Inhibition of Aldose Reductase Prevents Experimental Allergic Airway Inflammation in Mice

Umesh C. S. Yadav¹, Kota V. Ramana¹, Leopoldo Aguilera-Aguirre², Istvan Boldogh², Hamid A. Boulares³, Satish K. Srivastava³*

¹ Department of Biochemistry and Molecular Biology, ² Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas, United States of America, ³ Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center, New Orleans, Louisiana United States of America

Abstract

Background: The bronchial asthma, a clinical complication of persistent inflammation of the airway and subsequent airway hyper-responsiveness, is a leading cause of morbidity and mortality in critically ill patients. Several studies have shown that oxidative stress plays a key role in initiation as well as amplification of inflammation in airways. However, still there are no good anti-oxidant strategies available for therapeutic intervention in asthma pathogenesis. Most recent studies suggest that polyol pathway enzyme, aldose reductase (AR), contributes to the pathogenesis of oxidative stress-induced inflammation by affecting the NF-κB-dependent expression of cytokines and chemokines and therefore inhibitors of AR could be anti-inflammatory. Since inhibitors of AR have already gone through phase-III clinical studies for diabetic complications and found to be safe, our hypothesis is that AR inhibitors could be novel therapeutic drugs for the prevention and treatment of asthma. Hence, we investigated the efficacy of AR inhibition in the prevention of allergic responses to a common natural airborne allergen, ragweed pollen that leads to airway inflammation and hyper-responsiveness in a murine model of asthma.

Methods and Findings: Primary Human Small Airway Epithelial Cells (SAEC) were used to investigate the in vitro effects of AR inhibition on ragweed pollen extract (RWE)-induced cytotoxic and inflammatory signals. Our results indicate that inhibition of AR prevents RWE-induced apoptotic cell death as measured by annexin-v staining, increase in the activation of NF-κB and expression of inflammatory markers such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, Prostaglandin (PG) E₂, IL-6 and IL-8. Further, BALB/c mice were sensitized with endotoxin-free RWE in the absence and presence of AR inhibitor and followed by evaluation of perivascular and peribronchial inflammation, mucin production, eosinophils infiltration and airway hyperresponsiveness. Our results indicate that inhibition of AR prevents airway inflammation and production of inflammatory cytokines, accumulation of eosinophils in airways and sub-epithelial regions, mucin production in the bronchoalveolar lavage fluid and airway hyperresponsiveness in mice.

Conclusions: These results suggest that airway inflammation due to allergic response to RWE, which subsequently activates oxidative stress-induced expression of inflammatory cytokines via NF-κB-dependent mechanism, could be prevented by AR inhibitors. Therefore, inhibition of AR could have clinical implications, especially for the treatment of airway inflammation, a major cause of asthma pathogenesis.

Citation: Yadav UCS, Ramana KV, Aguilera-Aguirre L, Boldogh I, Boulares HA, et al. (2009) Inhibition of Aldose Reductase Prevents Experimental Allergic Airway Inflammation in Mice. PLoS ONE 4(8): e6535. doi:10.1371/journal.pone.0006535

Editor: Dominik Hartl, LMU University of Munich, Germany

Received April 10, 2009; Accepted June 21, 2009; Published August 6, 2009

This is an open-access article distributed under the terms of the Creative Commons Public Domain declaration which stipulates that, once placed in the public domain, this work may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose.

Funding: This study was supported by American Asthma Foundation (http://americanasthmafoundation.org) grant (AAF 08-0219) to SKS. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ssrivast@utmb.edu

Introduction

There has been a significantly increased prevalence of asthma over the last few decades, specifically in developing countries [1]. This appears to be related to changes in the environment that affects susceptible individuals, both in the induction and worsening of established disease [2]. Epidemiological studies identified multiple interacting risk factors, including inhaled pollutants such as environmental tobacco smoke, particulate matter, oxides of nitrogen, ozone, and repeated respiratory virus exposures, which induce and/or augment reactive oxygen species (ROS) generation in the airways [3]. Although lung has excellent antioxidative system, in the presence of excessive ROS the cells become oxidatively stressed leading to loss of intracellular redox homeostasis, additional ROS production, alterations in cellular signaling and pathological processes [4,5]. In addition, during inflammatory processes more ROS are generated by activated mast cells, macrophages, eosinophils, and neutrophils that have the potential to injure airway lining cells [6,7]. Cellular oxidative stress plays a fundamental role in inflammation through the activation of stress kinases such as MAPKs, which comprise a large family of protein kinases including ERK1 (p44MAPK)/ERK2 (p42MAPK) and JNK, which activate redox-sensitive transcription factors such as NF-κB and AP-1 [8]. The transcription factors bind to DNA and
transcribe inflammatory proteins such as cytokines, chemokines, iNOS and COX-2.

Our recent studies have shown that ROS-induced NF-κB activation is mediated by aldose reductase-catalyzed products of lipid aldehyde-glutathione conjugates [9,10]. Aldose reductase (AR; AKR1B1), a member of aldo-keto reductase superfamily, besides reducing glucose to sorbitol, efficiently reduces lipid aldehydes and their glutathione conjugates [11]. Most importantly, we have shown that AR-catalyzed reduced product of lipid aldehyde-glutathione conjugates such as glutathionyl-1,4-dihydroxynonane (GS-DHN) mediates NF-κB activation indicating that the inhibition of this enzyme could prevent inflammatory responses [10]. Pharmacological inhibition or siRNA ablation of AR attenuates TNF-α and growth factor-induced IkBa phosphorylation and degradation and resultant activation of NF-κB thereby preventing the cytotoxic effects in vascular smooth muscle cells (VSMC), vascular endothelial cells (VEC) and human lens epithelial cells (HLEC) [12–15]. Further, our studies have shown that hyperglycemia and endotoxin-induced increase in inflammatory cytokines and chemokines in both cellular and animal models (such as restenosis, colon cancer and uveitis) is efficiently prevented by AR inhibitors [9,16,17]. These results suggest that AR inhibitors, initially developed as anti-diabetic drugs, could be used as therapeutic intervention to prevent inflammation [11]. AR inhibitors such as zopolrestat and fidarestat have been found to be safe and passed in FDA’s Phase-I clinical trials for diabetic neuropathy but failed in Phase-III clinical trials as they have been shown to be not as effective, though they did not have any major side effects [18]. Our recent results demonstrate that AR inhibitors could have therapeutic use for the prevention and treatment of inflammatory disorders other than diabetic complications such as asthma, an airway inflammatory disease [11]. For such use a careful examination of the effect of AR inhibition in clinically relevant animal models is mandatory. We have used short ragweed (Ambrosia artemisiifolia) pollen extract, one of the most abundant aeroallergens that causes severe seasonal allergic symptoms in the United States, Canada and Europe, to elicit allergic responses via induction of oxidative stress that mimics airway inflammation in humans [19–21]. In the present study, we investigated whether treatment with AR inhibitors could prevent alterations in the cytokine and chemokine levels in a cellular model using human SAEC and a clinically relevant mouse model which mimics allergic airway inflammatory conditions in humans. Our results indicate that AR inhibitors are powerful repressors of the expression of major cytokines and chemokines in a mouse model of ragweed-induced allergic airway inflammation that leads to asthma.

