



Pulmonary, gastrointestinal and urogenital pharmacology

Potent ameliorating effect of Hypoxia-inducible factor 1 α (HIF-1 α) antagonist YC-1 on combined allergic rhinitis and asthma syndrome (CARAS) in Rats



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ABSTRACT

Recent studies have implicated that Hypoxia-inducible factor 1 α (HIF-1 α) plays an integral role in the pathogenesis of allergic rhinitis and asthma. In the present study, we showed that HIF-1 α antagonist YC-1, 3-(5-hydroxymethyl-2-furyl)-1-benzylindazole, elicited a potent allergy-ameliorating effect in a rat model of ovalbumin (OVA)-sensitized combined allergic rhinitis and asthma syndrome (CARAS). We revealed that YC-1 administration markedly impaired the total number and percentage of eosinophil in bronchoalveolar lavage fluid (BAL Fluid) of the rats, suggesting that YC-1 might attenuate lung and nasal mucosal inflammation in OVA-sensitized rats. Moreover, histological examination found that OVA-induced pathological alterations were evidently attenuated following YC-1 administration. In addition, immunohistochemical analysis indicated that YC-1 treatment decreased the expression of HIF-1 α in rat lungs and nasal mucosa. Notably, Nuclear factor kappa B (NF- κ B) p65 and Peroxisome proliferator-activated receptor α (PPAR α), two important regulators of inflammatory responses, were also significantly down-regulated following YC-1 administration. Real-time PCR analysis confirmed that YC-1 impaired the expression of HIF-1 α , NF- κ B and PPAR α in CARAS model. These findings together indicated that YC-1 exerted remarkable anti-allergic effects through the modulation of inflammatory pathways, implying that YC-1 may potentially serve as a novel anti-CARAS medicine in clinical patients.

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1. Introduction

Allergic rhinitis and asthma have become increasingly prevalent in recent decades all around the world. Accumulating evidence indicates that allergic rhinitis and asthma co-exist commonly in a large proportion of patients (Togias, 2003). Studies have found that 70–90% patients with asthma suffered with allergic rhinitis. Likewise, almost 40% patients with allergic rhinitis display asthmatic symptoms, suggesting a close relationship between the two symptoms (Leynaert et al., 2000; Pawankar et al., 2009). Therefore, combined allergic rhinitis and asthma syndrome (CARAS) has been defined to indicate concurrent rhinitis and asthma symptoms in clinical patients (Taramarcaz and Gibson, 2004; Togias, 2003). Despite the fact that CARAS has been increasingly recognized by clinical and basic researchers, the treatment of this syndrome has not yet received considerable attention.

The pathogenesis of CARAS involves immunologic responses to allergens in both upper and lower respiratory tracts. Although

allergic rhinitis and asthma occur in different tissues, they share the same molecular events and immunologic responses (Casale and Dykewicz, 2004). Mounting investigations implicate that chronic inflammation plays a vital role in the development of allergic rhinitis and asthma (Jeffery and Haahtela, 2006). Allergens stimulate the degranulation of mast cells, resulting in consequent recruitment of various inflammatory cell phenotypes and the release of pro-inflammatory mediators (Braunstaal et al., 2003). Eosinophilic airway inflammation has been observed in both upper and lower respiratory tract in patients with chronic rhinitis and asthma (Alvarez et al., 2000).

Allergic inflammation involves complicated molecular mechanisms. Recently, it was reported that Hypoxia-inducible factor-1 α (HIF-1 α) played a key role in the regulation of inflammatory responses. HIF-1 α is required for myeloid cell-triggered inflammation (Cramer et al., 2003; Walmsley et al., 2005). HIF-1 α promotes epithelial inflammation through cell autonomous NF- κ B activation (Scortegagna et al., 2008). Importantly, HIF-1 α directly mediates the transcription of iNOS and VEGF to induce vascular permeability and inflammatory responses (Ahluwalia and Taranowski, 2012; Peyssonnaud et al., 2005). Accordingly, inhibition of

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HIF-1 α ameliorates allergic phenotype in airway (Kim et al., 2010). In addition, airway hyper-responsiveness was significantly reduced after HIF-1 α inhibition. These findings together implicated that HIF-1 α might play an essential role in the pathogenesis of allergic rhinitis and asthma.

Because of the importance of HIF-1 α in immunological and allergic regulation, pharmacological agents that antagonize HIF-1 α activity may exert potential anti-allergic effects in the treatment of CARAS. Interestingly, 2-methoxyestradiol, a natural HIF-1 α antagonist, impaired the pathologies of both rhinitis and asthma, respectively (Kim et al., 2010; Zhou et al., 2012). However, 2-methoxyestradiol is unsuitable for in vivo therapy in human bodies, mainly due to extremely low level of bioavailability (Dahut et al., 2006). Given these findings, we analyzed the potential therapeutic merit of YC-1, a synthesized HIF-1 α antagonist that has been widely used in vivo, on CARAS. We established a rat model of CARAS by intraperitoneally injecting ovalbumin emulsified with complete Freund's adjuvant. Administration of YC-1 markedly ameliorated the allergic responses in ovalbumin-allergic rats. Our findings implicated that YC-1 might potentially serve as a therapeutic agent in the treatment of combined allergic rhinitis and asthma syndrome.

