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Aldose Reductase Inhibition Suppresses the Expression of Th2 Cytokines and Airway Inflammation in Ovalbumin-Induced Asthma in Mice

Umesh C. S. Yadav,* Amarjit S. Naura,§ Leopoldo Aguilera-Aguirre,† Kota V. Ramana,* Istvan Boldogh,‡ Sanjiv Sur,‡ Hamid A. Boulares,§ and Satish K. Srivastava2*

Airway inflammation induced by reactive oxygen species-mediated activation of redox-sensitive transcription factors is the hallmark of asthma, a prevalent chronic respiratory disease. In various cellular and animal models, we have recently demonstrated that, in response to multiple stimuli, aldose reductase (AR) regulates the inflammatory signals mediated by NF-κB. Because NF-κB-mediated inflammation is a major characteristic of asthma pathogenesis, we have investigated the effect of AR inhibition on NF-κB and various inflammatory markers in cellular and animal models of asthma using primary human small airway epithelial cells and OVA-sensitized/challenged C57BL/6 mice, respectively. We observed that pharmacological inhibition or genetic ablation of AR by small interfering RNA prevented TNF-α as well as LPS-induced apoptosis; reactive oxygen species generation; synthesis of inflammatory markers IL-6, IL-8, and PGE2; and activation of NF-κB and AP-1 in small airway epithelial cells. In OVA-challenged mice, we observed that administration of an AR inhibitor markedly reduced airway hyperresponsiveness, IgE levels, eicosanoids infiltration, and release of Th2 type cytokines in the airway. Our results indicate that AR inhibitors may offer a novel therapeutic approach to treat inflammatory airway diseases such as asthma. The Journal of Immunology, 2009, 183: 4723–4732.

Asthma is a complex chronic inflammatory disease that involves many kinds of inflammatory, structural, and epithelial cells of the airways. The airway epithelial cells, which are the point of first contact between the allergens and the respiratory system, play a key role in the asthmatic process by releasing inflammatory mediators (1). Exposure to different stimuli results in the generation of reactive oxygen species (ROS) in the airway epithelial cells, which produce inflammatory cytokines and chemokines and express adhesion molecules on their cell surface and cause airway inflammation, which involves narrowing of airways, secretion of large amounts of mucus, and infiltration of inflammatory cells (1–8).

Increasing evidences suggest that ROS play an important role in the pathogenesis of airway inflammation during asthma (2–3, 9–11). Beside the airway epithelial cells, activated inflammatory cells also produce ROS locally which disturb the redox homeostasis of the cells and activate various signaling molecules that further cause the activation of transcription factor NF-κB (12, 13). Increased activation of NF-κB has been demonstrated in lung tissues during asthma (14–16). It has been shown that ROS generated in response to inflammatory stimulus cause phosphorylation of signaling intermediates such as protein kinase C, MAPK, and IKK, which then phosphorylate IκB inducing its degradation and translocation of NF-κB to the nucleus (17, 18). In the nucleus, NF-κB binds to DNA and induces the expression of various genes including cytokines such as TNF-α, IL-1β, IL-4, and IL-5 chemokines such as MCP-1, and MIP-1, GM-CSF, IL-8, and inflammatory enzymes such as COX-2 and inducible nitric oxide synthase (iNOS) which regulate immune and inflammatory response (19–25). Various pharmacological regimes that inhibit the activation of signaling kinases or NF-κB have been suggested to control the airway inflammation in patients (26–35). However, these approaches have limitations in terms of partial effectiveness and serious side effects in long-term use. Therefore, novel approaches of therapeutic intervention that could reduce the oxidative stress-induced inflammation and block the synthesis and release of inflammatory intermediates are urgently required.

