



Hypoxia-inducible factor-1 α inhibition modulates airway hyperresponsiveness and nitric oxide levels in a BALB/c mouse model of asthma



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ABSTRACT

Hypoxia-inducible factor (HIF)-1 α is a master regulator of inflammation and is upregulated in alveolar macrophages and lung parenchyma in asthma. HIF-1 α regulates select pathways in allergic inflammation, and thus may drive particular asthma phenotypes. This work examines the role of pharmacologic HIF-1 α inhibition in allergic inflammatory airway disease (AIAD) pathogenesis in BALB/c mice, which develop an airway hyperresponsiveness (AHR) asthma phenotype. Systemic treatment with HIF-1 α antagonist YC-1 suppressed the increase in HIF-1 α expression seen in control AIAD mice. Treatment with YC-1 also decreased AHR, blood eosinophilia, and allergic inflammatory gene expression: IL-5, IL-13, myeloperoxidase and iNOS. AIAD mice had elevated BAL levels of NO, and treatment with YC-1 eliminated this response. However, YC-1 did not decrease BAL, lung or bone marrow eosinophilia. We conclude that HIF-1 α inhibition in different genetic backgrounds, and thus different AIAD phenotypes, decreases airway resistance and markers of inflammation in a background specific manner.

Capsule summary: Asthma is a common disease that can be difficult to control with current therapeutics. We describe how pharmacologic targeting of a specific transcription factor, HIF-1 α , suppresses *asthmatic* airway reactivity and inflammation.

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1. Introduction

Asthma is a highly prevalent disease with a lifetime incidence of 6.4% [1]. An inflammatory disorder of the lung, asthma is characterized by eosinophilia, lung inflammation, smooth muscle hypertrophy, airway

reactivity and airway obstruction [2]. Multiple cells participate in asthma pathogenesis, including eosinophils, alveolar macrophages, goblet cells, smooth muscle cells, T-cells, and epithelial cells, with involvement of hundreds of molecular pathways [3]. Furthermore, it is a disease process that exhibits heterogeneity with variable inflammatory phenotypes [4] and therapeutic responses [5,6].

Current therapeutics agents for asthma include systemic and topical steroids, leukotriene inhibitors, bronchodilators and immunobiologics targeting specific inflammatory proteins (anti-IgE anti-IL-5). However, a large portion of moderate and severe asthmatics do not respond to conventional therapies, and this is thought to be due to involvement of additional inflammatory pathways which are not modulated by current therapeutics. Suppression of pulmonary inflammation and airway reactivity via blockade of additional specific molecular pathways, and in specific cell-types, is an attractive therapeutic strategy. In this study, we demonstrate the potential of targeting hypoxia-inducible factor (HIF)-1 α as an additional therapeutic approach.

Abbreviations: HIF, hypoxia-inducible factor; AIAD, allergic inflammatory airway disease; WT, wild-type; KO, knockout; AHR, airway hyperresponsiveness; SCF, stem cell factor; BAL, bronchoalveolar lavage; NO, nitric oxide; BM, bone marrow; H&E, hematoxylin and eosin; PAS, periodic acid-Schiff; GCH, goblet cell hyperplasia; CCR and CCL, CC chemokine receptor and ligand (respectively); SCF, stem cell factor; MPO, myeloperoxidase; eNOS, extrinsic nitric oxide synthase; iNOS, intrinsic nitric oxide synthase.