2. Materials and methods

2.1. Establishing the rat model of CARAS

Adult male Sprague-Dawley (SD) rats (n=30, body weight=220 g) were obtained from Laboratory Animal Center, Nantong University. The rats were randomly divided into 3 groups, namely Control group (Con), OVA with aluminum hydroxide adjuvant group (OVA+Al(OH)₃) and OVA with complete Freund's adjuvant group (OVA+FA) (10 rats in each group). The rats were housed in a 12-h light/dark cycle and had free access to water and food. Both of food and water were sterilized before feeding. For the establishment of rat model of CARAS, rats were intraperitoneally injected with 20 mg/kg OVA and two different types of adjuvants, aluminum hydroxide or complete Freund's adjuvant, respectively. The rats received the same doses of OVA and aluminum hydroxide adjuvant (for OVA+Al(OH)₃ group) or incomplete Freund's adjuvant (for OVA+FA group) every other day for additional 7 times. 16 days later, the rats further received an additional daily dose of 20 mg/kg OVA through ultrasonic atomizing inhalation for 7 days. After atomizing inhalation of OVA for 7 days, allergic rhinitis reactions were examined through dropping 50 μ l 2% OVA into nasal cavities of the rats. Significant allergic rhinitis symptoms, including high frequency of nasal rubbing, sneezing and runny nose, were observed in every rat exposed to OVA+Al(OH)₃ or OVA+FA. Control rats received the same volume of 0.9% saline throughout the experiment. Animal care was performed in accordance with National of Health Guideline for the Care and Use of Laboratory Animals. The experimental procedures have been approved by the Animal Care and Use Committee of Nantong University of China.

2.2. Administration of YC-1 in OVA-sensitized rats

To analyze the ameliorating effect of HIF-1 α on allergic rhinitis and asthma, another 30 adult male Sprague-Dawley (SD) rats were employed and randomly divided into three groups, Control group, OVA group and OVA+YC-1 group (10 rats in each group; body weight=220 g). The rats were sensitized using OVA and incomplete Freund's adjuvant (OVA group and OVA+YC-1 group) as mentioned above. For YC-1 administration, at the 2th day and the 5th day of OVA atomizing inhalation, the rats in OVA+YC-1 group were intraperitoneally administrated with 2 mg/kg YC-1 (3-[5'-

hydroxymethyl-2'-furyl]-1-benzyl indazole) (AG Scientific, San Diego, CA). OVA and Control rats were administrated with the same volume of DMSO.

2.3. Immunohistochemical analysis

The rats were anesthetized using 10% chloral hydrate and perfused pericardially using 0.9% saline followed by 4% paraformaldehyde. The lung and front part of nasal cavity of each rat was removed and subjected to paraformaldehyde fixation for 12 h. Thereafter, the tissues were cryoprotected with 20% sucrose for 48 h followed by 30% sucrose for an additional 48 h. Next, the tissues were embedded in O.C.T. compound (Sakura, Sapporo, Japan) and 7- μ m frozen sections were prepared. The sections were blocked using a blocking solution containing 1% (w/v) bovine serum albumin, 10% donkey serum, 0.5% Triton X-100 and 0.2% Tween-20 for 2 h at 25 °C. Primary antibody incubation was conducted with rabbit anti-HIF-1 α (1:100; Cell Signal), anti-NF- κ B p65 (1:100; Santa Cruz) and anti-PPAR α (1:100; Santa Cruz) antibodies. Control sections were performed with rabbit normal serum. The sections were washed with PBS for three times, and incubated using biotinylated secondary antibody for 30 min. Later the sections were incubated with 0.02% diaminobenzidine tetrahydrochloride plus 3% H₂O₂ in PBS. Finally, the sections were dehydrated and coverslipped. All sections were examined using a Leica light microscope (Germany).

2.4. The detection of serum IgE level

Serum level of IgE in each rat was analyzed using an IgE ELISA Kit (Bethyl laboratories, Inc., Montgomery, TX) in accordance with the manufacturer's instructions.

2.5. The assessment of eosinophil number in bronchoalveolar lavage fluid (BAL Fluid)

The assessment of eosinophil in BAL Fluid was performed using a protocol described by Pope SM et al. (Pope et al., 2001). Briefly, the lungs of rats were lavaged with 2.0 ml PBS containing 1 mM EDTA for 4 times. The BAL Fluid was collected and centrifuged at 800 $\times$ g for 5 min at 4 °C. Next the cell pellet was resuspended using 1 ml PBS containing 1 mM EDTA. The total numbers of cells were counted using a hemacytometer. Furthermore, the cells were subjected to Wright-Giemsa staining, and differential cell counts were determined. At least 500 cells were calculated in each sample and the assay have been repeated for three times.

2.6. RNA isolation and real time PCR analysis

After euthanization, the tissues of the rats were taken and immediately frozen using liquid nitrogen. Next, adequate volume of Trizol reagent was added and the total RNA was isolated. The RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Logan, UT). Real-time PCR analysis was conducted using a LightCycler 480 qPCR System (Roche, Basel, Switzerland). The primers used for real-time PCR were as follow:

2.7. Statistical analysis

Statistical analysis were conducted using Stata 7.0 statistical software. Statistical significance was assessed via a one-way analysis of variance (ANOVA) and Tukey's post hoc multiple comparison tests. P-values less than 0.05 were considered to be statistically significant.

