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Review Article

Dry cod skin collagen oligopeptides ameliorate ovalbumin-induced asthma in a mouse model via inhibition of NLRP3 inflammasome

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Abstract: Background: The skin of many fish species contains bioactive and potentially therapeutic compounds, including anti-inflammatory peptides. The current study examined the effects of dry cod skin collagen oligopeptides (CP) on lung inflammation mediators in a mouse asthma model. Method: BALB/c mice were orally administered saline or CP. They were sensitized by OVA through intraperitoneal injections and exposed to aerosolized OVA. The lungs were analyzed for inflammatory cells and mediators, including cytokines and components of the NLRP3 inflammasome, via histological and immunohistochemical staining, RT-PCR, and Western blot analysis. Results: CP pretreatment significantly inhibited OVA-induced peri-bronchial inflammation, airway inflammatory cell recruitment, phosphorylation of NF-κBp65, activation of NLRP3 inflammasome, and production of some asthma-related cytokines in lung tissues. Conclusion: Present results identify several molecular mechanisms of action of CP, providing support for its use in alleviating asthma, as well as other diseases related with airway inflammation.

Keywords: Dry cod skin collagen oligopeptides, asthma, NLRP3 inflammasome, NF-κB, cytokine

Introduction

Asthma is a chronic and complex airway inflammatory disease. It results from aberrant infiltration and activation of bronchus airway epithelial cells and immune cells, as well as production of inflammatory mediators [1, 2]. The disease is characterized by increased airway hyperresponsiveness, smooth muscle hyperplasia, and inflammatory cell aggregation. It is predominantly treated with inhaled corticosteroids. Incidence rates of asthma, in the past few decades, have steadily increased and are still on the rise, highlighting the need for development of safer and more effective methods of treatment.

Inflammasomes are polyprotein complexes that regulate inflammatory response to a great many stressors. In numerous inflammasomes, the most well-featured and best well-recognized complex is the Nod-like receptor protein 3 (NLRP3) inflammasome [3]. It typically contains three components, NLRP3, ASC, and pro-caspase-1 [4]. Exposure of the NLRP3 inflammasome to stimulation signals, such as microbial- and danger-associated molecular patterns, induces recruitment of the caspase-1 precursor to NLRP3 via ASC, leading to hydrolysis and becoming the enzyme activity caspase-1 [5]. Conversely, activated caspase-1 splits the cytokines from their precursor forms, such as pro-interleukin (IL)-1β and pro-IL-18, to their active forms. Secretion production of such cytokines, related with inflammation, further stimulates the host immune cells to produce additional inflammatory cytokines, including interferon (IFN)-gama and tumor necrosis factor (TNF)-α [6]. Collectively, these activated cells and inflammatory factors play a critical role in the etiopathogenesis of many diseases, including asthma.

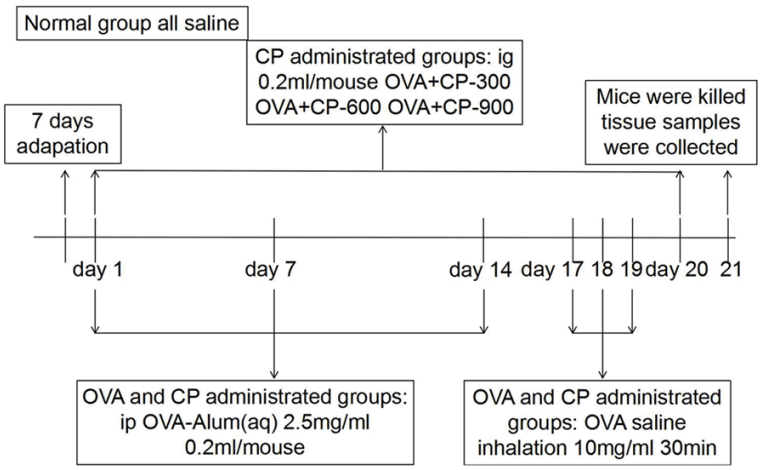

Figure 1. Protocol for animal experiments.

Dry cod skin collagen oligopeptide (CP) is a preparation of small proteins (average molecular weight <1 kDa) extracted from the dried skin of cod by biological enzymolysis, followed by lyophilization. The main components of CP are aspartic acid, glycine, and threonine, which enable human cells to fully replenish energy, nucleotides, electrolytes, and cell-signaling regulators. Recently, more and more studies have pointed out that CP increases the immune function of macrophages and lymphocytes. Other marine oligopeptides have also been confirmed to retard the progression of cancer and inflammatory diseases in animal models [7-12]. Chen et al. showed that collagen peptides decrease inflammation in a mouse burn model [7]. Alemán et al. demonstrated that squid gelatin hydrolysates produce anti-hypertensive, anti-cancer, and antioxidant activities in cell lines and animal models [8]. These studies have illustrated that CP may provide inflammation-suppression effects in various diseases, including asthma.