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HIF-1 α is a transcription factor that plays a crucial role in regulation of functional inflammatory and metabolic genes in mammalian cells. Recent evidence supports HIF-1 α as a key inflammatory regulator in asthma as well [7–11]. There is upregulation of HIF-1 α in the smooth muscle, submucosa and bronchoalveolar lavage (BAL) cells of asthmatic patients [7,10], while partial genetic knockout (KO) of HIF-1 α demonstrates reduction in eosinophilia [11]. HIF-1 α is a known regulator of inflammatory pathways in neutrophils and macrophages, which are myeloid cells [12–14]. HIF-1 α is thought to regulate inflammatory responses of another myeloid cell, the eosinophil, which is a major effector cell in allergic inflammatory airways disease (AIAD). Our previous studies in C57BL/6 mice, with either HIF-1 α lysMcre knockout (KO) or mice treated systemically with the HIF-1 α inhibitor YC-1, demonstrated reduced airway hyperresponsiveness (AHR) and reduced eosinophilia [15]. However, nitric oxide (NO) is not elevated in the phenotype seen in C57BL/6 OVA induced AIAD, which in the clinical setting is an important marker of disease activity in asthmatic patients [15]. Furthermore, data evaluating the role of HIF-1 α in various asthma phenotypes is lacking.

Because HIF has become a therapeutic target in many types of diseases, a variety of different compounds targeting specific α -subunits have become available. We chose YC-1 (Sigma) as the primary HIF antagonist to use in these studies because of published studies of its successful use in blocking HIF-1 α both *in vitro* and *in vivo* [16–19]. YC-1 is known to down-regulate both HIF-1 α and HIF-2 α at the post-translational level [20,21]. In addition, YC-1 inactivates the COOH-terminal transactivation domain (CAD) of HIF-1 α [22], and stimulates factor inhibiting HIF (FIH) binding, further suppressing HIF-1 α [23]. Pawpaw is a natural product-based anticancer botanical dietary supplement (Paw Paw Cell-Reg®) prepared from twig extracts of *Asimina triloba* (Annonaceae) that has been shown to inhibit HIF-1 activation through blocking hypoxic induction of nuclear HIF-1 α [24]. Pawpaw extracts and Pawpaw annonaceous acetogenins directly interfere with mitochondrial function, and are believed to suppress the hypoxia-induced mitochondrial ROS-mediated inactivation of HIF prolyl hydroxylases, thus promoting proteasomal HIF-1 α degradation under hypoxic conditions.

Utilizing an acute on chronic OVA mouse model of asthma in BALB/c mice, and pharmacological inhibition and agonism of HIF-1 α , we conducted a series of experiments to address the role of HIF-1 α in predominantly hyperreactive airway asthma and NO synthesis.

2. Methods

2.1. Reagents

Ovalbumin (OVA), aluminum hydroxide (alum), HIF-1 α inhibitor YC-1 and agonist L-mimosine were purchased from Sigma. HIF-1 α inhibitor Pawpaw was generously provided by Dale Nagle, University of Mississippi, and CHO-stem cell factor (SCF) cells by Mark Kamps, University of California at San Diego (UCSD). Eotaxin-1 and MIP-1 α (R&D), and IL-5 and FLT-3 L were purchased from Peprotech.

2.2. Mouse models of asthma

BALB/c 6–8 week old female mice (Jackson Laboratories, Bar Harbor, ME) underwent asthma induction as previously described [15] via intraperitoneal (i.p.) injection of OVA and alum on days 0 and 12. BALB/c mice were challenged with intranasal (i.n.) OVA in 50 μ L PBS (day 24, 27, 29, 36, and 38) with sacrifice on day 39. All groups were composed of seven mice, and all experiments were repeated three times. Pharmacological HIF-1 α antagonists (500 μ g YC-1 in 200 μ L PBS with 50% DMSO; 200 μ g Pawpaw in 200 μ L PBS with 2% isopropanol) were given i.p. one hour prior to i.n. OVA challenges as formerly described [15]. Control mice received either 50% DMSO or 2% isopropanol with PBS i.p. No differences between DMSO and isopropanol controls were

detected, thus only the 50% DMSO control data is included here. After sedation with ketamine (100 mg/kg) and xylazine (10 mg/kg), mice underwent tracheostomy and methacholine challenge for flexiVent measurements of airway resistance. Intracardiac bleed and BAL (800 μ L PBS) were performed, with 50 μ L of blood immediately transferred into 150 μ L of 5 mM EDTA and placed on ice. Bone marrow (BM) was flushed from the right femur with 1 mL of ice-cold PBS. The right lung was snap-frozen for RNA, and the left lung was fixed for histology. All animal protocols were approved by the Animal Care and Use Committee at UCSD.