3. Results

3.1. The establishment of ovalbumin-allergic rat model of combined allergic rhinitis and asthma syndrome

Although many previous reports have documented that injection of ovalbumin (OVA) could induce allergic asthma and rhinitis in rat models, the co-existence rat of the two symptoms has not been extensively reported. Therefore, we evaluated the rate of CARAS in Rats following OVA sensitization. Two groups (n=10) of rats were intraperitoneally injected with 20 mg/kg OVA and two different types of adjuvants, aluminum hydroxide and complete Freund's adjuvant, respectively. Additional injection of OVA and adjuvants was performed every other day for 7 times. Next, the rats received the same dose of ovalbumin exposure by ultrasonic

atomizing inhalation for the next 7 days. Finally, the symptoms of CARAS were evaluated within 30 min after ultrasonic atomizing inhalation through dropping 50 μ l 2% OVA into nasal cavities of the rats. We observed significant allergic responses in rats, including high frequency of nasal rubbing, sneezing and runny nose. To verify whether the rats suffered from allergy, the serum level of IgE was determined using an IgE ELISA kit. As shown in Fig. 1A, both the two groups of OVA-sensitized rats, especially the rats using Freund's adjuvant, exhibited remarkably elevated levels of serum IgE after OVA sensitization, whereas the serum IgE levels of control rats remained largely unchanged following 0.9% saline treatment. In addition, we found apparent pathological change in nasal mucosa of OVA-sensitized rats, including hyperemia, edema and necrosis (Fig. 1B, arrowheads). Furthermore, the lungs of OVA-sensitized rats also displayed elevated thickness of the alveolar