**Results**

**AR Inhibition prevents RWE-induced apoptosis in SAEC**

The airway epithelium plays an important function as a barrier to foreign particles, pollens, spores of fungi and other xenobiotics which disturb cellular redox homeostasis leading to apoptosis of airway epithelial cells [22–24,29,33]. Hence, we first determined the effect of AR inhibition on RWE-induced cell death of SAEC by Annexin-V binding assay. Annexin-V binds to the inverted phosphatidylinerine in the cells undergoing apoptosis. Propidium-iodide (PI) was used as the indicator of the cell mortality. In SAEC, RWE-treatment caused increased cell death (over 50%) in 18 h as compared to the control cells. Pre-incubation of the cells with AR inhibitor, zopolrestat, significantly (p<0.01) prevented RWE-induced cell death by >80% (Fig. 1B). Under similar conditions, AR inhibition alone did not cause apoptosis of SAEC.

AR inhibition decreases RWE-induced ROS formation in SAEC

It has been reported that addition of RWE to cultured epithelial cells or airways increases oxidative stress levels within minutes after exposure [21,25]. To examine the nature of the RWE-induced decrease in SAEC viability, we measured the level of ROS in RWE-induced SAEC and whether AR inhibitors could prevent it. As shown in Fig. 2, RWE (150 μg/mL) caused increase in cellular ROS levels as evident by increased fluorescence by ROS sensitive dihydroethidium (DHE). Pre-incubation of SAEC with two different AR inhibitors, sorbinil or zopolrestat, prevented these changes (Fig. 2). Under the similar conditions, AR inhibition alone caused no significant change in the ROS levels in SAEC. These results suggest that inhibition of AR increased antioxidant potential of cells and prevented RWE-induced increase in ROS and resultant cell death.

**AR inhibition prevents RWE-induced production of inflammatory cytokines and chemokines in SAEC**

Since RWE is known to elevate the levels of inflammatory markers in the airway epithelial cells that cause inflammation and aggravate the allergic condition [25], we next examined the effect of AR inhibition on the RWE-induced increase in the levels of selected inflammatory markers in SAEC culture medium. As shown in Fig. 3(I) A and B, treatment of SAEC with RWE (150 μg/mL) for 24 h caused 3 and 4-fold increase in the synthesis of IL-6, and IL-8, respectively, and inhibition of AR by pharmacological agents significantly prevented the increase. A more 2-fold increase in the RWE-induced PGE2 levels in SAEC was also significantly (>75%) prevented by AR inhibition (Fig. 3(I) C).

Although zopolrestat is a specific inhibitor of AR, to rule out its non-specific response in the biological system, we ablated AR message in SAEC by antisense oligonucleotides (AR siRNA) and studied whether phenotypic absence of AR will have similar effects in SAEC as did AR inhibitor in protection against RWE-induced inflammation. Transient transfection of SAEC with AR siRNA abolished AR protein by >95% (Fig. 3(II) A–C, inset) while with scrambled siRNA oligonucleotides AR expression did not change. We observed that siRNA ablation of AR also significantly prevented RWE-induced synthesis of cytokines such as IL-6, and chemokines IL-8 and PGE2 in SAEC (Fig. 3(II) A–C). We further examined the effects of AR inhibition on the expression of inflammatory markers at RNA levels using quantitative RT-PCR. As shown in Fig. 3(III) A&B, treatment of SAEC with RWE caused a 3–4 fold increase in the expression of IL-6 and IL-8 mRNA level and zopolrestat prevented it by >70% suggesting that AR could regulate the transcriptional activation of inflammatory marker genes. Also, since PGE2 is synthesized by inducible cyclooxygenase (COX)-2, we determined the effect of AR inhibition on the transcriptional activation of COX-2 by quantification of its mRNA in response to RWE in SAEC by RT-PCR. As shown in Fig. 3(III) C, RWE significantly increased the mRNA levels of COX-2 in SAEC and zopolrestat prevented it by more than 60%. These results suggest that AR regulates the synthesis as well as expression of inflammatory markers.

**AR inhibition prevents RWE-induced expression of inflammatory markers in SAEC**

Since biosynthesis of PGE2 and NO from their precursors is catalyzed by COX-2 and iNOS enzymes respectively, we next examined the effect of AR inhibition on RWE-induced COX-2 and iNOS expression in SAEC by immunoblotting. As shown in Fig. 4(I) A, treatment of SAEC with RWE significantly (~3-folds) increased COX-2 and iNOS protein expression and pre-treatment
Figure 1. Inhibition of AR prevents RWE-induced apoptosis and cell death in SAEC. (A) Growth-arrested SAEC, pretreated without or with zopolrestat (20 μM), were incubated with 150 μg/ml of RWE for 18 h to induce apoptotic cell death. The cells were stained with annexin-V FITC (FL-1) and propidium iodide (PI) (FL-2). R1 denotes dead cells (PI positive), R2 represents early apoptotic cells (annexin-V positive) and R1+R2 represents total dead cells. (B) The data from (A) has been plotted as bar diagram (n = 4, *p<0.01 Vs Control; **p<0.01 Vs RWE). RWE, ragweed pollen extract; zop, zopolrestat.

doi:10.1371/journal.pone.0006535.g001
of SAEC with AR inhibitor, zopolrestat, significantly (>90%) prevented the increase. This indicates that RWE-induced COX-2 and iNOS overexpression is mediated by AR, which is obligatory for RWE-induced PGE₂ and NO production that exacerbate allergic inflammation.

AR inhibition prevents RWE-induced alterations in pro- and anti-apoptotic proteins in SAEC

Since apoptosis is regulated by the fine balance between the pro-apoptotic and anti-apoptotic proteins, we next examined the effect of AR inhibition on the expression of these proteins. As shown in

Figure 2. Inhibition of AR prevents RWE-induced ROS generation in SAEC. Approximately 1 × 10⁶ cells were seeded on 2-chambered slides and starved in serum-free basal medium with or without AR inhibitors, sorbinil or zopolrestat, for 24 h. The cells were treated with RWE (150 μg/ml) for 16 h. The SAEC were washed with cold PBS (pH 7.2) and stained with ROS-sensitive dye, dihydroethidium (DHE) for 15 min at 37°C. The cells were washed again and mounted with floursave (with diamidino-2-phenylindole (DAPI)) mounting medium. Photomicrographs were acquired by a fluorescence microscope (Nikon). A representative picture is given (n = 4); Magnification 200×. ARI, aldose reductase inhibitor.

doi:10.1371/journal.pone.0006535.g002
Fig. 4. B RWE caused more than 2-fold increased expression of pro-apoptotic protein Bax while the expression of anti-apoptotic protein Bcl-XL decreased by 50%. The overall ratio of pro- and anti-apoptotic proteins in control cells was 1 which increased significantly (to approximately 4) in RWE-treated cells. Inhibition of AR not only controlled the expression of these proteins but it also maintained the ratio to approximately one. These results suggest that AR inhibition prevented the RWE-induced alteration in the ratio of pro- and anti-apoptotic proteins and thereby promoted cell survival and inhibited apoptosis in these cells.