We have recently shown that, in different cellular and animal models, pharmacological inhibition or genetic ablation of aldose reductase (AR; AKR1B1) prevents the activation of signaling kinases that eventually inhibit the activation of NF-κB and thereby block the release of inflammatory markers and resultant cytotoxicity (36–39). AR, a member of the aldo-keto reductase superfamily, is a cytosolic protein that catalyzes NADPH-dependent reduction of glucose to sorbitol in hyperglycemic conditions, which is suggested to be responsible for diabetic complications. Recently, increasing evidences implicate AR in the oxidative stress-induced activation of signaling molecules that activate transcription factor

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**Abbreviations used in this paper:** ROS, reactive oxygen species; AR, aldose reductase; GS-HNE, glutathione-4-hydroxynonenal; SAEc, small airway epithelial cell; SABM, small airway epithelial basal medium; E2F2, E2F transcription factor-2; DHE, dihydroethidium; PI, propidium iodide; H2DCF-DA, 5- (and 6-) carboxy-2′,7′-dichlorodihydrofluorescein diacetate; SEAP, pNF-κB-secretory alkaline phosphatase; BAL, bronchoalveolar lavage; KC, keratinocyte-derived chemokine; iNOS, inducible nitric oxide synthase; siRNA, small interfering RNA.

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2 Address correspondence and reprint requests to Dr. Satish K. Srivastava, Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX 77555-0647. E-mail address: ssrivast@utmb.edu

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4 Departments of *Biochemistry and Molecular Biology, and †Microbiology and Immunology, ‡Internal Medicine-Allergy, University of Texas Medical Branch, Galveston, TX 77555; and †Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center, New Orleans, LA 70112

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NF-κB and AP-1 (36–41). We have demonstrated that AR catalyzes the reduction of lipid-aldehydes such as 4-hydroxynonenal and their glutathione (GS) conjugates (GS-HNE) with high efficiency (42) and that the reduced product of GS-lipid aldehyde conjugates could be the activator of AR-mediated inflammatory signals (39). These results strongly suggest that AR inhibitors could be potential pharmacological agents in controlling the inflammation. Because asthma is an oxidative stress-induced inflammatory disease that involves the activation of NF-κB, we hypothesize that AR may be involved in the regulation of allergen-induced airway inflammation in asthma. We have, therefore, investigated the effect of pharmacological inhibition or small interfering RNA (siRNA) ablation of AR in the TNF-α as well as LPS-induced inflammation in primary human small airway epithelial cells (SAEC) and in OVA-induced airway inflammation in mice. Our results demonstrate that inhibition of AR prevents oxidant-induced inflammatory signals leading to cytotoxicity in SAEC and OVA-induced airway inflammation in mice, suggesting the use of AR inhibitors as potential therapeutic approach for airway inflammation in asthma.

Materials and Methods

Chemicals

Small airway epithelial basal medium (SABM), and small airway epithelial growth medium bullet kit, and one Reagent pack containing Trypsin 0.025%/EDTA 0.01%, Trypsin neutralizing solution, and HEPES buffered saline solution were purchased from Cambrex BioSciences. Aldose reductase inhibitors Sorbinil and Zopolrestat were gifts from Pfizer and Fidarestat was a gift from Sanwa Kagaku Kenkyusho. DMSO was obtained from Fischer Scientific. LPS from Escherichia coli was obtained from Sigma-Aldrich. TNF-α was purchased from R&D Systems, respectively. Abs against COX2, iNOS, Bcl-XL, Bax, GAPDH, cyclin-D1, and E2F transcription factor-2 were from Diaclone and R&D Systems, respectively. Abs against NF-κB p65 (H2DCF-DA) (Molecular Probes). Approximately 5,000 cells/well were plated in a 96-well plate and serum-starved for 24 h without or with AR inhibitors. The cells after washing with PBS were incubated with 10 μM LPS at 37°C for 24 h. The cells were then harvested with PBS twice and treated with TNF-α (2 nM) and LPS (1 μg/ml) in basal medium for 1 h. After washing the cells twice with PBS, serum-free medium was added and fluorescence was determined at 485 nm excitation and 538 nm emission wavelengths using a 96-well fluorescence plate reader.

ELISA for prostaglandin E2, IL-6, and IL-8 in cell culture medium

Approximately 2 × 10^5 SAEC were seeded per well in 6-well plates in triplicate for each group and incubated overnight. The cells were starved in serum-free basal medium containing sorbinil (20 μM) or carrier. The growth-arrested cells were treated with either TNF-α (2 μM), LPS (1 μg/ml), or carrier in serum-free medium for another 24 h. The medium was collected from each well, cleared by centrifugation, and the supernatant was analyzed for PGE_2, IL-6, and IL-8 (R&D Systems) by using respective ELISA kits according to the manufacturer’s instructions.