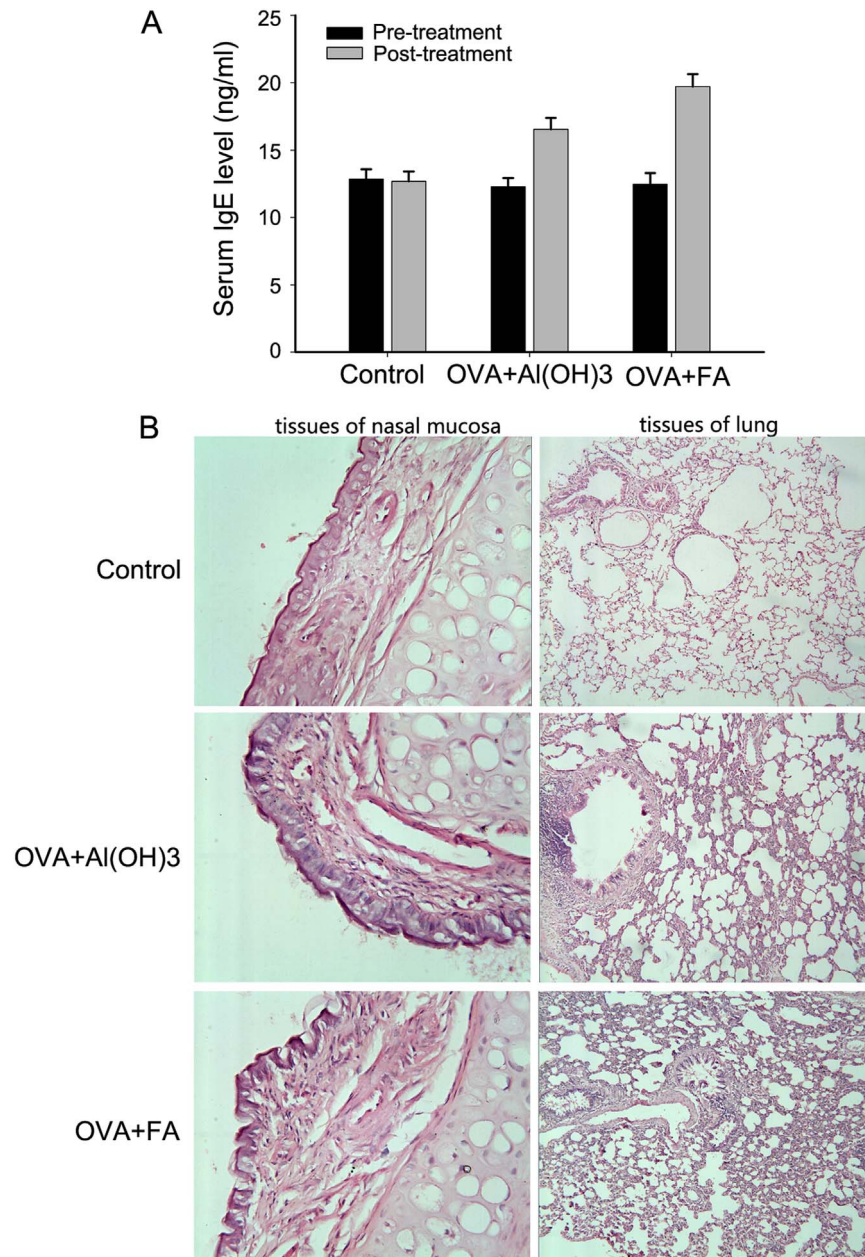


Fig. 1. The establishment of rat model of OVA-sensitized CARAS. Two groups of rats were treated with OVA combined with aluminum hydroxide or incomplete Freund's adjuvant, as was indicated in the materials and methods section. (A) Serum level of IgE was determined using ELISA assay in the indicated groups of rats before and after OVA or 0.9% saline treatment ($P < 0.05$). OVA+Al(OH)₃, OVA plus aluminum hydroxide treatment; OVA+FA, OVA plus incomplete Freund's adjuvant treatment. (B) Histological analysis of the pathological changes in rat lung and nasal mucosa following OVA sensitization. Left, hyperplasia in nasal mucosal epithelium following OVA treatment (Black arrowheads); Right, significant thickening of interstitial tissues and bronchial walls in OVA-sensitized rats (Black arrowheads).

Table 1

OVA treatment significantly increased the total number and the percentage of EOS in BAL Fluid (n = 10).

Group (n = 10)	Total number of cells ($\times 10^6 \text{ L}^{-1}$)	EOS%
Control	0.26 ± 0.21	2.14 ± 1.17
OVA + Al(OH) ₃	5.26 ± 0.51^a	34.25 ± 7.32^a
OVA + FA	5.90 ± 0.64^a	37.48 ± 6.27^a

^a $P < 0.05$, compared with control group.

interstitial matrix, compared with the lungs of control rats. The number of eosinophil in BAL Fluid of each group of rats were evaluated. As shown in Table 1, both the two groups of OVA-sensitized rats had evidently increased number of eosinophil in their BAL Fluid. These findings suggested that OVA-sensitized rats exhibited significant CARAS symptoms.

3.2. HIF-1 α inhibitor YC-1 ameliorated CARAS in OVA-sensitized rats

Next, we explored the effect of YC-1 administration on the pathological alterations in OVA-sensitized rats. Rats were mock