AR inhibition prevents RW-induced over-expression of cell cycle proteins in SAEC

As we showed above RWE induces epithelial cell injury and apoptosis that affects the cell cycle progression. The number of cells entering the cell division cycle is regulated by a fine balance of cell cycle proteins. We therefore examined whether AR inhibition will affect the level of cell cycle proteins in RWE-treated SAEC. As shown in Fig. 4C, RWE caused >3.5 fold increased expression of cyclin D1 and >2.5 fold increased expression in E2F2 proteins. Inhibition of AR by zopolrestat significantly (>90%) prevented the increase in the expression of cell cycle proteins. These results suggest that inhibition of AR is critical to maintaining the cell cycle under oxidative stress and prevents cells from undergoing apoptosis.

AR inhibition prevents RWE-induced activation of NF-κB and AP1

We next examined the effect of AR inhibition on RWE-induced activation of NF-κB and AP1 because these transcription factors are...
primarily responsible for the transcription of various inflammatory markers. As shown in Fig. 4(II) A, RWE caused approximately 4-fold activation of NF-κB and AP-1 and zopolrestat significantly (80%) prevented RWE-induced NF-κB activation and nuclear translocation. Zopolrestat alone did not affect the basal NF-κB activity in the SAEC. To further confirm NF-κB activation by RWE, we used NF-κB-dependent secretory alkaline phosphatase (SEAP) reporter assay and found that RWE significantly (>3-fold) induced NF-κB-dependent SEAP activity in SAEC and AR inhibitor, zopolrestat, caused >60% inhibition (Fig. 4(II) B). However, zopolrestat alone did not affect the NF-κB-SEAP activity. These results validated our measurement of DNA binding activity of NF-κB by gel-shift assay. Based on these observations, we conclude that inhibition of AR prevented RWE-induced activation of NF-κB, which could activate the expression and synthesis of inflammatory markers in SAEC.

AR inhibition prevents RWE-induced accumulation of eosinophils in mice airway

Since inflammatory response of RWE challenge to SAEC was blocked significantly by AR inhibition, we tested whether this approach would work in the animal model as well. We therefore sensitized and challenged the BALB/c mice with RWE- or carrier-treated without or with AR inhibitor. As shown in Fig. 5(I) there was a robust airway inflammation as measured by accumulation of inflammatory cells in bronchoalveolar lavage (BAL) fluid and subepithelium in RWE-sensitized and - challenged animals. In the mice treated with AR inhibitor there was significantly (p<0.001) less inflammation as determined by the number of eosinophils in BAL fluid. Similarly, perivascular and peribronchial inflammation and cell composition in the BAL fluid induced by RWE challenge was significantly prevented by AR inhibitor treatment (Fig. 5(II)).
AR inhibition prevents RWE-induced mucin levels and hyperreactivity in mice airway

Excessive mucin production by airway epithelium is characteristic of allergic asthma and its prevention is the main goal to treat allergic episodes in susceptible individuals [26]. Therefore, we next examined the mucin levels in the BAL fluid by ELISA using anti-MUC5ac monoclonal antibodies and found that mucin levels increased ~2.5-folds in RWE-challenged mice as compared to control and AR inhibition prevented it significantly (p<0.003). Similarly, mucin production in the epithelial cells as assessed by periodic acid Schiff (PAS)-staining of lung sections (Fig. 6(I), inset) was also prevented by AR inhibition. In addition, whole body unrestrained plethysmography was used to quantitatively measure airway responsiveness in mice after methacholine challenge. As shown in Fig. 6(II), enhanced pause (Penh) elevated dose-dependently in response to methacholine challenge as compared to control mice treated with PBS alone. Treatment of mice with AR inhibitor decreased the Penh values significantly (p<0.01) from methacholine (80 mg/ml) alone-challenged mice. These results indicate that AR inhibition significantly prevented the patho-physiological effects of allergic asthma in murine model.

AR inhibition prevents RWE-induced cytokines and chemokines secretion in mice airway

Th2 type cytokines such as IL-4, IL-5 and chemokines have been implicated in the pathology of allergic asthma [27]. Also, the

Figure 5. AR inhibition prevents RWE-induced accumulation of eosinophils in mice. (I) Determination of eosinophils in BAL. The Balb/c mice were sensitized and challenged with PBS, RWE, ARI alone, or ARI+RWE and 72 h later lungs were lavaged with ice-cold PBS and differential cell counts were performed on cytocentrifuge preparations stained with hematoxylin and eosin. Bars represent Mean±SD (n = 6-8), *p<0.002 Vs RWE. Inset: representative fields of hematoxylin and eosin preparations were photographed (magnification ×90). (II) Determination of eosinophils in peribronchial and subepithelial regions of lung. Sensitized BALB/c mice were challenged as in (I), 72 h later lungs were excised, fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned to 5 μm. Sections were stained with hematoxylin and eosin. Photomicrographs were acquired by photometrix CoolSNAP Fx camera mounted on a NIKON Eclipse TE 200 UV microscope. A representative field for each group is shown (magnification: ×30). PBS, phosphate buffer saline; RWE, ragweed pollen extract; ARI, aldose reductase inhibitor
doi:10.1371/journal.pone.0006535.g005
expression of Th2 cytokine mRNA have been shown to increase during allergic inflammation [28]. Therefore, we determined the effect of AR inhibition on the RWE-induced expression of Th2 type cytokines and chemokines in lung by quantitative RT-PCR. As shown in Table 1, RWE challenge caused increased expression of interleukin (IL)-4, IL-5, IL-6, IL-10 and IL-13, and chemokines such as Chemokine (C-C motif) ligand (CCL)-4 and CCL-5 which was significantly prevented by AR inhibition. There was no change in the level of expression of these cytokines and chemokines in AR inhibitor alone treated mice lungs.

Discussion

Among many factors, weed pollens are associated with the allergic asthma causing loss of human work hours and severe health problems, sometimes leading to life threatening conditions. Ragweed pollens are among many seasonal weed pollens that have been shown to induce severe allergic response in susceptible individuals. Ragweed pollens or their extract have been shown to possess intrinsic ROS producing machinery due to their intrinsic NAD(P)H oxidase [25,29]. We have shown that RWE adminis-
stress.

cytokines and chemokines in response to allergens [27,34] which Epithelial cells produce a large number of proinflammatory airway epithelial cells physiology plays a central role in innate and Thus, it is well recognized that oxidative stress-induced changes in the airways causing and exacerbating allergic asthma [30–33].

tration enhances ROS levels in the airway epithelium of murine factors such as N F -κ B binds to DNA and transcribes inflammatory

B [35,36] which translocates to the nucleus. In vascular smooth muscle cells DHN, which could be responsible for activation of inflammatory

dependent signaling, leading to cytotoxicity [10,47]. These data indicated a novel approach to prevent inflammation.