RT-PCR for the determination of IL-6, IL-8, and COX-2 expression

Approximately 3 × 10^5 SAEC were plated per well in 6-well plates. After ~80% confluence, cells were serum-starved in SABM containing sorbinil (20 μM) and carrier for 18 h, which was followed by carrier or for 24 h. The cells were then stimulated with TNF-α (2 nM) or LPS (1 μg/ml) for 6 h. Total RNA from SAEC was isolated by using RNeasy kit (Qiagen) as per the supplier’s instructions. RNA (1.0 μg) was reverse transcribed with Omniscript and Sensiscript reverse transcriptase one-step RT-PCR system with HotStar Taq DNA polymerase (Qiagen) at 55°C for 30 min followed by PCR amplification.

The oligonucleotide primer sequences were as follows: 5′-AT GAACGCTTCTTCTACAACGGCG-3′ (sense) and 5′-GAAGAGCCTCTCA GGCTGACTG-3′ (antisense) for IL-6; 5′-AGATCCCTCAAAGCTGGCC GTTGGCT-3′ (sense) and 5′-TCTACCTGTCTTACAATGAGAGGCC-3′ (antisense) for IL-8; 5′-TGAAACTCCACTCAACACAG-3′ (sense) and 5′-TCTACCTGTCTTACAATGAGAGGCC-3′ (antisense) for COX-2; and 5′-CTGGCCACCACATCTTACAATGAGCTG-3′ (sense) and 5′-CG TCTACTGCTTCTACATGTGACAT-3′ (antisense) for β-actin. PCR was conducted in a PCR Sprint thermal cycler (Thermo Electron Corporation) under the following conditions: initial denaturation at 95°C for 15 min followed by 35 cycles of 94°C 1 min, 62°C 1 min, and then 72°C 10 min for final extension. PCR products were electrophoresed with 1.5% agarose-1× Tris-aceate-EDTA gels containing 0.5 μg/ml ethidium bromide. The densitometry analyses of the blots were performed by using Kodak 1D image analysis software.

EMSA

The SAEC were plated in T-150 cm² culture flasks and incubated until 80% confluence and pretreated with AR inhibitor sorbinil (20 μM) or carrier for 24 h in basal medium, followed by treatment with TNF-α (2 nM) for 1 h or LPS (1 μg/ml) for 2 h at 37°C. The nuclear extracts were prepared and used for EMSA as described (38). In brief, SAEC were harvested and washed with cold PBS and suspended in 0.1 ml of hypotonic lysis buffer containing protease inhibitors for 10 min. The cells were then lysed with 5 μl of 10% Nonidet P-40. The homogenate was centrifuged at 6000 rpm, 1 min), and supernatant containing the cytoplasmic extract was stored frozen at ~80°C. The nuclear pellet was resuspended in 50 μl ice-cold nuclear extraction buffer. After 30 min of intermittent mixing, the extract was centrifuged (12000 rpm; 15 min), and supernatants containing nuclear extracts were secured. The protein concentration was measured by the Bradford method. If they were not used immediately, nuclear extracts were stored at −80°C. The consensus oligonucleotides for NF-κB and PA-1 transcription factors were 5′-end labeled using T4 polynucleotide kinase. EMSA was performed as described (38). The specificity of the
NF-κB-dependent secretory alkaline phosphatase reporter assay

The SAEC (1 × 10^5 cells per well) were plated in 24-well plates, serum starved in basal medium with either AR inhibitors, sorbinil, zopolrestat (20 μM), or carrier for 24 h, and transiently transfected with pRNF-κB-secretory alkaline phosphatase (SEAP) construct or control plasmid pTAL-SEAP DNA (Clontech) using the LipofectAMINE Plus reagent. After 6 h, transfection medium was replaced with fresh medium and cells were incubated with either TNF-α (2 nM), LPS (1 μg/ml), or carrier for 48 h. The cell culture medium was then harvested, complemented with normal mouse serum, and assayed for SEAP activity, essentially as described by the manufacturer (Clontech), using a 96-well chemiluminescence plate reader.