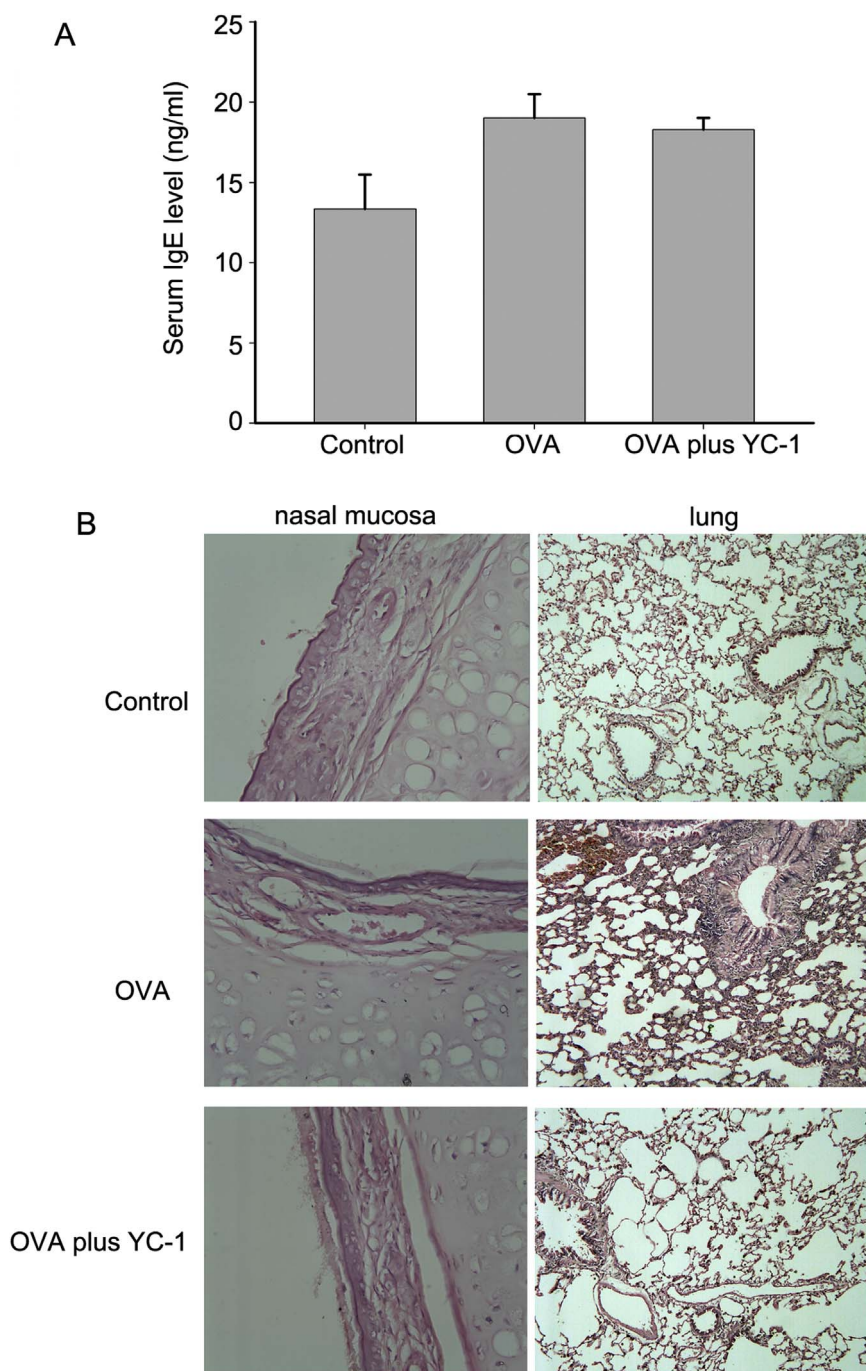


Fig. 2. YC-1 ameliorated OVA-induced eosinophil infiltration and pathological alterations in the lungs and nasal mucosa of rats. (A) The serum level of IgE in control, OVA-exposed and OVA + YC-1 (OVA + YC-1)-treated rats was determined using ELISA assay. (B) The pathological changes of rat lung and nasal mucosa were determined in the indicated groups of rats. Left, Arrowheads indicated that nasal mucosal epithelium in OVA-treated rats exhibited significant hyperplasia and hyperemia, which was not found in the other two groups; Right, arrowheads showed different thickness of bronchial walls among the indicated groups of rats.

Table 2

YC-1 administration significantly impaired the total number and the percentage of EOS in BAL Fluid following OVA treatment (n=10).

Group (n=10)	Total number of cells ($\times 10^6 \text{ L}^{-1}$)	EOS%
Control	0.24 ± 0.20	3.58 ± 0.32
OVA	5.73 ± 0.49^a	33.01 ± 2.15^a
OVA plus YC-1	2.20 ± 0.58^b	17.36 ± 2.32^b

^a $P < 0.05$, compared with control group.

^b $P < 0.05$, compared with OVA-treated group.

treated, treated with OVA or OVA plus YC-1 (OVA+YC-1). The serum level of IgE was firstly determined, which indicated that YC-1 administration did not significantly alter the serum level of IgE, compared with OVA-treated rats (Fig. 2A). Next, we analyzed the number of eosinophil in BAL Fluid in the indicated groups. Very interestingly, YC-1 administration markedly attenuated the level of total number and percentage of eosinophil in BAL Fluid (Table 1). In addition, the pathological changes of rat lungs and nasal mucosa were examined. As shown in Fig. 2B, OVA exposure induced significant pathological alterations in the lungs and nasal mucosa of rats, including bronchial hyperplasia, bronchial straitness, partial loss of alveolar epithelial cells, aberrant structure in Nasal mucosa epithelium et al. However, these pathological changes were totally attenuated in response to YC-1 treatment, suggesting that YC-1 administration could significantly ameliorate the pathology of CARAS (Table 2).

3.3. YC-1 administration attenuated OVA-induced HIF-1 α expression in rat lung and nasal mucosa

Because YC-1 mainly functions through negatively regulating the activity of HIF-1 α , we analyzed whether the expression of HIF-1 α was altered following YC-1 administration. Consistent with previous reports, the expression HIF-1 α was robustly induced following OVA exposure in rat lung and nasal mucosa (Fig. 3). Next, we investigated the influence of YC-1 on HIF-1 α expression. As predicted, YC-1 administration remarkably attenuated OVA-induced expression of HIF-1 α in rat rat lung and nasal mucosa. These findings indicated that YC-1 treatment resulted in the attenuation of HIF-1 α activation during CARAS.

3.4. YC-1 administration impaired the activation of NF- κ B and PPAR α in rat lung and nasal mucosa following OVA exposure

Because NF- κ B signaling plays a central role in chronic inflammation and allergy, we analyzed whether NF- κ B signaling was altered following YC-1 treatment. Immunohistochemical analysis revealed that OVA sensitization significantly triggered the expression of NF- κ B p65 in rat nasal mucosa and lung. Of note, YC-1 treatment led to significantly impaired expression of NF- κ B p65 in the both tissues (Fig. 4). These findings implied that YC-1 may ameliorate the inflammatory responses in rat nasal mucosa and lung through negatively modulating NF- κ B signaling.

PPAR family proteins have been also documented to play vital roles in the regulation of inflammation and allergy. Interestingly, it was reported that both PPAR- α and PPAR- γ was upregulated and

