65 and I $\kappa$ B $\alpha$  phosphorylation in LPS-induced BMDM cells (Fig. 6C-D). Immunofluorescent assay showed that  $\beta$ -HB visibly reversed LPS-induced NF- $\kappa$ B (p65) subunit translocation into the nucleus (Fig. 6E). Similarly, we examined the effect of  $\beta$ -HB on LPS-induced NLRP3 inflammasomes. Our results suggested that  $\beta$ -HB treatment markedly decreased LPS-induced expression of NLRP3, Caspase-1 and IL-1 $\beta$  protein (Fig. 6F-G). Taken together, these findings verified that  $\beta$ -HB treatment resulted in attenuation of pulmonary inflammation *in vitro*.

### 3.7 $\beta$ -HB regulated the GSK3 $\beta$ /AMPK/Nrf2 signaling pathways in BMDM cells

ROS are known to play an important role in the inflammatory signaling pathway. Thus, ROS generation was measured by DCFH-DA assay (Fig. 7A, Fig. S2). The results suggested that LPS increased ROS formation, whereas  $\beta$ -HB significantly inhibited the ROS production. Immunofluorescent staining revealed that  $\beta$ -HB obviously promoted nuclear accumulation and stabilization of Nrf2 (Fig. 7B).  $\beta$ -HB significantly promoted the nuclear translocation of Nrf2 in a dose-dependent manner (Fig. 7C-D). In addition,  $\beta$ -HB significantly increased the expression of p-AMPK, p-GSK3 $\beta$  and Nrf2 (Fig. 7E-F). SNP, a GSK3 $\beta$  phosphorylation inhibitor, significantly decreased  $\beta$ -HB-induced AMPK phosphorylation, and reduced GSK3 $\beta$  phosphorylation and total protein expression of Nrf2, indicating that GSK3 $\beta$  might act as upstream regulator of Nrf2 (Fig. 7G-H). In addition, Compound C (an inhibitor of AMPK) significantly reversed  $\beta$ -HB-induced upregulation of p-AMPK and Nrf2, while Compound C

did not affect  $\beta$ -HB-induced GSK3 $\beta$  phosphorylation, implying that GSK3 $\beta$  might act as upstream regulator of AMPK. Trigonelline (an inhibitor of Nrf2) had no significant effects on both  $\beta$ -HB-mediated AMPK and GSK3 $\beta$  phosphorylation (Fig. 7I-J). Taken together, these results indicated that  $\beta$ -HB treatment enhanced the GSK3 $\beta$  phosphorylation which upregulated the AMPK phosphorylation. Then, the enhanced AMPK phosphorylation promoted nuclear accumulation and stabilization of Nrf2.

### *3.8 Increase of $\beta$ -HB-induced Nrf2 expression played a vital role in antioxidant and anti-inflammatory activities in LPS-stimulated BMDM cells*

We verified whether Nrf2 is essential to the antioxidant effect of  $\beta$ -HB. As shown in Fig. 8A-B, cells transfected with Nrf2 siRNA remarkably decreased the protein level of HO-1 and Nrf2 compared to  $\beta$ -HB-treated negative control cells. Meanwhile, the inhibitory effect of  $\beta$ -HB treatment on LPS-induced ROS production was attenuated after Nrf2 being silenced (Fig. 8C, Fig. S3). Next, we evaluated the role of Nrf2 signal in the anti-inflammatory effects of  $\beta$ -HB on LPS-treated BMDM. The results demonstrated that Nrf2 knockdown significantly counteracted the inhibitory effects of  $\beta$ -HB on COX-2, IL-6 and iNOS (Fig. 8D). Thus, the results demonstrated that Nrf2 activation was crucial to the antioxidant and anti-inflammatory properties of  $\beta$ -HB.

### *3.9 $\beta$ -HB treatment protected against OVA-induced cough variant asthma in partly dependent on Nrf2 activation*

We further confirmed the crucial role of Nrf2 in the antioxidant effect of  $\beta$ -HB *in vivo*. CVA rats were exposed to trigonelline with or without  $\beta$ -HB treatment.  $\beta$ -HB partly ameliorated pulmonary function (Fig. 9A), inhibited production of COX-2, IL-6 and iNOS in lung (Fig. 9B) and attenuated severe histopathological changes (Fig. 9C-D). But these changes were significantly reversed by i.p. of trigonelline. Western blotting and pathological immunohistochemistry of the lung tissue showed that  $\beta$ -HB treatment mediated increase in Nrf2 and decrease in p65 phosphorylation and I $\kappa$ B $\alpha$  phosphorylation were evidently impeded by i.p.

of trigonelline (Fig. 9E-H). Our results revealed that  $\beta$ -HB inhibited NF- $\kappa$ B signaling pathway in CVA rats, which might be dependent upon upregulation of Nrf2. In summary, these results indicated that the protective effect of  $\beta$ -HB on CVA rats might be partly dependent upon upregulation of Nrf2.

#### 4. Discussion

Consistent with the previous research (Qin et al., 2019; Tong et al., 2019), our findings suggested that Suhuang, a Chinese patent medicine, could significantly improve pulmonary dysfunction caused by CVA. In this study, we elucidated the metabolic regulatory effect of Suhuang based on the metabolomics on the CVA rat model. Suhuang treatment modulated metabolic pathways including lactose degradation, galactose metabolism and sphingolipid metabolism, which also coincided with the changes in metabolic characteristics in the guinea pig model of allergic asthma in the previous studies (Kertys et al., 2020; Villasenor et al., 2021).  $\beta$ -HB, an endogenous histone deacetylase inhibitor, has been reported to block NLRP3 inflammasome-mediated inflammatory disease and inhibit oxidative stress (Shimazu et al., 2013; Youm et al., 2015). Besides,  $\beta$ -HB also exhibits other biological activities such as anti-aging and anti-tumor (Fang et al., 2021; Feng et al., 2019). The obvious increase in the level of  $\beta$ -HB in the serum of the Suhuang group attracted our attention. Previous studies showed that activation of PPAR $\alpha$  induced  $\beta$ -HB production in mouse liver (Feng et al., 2019; Ruppert et al., 2019). We found that Suhuang upregulated the expression of PPAR $\alpha$ , which might be responsive for the increase in the level of  $\beta$ -HB in the Suhuang group. In addition, we demonstrated that  $\beta$ -HB could protect against pulmonary dysfunction in OVA-induced CVA rats for the first time.

The pathogenesis of CVA is similar to that of classic asthma, includes following characteristics: airway inflammation, airway remodelling, and airway hyperresponsiveness. Airway inflammation can lead to pulmonary dysfunction (Sagani et al., 2015), including the increase in eosinophils and neutrophils, and release of inflammatory mediators and a series of pathological manifestations (Li et al., 2021). Our results showed that exposure to  $\beta$ -HB could reduce cough frequency, eosinophil and neutrophil infiltration and improve OVA-induced

histopathological damage (Wang et al., 2020). Furthermore, the activation and recruitment of macrophages in the lungs have been reported to play a key role in the pathophysiology of asthma (Chen et al., 2022), not only promoting the release of various inflammatory mediators but also increasing the production of ROS, which is closely related to disease severity (Qin et al., 2021). In this study,  $\beta$ -HB treatment significantly reduced the secretion of COX-2, IL-6 and iNOS in the CVA rats and LPS-induced BMDM cells. Taken together,  $\beta$ -HB significantly improved pulmonary dysfunction by reducing inflammation and oxidative stress *in vivo* and *in vitro*, which prompted us to further explore the mechanism of the protective effect of  $\beta$ -HB on OVA-induced pulmonary dysfunction.