RNA interference ablation of AR in SAEC

The SAEC (1 × 10^5 cells per well) were plated in a six-well plate and incubated overnight. The cells (75% confluent) were transfected with human AR-siRNA (AAC GCA TTG CTG AGA ACT TTA) or scrambled siRNA (AAC AGC GCT TGA ATG ACT ATA; control) using the RNAiFect transfection reagent (Qiagen) as per the manufacturer’s instructions. The cells were incubated for 48 h at 37°C and AR expression was determined by Western blot analysis using anti-AR Abs. For treatment with TNF-α or LPS, cells were starved after 24 h of transfection and stimulated at 48 h posttransfection when expression of AR was found lowest.

Western blotting

The cells were washed with ice-cold PBS and lysed in RIPA lysis buffer. Thelysate was cleared by centrifugation and 40 μg of cytoplasmic proteins were resolved on 10% SDS-PAGE gel. After electrophoresis, the proteins were electro-transfered to a nitrocellulose membrane, blocked with 5% nonfat milk in TBST, and probed with Abs against COX-2, iNOS, Bcl-XL, Bax, cyclin D1, E2F2 (1/1,000 dilution), and GAPDH (1/10,000 dilution). The blots were then washed, exposed to HRP-conjugated second-
We therefore examined the effect of AR inhibition on TNF-α- or LPS-induced increase in the levels of various inflammatory markers in the SAEC culture medium. As shown in Fig. 3A, a and b, treatment of SAEC with TNF-α (2 nM) for 24 h caused ~4-fold increase in the levels of IL-6, and >19-fold increase in IL-8 and inhibition of AR significantly (>80%) prevented these changes. Approximately 2.5-fold increase in the PGE2 secretion was observed in TNF-α-treated cells, which was significantly prevented by AR inhibition (Fig. 3Ac). Similarly, LPS treatment also increased (1.5- to 3-fold) the levels of various cytokines and chemokines, which were also significantly prevented by AR inhibition (Fig. 3A).

To rule out nonspecific effects of zopolrestat in the biological system, we silenced AR in SAEC by small interfering oligonucleotides (AR siRNA) to investigate whether phenotypic absence of AR will have similar effects in SAEC as it does with AR inhibitors. We observed that transfection of SAEC with AR siRNA abolished AR protein by >95% (Fig. 3B, insets) while with scrambled (control) antisense oligonucleotides, AR expression remained at the basal level as in control cells. As shown in Fig. 3B, a, b, and c anti-sense ablation of AR significantly prevented the TNF-α- as well as LPS-induced IL-6, IL-8, and PGE2 production in SAEC which was similar to the pharmacological inhibition of AR. The inhibitory effect of AR inhibition on the expression of inflammatory proteins was further confirmed by their expression at RNA level using RT-PCR. As shown in Fig. 3Ca, treatment of SAEC with TNF-α or LPS caused >3-fold increase in the expression of IL-6 mRNA and AR inhibition prevented it by >60%. Similarly, TNF-α and LPS, respectively, caused ~8- and 4-fold increase in the expression of IL-8 mRNA and inhibition of AR prevented these changes by >70% (Fig. 3Cb). These results suggest that AR mediates the transcriptional activation of inflammatory genes. Increased expression of COX-2 corresponds to increased production of PGE2 in oxidative stress, therefore we determined the effect of AR inhibition on transcriptional activation of COX-2 by quantifying its mRNA in SAEC by RT-PCR in response to TNF-α and...
LPS. As shown in Fig. 3C, both TNF-α and LPS significantly increased the mRNA levels of COX-2 by ~3.5- and 2-fold, respectively, in SAEC and sorbinil significantly prevented the increase by 60%.

**AR inhibition prevents TNF-α- and LPS-induced synthesis of inflammatory marker proteins in SAEC**

The biosynthesis of PGE₂ and NO from their precursors is catalyzed by inducible enzymes COX-2 and iNOS, respectively. Therefore, we examined the effect of AR inhibition on TNF-α- and LPS-induced expression of COX-2 and iNOS proteins in SAEC. The SAEC stimulation by TNF-α as well as LPS significantly increased the expression of COX-2 and iNOS proteins by ~3.5- and 2-fold, respectively, and AR inhibition significantly prevented the increase (Fig. 4Aa). These results indicate that AR-dependent COX-2 and iNOS over-expression is necessary for PGE₂ and iNOS production which cause cytotoxicity and tissue damage during airway inflammation.