2.3. Quantification of cell types

Blood, BAL and BM samples underwent pelleting at 3000 rpm for 4 min. Blood pellets were resuspended with ACK red blood cell lysis solution, incubated at room temperature for 7 min, and then spun at 3000 rpm for 4 min. All pellets were resuspended in 1 mL PBS, 200 μ L of BAL, 200 μ L blood, and 20 μ L BM + 180 μ L PBS were cytospun onto Superfrost slides at 1000 rpm for 3 min. Slides were stained with Giemsa Wright, de-identified and randomized. From each BAL slide, ≥ 400 cells were counted via light microscopy. From each blood and BM slide, ≥ 100 cells were counted via light microscopy.

2.4. Analysis of cytokines and nitric oxide in BAL and IgE in serum

ELISA DuoSets (R&D) was used to assay for IL-4, IL-5, IL-9 and IL-13 from undiluted BAL. Concentration of nitric oxide (NO) in undiluted BAL was obtained using Sievers NO Analyzer (NOA; GE), which measured total nitrates, including NO, nitrite, nitrate and nitrosothiols. All samples were centrifuged through a 10 kDa column to remove cell debris and high molecular weight proteins. The filtrate was injected into the reaction vessel containing vanadium chloride in HCl, heated to 95 °C. This reduced all nitrates to NO (gas phase) to enable detection by the NOA. The millivolt output equates to μ M nitrate from a pre-loaded standard curve. Serum was diluted 1:100 for total IgE determination, and 1:10 for OVA-specific IgE quantification (Chondrex) as previously described [15].

2.5. Quantitative RT-PCR analysis

As previously described in detail in [15], the total RNA was extracted and evaluated for purity. cDNA was prepared and amplified with primers. Relative gene expression was determined via $\Delta\Delta$ Ct analysis utilizing non-OVA controls and OVA mouse groups for comparison.

2.6. Statistical tests

ANOVA one-way analysis and Bonferroni's multiple comparison were used for NO levels, and ELISA. Airway resistance and BAL cellularity were evaluated with two-way repeated measures ANOVA and qPCR by two-way ANOVA, thereafter followed by Bonferroni post-tests.

3. Results

3.1. Treatment of OVA induced asthma in BALB/c mice with HIF-1 α antagonist YC-1 diminished airway reactivity and lung elastance

Allergic inflammatory airway disease (AIAD) phenotypes differ between mouse strains. C57BL/6 mice have eosinophil predominant disease while BALB/c mice have an airway hyperresponsive (AHR) phenotype. In addition, prolonged or chronic induction of AIAD (>4 weeks) leads to different inflammatory changes compared to short-term models (2–4 weeks). Here we used a chronic model of asthma in BALB/c mice. BALB/c OVA (AIAD) mice treated with the HIF-1 α inhibitor YC-1 prior to each OVA challenge had similar AHR compared to negative (non-AIAD) controls, while OVA mice had significantly higher AHR than non-AIAD controls at methacholine 6 and 12 mg/mL ($n = 7$; Fig. 1A).