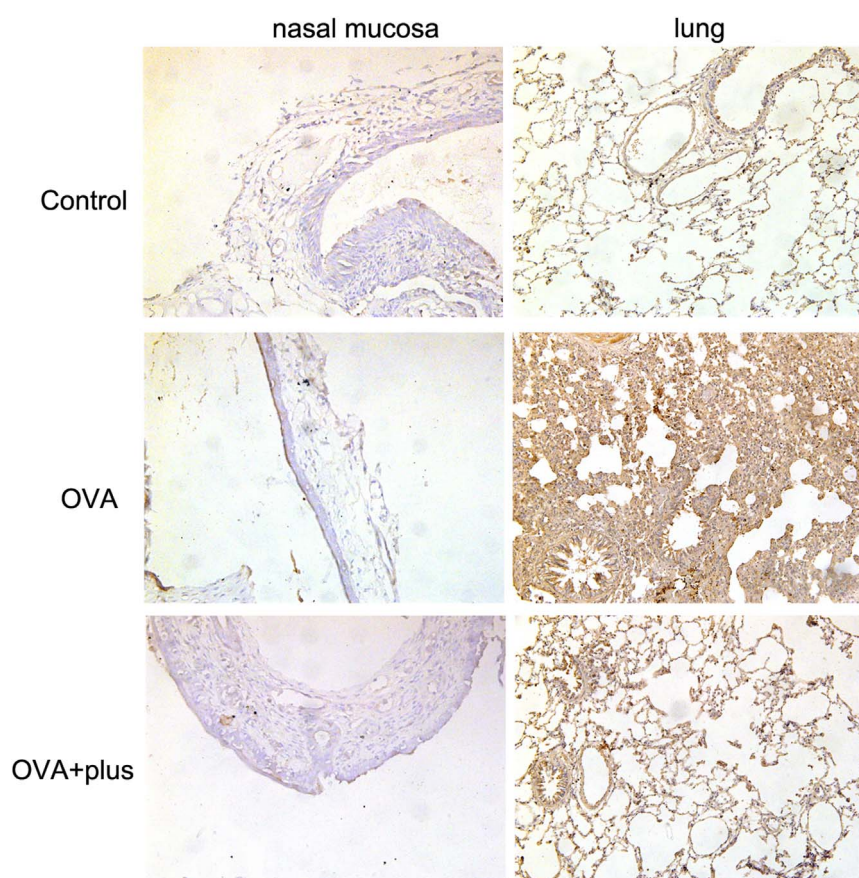


Fig. 3. YC-1 treatment impaired the expression of HIF-1 α in the lungs and nasal mucosa of OVA-exposed Rats. Left, Immunohistochemical analysis of HIF-1 α expression in the lungs of Rats with mock, OVA and OVA+YC-1 treatments. Right, Immunohistochemical analysis of HIF-1 α expression in the nasal mucosa of Rats with control, OVA and OVA+YC-1 treatments.

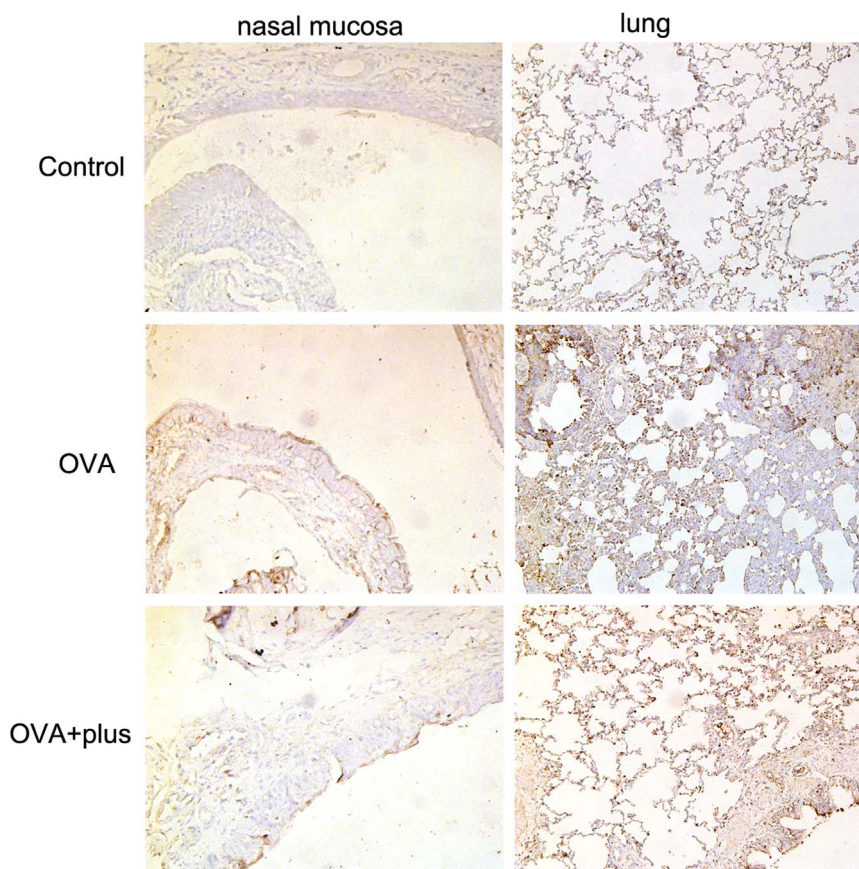


Fig. 4. Immunohistochemical analysis of the expression of NF- κ B p65 in the lungs and nasal mucosa of the indicated groups of rats. The lungs and nasal mucosa of Control, OVA and OVA + YC-1 groups of rats were subjected to immunohistochemical assay to determine the expression of NF- κ B p65 in the indicated groups.

could suppress allergic inflammation and eosinophil activation (12900517). As such, we analyzed the expression pattern of PPAR- α expression in rat lung and nasal mucosa. We found that PPAR- α was evidently upregulated in allergic lung and nasal mucosa (Fig. 5). Notably, YC-1 administration led to impaired expression of PPAR- α in both OVA-exposed rat lung and nasal mucosa. These findings together implicated that PPAR- α expression was indicative in the content of allergic rhinitis and asthma, and YC-1 expression could ameliorate the pathology of CARAS.