**AR inhibition prevents TNF-α- and LPS-induced imbalance in the ratio of pro- and anti-apoptotic proteins in SAEC**

It is well understood that cellular apoptosis is regulated by a fine balance between the proapoptotic (e.g., Bax) and anti-apoptotic (e.g., Bcl-XL) proteins. Therefore, we next examined the effect of AR inhibition on the expression of pro- and anti-apoptotic proteins. Both TNF-α and LPS caused ~2-fold increased expression of proapoptotic protein, Bax, while the expression of anti-apoptotic protein, Bcl-XL, decreased by approximately half. In the control cells, we observed that the ratio of pro- and anti-apoptotic proteins remained approximately one, but increased by ~3-fold in TNF-α- and LPS-treated cells. Inhibition of AR controlled the expression of both pro- and anti-apoptotic proteins and maintained their ratio to less than one (Fig. 4Ab). These results suggest that AR inhibition could prevent the TNF-α- and LPS-induced apoptosis in SAEC by regulating the expression of pro- and anti-apoptotic proteins.

**AR inhibition prevents TNF-α- and LPS-induced over-expression of cell cycle proteins in SAEC**

Under oxidative stress, the cell cycle progression is affected as the expression of key cell cycle proteins is altered. Therefore, we examined whether AR inhibition will affect the expression of cell cycle proteins in SAEC under oxidative stress. As shown in Fig. 4Ac, both TNF-α and LPS caused ~2.5-fold increase in the expression of cyclin D1 and E2F2 proteins and inhibition of AR significantly (>90%) prevented it indicating that inhibition of AR is critical to maintaining the cell cycle under oxidative stress.

**AR inhibition prevents TNF-α- and LPS-induced activation of NF-κB and AP-1 in SAEC**

The redox-sensitive transcription factors such as NF-κB and AP-1 are responsible for the transcription of various cytokines and chemokines that cause inflammation and tissue injury (36–40). We therefore examined the effect of AR inhibition on TNF-α- and LPS-induced activation and DNA binding activity of NF-κB and AP-1 by EMSA. As shown in Fig. 4B, TNF-α and LPS, respectively, caused ~3 and 2-fold increased DNA binding of NF-κB as well as AP-1 as compared with control and AR inhibition significantly prevented it. The basal DNA binding activity of NF-κB and AP-1 was not affected by AR inhibitor in the SAEC.

For additional confirmation of NF-κB activation by TNF-α or LPS, we used NF-κB-dependent SEAP reporter assay. As shown in Fig. 4Bb, TNF-α and LPS, respectively, caused ~7- and 2.5-fold increase in NF-κB-dependent reporter (SEAP) activation in SAEC. Two structurally different inhibitors of AR, sorbinil and zopolrestat, inhibited (60%) NF-κB-dependent SEAP activity (Fig. 4Bb). However, sorbinil and zopolrestat alone did not significantly affect the basal NF-κB-SEAP activity. These results validate our measurement of DNA binding activity of NF-κB by EMSA. Our observations thus demonstrate that inhibition of AR could prevent TNF-α- as well as LPS-induced activation of NF-κB and thereby production of inflammatory markers and resultant cytotoxicity during airway inflammation.

**AR inhibition blocks eosinophils infiltration in OVA-challenged mice**

To validate our findings in the cellular model, we used OVA-induced murine model of airway inflammation. Fig. 5Aa shows that OVA-sensitization and challenge induced a clear and marked perivascular and peribronchial infiltration of eosinophils into the lungs of C57BL/6 mice, a trait of allergic airway inflammation.
Such infiltration of inflammatory cells into the airways of OVA-challenged mice was greatly reduced in mice pretreated with AR inhibitor. We further determined the infiltration of total inflammatory cells and specifically eosinophils in the BAL fluid. There was a significant (p < 0.001) increase in the total inflammatory cells (Fig. 5Bb) and eosinophils infiltration (Fig. 5Ac) in the BAL fluid after 48 h of OVA-challenge, which was reduced significantly by the AR inhibitor treatment. The control (unsensitized and unchallenged) mice exhibited no eosinophil recruitment in the BAL fluid.