Antioxidants and ROS scavengers such as ascorbic acid, N-acetyl-L-cysteine (NAC), and superoxide dismutase (SOD) have been shown to be effective in controlling ROS-mediated airway inflammation [48,49]. Increased dietary intake of ascorbic acid has been shown to improve lung functions in asthma patients [50]. Similarly, NAC has been shown to have protective effects both in vitro and in vivo against oxidative stress in airway inflammation [51–53]. These evidences suggest that quenching ROS or blocking its effect on airway epithelial cells could be a potential strategy to prevent inflammation and tissue injury during allergic asthma. We observed that in SAEC, AR inhibition significantly prevents ROS formation by RWE challenge and thereby prevents ROS-induced cytotoxicity, including cellular apoptosis. These results suggest that AR inhibition could successfully attenuate the effect of ROS as well as ROS formation in SAEC.

We have shown earlier that AR inhibition prevents oxidative stress-induced signaling cascade that activates transcription factors such as NF-κB and AP-1 [9–14] which could prevent the ROS-mediated airway inflammation. The airway epithelial cells are known to produce ROS in response to many stimuli including weed pollens and virus [25,44]. The locally produced ROS oxidize the cell membrane phospholipids and generate lipid hydroperoxides which impair membrane function such as membrane transport, dysfunction of channel proteins, and loss of fluidity leading to tissue injury [3]. The lipid peroxidation products, including the most abundant and reactive lipid aldehydes such as 4-HNE, have been shown in the airways of experimental animals [3,25]. 4-HNE is known to induce various cellular events including activation of signaling cascade leading to cytotoxicity [43,46]. Also, 4-HNE readily forms conjugates with glutathione (GS), GS-HNE. In vascular smooth muscle cells and murine macrophages we have recently shown that AR-catalyzed reduced product of GS-HNE conjugates, glutathionyl-1,4-dihydroxynonane (GS-DHN), caused activation of NF-κB-dependent signaling, leading to cytotoxicity [10,47]. These data designated a novel role for AR-catalyzed reduced product, GS-DHN, which could be responsible for activation of inflammatory signaling during oxidative stress induced by various stimuli including pathogens, allergens and environmental pollutants. These results indicate that inhibition of AR that blocks the reduction of glutathione-lipid aldehyde conjugate could be a novel approach to prevent inflammation.

ROS oxidation of the cell membrane phospholipids and generate lipid hydroperoxides which impair membrane function such as membrane transport, dysfunction of channel proteins, and loss of fluidity leading to tissue injury [3]. The lipid peroxidation products, including the most abundant and reactive lipid aldehydes such as 4-HNE, have been shown in the airways of experimental animals [3,25]. 4-HNE is known to induce various cellular events including activation of signaling cascade leading to cytotoxicity [43,46]. Also, 4-HNE readily forms conjugates with glutathione (GS), GS-HNE. In vascular smooth muscle cells and murine macrophages we have recently shown that AR-catalyzed reduced product of GS-HNE conjugates, glutathionyl-1,4-dihydroxynonane (GS-DHN), caused activation of NF-κB-dependent signaling, leading to cytotoxicity [10,47]. These data designated a novel role for AR-catalyzed reduced product, GS-DHN, which could be responsible for activation of inflammatory signaling during oxidative stress induced by various stimuli including pathogens, allergens and environmental pollutants. These results indicate that inhibition of AR that blocks the reduction of glutathione-lipid aldehyde conjugate could be a novel approach to prevent inflammation.

Table 1. AR inhibition prevents RWE-induced expression of cytokine and chemokines in mice lungs.

| Cytokine and chemokines | Abbrev. | ARI alone (n = 5) | RWE alone (n = 5–6) | RWE-ARI (n = 7) | P value |
|------------------------|---------|------------------|-------------------|----------------|---------|
| Interleukin 4          | IL-4    | 1.07±0.13        | 15.37±0.71        | 3.84±0.44      | P=0.0012 |
| Interleukin 5          | IL-5    | 1.09±0.09        | 6.84±0.63         | 2.84±0.14      | P=0.0023 |
| Interleukin 6          | IL-6    | 0.87±0.16        | 3.11±0.21         | 1.94±0.53      | P=0.017  |
| Interleukin 8          | IL-8    | 0.97±0.03        | 4.58±0.77         | 2.18±0.31      | P=0.0019 |
| Interleukin 10         | IL-10   | 0.93±0.011       | 6.64±0.21         | 3.18±0.21      | P=0.022  |
| Interleukin 13         | IL-13   | 1.17±0.23        | 9.14±0.29         | 3.18±0.66      | P=0.017  |
| Interleukin 17         | IL-17   | 1.09±0.019       | 6.24±0.27         | 2.50±0.10      | P=0.031  |
| Interleukin 18         | IL-18   | 1.17±0.011       | 2.64±0.47         | 1.64±0.47      | P=0.027  |
| Chemokine (C-C motif) ligand 4 | CCL4 | 1.01±0.09       | 5.18±0.19         | 1.18±0.59      | P=0.019  |
| Chemokine (C-C motif) ligand 5 | CCL5 | 1.12±0.22       | 7.38±0.29         | 4.18±0.79      | P=0.033  |
| Tumor necrosis factor alpha | TNF-α | 0.96±0.14       | 4.83±0.53         | 2.33±0.41      | P=0.029  |

Total RNA was isolated from homogenized lungs (n = 5–7), one microgram of total RNA from each sample was transcribed into first-strand cDNA and quantitative RT-PCR was conducted for selected genes using specific forward and reverse primers. The levels of RNA for the target sequences were determined by melting curve analysis. The values presented here are fold-change over the control. RWE, ragweed pollen extract; ARI, aldose reductase inhibitor.

doi:10.1371/journal.pone.0006535.t001

Antioxidants and ROS scavengers such as ascorbic acid, N-acetyl-L-cysteine (NAC), and superoxide dismutase (SOD) have been shown to be effective in controlling ROS-mediated airway inflammation [48,49]. Increased dietary intake of ascorbic acid has been shown to improve lung functions in asthma patients [50]. Similarly, NAC has been shown to have protective effects both in vitro and in vivo against oxidative stress in airway inflammation [51–53]. These evidences suggest that quenching ROS or blocking its effect on airway epithelial cells could be a potential strategy to prevent inflammation and tissue injury during allergic asthma. We observed that in SAEC, AR inhibition significantly prevents ROS formation by RWE challenge and thereby prevents ROS-induced cytotoxicity, including cellular apoptosis. These results suggest that AR inhibition could successfully attenuate the effect of ROS as well as ROS formation in SAEC.