More and more studies have been conducted on the NLRP3 inflammasome. Evidence suggests that the NLRP3 inflammasome active is associated with the development of pulmonary inflammation in respiratory diseases, including asthma [13-15]. It may play a pivotal regulatory role in chronic obstructive pulmonary disease [6]. Investigating this in more detail, the current study examined influences of CP pretreatment on inflammation, as well as lung pathophysiology, in a mouse asthma model.

Material and methods

Animals

Male BALB/c mice, aged six-to-eight-weeks, were provided by the Experimental Animal Center of Yanbian University (YanJi, China). Before the experiment began, the mice were fed under standard experiment facility conditions (20-24°C, 12-hour light/dark for one day) for 7 days. All experiments associated with animals were carried out in accordance with requirements of the Yanbian University Animal Care Ethics Committee. All effort was made to decrease suffering.

Experimental design

After 7 days of acclimation, the mice were separated into five groups, with 10 mice in each group. These included normal, ovalbumin (OVA; Sigma, Darmstadt, Germany), OVA+CP-300, OVA+CP-600, and OVA+CP-900 groups. The CP groups were pretreated with daily administration of 300, 600, and 900 mg/kg body weight by oral gavage for 20 consecutive days (days 1 to day 20). Normal and OVA groups were orally administered an equal volume of saline on the same days. All OVA groups were immunized intraperitoneally with an OVA-alum solution. The concentration was 2.5 mg/mL (25 mg OVA plus 4 mL aluminum hydroxide adjuvant, plus 6 mL saline) on days 1, 7, and 14. Afterward, the mice that were immunized were stimulated through inhaled 1% OVA-saline aerosol. This lasted for 30 minutes from day 17 to 19 (see **Figure 1** for details). The mice were sacrificed on day 21 and processed as described below.

Sample collection

After the animals were sacrificed, the lungs were collected and separated into two portions. One part was fixed with 4% formaldehyde and inserted in paraffin. Fixed lung tissues were chipped into 4-μm sections and adsorbed on slides for histological staining. Regarding immunohistochemistry, some sections were deparaffinized and hydrated before staining, as

Table 1. Primer sequences for RT-PCR

Target genes	Primer sequences	Product length
NLRP3	F: GAGTTCTTCGCTGCTATGT	107 bp
	R: ACCTTCACGTCTCGGTTC	
Caspase-1	F: TATCCAGGAGGGAATATGTG	170 bp
	R: ACAACACCACTCCTTGTTTC	
ASC	F: ACACTTTGTGGACCAGCACA	116 bp
	R: CACGAACTGCCTGGTACTGT	
IL-1β	F: CAACCAACAAGTGATATTCTCCATG	152 bp
	R: GATCCACACTCTCCAGCTGCA	
IL-4	F: TCATCGGCATTTTGAACGAGGT	224 bp
	R: GCATCGAAAAGCCCGAAAGAG	
IL-5	F: AGCACAGTGGTGAAAGAGACCTT	117 bp
	R: TCCAATGCATAGCTGGTGATTT	
IL-6	F: AGTTGCCTTCTTGGGACTGA	159 bp
	R: TCCACGATTTCCCAGAGAAC	
IL-13	F: CCTGGCTCTTGCTTGCCTT	116 bp
	R: GGTCTTGTGTGATGTTGCTC	
IL-17	F: CTCAAAGCTCAGCGTGTCCA	171 bp
	R: TATCAGGGTCTTCATTGCGGTGGA	
IL-18	F: AGTAAGAGGACTGGCTGTGACC	214 bp
	R: TTGGCAAGCAAGAAAGTGTC	
IFN-γ	F: TGAGCTGATTGAATGCTTGG	148 bp
	R: GCCATCAGCAACAACATAAGC	
TNF-α	F: GGCAGGTCTACTTTGGAGTCATTG	299 bp
	R: ACATTCGAGGCTCCAGTCAATTCGG	
β-actin	F: TCTGGTCGTACCACAGGCAT	329 bp
	R: CGCTCGTTGCCAATAGTGAT	

described below. The remaining lung tissue specimens were frozen in liquid nitrogen and transferred to -80°C refrigerator for mRNA and protein analysis.

Histological analysis

Prepared lung sections were dyed with hematoxylin and eosin (H&E), estimating the infiltration of inflammatory cells into lung tissues. Periodic acid-Schiff (PAS) staining was used to detect the proliferation of goblet cells. Masson's trichrome staining was used to detect increases in collagen fibers. The sections were observed by light microscopy at the original magnification 200 ×.

Immunohistochemical (IHC) analysis

After deparaffinization and rehydration, prepared lung tissue sections were incubated overnight with rabbit antibodies against mouse NF-κB p65, Cox2 (both from CST, MA, USA), NLRP3, or caspase-1 (both from Abcam, Cambridge, UK) at 4°C. The sections were slowly washed with phosphate-buffered saline and incubated at an appropriate temperature with anti-rabbit IgG (ZSGB-BIO, Beijing, China), lasting for 20 minutes. Color development was achieved by incubation of sections with 3,3'-diaminobenzidine for 4 minutes in a black environment. Lastly, the sections were dehydrated, sealed, and observed by light microscopy at an original magnification 200 × or 400 ×.