AR inhibition prevents OVA-specific IgE production and airway hyperresponsiveness in OVA-challenged mice

Because an increased IgE level in the BAL fluid is the hallmark of allergic asthma, we determined the level of OVA-specific IgE in the BAL fluid of mice. As shown in Fig. 5Ba OVA-challenge significantly (53 ± 14.8 ng/ml) increased OVA-specific IgE. Interestingly, AR inhibitor decreased the IgE levels by ~50% (24 ± 13.9 ng/ml). Further, airway hyperresponsiveness, a characteristic of asthma, determined using whole body unrestrained plethysmography, showed a significant increase in the “enhanced pause” (Penh) in response to methacholine in OVA-challenged mice (Fig. 5Bb). Treatment with AR inhibitor, Fidarestat, significantly (p < 0.001) decreased Penh. These results indicate that inhibition of AR could prevent the IgE levels and hyperreactivity associated with the Ag-challenge in murine model of asthma.

AR inhibition prevents Th2 cytokine production in OVA-challenged mice

Th2 cytokines, involved in inflammation and known to increase in allergic asthma, are targets of anti-asthmatic drugs. Therefore, we quantified in BAL fluid various Th2 cytokines (IL-4, IL-5, IL-10, and IL-13) and chemokines (KC, G-CSF, and MCP-1) which immunomodulate inflammation and play a significant role in allergic response. In addition, we determined the effect of OVA-challenge and AR inhibition on IL-6 levels in BAL fluid as it is secreted by cells of the innate immunity and known to induce the expansion of
the Th2 cells (45). As shown in Fig. 6, various inflammatory cytokines and chemokines were found to be significantly \((p < 0.001)\) increased in the BAL fluid of OVA-challenged mice and treatment with AR inhibitor significantly \((p < 0.005)\) decreased them. Further, there was a significant increase in the IFN-\(\gamma\) level in the BAL fluid of OVA-challenged mice which was undetectable in AR inhibitor-treated mice as well as in control mice (Fig. 6).

**Discussion**

According to a recent estimate, asthma is one of the common chronic disorders worldwide, affecting ~300 million individuals (46). In U.S. alone, over 22 million people (7.7\% of the total population) suffer from asthma and ~5000 people die due to asthma each year (47). The annual direct medical expenditure attributable to asthma treatment increased from ~$11.3 billion in 1998 to an estimated ~$37.2 billion in 2007 (48, 49). Beside the growing trend in the prevalence of asthma, the uncertainty remains regarding the cause of asthma. However, there is growing realization that ROS produced by different stimuli could be responsible for the onset as well as exacerbation of asthma (2, 3, 9–11). The ROS are known to disturb the cellular redox homeostasis and induce inflammatory signals that activate transcription factors such as NF-\(\kappa\)B leading to the expression of cytokines, chemokines, and other inflammatory markers such as PGE\(_2\), NO, and adhesion molecules that promote inflammatory cells infiltration accentuating the oxidative stress and cytotoxicity (15, 16, 19–25).

With growing understanding of the role of ROS in mediating the airway inflammation, various studies have suggested the use of antioxidants to treat such inflammation (50). Although the antioxidant capacity of airway epithelial cells is excellent, upon repeated and continued exposure to allergens, the antioxidant capacity decreases. This further augments the ROS generation and inflammation. Therefore, antioxidant(s) or the compounds that could block the inflammatory signals and/or the transcription of inflammatory markers could be excellent drugs to treat airway inflammation. Increased dietary intake of ascorbic acid has been shown to improve lung function in asthma patients (51). Similarly, recent studies using \(n\)-acetylcysteine have suggested its protective effects both in vitro and in vivo against oxidative stress (52–55). Various other anti-inflammatory agents have also been tried to inhibit specific kinases and/or transcription factors for the amelioration of respiratory inflammation in asthma (26, 27, 30, 32). However, these efforts have
not resulted in the development of effective therapeutic intervention. We have recently demonstrated that inhibition of a polyol pathway enzyme, AR, could attenuate the oxidative stress-induced inflammation and expression of inflammatory markers in various cellular and animal models. Our results also show that AR inhibition efficiently prevents the transcription of cytokines.