Increased levels of inflammatory markers including cytokines such as interleukins (IL-6), chemokines such as IL-8 and other inflammatory mediators such as PGE2 by airway epithelial cells are the hallmarks of inflammatory response in allergic asthma [54]. Primarily, these inflammatory markers are released by...
airway epithelial cells in response to allergens in order to remove and dispose off the allergens by recruiting immune cells. However, excessive allergen exposure causes an overwhelming response by epithelial cells resulting in increased ROS formation leading to activation of redox-sensitive transcription factors such as NF-κB and AP-1 [36,42] which transcribe various inflammatory markers that cause cytotoxicity and damage to airway resulting in the pathogenesis of allergic asthma. Therefore, blocking the synthesis and release of the inflammatory markers could potentially prevent asthma pathogenesis. Anti-inflammatory and antioxidant treatment has been suggested to have considerable therapeutic potential for asthma and allergy [55–58]. Similarly, various studies have shown increased iNOS expression in airway epithelium of asthma patients [59,60]. COX-2 has also been found to be elevated during asthma [60]. COX-2 and iNOS catalyze oxidative stress-inducible production of PGE2 and NO, respectively which play important role in the pathogenesis of asthma and have been suggest as possible therapeutic targets [60]. In the present study we have shown that pharmacological inhibition as well as genetic ablation of AR message significantly (70–90%) inhibited RWE-induced expression of these inflammatory markers in SAEC which suggests that AR is a key mediator in RWE-induced inflammation and inhibition of AR, that we have shown earlier prevents the oxidative stress-induced generation of COX-2 and iNOS, would prevent allergen-induced airway inflammation.

Since our ultimate goal is to develop an effective therapeutic intervention to allergic respiratory disorders, we tested whether significant prevention of inflammatory response of RWE challenge in in-vitro cellular model would work in the animal model of allergic asthma as well. The recruitment of eosinophils in the airways plays an important role in the pathogenesis of allergic asthma [41,43]. Studies with RWE-challenged mice have shown that inflammatory cells including eosinophils infiltrate the airway [25,49]. A study using suicidal signals specific to eosinophils in mice showed that eosinophils infiltration is mandatory for the pathology of asthma [61]. We, using murine model of RWE-induced airway inflammation, observed that treatment with AR inhibitor significantly prevented the airway inflammation and recruitment of eosinophils in BAL fluid and subepithelial spaces. In addition, RWE-induced hyperresponsiveness and excessive mucin production by airway epithelium and Th2 type cytokines and chemokines gene expression in sensitized mice were also significantly prevented by AR inhibition. These data with animal model of RWE-induced airway inflammation confirm our in-vitro finding with human primary airway epithelial cells and suggest that AR inhibitors could be initiated for controlled clinical trial in allergic asthma patients. A recent report suggests that AR inhibitors such as zopolrestat and fiderastat have been found to be safe in clinical trials and did not show any major side effects [18].

Thus, with predicted increase in the ragweed pollen production under increased environmental CO2 condition in future climate there is strong likelihood of prevalence of allergic disorders [62]. In view of limitations and side effects of present day anti-inflammatory medication to treat allergic disorders, it is imperative to search for a pharmacological agent with minimal side effects. The evidences presented in this report indicate that AR inhibitors could be a novel approach to control the allergen-induced inflammation in the airway epithelial cells. In summary, we have presented the evidence that AR mediates the respiratory inflammatory response during ragweed pollen-induced allergic asthma and have provided a novel concept that inhibition of AR could be a therapeutic approach in the treatment of allergic airway inflammation.

Materials and Methods

Reagents

Small airway epithelial basal medium (SABM), and small airway epithelial growth media (SAGM™), bulletkit; and one Reagent-pack™ containing Trypsin 0.025%/EDTA 0.01%, Trypsin neutralizing solution and HEPES buffered saline solution were purchased from Cambrex Bio Sciences Walkersville, Inc. (Walkersville, MD). Sorbinil and Zopolrestat were obtained as gift from Pfizer (New York, NY). Dimethyl sulfoxide (DMSO) was obtained from Fischer scientific (Pittsburg, PA). Ragweed pollen extract (RWE) was purchased from Greer’s laboratory (Lenoir, NC). Nitrite/Nitrate and PGE2 assay kits were obtained from Cayman Chemical Inc (Ann Arbor, MI). Human IL-6 and IL-8 ELISA kits were from Diaclone (Stamford, CT) and R&D systems, respectively. Antibodies against COX-2, iNOS, Bcl-XL, Bax, GADPH, cyclin-D1 and E2F2 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); and antibodies against phospho-IkB were from Cell signaling (Danvers, MA). Dihydroethidium (DHE) fluorescent dye was purchased from Molecular Probes, Invitrogen (Carlsbad, CA) and polyclonal antibodies against human recombinant AR were made for us by Alpha diagnostic Intl. (San Antonio, TX). The reagents used in the electrophoretic mobility shift assay (EMSA) and Western blot analysis were obtained from Sigma. All other reagents used were of analytical grade.

Cell Culture

Primary human Small Airway Epithelial Cells (SAEC) obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD) were normal human SAEC harvested from distal airspace of 18 yrs old male donor. The cells were cultured according to the supplier’s instructions at 37°C in humidified atmosphere containing 95% air and 5% CO2 in small airway epithelial basal medium (SABM) with supplements containing 32 µg/ml bovine pituitary extract, 0.5 ng/ml human recombinant epidermal growth factor (EGF), 0.5 µg/ml epinephrine, 1 µg/ml hydrocortisone, 10 µg/ml transferrin, 5 µg/ml insulin, 0.1 ng/ml retinoic acid (RA), 6.5 ng/ml triiodothyronine, 50 µg/ml Gentamicin/Amphotericin-B (GA-1000), and 50 µg/ml fatty acid-free bovine serum albumin (BSA).

Annexin-V Staining and Flow Cytometry

Approximately 2×105 SAEC per well were plated in 6-well plates in triplicate for each group. The medium was replaced with serum-free SABM with or without zopolrestat (20 µM) and incubated for 24 h. The cells were treated by RWE (150 µg/mL) and incubated for 18 h. Apoptotic cell death was examined using the annexin-V-FITC/propidium iodide (PI) [molecular probes, Invitrogen] according to the manufacturer’s instructions. Twenty thousand events were acquired for each sample and analyzed by flow cytometry using the LYSIS II software (FACScan, BD Pharmingen).

In situ detection of superoxide

Dihydroethidium (DHE, Molecular Probes) staining was carried out to assess the ROS production. Briefly, Approximately 1×105 SAEC were seeded on chambered slides and starved in serum-free SABM with or without AR inhibitor for 24 h. The cells were treated with RWE (150 µg/mL) for 16 h. SAEC were rinsed with cold PBS and incubated in PBS containing DHE (2.5 µmol/L) at 37°C for 15 minutes. Cells were rinsed in PBS and mounted with mounting medium with DAPI (Vector Laboratories Inc., Burlingame, CA). The image of ethidium staining was observed and photomicrographs were acquired with a Nikon epifluorescence microscope with
control) to a final concentration of 100 nM using RNAiFect®
scrambled siRNA (AAC ACG GCT TGA ATG ACT ATA; AR-siRNA (AAC GCA TTG CTG AGA ACT TTA) or
starving medium, followed by treatment with RWE (50 µg/ml) for
2 h at 37°C. The nuclear extracts were prepared as described [63].
The Consensus oligonucleotides for NF-κB and AP-1 transcription
factors were 5′-end labeled using T4 polynucleotide kinase. EMSA
was performed as described earlier [64].