3.5. YC-1 administration attenuated the mRNA levels of HIF-1 α , NF- κ B p65 and PPAR- α in OVA-exposed rat lung and nasal mucosa

To verify the altered expression of HIF-1 α , NF- κ B p65 and PPAR- α , real-time PCR analysis was employed to determine the expression of HIF-1 α , NF- κ B p65 and PPAR- α mRNA in the lung and nasal mucosa of the indicated groups of rats. As shown in Fig. 6, real-time PCR assay revealed that the mRNA levels of HIF-1 α , NF- κ B p65 and PPAR- α was altered in similar tendencies with that HIF-1 α , NF- κ B p65 and PPAR- α proteins. Therefore, our results clearly indicated that YC-1 could exert significant allergy-ameliorating effects in rat model of CARAS.

4. Discussion

Despite remarkable progress in the diagnosis and management of chronic allergic rhinitis and asthma, severe allergic asthma remains a life-threatening disease with limited therapeutic strategies. In addition, the co-incidence of chronic rhinitis and asthma raises the hypothesis that the two symptoms may share a common

molecular basis. In this aspect, HIF-1 α has been critically implicated in the pathogenesis of both rhinitis and asthma, suggesting that HIF-1 α might be a promising therapeutic target of CARAS (Huerta-Yepez et al., 2011). In the present study, we showed that YC-1, a synthesized HIF-1 α antagonist, exerted powerful beneficial effect in the treatment of CARAS. Moreover, we uncovered that the allergy-relieving effect of YC-1 involved the modulation of inflammatory pathways. These findings together implicated that YC-1 may elicit an anti-inflammatory role in the prevention of CARAS, shedding new light on the treatment of severe CARAS.

Mounting studies have pointed to a central role of inflammation in chronic allergic rhinitis and asthma. In consistent with the notion, studies have proved that pharmaceutical inhibition of inflammation significantly ameliorated rhinitis and asthma in human and rodents. Therefore, it is important to explore the clinical merit of drugs that target inflammatory regulators in the treatment of chronic allergic rhinitis and asthma. In this regard, HIF-1 α has been proposed to be a promising target of anti-inflammatory medicines. It was revealed that myeloid cell HIF-1 α played an important role in the regulation of eosinophil activity, thus contributing to airway inflammation during chronic asthma (Crotty Alexander et al., 2013). Another study showed that hypoxia potentiated airway inflammation and remodeling in OVA-induced mouse model of asthma through the activation of HIF-1 α (Baek et al., 2013). In line with these findings, studies have showed that HIF-1 α was directly implicated in the pathogenesis of chronic rhinitis and asthma, and a natural HIF-1 α inhibitor 2-methoxyestradiol ameliorated the severity of both rhinitis and asthma, respectively (Kim et al., 2010; Zhou et al., 2012). However, clinical trials revealed that 2-methoxyestradiol exhibited limited

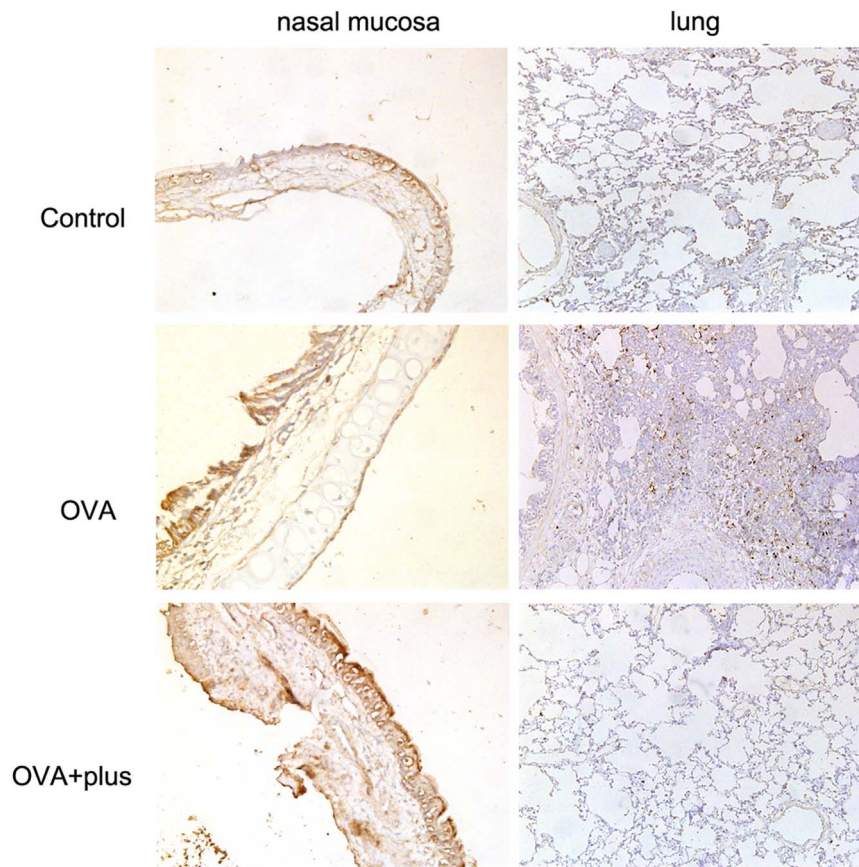


Fig. 5. Immunohistochemical analysis of the influence of YC-1 treatment on PPAR α expression following OVA exposure. The lungs and nasal mucosa of rats were subjected to immunohistochemical assay to determine the expression of PPAR α in the indicated groups.