RT-PCR

TC-512 PCR System (TECHNE, UK) was used to perform PCR, with the following amplification program: 95°C for 5 minutes, 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, for 30 cycles, as well as 72°C for 5 minutes and extension at 4°C for 30 minutes. Primer sequences for cytokines and the NLRP3 inflammasome are listed in **Table 1**.

Western blotting

Frozen lung tissues were removed from the -80°C refrigerator and thawed. When the tissues become soft, they were homogenized in a buffer containing protease inhibitors. A BCA assay kit (Biotime, Jiangsu, China) was used to test protein concentrations. Aliquots of homogenates (equivalent to 60 mg protein) were separated by 10-15% SDS-PAGE (Bio-Rad, Munich, Germany) and transferred to nitrocellulose membranes (Millipore, Burlington, MA, USA). Protein analysis was carried out by incubation of the membranes overnight with rabbit primary antibodies against mouse NF-κB p65 (1:1000), β-actin (1:1000), phosphorylated NF-κB-p65 (Ser 536) (1:500), NLRP3 (1:300), ASC (1:1000), IL-17A, TNF-α (1:200), IL-6 (1:1000), Cox2 (1:1000), caspase-1 (1:1000), and IL-18 (1:1000) or rat primary antibodies against mouse IL-5 (1:1000) (Novus Biologicals, USA), IFN-gama (1:1000), IL-4 (1:1000), and IL-13 (1:1000) (R&D Systems, MN, USA).

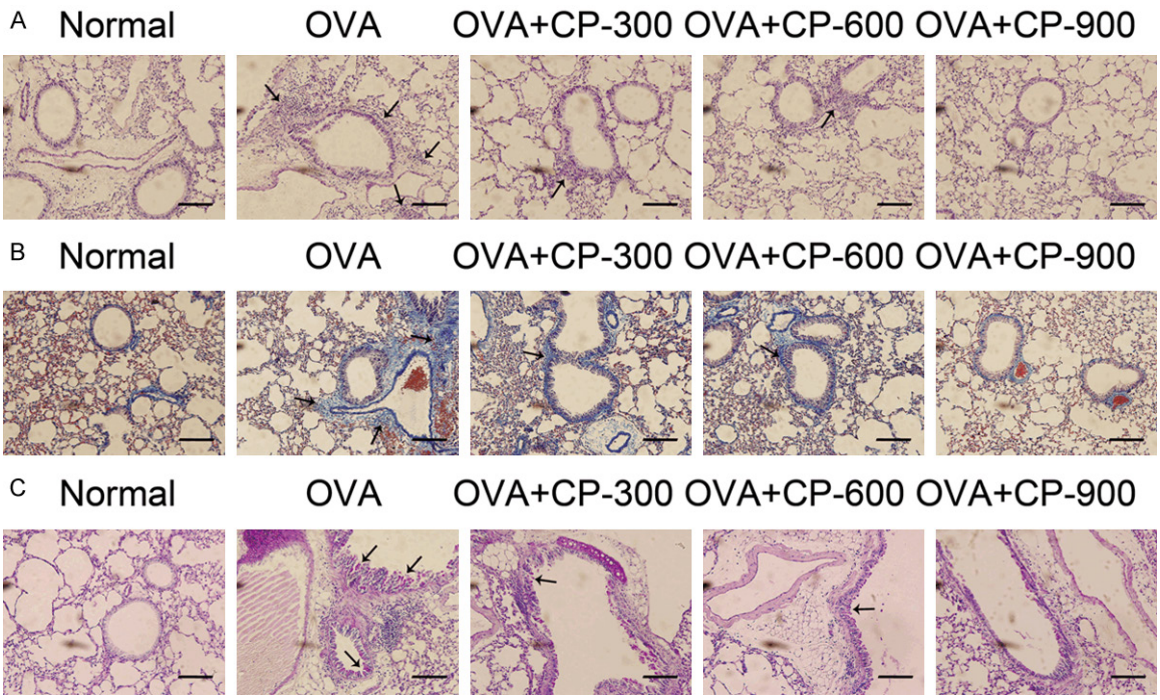

Figure 2. Effects of CP on histological changes in mouse lung tissues. A. H&E staining; B. Masson's trichrome stain; C. Periodic acid-Schiff's stain. Magnification 200 ×. Scale bars, 50 μm.

Nitrocellulose membranes were then slowly washed and covered with peroxidase-conjugated goat anti-rabbit or goat anti-rat IgG (1:5000) (Absin, Shanghai, China) at an appropriate temperature lasting for 1 hour. They were then washed to remove surplus reagent. Finally, an ECL detection system (Millipore) was used, following manufacturer instructions, to detect antibody binding.

Statistical analysis

Data are presented as mean ± SD and were analyzed with SPSS 14.0 (SPSS Inc, Chicago, USA). Two-tailed Student's t-tests were chosen to determine statistical significance ($p<0.05$ indicates significant differences).