The previous study suggested that NF- $\kappa$ B-mediated multiple inflammatory mediators play key roles in the pathogenesis of chronic respiratory diseases (Ma et al., 2018). In this study,  $\beta$ -HB potently inhibited phosphorylation of p65 and I $\kappa$ B $\alpha$  in the CVA rats and LPS-induced BMDM cells. It has been reported that  $\beta$ -HB could activate NLRP3 to improve inflammatory diseases (Youm et al., 2015). We also found that  $\beta$ -HB inhibited the activation of NLRP3 to prevent the progression of the CVA, which is manifested by down-regulation of NLRP3, Caspase-1 and IL-1 $\beta$  secretion. Based on the evidences mentioned above, the protective effect of  $\beta$ -HB on CVA might be closely related to its anti-inflammatory effect.

Multiple signaling pathways are involved in the pathophysiological process of asthma. Nrf2, as the coordinator of multiple signaling pathways, is closely related to the airway inflammation in asthma (Chen et al., 2021). In the present study,  $\beta$ -HB treatment was found to significantly upregulate the expression of Nrf2 *in vivo* and *in vitro*, while knockdown of Nrf2 abolished anti-inflammatory and antioxidant effects of  $\beta$ -HB. The previous study showed that the activation of Nrf2 could induce the expression of downstream antioxidant enzymes, such as HO-1 (Miyauchi et al., 2019), and overexpression of HO-1 had a protective effect on lung dysfunction. Our results demonstrated that  $\beta$ -HB significantly induced nuclear accumulation of Nrf2 in BMDM cells and CVA rats.

A recent study reported that phosphorylation of AMPK can lead to accumulation of Nrf2 nuclear transcripts (Matzinger et al., 2020). However, inhibition of GSK3 $\beta$  increased AMPK activity to reduce proinflammatory immune activation (Zhou et al., 2018). In our study,  $\beta$ -HB treatment clearly increased phosphorylation of AMPK and GSK3 $\beta$ . Therefore, in order to

further study the specific mechanism of  $\beta$ -HB, our results suggested that SNP (a GSK3 $\beta$  phosphorylation inhibitor) promoted GSK3 $\beta$  activation and inhibited phosphorylation of AMPK, indicating that GSK3 $\beta$  is an upstream regulator of AMPK. Similarly, Compound C (an inhibitor of AMPK) significantly reversed  $\beta$ -HB-induced upregulation of p-AMPK and Nrf2, whereas trigonelline (an inhibitor of Nrf2) only reversed  $\beta$ -HB-induced upregulation of Nrf2. In addition, we found that the protective effects of  $\beta$ -HB on the CVA rats were abolished by trigonelline treatment. Moreover,  $\beta$ -HB-mediated phosphorylation of p65 and I $\kappa$ B $\alpha$  were also significantly blocked after trigonelline treatment. Taken together, the protective effect of  $\beta$ -HB on CVA was closely related to the activation of Nrf2 through the GSK3 $\beta$ /AMPK axis.

The limitation of this study is that the metabolic changes of human serum after Suhuang administration were not carried out. Further studies need to collect with human biological samples to observe the metabolic changes of people after Suhuang administration, which will help to further determine that dietary or pharmacological approaches for elevating  $\beta$ -HB levels might prevent the progression of CVA.

In conclusion, we found that Suhuang could up-regulate the level of the endogenous metabolite  $\beta$ -HB, which directly inactivated NLRP3 inflammasome, and simultaneously inactivated GSK3 $\beta$ , inhibition of GSK3 $\beta$  activated AMPK to up-regulate Nrf2 and inhibited NF- $\kappa$ B signaling pathway, thereby protecting CVA from oxidative stress and inflammatory damage (Fig. 10). Therefore, dietary or pharmacological approaches to elevate  $\beta$ -HB levels are expected to prevent the progression of CVA.

#### **Data availability**

The additional data collected during this study are available from the corresponding author upon reasonable request.

#### **Ethics approval and consent to participate**

The animal experiment was overseen and approved by the Animal Ethics Committee of China Pharmaceutical University.

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#### **CRedit authorship contribution statement**

**Hong Jiang:** Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing - review and editing. **Ziyu Bai:** Investigation, Formal analysis, Writing - original draft. **Yongyu Ou:** Formal analysis, Writing – original draft. **Huiling Liu:** Investigation. **Zilin Si:** Investigation. **Yafang Liu:** Formal analysis. **Xiaoqiong Liu:** Formal analysis. **Xiaoqing Liu:** Conceptualization. **Zhihao Zhang:** Conceptualization, Writing - review and editing, Supervision. **Ninghua Tan:** Conceptualization, Resources, Writing - review and editing, Supervision, Project administration.

#### **Competing of Interest Statement**

The authors declare no conflict of interest related to this paper.

#### **Appendix A. Supporting information**

Supplementary data associated with this article can be found in the online version at

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## Figure legends

**Fig. 1.** Experimental protocol for the establishment of OVA-induced CVA model and the treatment schedule. Protocol of induction of CVA in which rats were sensitized by intraperitoneal injection of ovalbumin or saline, two weeks later, the rats were administered drug and nebulized ovalbumin or saline.

**Fig. 2.** Metabolic changes in serum after Suhuang treatment. Metabolite changes in serum were analyzed by orthogonal partial least squares discriminant analysis (OPLS-DA) models (A) (n=6). Heat map of differential metabolites after Suhuang treatment (B) (n=6). Analysis of the effect of Suhuang on CVA metabolism regulation (C) (n=6). Peak areas of  $\beta$ -HB in serum were determined by gas chromatography-mass spectrometry (D) (n=6). PPAR $\alpha$  expression was detected by Western blot (E) (n=3).  $^{\#}p < 0.05$ ,  $^{###}p < 0.001$  vs. the control,  $^{*}p < 0.05$ ,  $^{**}p < 0.01$  vs. the OVA group. All data are presented as means  $\pm$  SEM of three independent experiments.

**Fig. 3.** Protective effects of  $\beta$ -HB treatment in OVA-induced CVA rat. Number of coughs and cough latencies were recorded in the capsaicin challenge before sacrifice (A). Lung tissues sections were stained with H&E and Masson, and the lung injury score was determined (B and C). The protein expressions of pro-inflammatory cytokines were measured by ELISA (COX-2, IL-6 and iNOS) (D). Counts of suspension immune cells in the BALF (E). Scale bars: 100  $\mu$ m, n=5,  $^{\#}p < 0.05$ ,  $^{###}p < 0.001$  vs. the control,  $^{*}p < 0.05$ ,  $^{***}p < 0.001$  vs. the OVA group. Each data point represents the mean  $\pm$  SEM of three independent experiments.

**Fig. 4.** Effects of  $\beta$ -HB treatment on NLRP3 inflammasome and NF- $\kappa$ B signal pathways in

CVA rats. Rats were intraperitoneally injected with different concentrations of  $\beta$ -HB (87.5 or 175 mg/kg) prior to OVA nebulization stimulation, and then lung tissues were collected. Protein samples in the lung tissue were extracted for western blot assays. The protein levels of NLRP3, pro-caspase-1, caspase-1 and IL-1 $\beta$  were determined by western blotting assay and quantified by densitometry (A and B). Protein expressions of P-p65, p65, P-I $\kappa$ B $\alpha$  and I $\kappa$ B $\alpha$  were characterized by western blot assays and quantified by densitometry (C and D). The nuclear translocation of p65 in the lung tissue was showed by immunohistochemistry and the positive expression score was calculated, the red arrow indicates the positive expression of p65 in macrophage nuclear and the blue arrow indicates the positive expression of p65 in macrophage cytoplasm. (E and F). Scale bars: 50  $\mu$ m, n=3,  $^{\#}p < 0.05$ ,  $^{###}p < 0.001$  vs. the control,  $^*p < 0.05$ ,  $^{**}p < 0.01$ ,  $^{***}p < 0.001$  vs. the OVA group. Values are presented as mean  $\pm$  SEM of three independent experiments.