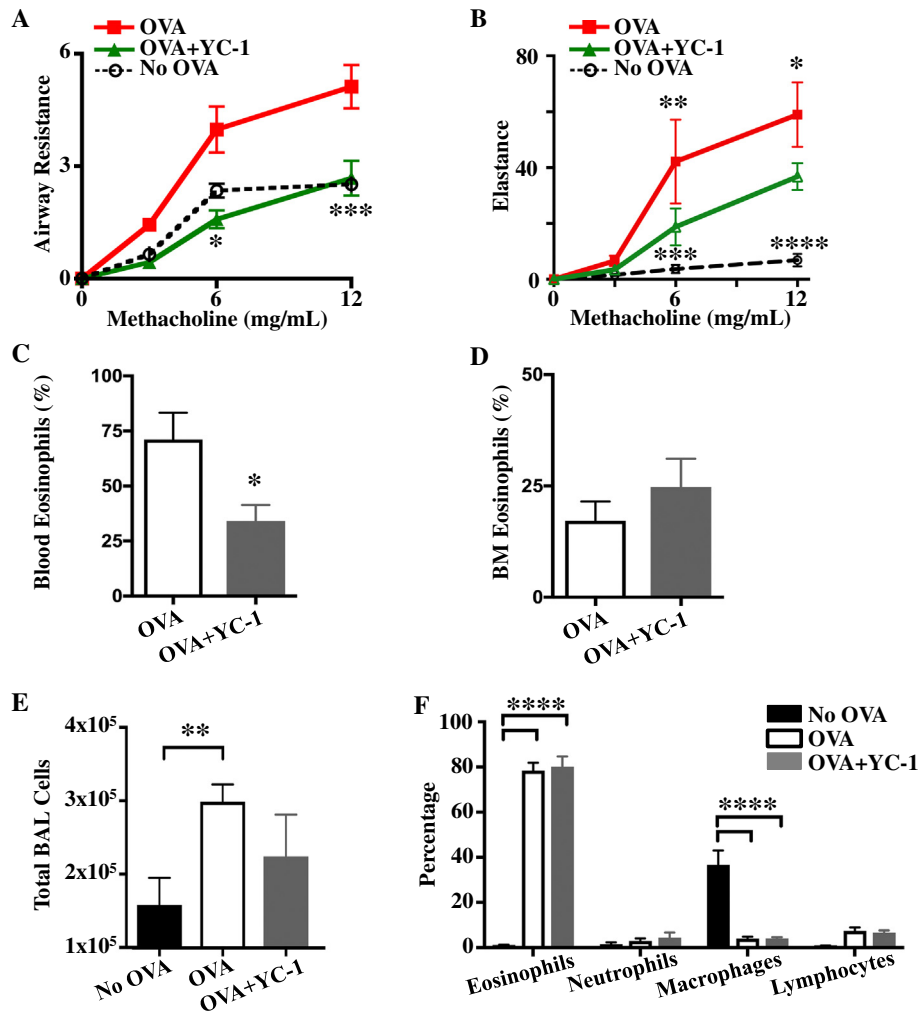


Fig. 1. Treatment with HIF-1 α antagonist YC-1 decreased airway hyperreactivity, lung elastance, blood eosinophilia and total BAL cells in a BALB/c OVA asthma model. A, AHR was no different in YC-1 treated OVA mice compared to no-OVA controls, while untreated OVA mice had significantly higher AHR compared to no-OVA controls. B, Untreated OVA mice had significantly higher elastance (lower lung compliance) than no-OVA controls at MCH 6 and 12 mg/mL. YC-1 treatment decreased AHR at both MCH 6 and 12 mg/mL compared to untreated OVA mice. C, Blood eosinophils decreased by 50% with YC-1 treatment. D, Production of eosinophils in the bone marrow was unchanged by HIF inhibition. E, Total number of immune cells in the BAL increased with AIAID induction with OVA, but there was no difference in BAL cell counts between non-OVA controls and OVA mice treated with YC-1. F, YC-1 treated OVA mice had the same level of eosinophilia in BAL compared to untreated OVA mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

BALB/c OVA mice treated with YC-1 had lower lung elastance (higher lung compliance) compared to untreated OVA mice (Fig. 1B).

3.2. HIF-1 α inhibition decreased blood but not BM, airway or lung eosinophilia

BALB/c OVA + mice had 71% eosinophils in blood, while YC-1 treated OVA + mice showed a half-fold reduction, with 34% total blood eosinophils ($p = 0.0395$; Fig. 1C). In the bone marrow (BM), OVA + mice had 17% mature and immature eosinophils, compared to 25% in YC-1 treated OVA + mice (non-significant difference; Fig. 1D).