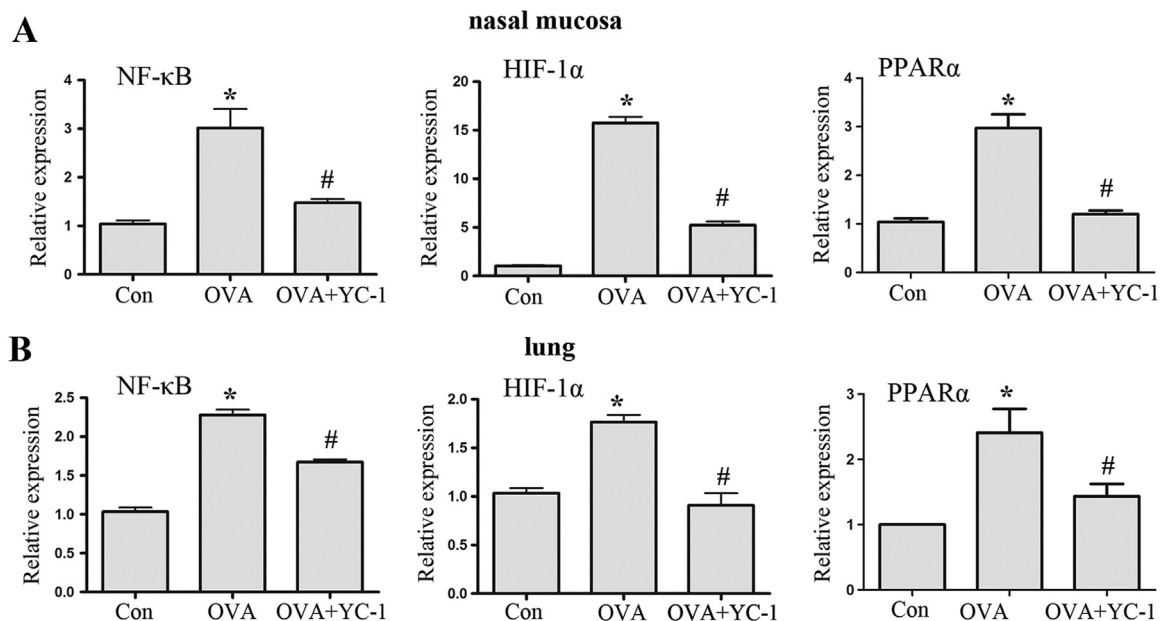


Fig. 6. Real-time PCR analysis of HIF-1 α , NF- κ B p65 and PPAR α expression in the nasal mucosa and lungs of control, OVA-exposed and OVA+YC-1-exposed rats. (A) The mRNA levels of HIF-1 α , NF- κ B p65 and PPAR α in the nasal mucosa of the indicated groups of rats were determined using Real-time PCR analysis. (B) The mRNA levels of HIF-1 α , NF- κ B p65 and PPAR α in the lungs of the indicated groups of rats was analyzed through Real-time PCR assay.

therapeutic merit because of its extremely low plasma levels following high doses of administration (Dahut et al., 2006). On the other side, YC-1 is currently undergoing preclinical trials, and has been widely documented to exert considerable beneficial effects in the treatment of tumors and inflammation-related

diseases in mouse models (Carroll et al., 2013; Pan et al., 2005; Yeo et al., 2003). YC-1 has been shown to suppress HIF-1 α activity through multiple mechanisms. YC-1 treatment led to reduced translation of HIF-1 α mRNA (Hu et al., 2013; Meijer et al., 2012). YC-1 may induce rapid degradation of HIF-1 α through

ubiquitination-mediated proteolysis (Kim et al., 2006; Lau et al., 2006). YC-1 may also directly inactivate the C-terminal transactivation domain (CAD) of HIF-1 α and prevent the recruitment of transcription activator p300 to HIF-1 α target genes in mammalian cells (Hu et al., 2013; Li et al., 2008). Through these mechanisms, YC-1 may inhibit the accumulation and activation of HIF-1 α , and consequently impaired the process of allergic inflammation. These findings implicated the potential of YC-1 and its derivatives as novel therapeutic agents of allergic diseases in human population.

In summary, our present study revealed that HIF-1 α antagonist YC-1 could strongly ameliorate the pathology of combined allergic rhinitis and asthma symptoms through the modulation of inflammatory responses. YC-1 reduced OVA-induced eosinophil infiltration both in rat lung and nasal mucosa. Moreover, our findings indicated that YC-1 administration suppressed the activation of multiple inflammatory pathways, including HIF-1 α , NF- κ B and PPAR α . These data implicated that YC-1 may be of important clinical merit in the treatment of allergic rhinitis and asthma.

Conflict of interest disclosure

The authors have no conflict of interest to declare.

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