**Fig. 5.** Effects of  $\beta$ -HB treatment on the expression of AMPK, GSK3 $\beta$  phosphorylation and Nrf2 in OVA-induced CVA rats. After two weeks of sensitization with OVA-alum adjuvant mixture, rats were challenged with 1% ovalbumin. Before the challenge, different concentrations of  $\beta$ -HB (87.5 or 175 mg/kg) were injected intraperitoneally for 13 days, and then the lung tissues were collected. The levels of the indicated proteins (Nrf2/GAPDH, HO-1/GAPDH, P-GSK3 $\beta$ /GSK3 $\beta$  and P-AMPK/AMPK) were performed by western blotting assay and quantified by densitometry (A and B). The nuclear translocation of Nrf2 and expression of HO-1 were performed with immunohistochemical staining and the positive expression score was calculated, the red arrow indicates the positive expression of Nrf2 in macrophage nuclear and the blue arrow indicates the positive expression of Nrf2 in macrophage cytoplasm. (C and D). Scale bars: 50  $\mu$ m, n=3,  $^{\#}p < 0.05$ ,  $^{###}p < 0.001$  vs. the control,  $^*p < 0.05$ ,  $^{**}p < 0.01$ ,  $^{***}p < 0.001$  vs. the OVA group. Data are presented as mean  $\pm$  SEM of three independent experiments.

**Fig. 6.** Effects of  $\beta$ -HB exposure on LPS-induced inflammatory responses in BMDM cells. After BMDM cells were pretreated with different concentrations of  $\beta$ -HB for 1 h, and stimulated with LPS (1  $\mu$ g/mL) for another 6 h. Cell viability was detected by an MTT assay

after 8h exposure to  $\beta$ -HB (A). The mRNA expression levels of pro-inflammatory cytokines *COX-2*, *IL-6*, *iNOS* were analyzed by qPCR (B). Effects of  $\beta$ -HB on the P-p65, p65, P-I $\kappa$ B $\alpha$  and I $\kappa$ B $\alpha$  protein expression were determined by western blot analysis and quantified by densitometry (C and D).  $\beta$ -HB effects on nuclear translocation of p65 was characterized by immunofluorescent assay (E). Effects of  $\beta$ -HB on the NLRP3, pro-caspase-1, caspase-1 and IL-1 $\beta$  protein expression were determined by western blot analysis and quantified by densitometry (F and G). Scale bars: 20  $\mu$ m,  $^{\#}p < 0.05$ ,  $^{\#\#}p < 0.01$ ,  $^{\#\#\#}p < 0.001$  vs. the control,  $^*p < 0.05$ ,  $^{**}p < 0.01$  vs. the LPS group. The results are expressed as the mean  $\pm$  SEM of three independent experiments.

**Fig. 7.** Effects of  $\beta$ -HB exposure on GSK3 $\beta$ /AMPK/Nrf2 signaling pathways in BMDM cells. Cells were pretreated with various concentrations of  $\beta$ -HB for 1 h, and treated with LPS for another 6 h (A-F), and then stained with 10  $\mu$ M DCFH-DA for 30 min to stimulate ROS production. ROS was examined under laser confocal microscopy and fluorescence intensity was used for densitometry quantification analysis (A). The effect of  $\beta$ -HB on LPS-induced Nrf2 nuclear translocation is measured by immunofluorescent assay (B). Effect of  $\beta$ -HB on nuclear aggregation of Nrf2 was examined by western blot assay and densitometry quantification analysis (C and D). AMPK and GSK3 $\beta$  phosphorylation and Nrf2 protein expression were detected by western blotting assay and quantified by densitometry (E and F). Cells were exposed to SNP (a GSK3 $\beta$  activator) for 3h, and then were incubated with  $\beta$ -HB for another 3h, P-AMPK, P-GSK3 $\beta$  and Nrf2 protein levels were detected by western blotting assay and quantified by densitometry (G and H). Cells are incubated with Compound C (an inhibitor of AMPK) or trigonelline (an inhibitor of Nrf2) for 18 h, and then treated with  $\beta$ -HB (20 mM) for 6 h, P-AMPK, P-GSK3 $\beta$  and Nrf2 protein levels were detected by western blotting assay and quantified by densitometry (I and J). Scale bars: 20  $\mu$ m,  $^{\#}p < 0.05$ ,  $^{\#\#}p < 0.01$ ,  $^{\#\#\#}p < 0.001$  vs. the control,  $^*p < 0.05$ ,  $^{**}p < 0.01$ ,  $^{***}p < 0.001$  vs. the LPS group or the  $\beta$ -HB group. Results are expressed as mean  $\pm$  SEM of three independent experiments.

**Fig. 8.** Activation of Nrf2 is essential for the anti-inflammatory and antioxidant effects of  $\beta$ -HB. Cells were transfected with Nrf2 siRNA or NC siRNA for 6 h, then treated with  $\beta$ -HB for

6 h, and Nrf2, HO-1 protein expression were analyzed by western blot and quantified by densitometry (A and B). Cells were transfected with Nrf2 siRNA or NC siRNA for 6 h, then  $\beta$ -HB pretreated for 1 h, and then stimulated with LPS for another 6 h for subsequent experiments. Cells were incubated with DCFH-DA for 30 min for intracellular ROS assay (C). The mRNA expression levels of *COX-2*, *IL-6*, *iNOS* were determined by qPCR (D). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. the NC +  $\beta$ -HB group. Results are expressed as mean  $\pm$  SEM of three independent experiments.

**Fig. 9.** Protective effects of  $\beta$ -HB-mediated Nrf2 on OVA-induced CVA rats. CVA rats were intraperitoneally injected with  $\beta$ -HB (175 mg/kg) and an Nrf2 inhibitor (trigonelline, 20 mg/kg) before receiving OVA nebulization stimulation. Number of coughs and cough latencies were recorded in capsaicin evoked challenge before sacrifice (A) (n=5). The protein expression of pro-inflammatory cytokines was measured by ELISA (COX-2, IL-6, iNOS) (B) (n=5). Lung tissues sections were stained with H&E and Masson, and the degree of lung injury and fibrosis was scored (C and D) (n=5). Total proteins from lung tissues were processed for western immunoblot analysis for Nrf2, P-I $\kappa$ B $\alpha$ , I $\kappa$ B $\alpha$ , P-p65 and p65 (E and F) (n=3). Immunohistochemistry was used to characterize nuclear translocation of p65 and Nrf2 and the positive expression score was calculated, the red arrow indicates the positive expression of p65, Nrf2 in macrophage nuclear and the blue arrow indicates the positive expression of p65, Nrf2 in macrophage cytoplasm. (G and H) (n=3). Scale bars: 50  $\mu$ m, # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  vs. the control, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. the OVA group or the OVA +  $\beta$ -HB group. Each data point represents the mean  $\pm$  SEM of three independent experiments.

**Fig. 10.** Schematic diagram.  $\beta$ -HB effectively protected OVA-induced CVA rats from oxidative stress and inflammatory injury, which was largely dependent on upregulating Nrf2-mediated antioxidant pathways through activation of GSK3 $\beta$ /AMPK and inhibiting NLRP3 inflammasome activation.