Results

CP attenuates lung injuries in the mouse asthma model

To determine whether CP can alleviate and/or prevent asthma, the current study employed a well-known OVA-induced mouse asthma model. In this model, the mice are sensitized by OVA injections. Asthma is induced by OVA inhalation. Groups of mice were pretreated with CP by oral administration of 0 (saline for normal and OVA control groups), 300, 600, or 900 mg/kg daily for 20 days during the period of OVA sensitization and challenge. Histological analysis of the mouse lungs indicated that OVA induced marked pathological alterations, compared with lungs of the normal mouse group, including inflammatory cell aberrant infiltration, lumen stenosis, epithelial collagen fiber deposition, goblet cell hyperplasia, and mucus secretion (**Figure 2**). However, these changes were virtually absent from the lungs of mice pretreated with 300-900 mg/kg CP and challenged with OVA (**Figure 2**). Results suggest that CP administration markedly reduced asthma-associated lung injuries.

CP attenuates inflammatory cytokine mRNA expression in the mouse asthma model

Next, this study asked whether the beneficial effects of CP were accompanied by a reduction in inflammatory cytokine expression in the injured lungs. For this, RT-PCR analysis was conducted for cytokine mRNAs in homogenized lung tissues. As shown in **Figure 3**, OVA injections and inhalation significantly increased mRNA levels of nearly all cytokines, compared

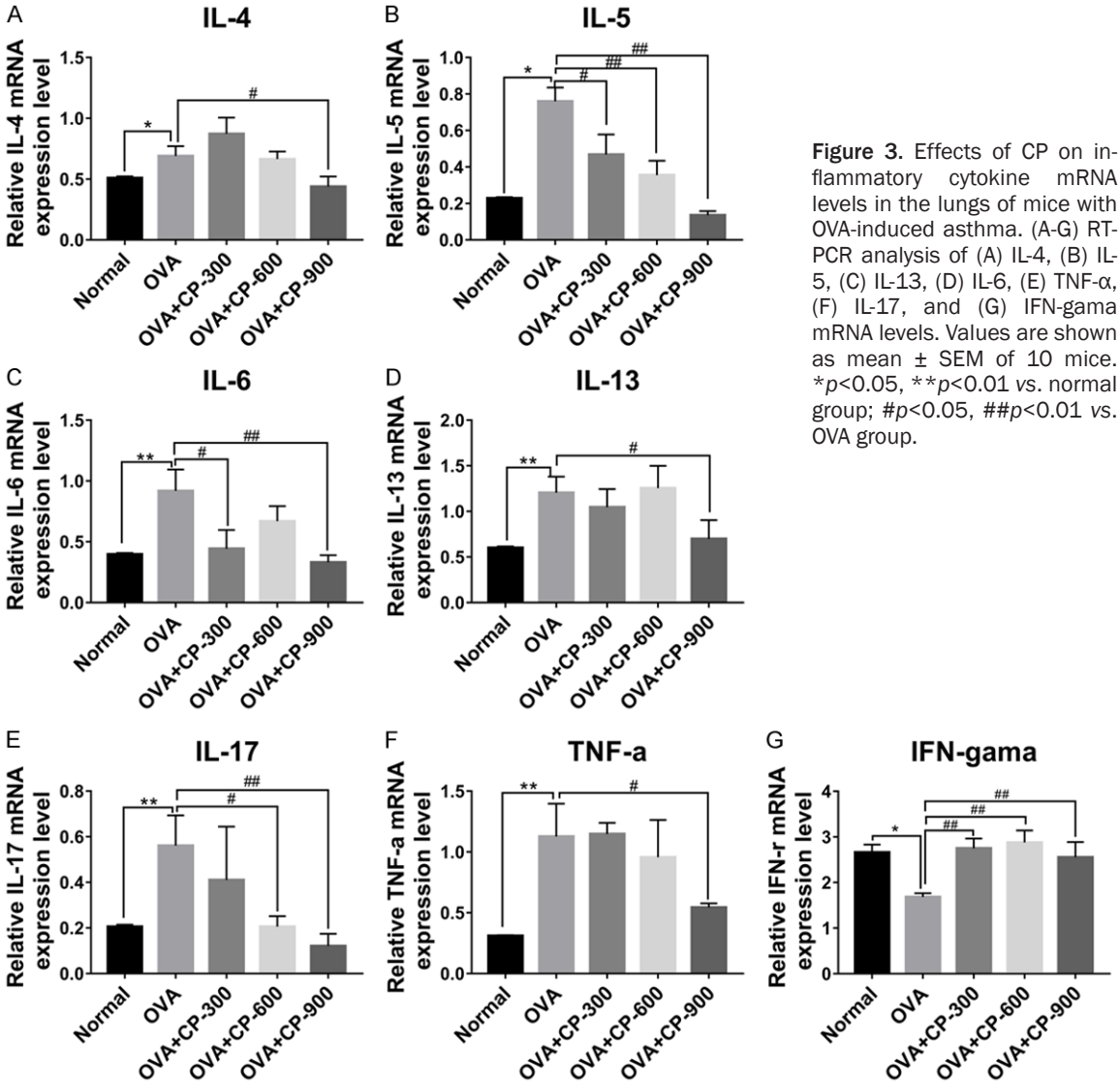

Figure 3. Effects of CP on inflammatory cytokine mRNA levels in the lungs of mice with OVA-induced asthma. (A-G) RT-PCR analysis of (A) IL-4, (B) IL-5, (C) IL-13, (D) IL-6, (E) TNF-α, (F) IL-17, and (G) IFN-gama mRNA levels. Values are shown as mean ± SEM of 10 mice. **p*<0.05, ***p*<0.01 *vs*. normal group; #*p*<0.05, ##*p*<0.01 *vs*. OVA group.