NF-κB-dependent secretory alkaline phosphatase
expression (SEAP) reporter assay
Approximately 1×10⁵ SAEC per well were plated in 6-well plates and
grown until 80% confluency. The cells were transfected with
AR-siRNA (AAC GCA TTG CTG AGA ACT TTA) or
scrambled siRNA (AAC ACG GCT TGA ATG ACT ATA) using the Lipofectamine™ Plus reagent. After 6 h of
transfection, the medium was replaced with fresh medium and cells
were treated with RWE (50 µg/ml) for 48 h. The cell culture medium
was harvested, centrifuged and supernatant was analyzed for SEAP
activity, essentially as described by the manufacturer (Invitrogen, CA),
using a 96-well chemiluminescence plate reader.

RNA interference ablation of AR in SAEC
Approximately 2×10⁵ SAEC were plated in 6-well plates and
grown until ~80% confluency. The cells were transfected with
AR-siRNA (AAC GCA TTG CTG AGA ACT TTA) or
scrambled siRNA (AAC ACG GCT TGA ATG ACT ATA; control) to a final concentration of 100 nM using RNAiFect™
transfection reagent (Qiagen). The cells were further incubated for
48 h at 37°C, and AR expression was determined by measuring AR
protein by Western blot analysis using rabbit anti-AR polyclonal
antibodies.

Determination of inflammatory markers IL-6, IL-8 and
PGE₂
The SAECs were plated in 6-well plates at a density of 2×10⁵
cells per well in triplicate for each group. After 24 h, the cells were
pretreated in serum-free SABM with or without zopolrestat
(20 µM) or transfected with AR siRNA as described above. The
growth-arrested and AR ablated cells were stimulated with RWE
(150 µg/ml) for another 24 h in serum-free medium. The
medium was collected from each well, centrifuged and supernatant
was analyzed for cytokines. For the evaluation of inflammatory
markers, ELISA kits for IL-6 (Diaclone, France), IL-8 (R&D systems Inc, Minneapolis, MN) and
PGE₂ (Cayman Chemical Co., Ann Arbor, Michigan) were used
according to the manufacturer’s instructions.

Determination of gene expression of IL-6, IL-8 and COX-2
by RT-PCR
The SAEC were grown in 6-well plates at a density of approximately
2×10⁵ cells per well. After approximately 80% confluence, cells were
serum-starved in the presence or absence of zopolrestat (20 µM) for
24 h and then stimulated with 150 µg/ml RWE for 6 h. Total RNA from
SAEC was isolated by using RNAeasy kit (Qiagen) as per supplier’s instructions. Aliquots of
RNA (0.5–1.0 µg) isolated from each sample were reverse
transcribed with Omniscript and Sensiscript reverse transcriptase.
One-step RT-PCR system with HotStar Taq DNA polymerase
(Qiagen) was used. The PCR was performed with HotStar Taq DNA
polymerase (Clontech, Palo Alto, CA) using a 96-well chemiluminescence plate reader.

Animals
BALB/c mice were purchased from Harlan Sprague-Dawley
(San Diego, CA, USA). All animal experiments were performed
according to the National Institutes of Health Guide for Care and
Use of Experimental Animals and approved by the University of
Texas Medical Branch Animal Care and Use Committee.

Sensitization and challenge of animals
Eight-week-old female animals were sensitized with RWE as
we have previously described [21,25,28]. Briefly, mice were
sensitized with two intraperitoneal administrations of endotoxin-
free RWE 130 µg/100 µl combined with Alum adjuvant in a 3:1
ratio, on days 0 and 4. On days 9 and 10, animals were treated
with AR inhibitor (i.p. 25 mg/kg body weight) every 12 h for total
duration of 48 h. On day 11, parallel groups of mice (n = 6–8) were
challenged intranasally with RWE (100 µg), along with AR
inhibitor. Control groups of mice were challenged with equivalent
volumes of PBS.

Evaluation of allergic inflammation
To evaluate inflammation, animals from all experimental
groups were euthanized on day 14 with ketamine (135 mg/kg
body wt) and xylazine (15 mg/kg body wt), and the lungs were
lavaged with two 0.8 ml aliquots of ice-cold PBS. The cells were
collected by centrifugation (1000 g, for 10 min at 4°C), resuspended
in one ml PBS and total cell counts were determined.

Differential cell counts were performed using a centrifuge.
Preparations stained with hematoxylin and eosin. To evaluate
alveolar lavage (BAL), the lungs were fixed with 4% paraformal-
dehyde, embedded in paraffin, and sectioned to 5 µm. Lung
sections were stained with hematoxylin and eosin [25]. Perivas-
cular and peribronchial inflammation and cell composition in the

a 585 nm long-pass filter. Generation of superoxide in the cells was
demonstrated by strong red fluorescent labeling.

Electrophoretic mobility shift assay (EMSA)
Approximately 90% confluent SAEC in T-150 cm² culture
flasks were incubated with AR inhibitor or carrier for 24 h in
starving medium, followed by treatment with RWE (50 µg/ml) for
2 h at 37°C. The nuclear extracts were prepared as described [63].
Approximately 90% confluent SAEC in T-150 cm² culture
flasks were incubated with AR inhibitor or carrier for 24 h in
starving medium, followed by treatment with RWE (50 µg/ml) for
2 h at 37°C. The nuclear extracts were prepared as described [63].
Approximately 90% confluent SAEC in T-150 cm² culture
flasks were incubated with AR inhibitor or carrier for 24 h in
starving medium, followed by treatment with RWE (50 µg/ml) for
2 h at 37°C. The nuclear extracts were prepared as described [63].
Approximately 90% confluent SAEC in T-150 cm² culture
flasks were incubated with AR inhibitor or carrier for 24 h in
starving medium, followed by treatment with RWE (50 µg/ml) for
2 h at 37°C. The nuclear extracts were prepared as described [63].
Approximately 90% confluent SAEC in T-150 cm² culture
flasks were incubated with AR inhibitor or carrier for 24 h in
starving medium, followed by treatment with RWE (50 µg/ml) for
2 h at 37°C. The nuclear extracts were prepared as described [63].
BAL were evaluated by a pathologist, blinded to treatment groups, to obtain data for each lung. The representative fields were photographed with a Photometric CoolSNAP Fx camera mounted on a NIKON Eclipse TE 200 UV microscope.

Mucin production by the epithelial cells was assessed by periodic acid Schiff (PAS)-staining of formalin-fixed, paraffin-embedded lung sections. The stained sections were analyzed as above and representative fields were photographed with a Photometric CoolSNAP Fx camera mounted on a NIKON Eclipse TE 200 UV microscope [25,28].

To determine mucin levels, BAL was centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatants were kept at −80 °C until assayed. MUC5ac levels in the BAL were assessed by ELISA using commercially available anti-MUC5ac monoclonal antibody (I-15M1) (Lab Vision, Fremont, CA, USA). Briefly, MUC5ac present in the BAL was captured to a microtiter plate and a second antibody conjugated to biotin was added. After 30 min incubation with streptavidin-horseradish peroxidase (HRP) plates were washed and peroxidase substrate was added to obtain colorimetric product, which was quantified by spectrometry. Data are expressed as arbitrary units relative to a MUC5ac standard curve that was included on each plate [25].