**Fig. 1.**

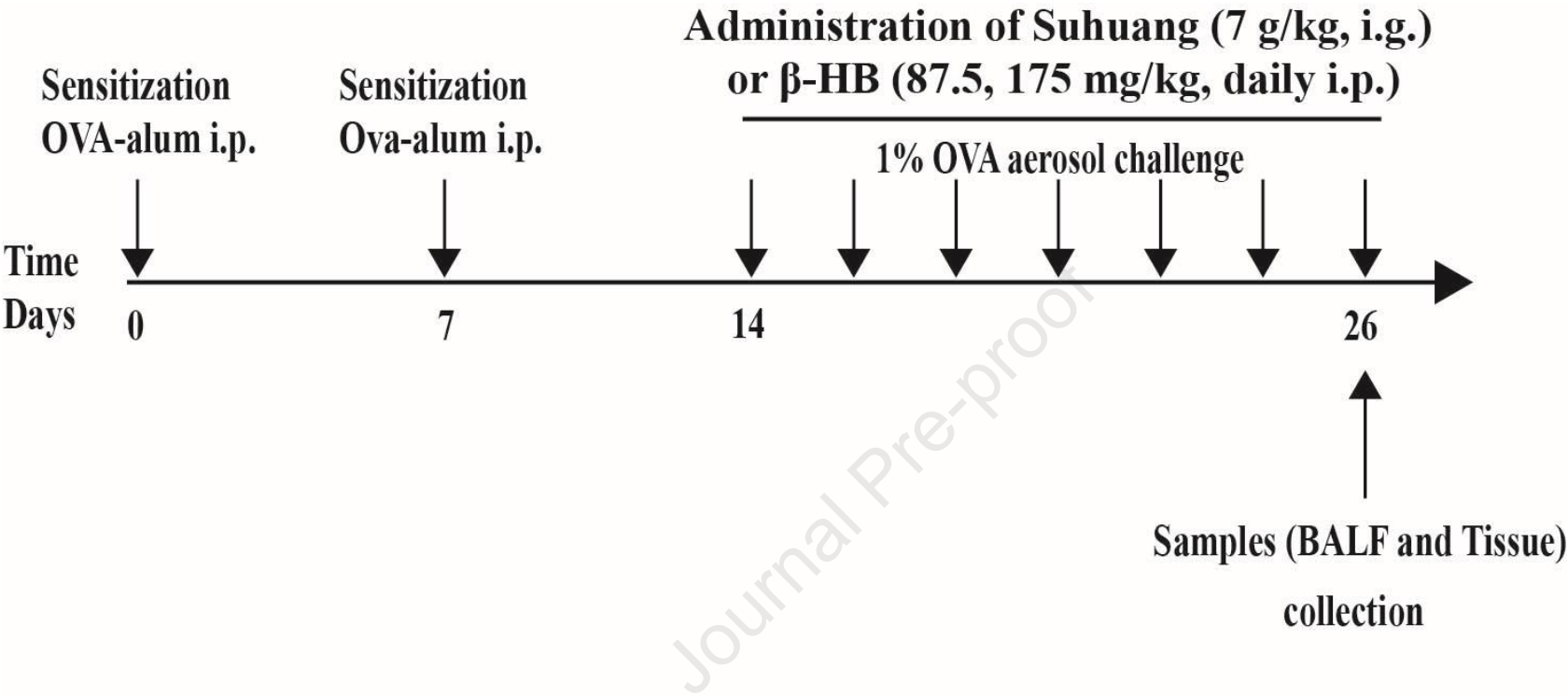


Fig. 2.

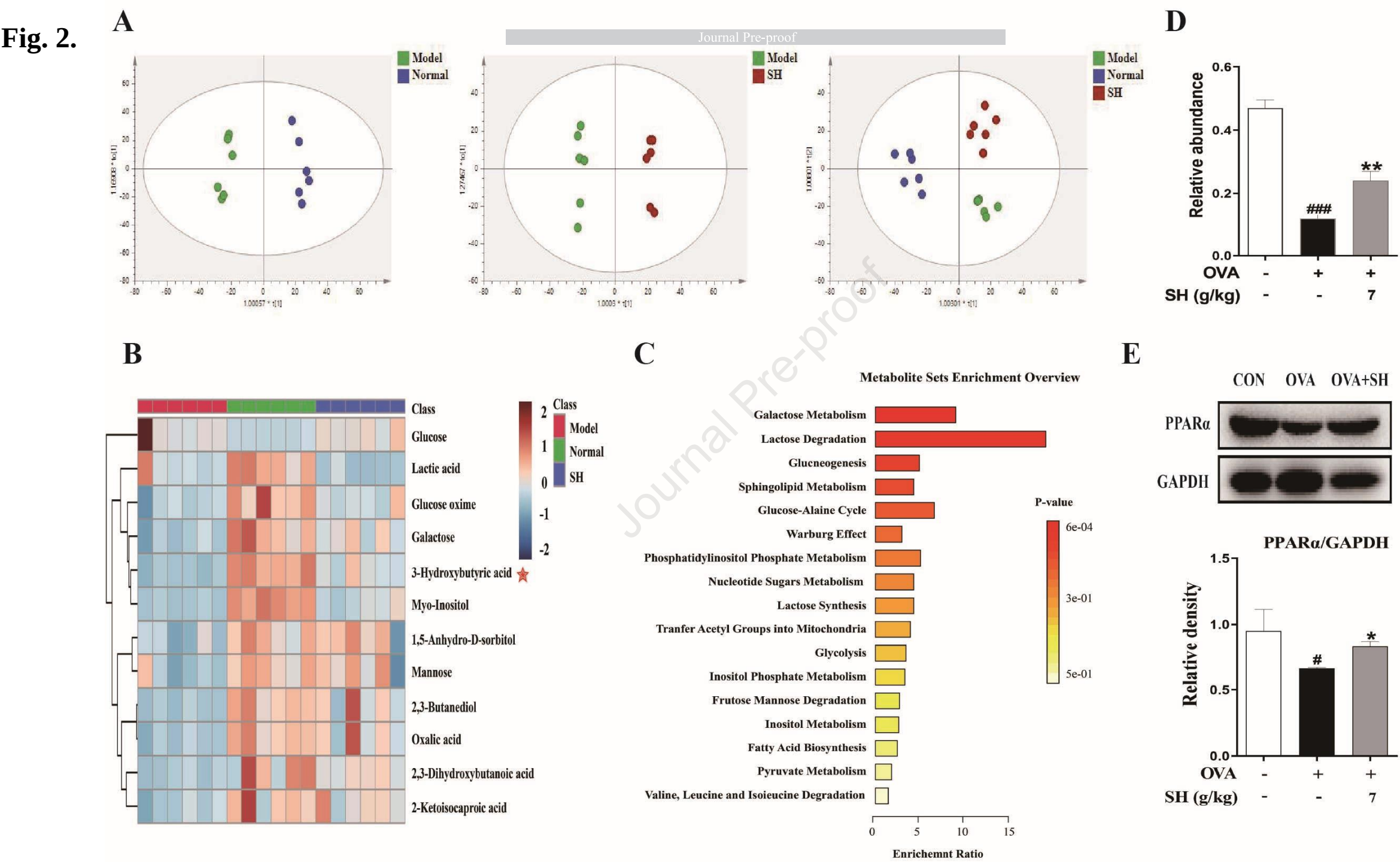


Fig. 3.