with the normal group. However, these changes were all obviously inhibited in mice exposed to OVA and pretreated with CP-900 group ($p<0.05$, **Figure 3A-F**). In the CP-300 group, IL-5 and IL-6 mRNA levels were downregulated ($p<0.05$, **Figure 3B**, **3C**). In the CP-600 group, only IL-5 and IL-17 mRNA levels were inhibited, compared with the OVA group ($p<0.05$, **Figure 3B**, **3E**). Results suggest that the CP-900 group had the best results, while the other two groups have partial effects. Notably, IFN-gama mRNA levels were decreased after induction of asthma. However, levels were significantly increased by CP treatment ($p<0.05$, **Figure 3F**). Thus, CP attenuates the transcription of inflammatory cytokines in the lungs of asthmatic mice.

CP attenuates inflammatory cytokine protein expression in the mouse asthma model

After the effects of CP treatment on cytokine mRNA levels were confirmed, Western blot analysis was conducted to observe protein levels in the lung tissues. IL-4, 5, 6, 13, 17A, and TNF-α protein levels were obviously upregulated in the OVA group, compared to the normal group. However, as observed for mRNA levels, protein upregulation was significantly inhibited by CP treatment for all cytokines ($p<0.05$, **Figure 4A-G**), except IFN-gama, which was significantly increased by CP treatment ($p<0.05$, **Figure 4H**). Results confirm that CP can attenuate OVA-induced inflammatory cytokine expression in this asthma model.