BALB/c OVA + control mice had higher total cell numbers in the airways than non-OVA control mice ($p < 0.01$; Fig. 1E). YC-1 treated OVA + mice had no significant difference in airway cell numbers as compared to either non-OVA control or OVA + mice. Both YC-1 treated OVA + and untreated OVA + mice were found to have similar airway cell composition, with 78–80% eosinophils ($p < 0.0001$; Fig. 1F), and a smaller percentage of airway macrophages compared to non-OVA controls ($p < 0.0001$; Fig. 1F). Total airway macrophage numbers in non-OVA controls (2.1×10^5 cells) were not significantly different than YC-1 treated OVA + or untreated OVA + groups (2.5×10^4 and 2.4×10^4 cells,

respectively; $p = 0.12$ and $p = 0.07$, respectively). Parenchymal eosinophilia was also not significantly different between YC-1 treated OVA + and untreated OVA + mice, with 25% of OVA + lung parenchyma, and 12.5% of YC-1 treated OVA + mice having eosinophilic infiltrates, by blinded histopathologic evaluation ($p > 0.05$, n.s.).

3.3. YC-1 treatment decreased markers of allergic inflammation

IL-5 is associated with allergic, T_H2 inflammation, and is one of the key eosinophil chemokines. BAL IL-5 levels were elevated in OVA mice, and treatment with YC-1 diminished IL-5 levels (Fig. 2A). Additional T_H2 cytokines include IL-4, IL-9 and IL-13. BAL IL-4 was lowered by YC-1 treatment (Fig. 2B), while BAL levels of IL-9 and IL-13 were unchanged compared to OVA mice. These data suggest that HIF-1 α is modulating T_H2 pathways governing IL-4 and IL-5 secretion into the airways, but not IL-9 and IL-13.

Systemic allergic inflammation leads to increased levels of antigen-specific IgE antibodies. OVA-specific IgE levels in mice treated with HIF-1 α antagonists [YC-1 and Pawpaw; Pawpaw is a natural product that inhibits HIF-1 α [25]] were similar to untreated mice. To assess whether HIF may have a larger role in driving antigen-specific IgE