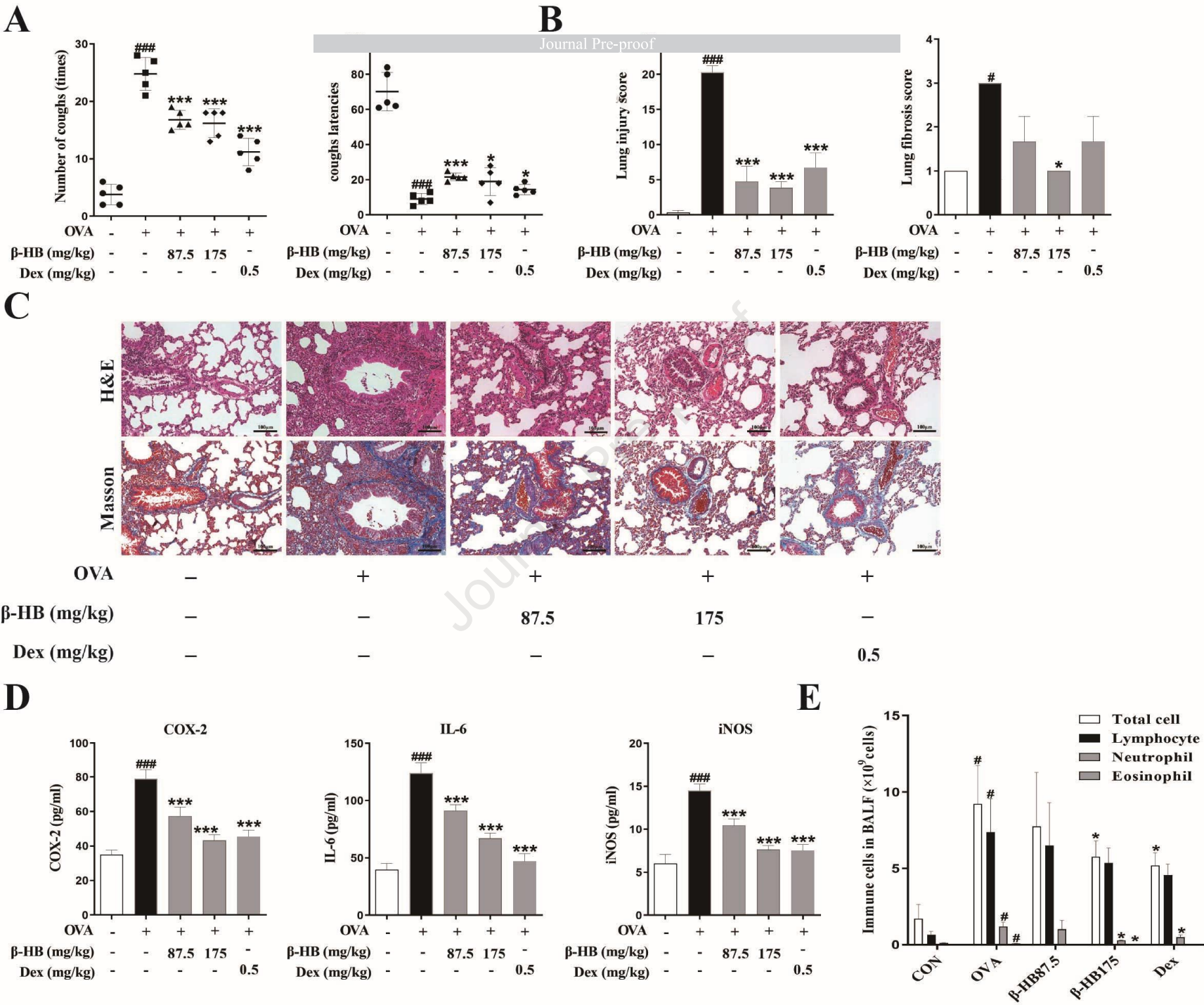


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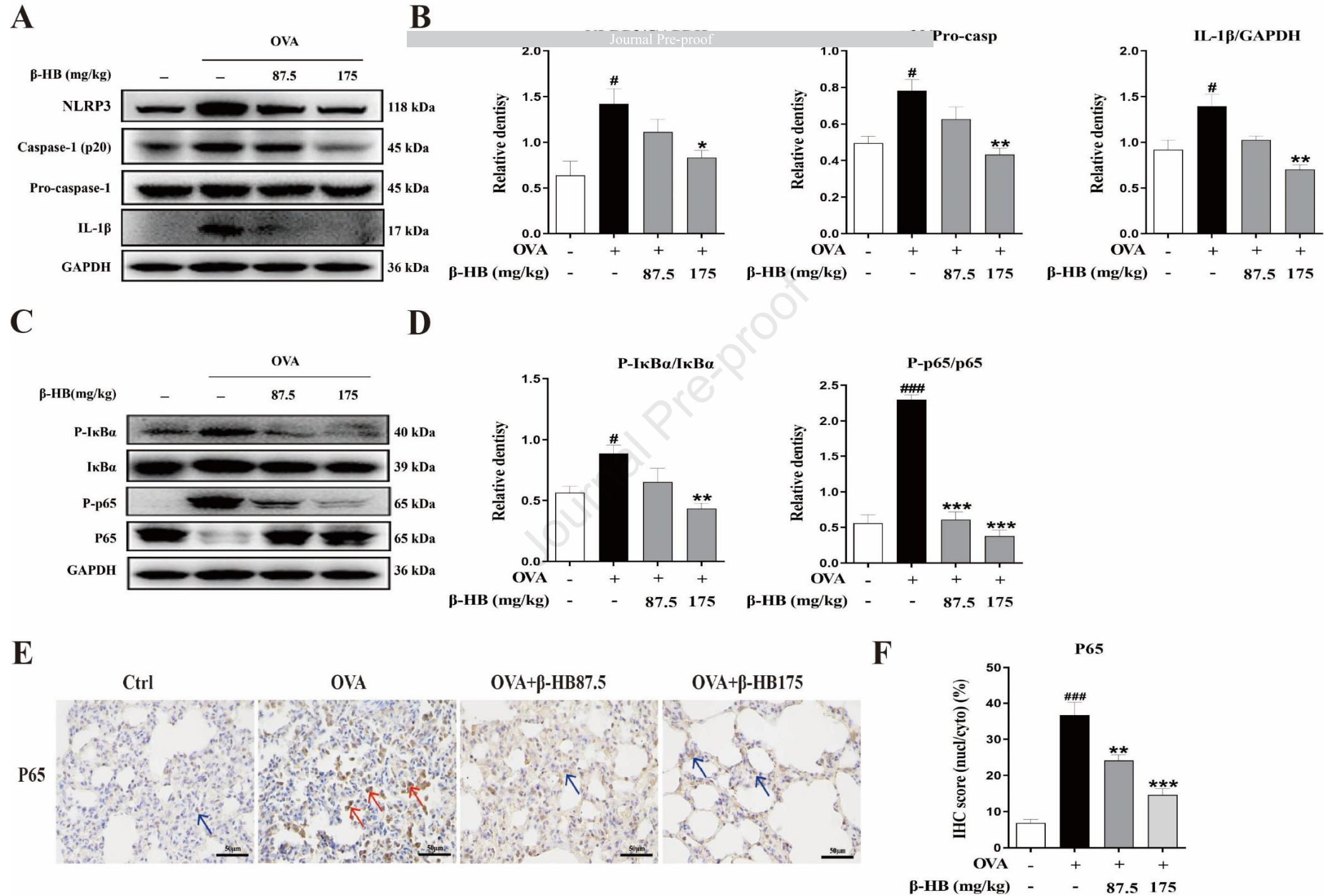


Fig. 5.