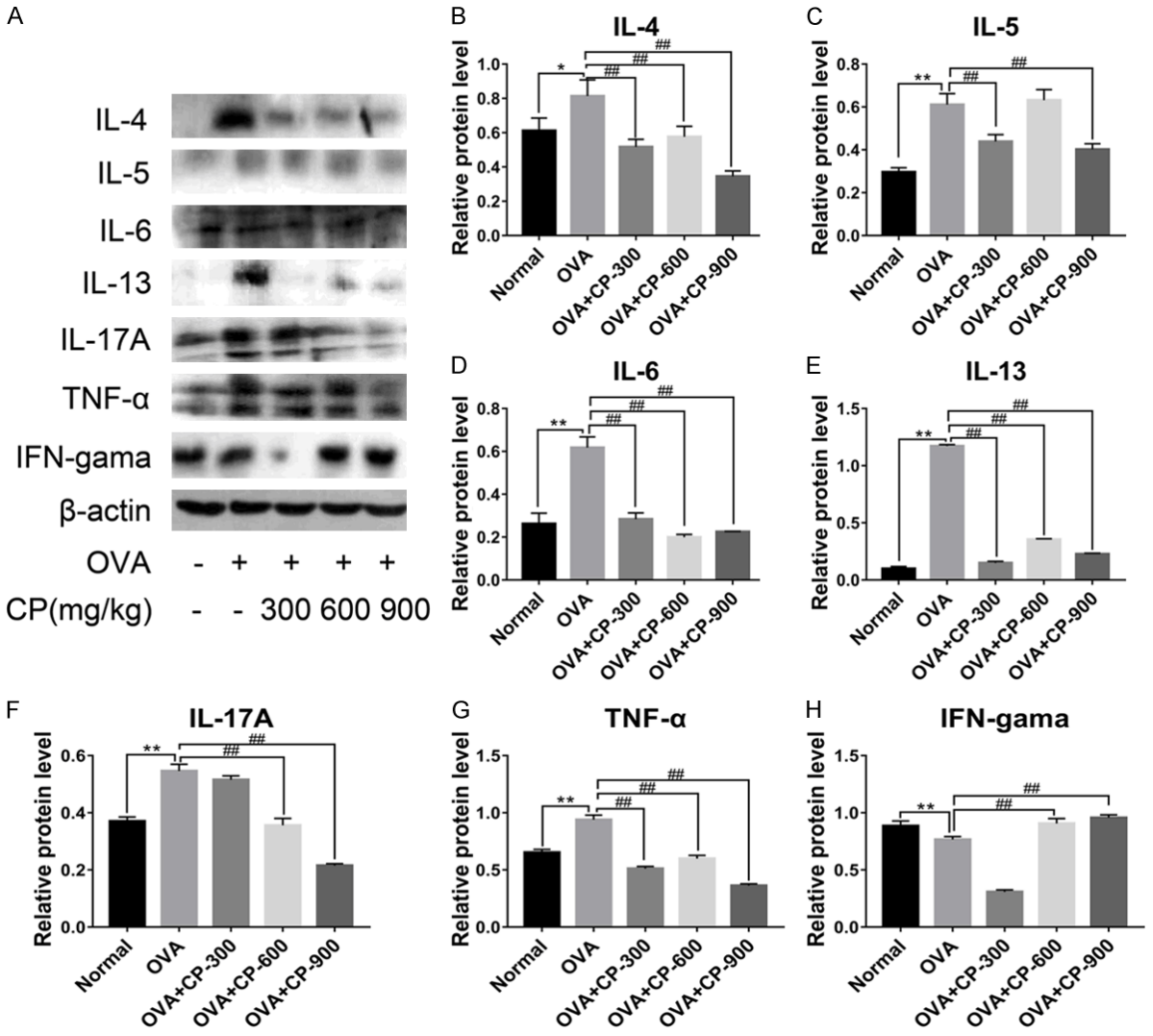

Figure 4. Effects of CP on inflammatory cytokine protein levels in the lungs of mice with OVA-induced asthma. (A-H) Western blot analysis of (B) IL-4, (C) IL-5, (D) IL-6, (E) IL-13, (F) IL-17A, (G) TNF-α, and (H) IFN-gama. Values are shown as mean ± SEM of 10 mice. **p*<0.05, ***p*<0.01 *vs*. normal group; #*p*<0.05, ##*p*<0.01 *vs*. OVA group.

CP reduces NF-κB signaling in the mouse asthma model

NF-κB is a well-known part of inflammation-related transcription genes [16]. To evaluate the effects of CP on this transcription factor, the current study analyzed expression levels of the serine 536-phosphorylated (active) form of NF-κB p65 and one of its downstream target genes, cyclooxygenase enzyme Cox2 [17]. It was found that asthma was associated with an increase in NF-κB activity, as evidenced by elevated phospho-NF-κB p65 and Cox2 protein levels in the lung tissues isolated from OVA-treated mice, compared with mice in the normal group (**Figure 5A-C**). It was observed that CP treatment markedly reduced expression levels of both phospho-NF-κB p65 and Cox2 in the CP-900 group ($p<0.05$, **Figure 5A-C**). To verify, IHC staining of lung tissues was performed. Analysis revealed a higher level of phospho-NF-κB p65 in the nucleus of asthma model mice lung cells, compared to normal mice. Protein levels were markedly decreased in the lungs of CP-treated mice (**Figure 5D**). Consistent with these findings, Cox2 was also increased in the lung tissues of asthma model mice, compared to normal mice. OVA effects were reversed by CP treatment (**Figure 5E**).

CP reduces activation of NLRP3 inflammasome in the mouse asthma model

NF-κB activation leads to transcription of NLRP3 and is the first initiate signal of NLRP3 inflammasome [18-20]. Given the finding that