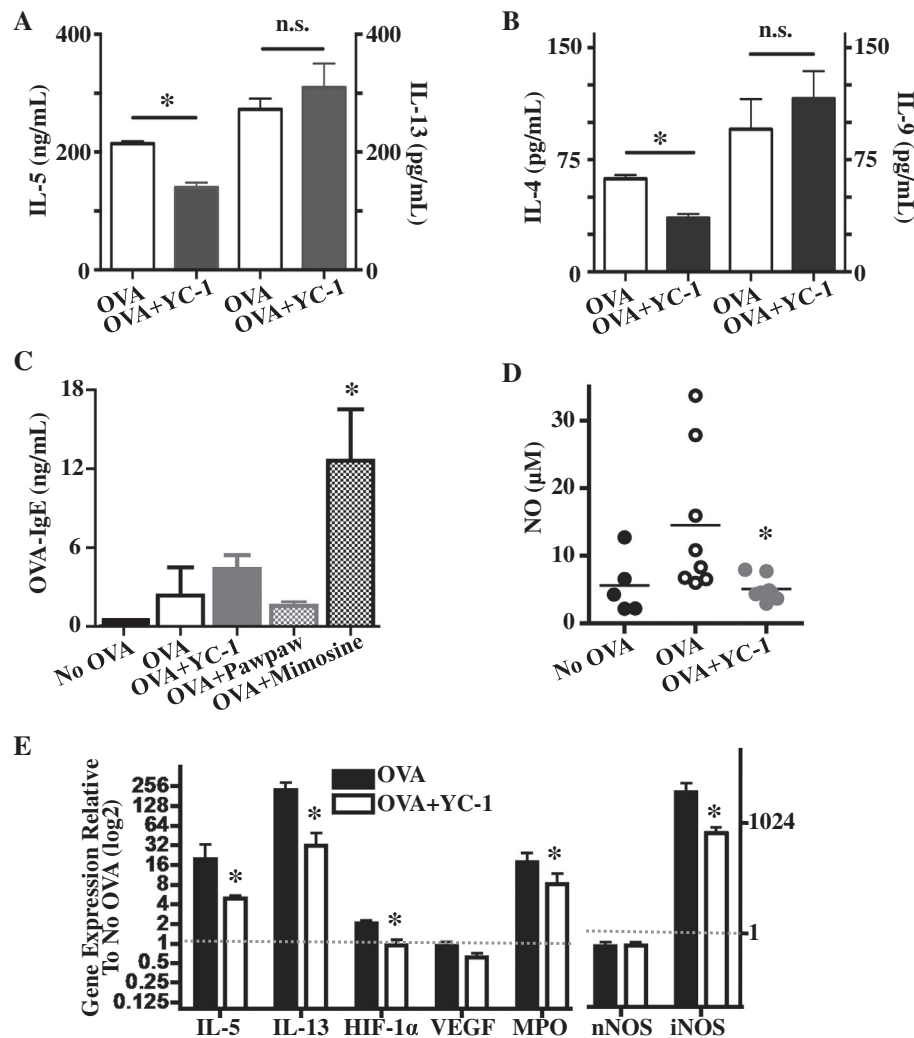


Fig. 2. Treatment with YC-1 diminished T_H2 markers of allergic inflammation, exhaled NO and expression of inflammatory genes in mice with allergic inflammatory airways disease. A, BAL levels of IL-5 were lower in YC-1 treated OVA mice compared to untreated OVA mice. BAL IL-13 levels were unchanged by YC-1 treatment. B, BAL levels of IL-4 were lower in YC-1 treated OVA mice compared to untreated OVA mice. BAL IL-9 levels were unchanged by YC-1 treatment. C, OVA-specific IgE levels were elevated in mice treated with HIF-1 α agonist mimosine, but similar in OVA mice untreated and treated with HIF-1 α antagonists YC-1 and Pawpaw. D, NO BAL levels were decreased in YC-1 treated OVA mice, and were similar to no-OVA controls. E, YC-1 treated OVA mice had decreased lung parenchymal mRNA expression of IL-5, IL-13, HIF-1 α , iNOS, and MPO. * $p < 0.05$.

production, mice were treated with the HIF agonist L-mimosine (mimosine). Mimosine stabilizes HIF-1 α by inhibiting prolyl hydroxylases, leading to higher levels of HIF-1 α in cells. OVA mice treated with mimosine developed higher OVA-specific IgE levels (Fig. 2C).

NO levels in BAL were higher in BALB/c OVA mice compared to no-OVA controls. Many human asthmatics have increased exhaled NO, which can be used as a marker of their disease activity. Our data demonstrate that the BALB/c OVA mouse model of asthma has activation of similar molecular inflammatory pathways as human asthma, and thus may be a useful model for therapeutics targeting pathways leading to increased exhaled NO in particular. Treatment of OVA mice with the HIF antagonist YC-1 led to lower NO levels in BAL than untreated OVA mice (Fig. 2D).

The lung parenchyma of BALB/c OVA mice treated with YC-1 had greater than two-fold lower gene expression of IL-5, IL-13, HIF-1 α , iNOS, and MPO, compared to untreated OVA mice (Fig. 2E). These decrements in gene expression align with the decreased IL-5 and NO found in BAL, and with the trend towards decreased lung parenchymal eosinophils (12.5% in YC-1 treated OVA mice compared to 25% in untreated OVA controls; $p = n.s.$).