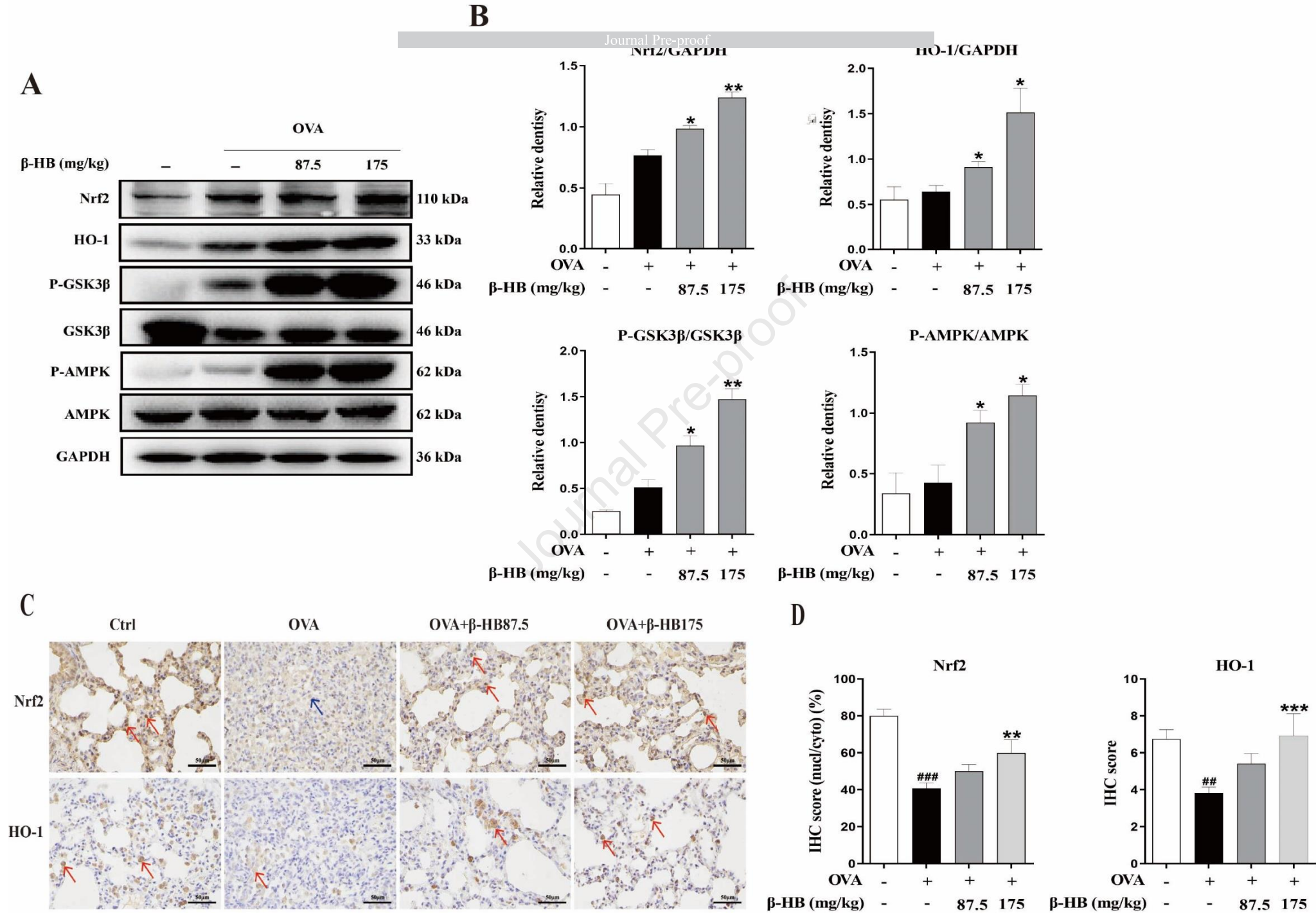


Fig. 6.

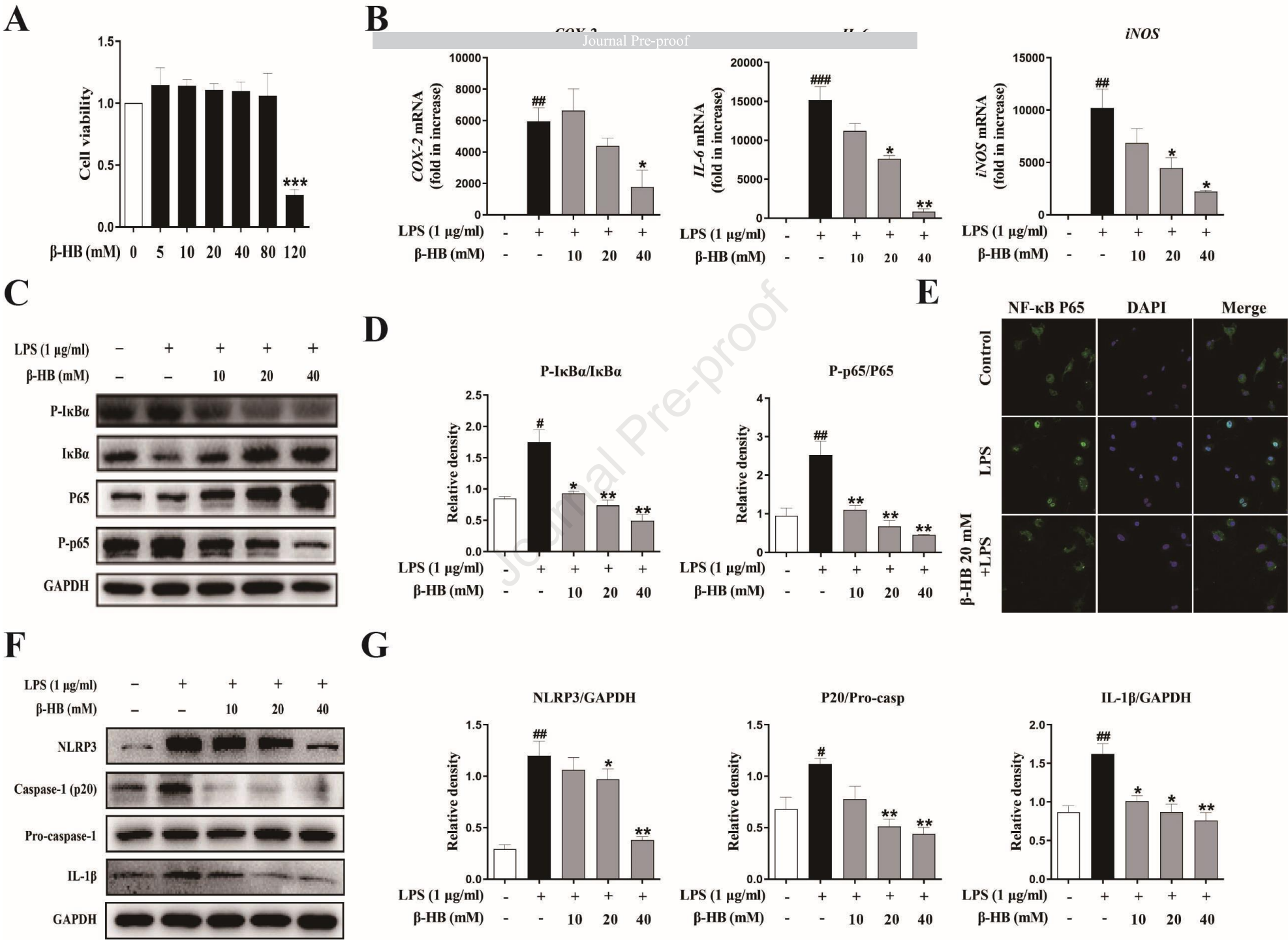


Fig. 7.