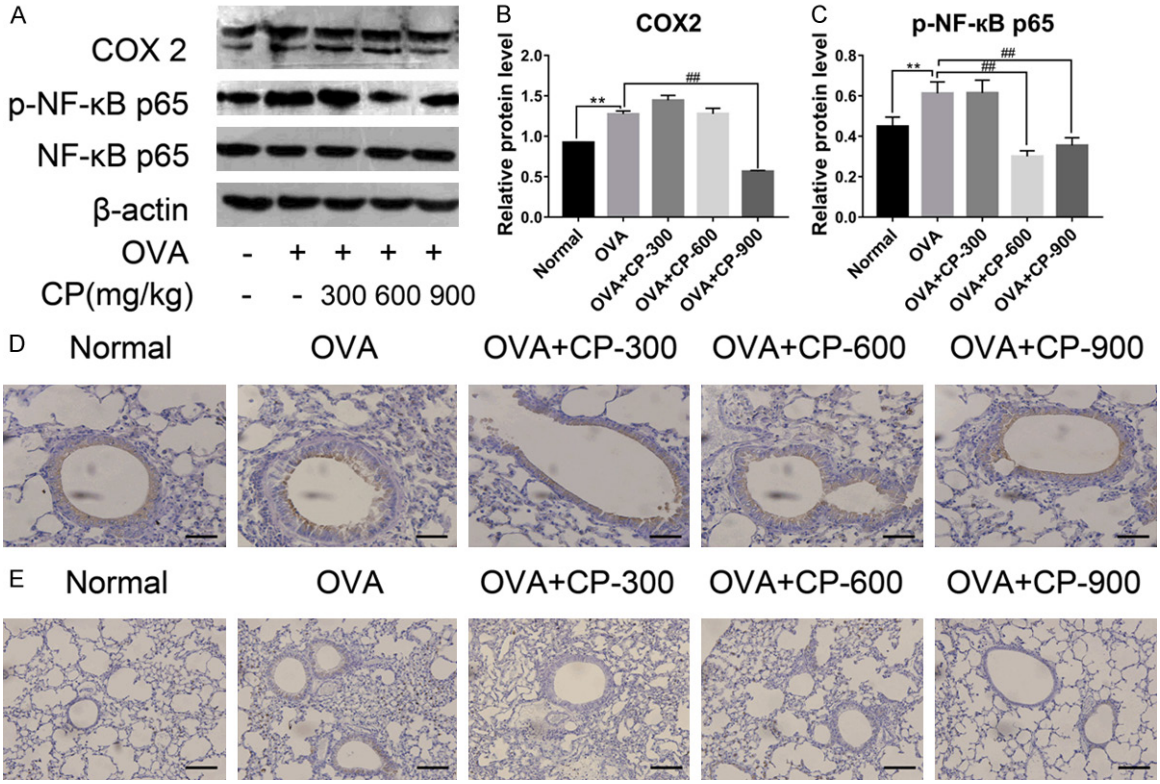

Figure 5. Effects of CP on NF-κB p65 and Cox2 protein expression in the lungs of mice with OVA-induced asthma. (A-C) Western blot analysis of (A) phosphorylated NF-κB p65 and Cox2, (B) total Cox2, and (C) total NF-κB p65. (D and E) Immunohistochemistry analysis of NF-κB p65 (D) and Cox2 (E) protein expression in lung tissue. Magnification 200 × (Cox2) and 400 × (NF-κB). Scale bars, 50 μm (Cox2) and 25 μm (NF-κB). Values are shown as mean ± SEM of 10 mice. *p<0.05, **p<0.01 *vs*. normal group; #p<0.05, ##p<0.01 *vs*. OVA group.

CP inhibited transcription factor NF-κB activation in OVA-challenged mice lungs tissues, expression levels of compositions of NLRP3 inflammasome were examined. It was discovered that NLRP3, ASC caspase-1, and downstream cytokine IL-18 and IL-1β mRNA levels had a marked upregulation in the lungs from OVA-exposed mice. Consistent with the effects on NF-κB, these increases were significantly inhibited by CP treatment (p<0.05, **Figure 6A-E**). Protein analysis revealed that OVA and CP had the same effects on expression of the corresponding proteins (p<0.05, **Figure 6F-J**). Finally, IHC analysis of lung tissues was conducted. As expected, in OVA-exposed mice, NLRP3 and caspase-1 were increased, compared with normal mice (**Figure 6K**, **6L**). However, levels were reduced in mice treated with CP concomitantly with OVA sensitization and challenge (**Figure 6K**, **6L**).

Discussion

Asthma is a complex respiratory disorder disease [21]. It is expressed by lung injuries and persistent airway inflammation associated with hyper-responsiveness [22]. It has become one of the most widespread diseases, worldwide, especially among children. Although mortality rates from asthma are relatively low compared with other diseases, it is a protracted illness accompanied by repeated attacks. It has a difficult healing process, severely impairing quality of life. Therefore, it is necessary to discover safe and effective treatments for this disease. The current study investigated influences and underlying mechanisms of action of CP, establishing an OVA-induced *in vivo* model of mouse asthma. Present data shows that CP significantly ameliorates OVA-induced changes in lung histopathology and inflammatory cytokine production in lung tissues, providing support for the possible function of CP for asthma therapy.

Inflammation is a pivotal part of asthma occurrence and development. Asthma has always been an allergic disease with typical Th2 inflammatory characteristics [23]. When asthma occurs, Th2 cells have been shown to secrete