4. Discussion

Many mouse strains have been used to model human asthma, through the induction of allergic inflammatory airways disease (AIAD). Similar to human asthma, different genetic backgrounds and different patterns and durations of allergen exposures in mice lead to a variety of asthma phenotypes. The C57BL/6 mouse model of asthma typically has an eosinophilic phenotype with modest AHR, whereas BALB/c animals typically have modest eosinophilia and severe AHR [25,26]. Many groups have studied both C57BL/6 and BALB/c mice and defined the AIAD phenotype produced by various OVA induction and challenge protocols [27–29]. Some researchers and clinicians have questioned the human relevance of mouse models of asthma [30,31], but typically have concluded that utilizing certain genetic backgrounds and AIAD protocols to accurately mimic human disease may help in understanding the molecular underpinnings of asthma, and help study potential therapeutics targeting specific inflammatory pathways.

In our prior studies, we demonstrated that asthma treatment with HIF antagonist (YC-1) in the C57BL/6 background successfully decreased eosinophils in BAL, and diminished allergic lung inflammation

[15]. Such findings suggested potential importance of HIF as a therapeutic target in asthma. However, the C57BL/6 OVA mouse model of asthma did not show NO elevation [15], which raised the question that it may not be the ideal system for studying NO targeted therapies, given that exhaled NO is a known important marker of disease activity in human asthmatics [32].

In our current study, BALB/c OVA mice exhibited elevated NO levels that were reduced when mice were treated with HIF-1 α antagonists (YC-1 and PawPaw), consistent with decreased inflammation and decreased iNOS – a key target gene regulated by HIF-1 α [33]. Transcription of HIF-1 α itself was diminished by YC-1 treatment, which may have played a significant role in reduction of iNOS transcription and thus NO production. Diminishment of inflammation by YC-1 treatment may have decreased HIF-1 α gene transcription via decreased feedback through inflammatory pathways to HIF gene transcription regulators, which we have shown in prior studies [34]. YC-1 has been shown to suppress the PI3K/Akt/mTOR/4E-BP pathway, which regulates HIF-1 α expression at the translational step [35]. Thus, YC-1 treatment may diminish HIF-1 α gene expression through suppression of this pathway, or by changing the inflammatory milieu, or both. NO has a very short physiologic half-life, <1 s in blood, therefore assessment of NO metabolites in biological samples is necessary [36]. In these studies, total nitrites and nitrosothiols were not separated from nitrates in the measurement of BAL samples, thus the NO measured in our system is a summation of the amount of nitrates, NO $_3^-$ – the major oxidation product of NO, formed when NO reacts with O $_2^-$ – and nitrites, NO $_2^-$ – the major oxidation product of NO in the presence of dissolved O $_2$ – plus nitrosothiols – produced by the interaction of NO and NO-derived metabolites with protein thiols – in the liquid lining the airways. One limitation of these data is that it does not differentiate the NO produced by iNOS in lung parenchyma, from other sources. However, all NO metabolites are thought to be important for the end-effects of NO *in vivo*. Thus, the return of NO metabolites to baseline (non-AIAD) levels in HIF-1 α antagonist (YC-1 and Pawpaw) treated OVA + mice is consistent with suppression of inflammation, including the iNOS pathway.

Even though YC-1 is a known HIF-1 α antagonist, and HIF-1 α is a known regulator of VEGF expression, treatment with YC-1 did not suppress VEGF gene expression in our model. It has been hypothesized that regulation of VEGF by HIF-1 α primarily occurs during hypoxia, and even under hypoxic conditions binding of multiple promoters by key factors in addition to HIF-1 α may be required to increase VEGF expression [37]. One group studied the effects of HIF-1 α inhibition with YC-1 *in vitro* and found no change in VEGF levels when cells were treated with YC-1 for 6, 24 and 48 h [38]. Thus, in our mouse model of asthma, where inflammation and not hypoxia is the primary process occurring the lungs, it is probable that inhibition of HIF-1 α would not alter VEGF expression.