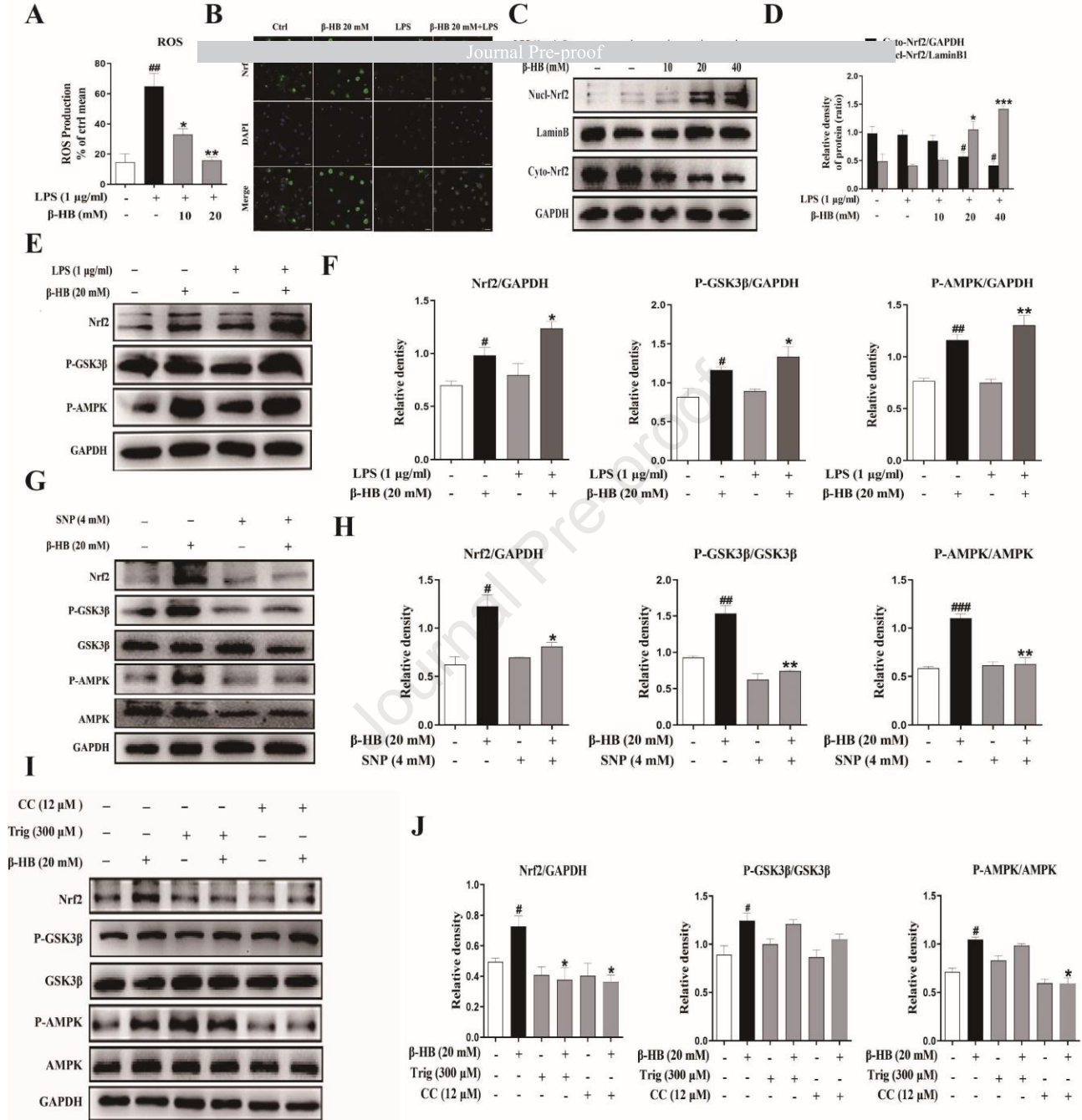


Fig. 8.

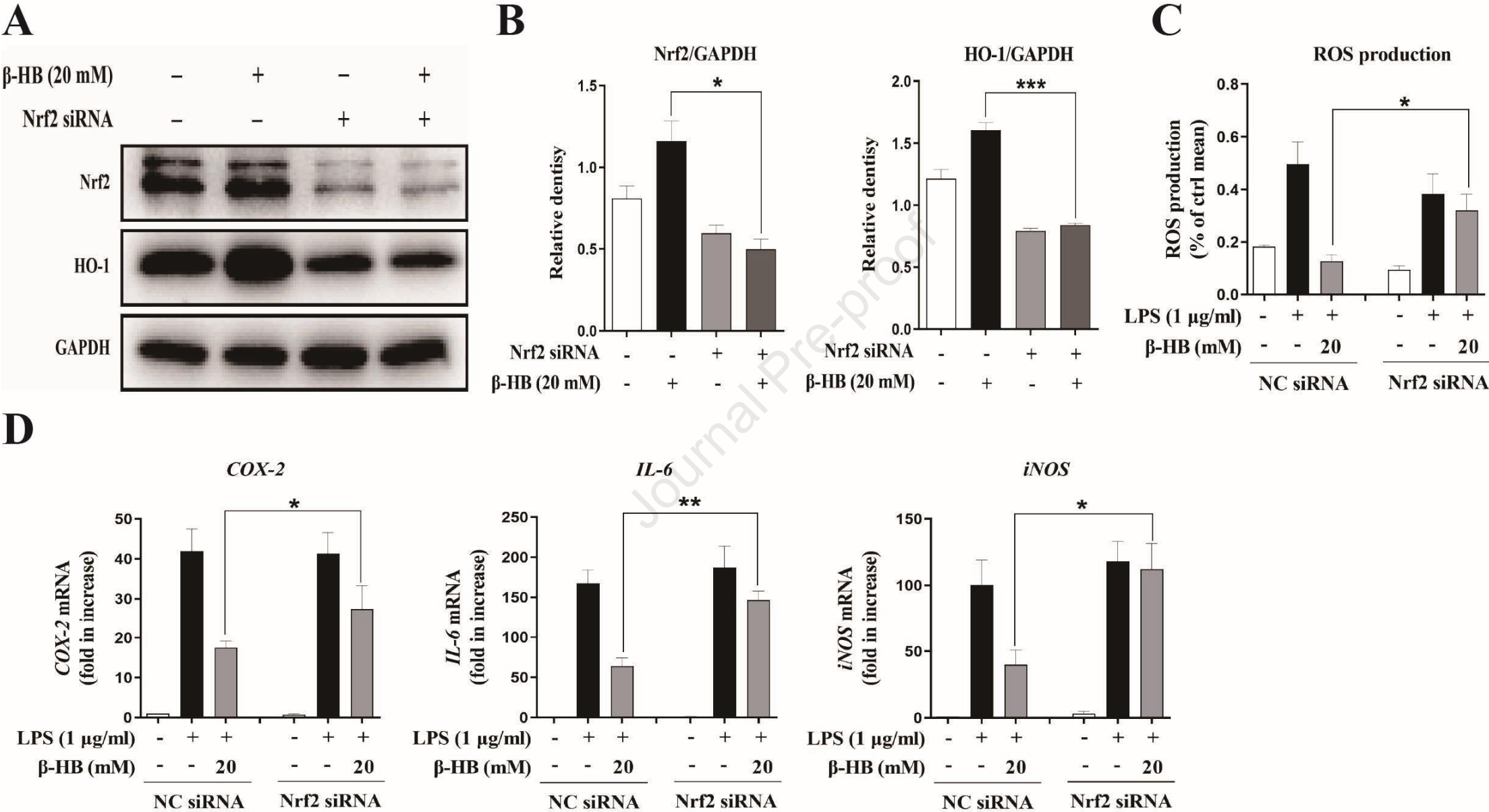


Fig. 9.