Airway responsiveness was measured in unrestrained, conscious mice 3 days after the last challenge. Mice were placed in a barometric plethysmographic chamber, and baseline readings were taken and averaged for 3 min. Aerosolized methacholine in increasing concentrations (from 10 to 80 mg/ml) were nebulized through an inlet of the main chamber for 3 min. Readings were taken and averaged for 3 min after each nebulization and ‘enhanced pause’ (PENH) was determined. PENH, a dimensionless value, represents a function of the ratio of peak expiratory flow to inspiratory flow and a function of the timing of expiration, was calculated as (expiratory time/relaxation time) × (peak expiratory flow/peak inspiratory flow) according to the manufacturers’ protocol. PENH correlates with pulmonary airflow resistance or obstruction and was used as a measure of airway responsiveness to methacholine.

RNA isolation
Total RNA was isolated from homogenized lungs of sensitized, RWE-challenged mice (with and without ARI) using Ambion’s RNAqueous Kit (Ambion, Austin, TX) according to manufacturer’s recommendations. RNA concentration was quantified using a DU530 Beckman spectrophotometer (Beckman Coulter, Fullerton, CA) at 260 nm. The 260/280 nm absorbance ratio was used to assess the purity of isolated RNA.

Quantitative RT-PCR
One microgram of total RNA from each sample was transcribed into first-strand cDNA using SYBR GreenER two step qRT-PCR kit from ABI Prism (Invitrogen, Carlsbad, CA). For selected genes forward and reverse primers were purchased from SABiosciences Corporation (Frederick, MD). Interleukin 5 (Cat # PPM03012E); Interleukin 4 (Cat # PPM03013E); Interleukin 5 (Cat # PPM03014E); Interleukin 8 receptor (Cat # PPM03086D); Interleukin 10 (Cat # PPM03017B); Interleukin 13 (Cat # PPM03021A); Interleukin 17B (Cat # PPM03540A); Interleukin 18 (Cat # PPM03112B); Chemokine (C-C motif) ligand 4 (Cat # PPM02948F); Chemokine (C-C motif) ligand 5 (Cat # PPM02960E); tumor necrosis factor alpha (Cat # PPM03113F); Interferon gamma (Cat # PPM03113F). Internal controls were hypoxanthine guanine phosphoribosyl transferase 1 (Cat # PPM03559E) and glyceraldehyde-3-phosphate dehydrogenase (Cat # PPM02946E). PCR was conducted under the following conditions: denaturation at 95 °C for 10 min, 1 cycle, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and elongation at 60 °C for 60 s. The levels of RNA for the target sequences were determined by melting curve analysis using the ABI PRISM 7000 sequence Detector software (Applied Biosystems, Foster City, CA).

Statistical analysis
For the cell culture experiments data presented are mean ± SD and P values were determined by unpaired, two-tailed Student’s t test. For animal studies, data collected from in vitro and in vivo experiments were analyzed by ANOVA, followed by Bonferroni post-hoc analyses for least significant difference. For quantitative RT-PCR data statistical analysis was done by unpaired, two-tailed t-test using Graph-pad prism software. p<0.05 was considered as statistically significant.

Author Contributions
Conceived and designed the experiments: KVR SKS. Performed the experiments: UGY LAA IB. Analyzed the data: KVR IB HAB. Contributed reagents/materials/analysis tools: HAB. Wrote the paper: KVR.