Furthermore, our model showed prevention of AHR with YC-1, as it did in our previous studies in C57BL/6 mice [15]. In contrast to the C57BL/6 OVA model, the BALB/c OVA model showed no alteration in BAL eosinophilia with YC-1 treatment, implying that in this genetic background, YC-1 based HIF inhibition does not significantly modulate eosinophil chemokine release by lung cells or eosinophil chemotaxis. However, AIAD induction in this model led to fewer airway macrophages, which persisted with YC-1 treatment. Airway macrophages are known to regulate AIAD [39]. Depletion of macrophages promotes inflammation [40], while transfer of un-sensitized and OVA-sensitized macrophages confers decreased and increased inflammation, respectively, in BALB/c OVA models of asthma [41]. Therefore, OVA sensitization and induction of AIAD may cause diminishment of the airway macrophage population, through apoptosis or decreased recruitment, leading to dysregulation of inflammatory pathways, and ultimately an asthmatic phenotype. In these studies, with our acute on chronic mouse model of asthma, eosinophils made up 78–80% of total BAL immune cells. Prior AIAD studies in BALB/c mice had lower BAL eosinophilia, between 25 and 50%, however those studies were done in male mice

and utilized different AIAD induction and challenge protocols, either of which could be the etiology of the lower eosinophilic response [42, 43]. Groups utilizing female BALB/c mice have found higher BAL eosinophilia, up to 63%, depending on the AIAD protocol used [44–46]. The generation of 80% BAL eosinophils by our AIAD induction protocol suggests that it may be an ideal model to study anti-eosinophil and anti-eosinophil chemokine therapeutics.

In both C57BL/6 and BALB/c backgrounds, treatment with YC-1 showed reduction in crucial inflammatory and physiologic markers of asthma, but with particular profiles that are likely dependent on the genetic background and induction protocol. These data attest to the significant differences in response to treatments dependent on genetic mice strains that is reflective of the various therapeutic responses underscored by genetic and phenotypic differences in human asthma. We demonstrate here that not only do these mouse strains differ phenotypically, they also differ in their response to administration of HIF-1 α antagonists.

Treatment of AIAD mice with the natural product Pawpaw did not significantly modulate AHR, but did reduce exhaled NO to a similar degree as YC-1 treatment. YC-1 is known to decrease both HIF-1 transactivation and increasing factor inhibiting HIF (FIH) binding to HIF-1 α . Pawpaw mainly interferes with the mitochondria-mediated hypoxic induction of nuclear HIF-1 α protein, most likely by promoting HIF prolyl hydroxylase activity and subsequent proteasomal HIF-1 α degradation, even under hypoxic conditions. Thus, under certain circumstances, YC-1 may act as a more powerful HIF-1 α antagonist than Pawpaw. Because pharmacokinetic differences between these small molecule-based HIF inhibitors may also contribute to their observed activities, further studies of Pawpaw using different formulations and patterns of dosing may yield greater therapeutic effects than those defined here.

Effects on systemic allergic inflammation were evaluated via quantification of circulating OVA-specific IgE antibodies. Treatment with HIF antagonists YC-1 and Pawpaw had no effect on OVA-IgE levels. However, treatment with HIF agonist mimosine increased OVA-IgE levels 4-fold compared to untreated OVA mice. Other researchers have found HIF antagonists to diminish OVA-IgE production in mouse models of allergy [8,47,48], but did not find significant increases in OVA-IgE production with another HIF agonist CoCl $_2$ [48]. Overall, this suggests that each HIF drug, whether agonist or antagonist, affects particular and disparate pathways, which may or may not lead to alterations in allergic inflammatory antibody production. Thus, each type of HIF modulator will need to undergo individual activity profiling to determine whether it is a potential therapeutic drug candidate.

5. Conclusions

In summary, we have further demonstrated that HIF-1 α plays a key role in allergic inflammation, asthma pathogenesis and AHR, with various patterns of inflammation based on genetic and phenotypic backgrounds. HIF modulating drugs act on allergic inflammatory pathways and responses in a variable manner, likely related to the mechanism by which they modulate HIF levels and activity. As found with many other targeted asthma therapeutics – anti-IgE, anti-IL5, anti-IL13 and leukotriene inhibitors – we predict that the clinical success of HIF-1 α inhibitors will be dependent on their use in the correct asthma phenotype.

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