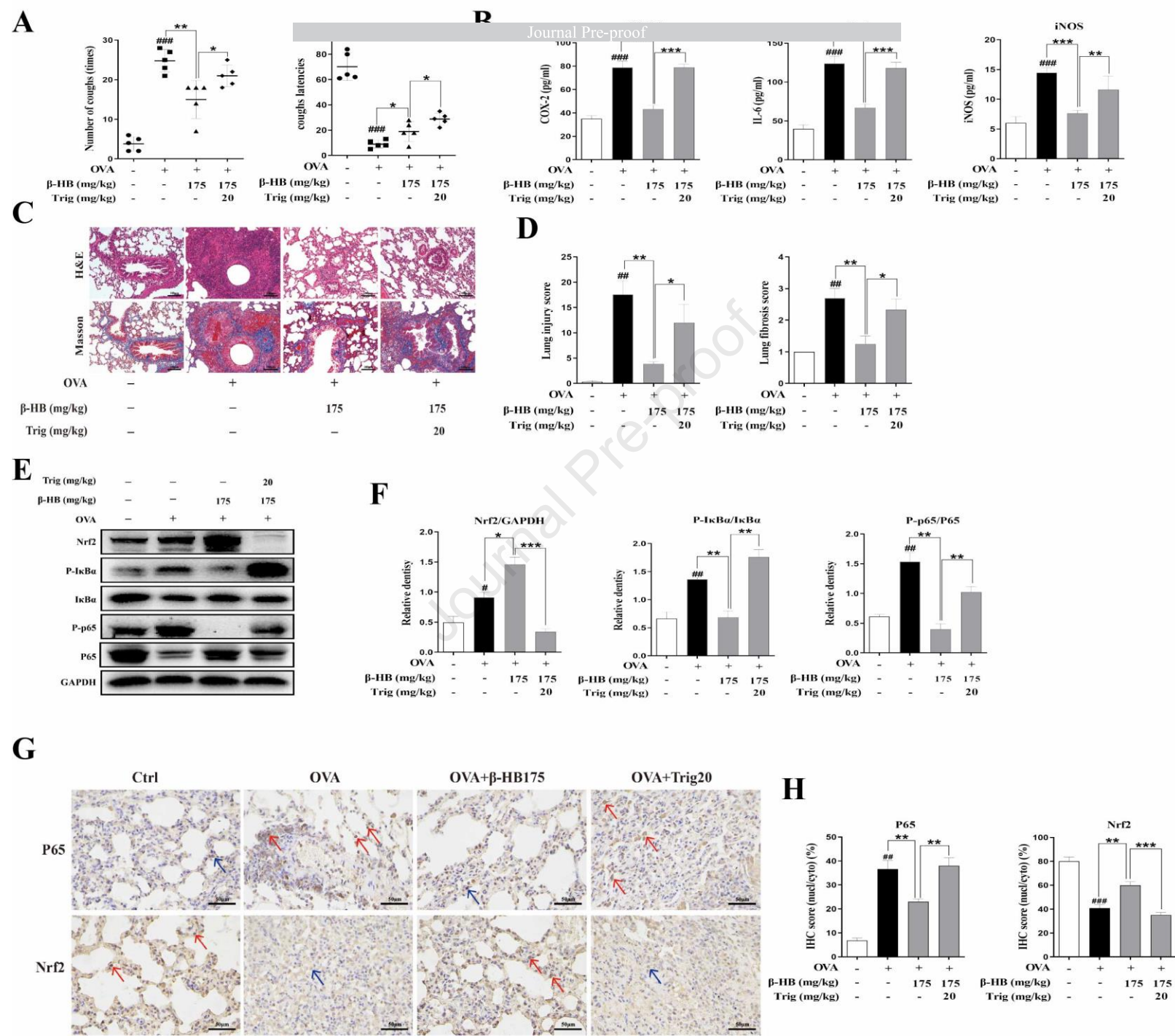
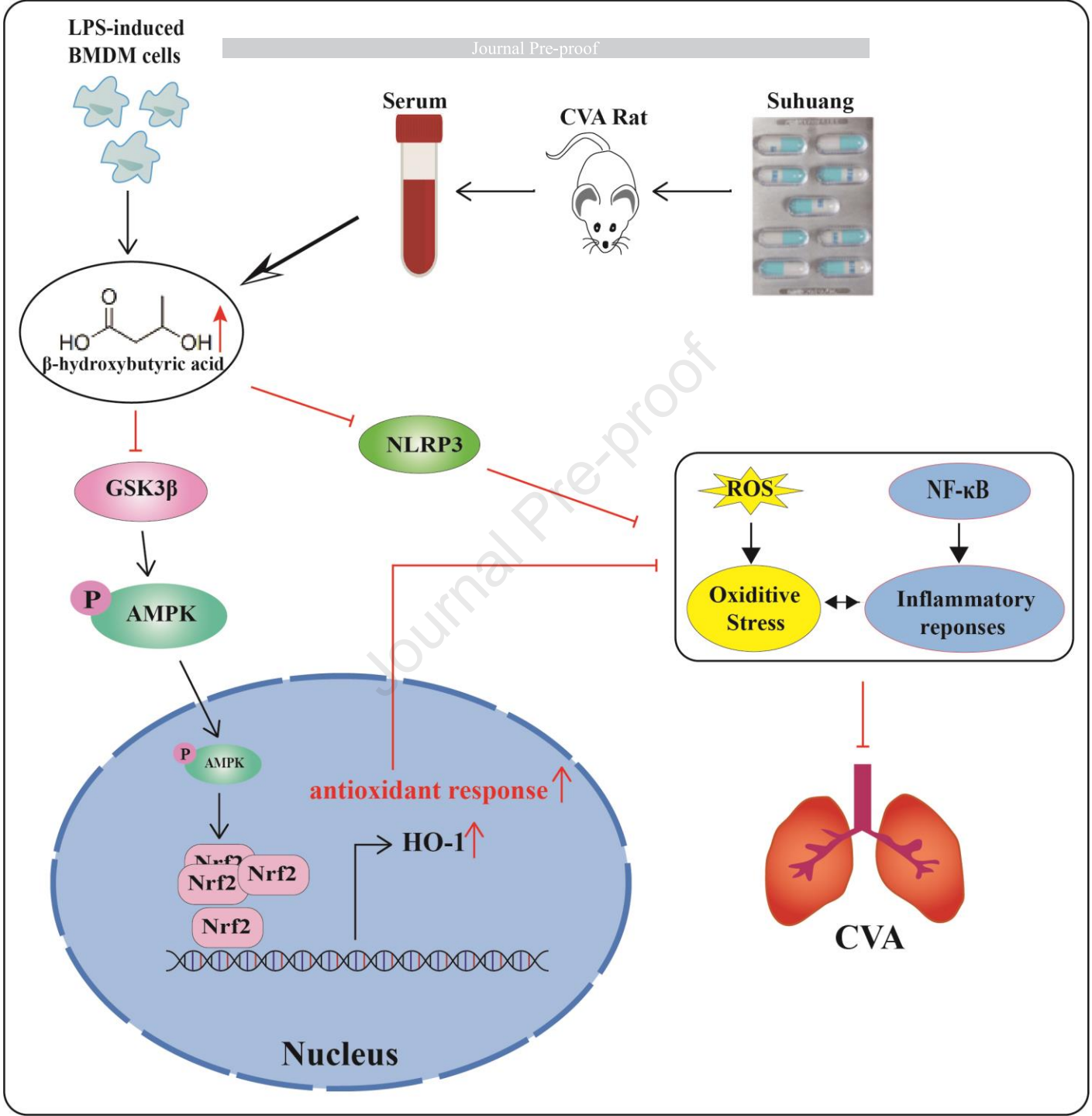


Fig. 10.



## Highlights

1. Suhuang treatment significantly increases the serum level of  $\beta$ -hydroxybutyric acid ( $\beta$ -HB).
2.  $\beta$ -HB protects against cough variant asthma (CVA) *in vitro* and *in vivo*.
3.  $\beta$ -HB ameliorates CVA by regulation of GSK3 $\beta$ /AMPK-Nrf2 signal axis and NLRP3 inflammasome.

The authors declare no conflict of interest related to this paper.