References

1. Pearce N, Ait-Khaled N, Beasley R, Mallol J, Keil U, et al. (2007) Worldwide trends in the prevalence of asthma symptoms: Phase III of the International Study of Asthma and Allergies in Childhood (ISAAC). Thorax 62: 758–766.
2. Holgate ST (1999) The epidemic of allergy and asthma. Nature 402: B2–4.
3. Ciureanu EI, Trivedi S, Kleeberger SR (2008) Oxidants and the pathogenesis of lung diseases. J Allergy Clin Immunol 122: 456–468.
4. Hendry K, Robinson KA, Gabbita SP, Salmans H, Foy RA (2000) Reactive oxygen species, cell signaling, and cell injury. Free Radic Biol Med 28: 1456–1462.
5. Dovzuk R (2000) Oxidant stress in asthma. Thorax 55: 851–853.
6. Kay AB, Corrigian CJ (1992) Asthma. Eosinophils and neutrophils. Br Med Bull 48: 51–64.
7. Schindlmann HJ, Muni P, Friedenreich JM, Armstrong D, Browne R, et al. (1997) Oxidative stress and lung function. Am J Epidemiol 146: 939–948.
8. Wang CC, Lin WN, Lee CW, Lin CC, Luo SF, et al. (2005) Involvement of p42/p44 MAPK, p38 MAPK, JNK, and NF-kappaB in IL-1beta-induced VCAM-1 expression in human tracheal smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 288: L227–237.
9. Ramana KV, Friedrich B, Srivastava S, Bhatnagar A, Aggarwal BB, et al. (2002) Aldose reductase mediates mitogenic signaling in vascular smooth muscle cells. J Biol Chem 277: 32063–32070.
10. Srivastava SK, Ramana KV, Bhatnagar A (2005) Role of aldose reductase and oxidative damage in diabetes and the consequent potential for therapeutic options. Endocr Rev 26: 389–392.
11. Srivastava SK, Ramana KV, Bhatnagar A, Aggarwal BB, et al. (2002) Aldose reductase mediates mitogenic signaling in vascular smooth muscle cells. J Biol Chem 277: 32063–32070.
12. Ramana KV, Huang D, Srivastava S, Bhatnagar A, Aggarwal BB, et al. (2002) Aldose reductase mediates mitogenic signaling in vascular smooth muscle cells. J Biol Chem 277: 32063–32070.
13. Ramana KV, Bhatnagar A, Srivastava SK (2004) Aldose reductase regulates TNF-alpha-induced cell signaling and apoptosis in vascular endothelial cells. FEBBS Lett 570: 189–194.
14. Ramana KV, Friedrich B, Bhatnagar A, Srivastava SK (2003) Aldose reductase mediates cytotoxic signals of hyperglycemia and TNF-alpha in human lens epithelial cells. FASEB J 17: 315–317.
15. Yadav UC, Ighani-Hoseinabadi F, van Kuik FJ, Srivastava SK, Ramana KV (2009) Prevention of posterior capsular opacification through aldose reductase inhibition. Invest Ophthalmol Vis Sci 50: 752–759.
16. Tammali R, Ramana KV, Singh SS, Awasthi S, Srivastava AK, et al. (2006) Aldose reductase mediates the lipopolysaccharide-induced release of inflammatory mediators in RAW264.7 murine macrophages. J Biol Chem 281: 33019–33029.
17. Tammali R, Ramana KV, Singhal SS, Awasthi S, Srivastava AK, et al. (2006) Aldose reductase mediates the lipopolysaccharide-induced release of inflammatory mediators in RAW264.7 murine macrophages. J Biol Chem 281: 33019–33029.
19. King TP (1976) Chemical and biological properties of some atopic allergens. Adv Immunol 23: 77–105.
20. Raffait, T, Griffiths, T, Kuo, MC, Bond, JF, et al. (1991) Cloning of Amb a 1 (an antigen, Ee, the major allergen family of short ragweed pollen. J Biol Chem 266: 1229–1236.
21. Bacsi A, Chauriaya N, Choudhury BK, Sur S, Boldogh I (2005) Effect of pollen-mediated oxidative stress on immediate hypersensitivity reactions and late-phase inflammation in allergic conjunctivitis. J Allergy Clin Immunol 116: 836–843.
22. Holgate ST (2007) Epithelial dysfunction in asthma. J Allergy Clin Immunol 120: 1233–1244.
23. Holgate ST (2008) The airway epithelium is central to the pathogenesis of asthma. Allergol Int 57: 1–10.
24. Puchelle E, Zahm JM, Tournier JM, Coraux C (2006) Airway epithelial repair, regeneration, and remodeling after injury in chronic obstructive pulmonary disease. Proc Am Thorac Soc 3: 726–733.
25. Boldogh I, Bacsi A, Chauriaya N, et al. (2005) ROS generated by pollen NADPH oxidase provide a signal that augments antigen-induced allergic airway inflammation. J Clin Invest 115: 2169–2179.
26. Hauber HP, Zabel P (2008) Emerging mucus regulating drugs in inflammatory and allergic lung disease. Inflamm Allergy Drug Targets 7: 30–34.
27. Lordan JG, Buccheri F, Richter A, Konstantinidou A, Holloway JW, et al. (2002) Cooperative effects of Th2 cytokines and allergens on normal and asthmatic bronchial epithelial cells. J Immunol 169: 407–414.
28. Robinson D, Hamid Q, Bendle A, Ying S, Kay AB, et al. (1993) Activation of nuclear factor kappaB in the induction of eosinophilia in allergic airway inflammation. J Exp Med 188: 1739–1750.
29. Lin T, Castro S, Brasier AR, Jamialahmadi M, Garofalo RP, et al. (2004) Reactive oxygen species mediate virus-induced STAT activation: role of tyrosine phosphatases. J Biol Chem 279: 2461–2469.
30. Awschl YC, Sharma R, Cheng JZ, Yang Y, Sharma A, et al. (2003) Role of 4-hydroxyenonal in stress-mediated apoptosis signaling. Mol Aspects Med 24: 210–230.
31. Awasthi YC, Sharma R, Sharma A, Yadav S, Singhal SS, et al. (2008) Self-regulatory role of 4-hydroxyenonal in signaling for stress-induced programmed cell death. Free Radic Biol Med 45: 111–118.
32. Ramirez KV, Bhanshara A, Srivastava S, Yadav UC, et al. (2006) Mitogenic responses of vascular smooth muscle cells to lipid peroxidation-derived aldehyde 4-hydroxy-trans-2-nonenal (HNE): role of aldose reductase-catalyzed reduction of the HNE-glutathione conjugates in regulating cell growth. J Biol Chem 281: 17652–17660.
33. Kirkham P, Rahman I (2005) Oxidative stress in asthma and COPD: antioxidants as a therapeutic strategy. Pharmacol Ther 111: 476–494.
34. Bacsi A, Chauriaya N, Boldogh I, et al. (2007) Inhibiting pollen reduced nicotinamide adenine dinucleotide phosphate oxidase-induced signal by intrapulmonary administration of antioxidants blocks allergic airway inflammation. J Allergy Clin Immunol 119: 646–653.
35. Schwartz J, Weiss ST (1994) Relationship between dietary vitamin C intake and pulmonary function in the First National Health and Nutrition Examination Survey (NHANES I). Am J Clin Nutr 59: 110–114.
36. Dekhuijzen PN (2004) Antioxidant properties of N-acetylcycteine: their relevance in relation to chronic obstructive pulmonary disease. Eur Respir J 23: 629–636.
37. De Benedetto F, Aceto A, Dragani B, Spacone A, Formisano S, et al. (2005) Longterm oral N-acetylcysteine reduces exhaled hydrogen peroxide in stable COPD. Pulm Pharmacol Ther 18: 41–47.
38. Sadowska AM, van Overveld FJ, Gerecke D, Zdral A, et al. (2005) The interrelationship between markers of inflammation and oxidative stress in chronic obstructive pulmonary disease: modulation by inhaled steroids and antioxidant. Respir Med 99: 241–249.
39. Yamashita M, Onodera A, Nakayama T (2007) Immune mechanisms of allergic airway disease: regulation by transcription factors. Crit Rev Immunol 27: 539–546.
40. Shapovalovskiy FR, Venzor J 3rd, Barrios R, Leong KP, Huston DP (1999) Therapeutic efficacy of an anti-IL-5 monoclonal antibody delivered into the respiratory tract in a murine model of asthma. Am J Respir Crit Care Med 160: 212–221.
41. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O’Connor BJ, et al. (2000) Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyperresponsiveness, and the late asthmatic response. Lancet 356: 2141–2148.
42. Yang G, Li L, Volk A, Emmell E, Peley T, et al. (2005) Therapeutic dosing with anti-interleukin-13 monoclonal antibody inhibits asthma progression in mice. J Pharmacol Exp Ther 313: 8–15.
43. Cheng G, Arima M, Honda K, Hirata H, Eda F, et al. (2002) Anti-interleukin-9 antibody treatment inhibits airway inflammation and hyperreactivity in mouse asthma model. Am J Respir Crit Care Med 166: 495–506.
44. Hamid Q, Springall DR, Riveros-Moreno V, Chanez P, Howarth P, et al. (1999) Inhibition of aldose reductase prevents lipopolysaccharide-induced inflammation. J Exp Med 188: 1739–1750.
45. Langham JF, Zeev E, Manjunath R, Matsui K, et al. (2006) Increased prostaglandin E2 concentrations and cyclooxygenase-2 expression in asthmatic subjects with spasm eosinophilia. J Allergy Clin Immunol 112: 709–716.
46. Ramas I, Brieque G, Lorent J, Jares P, Quesada P, et al. (2000) Constitutive nuclear factor-kappaB activity in human upper airway tissues and nasal epithelial cells. Eur Respir J 15: 582–589.
47. Zhao Y, Utsayuk PV, Gershovka IA, He D, Wang T, et al. (2009) Regulation of COX-2 expression and IL-6 release by particulate matter in airway epithelial cells. Am J Respir Cell Mol Biol 40: 19–30.
48. Gonzalo JA, Lloyd CM, Krerem LR, Efrin E, Martinez-A C, et al. (1996) Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. J Clin Invest 98: 2312–2315.
49. Pantano C, Athey JL, Alcorn JF, Peytner ME, Brown AL, et al. (2008) Nuclear factor-kappaB activation in airway epithelium induces inflammation and hyperresponsiveness. Am J Respir Crit Care Med 177: 959–969.