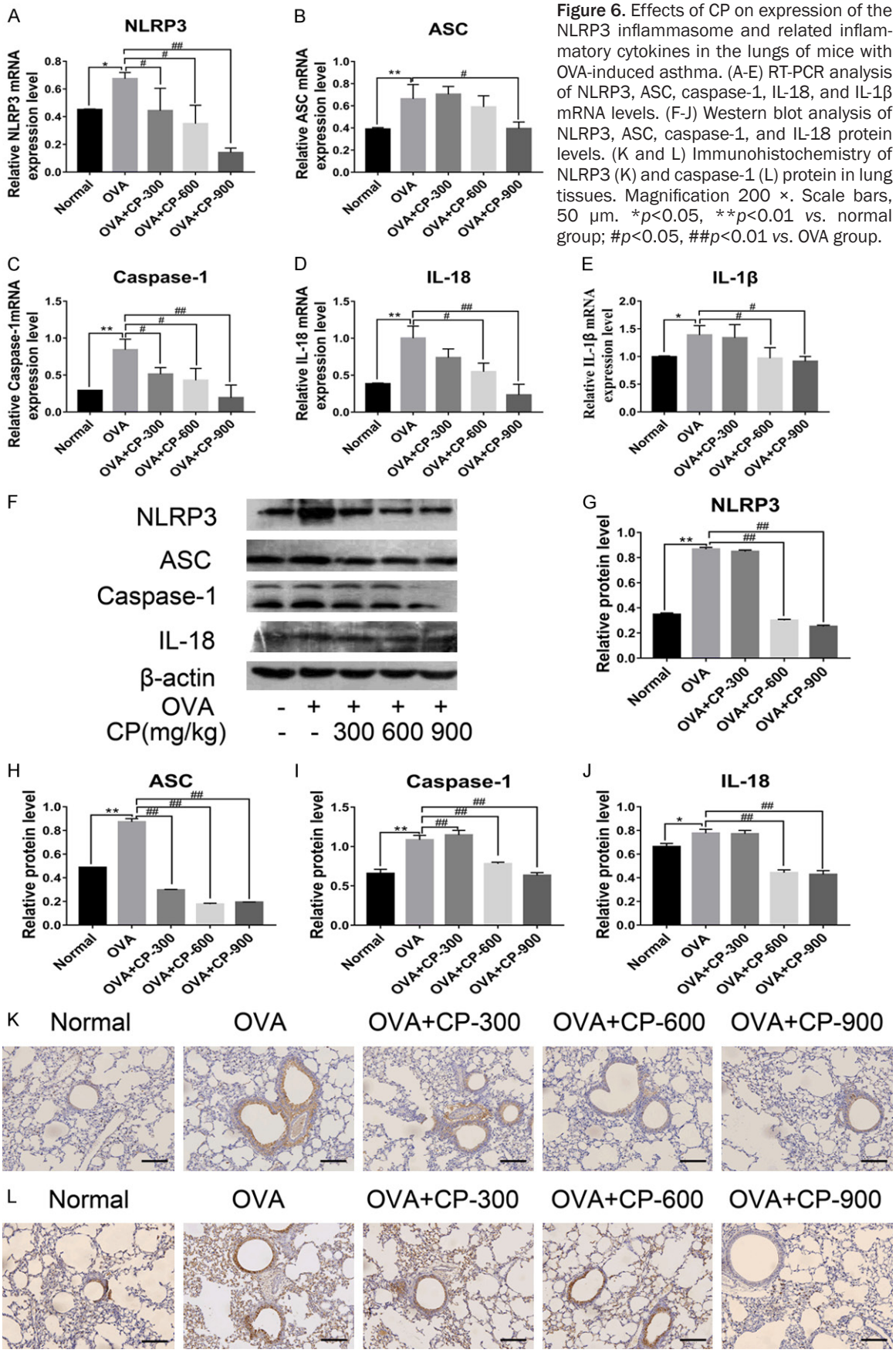

Figure 6. Effects of CP on expression of the NLRP3 inflammasome and related inflammatory cytokines in the lungs of mice with OVA-induced asthma. (A-E) RT-PCR analysis of NLRP3, ASC, caspase-1, IL-18, and IL-1β mRNA levels. (F-J) Western blot analysis of NLRP3, ASC, caspase-1, and IL-18 protein levels. (K and L) Immunohistochemistry of NLRP3 (K) and caspase-1 (L) protein in lung tissues. Magnification 200 ×. Scale bars, 50 μm. *p<0.05, **p<0.01 *vs*. normal group; #p<0.05, ##p<0.01 *vs*. OVA group.

IL-4, 5, 6, and 13 cytokines [24, 25], consistent with current findings. In the current study, Th1 cytokine IFN-gama was decreased. Expression was increased after treatment with CP in the lung tissues of model mice. This finding is related with the typical opposing response of Th1 and Th2 cytokines in asthma resulting from hyperactivity of Th2 cells relative to Th1 cells [26]. This leads to an imbalance in Th1/Th2 response, resulting in chronic airway inflammation [28]. IFN-gama and IL-4 have been considered as the characteristic Th1 and Th2 cytokines, respectively, playing opposing positive and negative roles in the pathology of asthma [29]. Moreover, IL-4 promotes IgE synthesis, downregulates IFN-γ secretion, and participates in airway mucosal maintenance [27].

NF-κB is a crucial transcription factor in inflammatory diseases because it regulates the activity of many pro-inflammatory signaling pathways [30], including activation of the NLRP3 inflammasome [31]. Thus, NF-κB acts as the start-up priming signal for activation of this inflammasome [32, 33]. The current study confirmed that levels of active (phosphorylated) NF-κB p65 were increased in OVA-induced asthma model mice lung tissues. Levels were significantly inhibited by CP treatment. Similarly, results showed OVA-induced elevated expression of NLRP3 inflammasome in lungs of asthmatic mice. Caspase-1 is produced through pro-caspase-1 self-cleavage [34] and some other cytokine precursors. For example, pro-IL-18 and pro-IL-1β could be cleaved by enzymatically active caspase-1 and changed to be the active forms [35]. Present results are precisely consistent with this scenario, in which IL-1β and IL-18 protein expression levels were noticeably increased in OVA-challenged mice lung tissues. CP treatment significantly inhibited expression levels.

Conclusion

In summary, present results suggest that CP inhibits lung injuries and inflammatory changes associated with asthma in an OVA-induced mouse model. The current study also demonstrates that the mechanisms of action of CP involve suppression of NF-κB activation and suppression of NLRP3 inflammasome activity, as well as upregulation of asthma-related inflammatory cytokines. Therefore, current results supply strong support the potential clinical utility of CP as an asthma therapy medicine, as well as other inflammatory diseases.

Acknowledgements

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Disclosure of conflict of interest

None.

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