FIGURE 5. AR inhibition blocks inflammatory cells infiltration, OVA-specific IgE secretion and airway hyperresponsiveness in OVA-challenged mice. A. Fixed lungs from the different experimental groups were sectioned and stained with H&E. Arrowheads indicate sites of inflammatory cells infiltration induced by OVA-challenge (Ao). The cells in the BAL fluid were counted 48 h after OVA-challenge. The total cells (Ab) and eosinophils (Ac) per ml BAL fluid are shown as mean ± SD (n = 6). *, p < 0.001 vs control; #, p < 0.005 vs OVA-challenged group. Ba, IgE levels in the BAL fluid are shown as mean ± SD (n = 4–6). *, p < 0.001 vs control; #, p < 0.05 vs OVA-challenged group. Bb, The changes in pause of breathing “enhanced pause” (Penh), an index of airway obstruction, were measured by whole-body plethysmography. Mice were placed in a barometric plethysmographic chamber and Penh was determined and plotted against the increasing concentration of methacholine. Each data point represents mean ± SD of seven mice for each group. #, p < 0.01 vs control (PBS); ##, p < 0.001 vs control (PBS); *, p < 0.01 vs OVA-challenged mice; **, p < 0.001 vs OVA-challenged mice. BL, baseline; ARI, aldose reductase inhibitor; Fidarestat; PBS, PBS.

FIGURE 6. AR inhibition blocks cytokine and chemokine production in BAL fluid of OVA-challenged mice. Cytokines and chemokines in the BAL fluid were measured using the Bio-Rad Bioplex system and specific ELISA kits. Data are given as means ± SD (n = 4). *, p < 0.001 vs unchallenged mice; #, p < 0.005 vs OVA-challenged mice; ##, p < 0.05 vs OVA-challenged mice. OVA, OVA, ARI, aldose reductase inhibitor.
and chemokines by blocking the signals downstream of ROS that activate transcription factors NF-κB and AP-1 (36–39). We therefore hypothesized that blocking the ROS-mediated activation of signal cascades by AR inhibition could block airway inflammation.

In asthma, several stimuli including allergens activate airway epithelial cells that produce ROS which cause cell death and tissue injury (2, 3, 9–11, 56). Using human primary small airway epithelial cells as an in vitro model, we have demonstrated for the first time that inhibition of AR significantly prevented TNF-α as well as LPS-induced apoptosis in the SAEC. Moreover, increased ROS level in SEAC was also prevented by AR inhibition, which indicates that inhibition of AR could play antioxidant role and prevent the ROS production and thereby block the SEAC apoptosis. When exposed to allergens, airway epithelial cells release various cytokines and chemokines, and activate inflammatory enzymes such as COX-2 and iNOS, which attract the inflammatory cells like eosinophils and macrophages into the airway. The infiltrated cells secrete more cytokines and inflammatory mediators, which aggravate and exacerbate the asthma (12). Studies have suggested that use of Abs against the inflammatory markers such as interleukins 33–35, 57–59, or inhibitors of enzymes such as NF-κB and MAPK (26, 29, 31), PI-3K (27), glycogen synthase kinase-3β (28), or inhibitors of transcription factors NF-κB and AP-1 (30) could be helpful in the treatment of asthma. We have demonstrated that the increased expression of inflammatory markers such as IL-6, IL-8, and COX-2 at both protein and mRNA levels in SAEC in response to LPS as well as TNF-α were prevented by inhibiting AR by pharmacological inhibition as well as by genetic ablation of AR message. AR inhibition also blocked both TNF-α- and LPS-induced activation of transcription factors NF-κB and AP-1 in SAEC. These results are in concert with our earlier findings in other cellular and animal models suggesting the ROS-induced activation of NF-κB and resultant release of inflammatory markers is the main cause of inflammation and cytotoxicity in airway epithelial cells and that it could be prevented by inhibition of AR. Though the exact molecular mechanism as to how AR mediates inflammation is not yet clear, we have recently identified that AR-catalyzed reduced product of GS-HNE (i.e., glutathionyl-4 dihydroxynonene) activates NF-κB via a cascade of kinases that transcribe a number of inflammatory proteins and inhibition of AR prevented it (39). Further studies are required to ascertain the precise role of AR in oxidative stress-induced inflammation.

The results observed in cell culture model are not always corroborated in living systems. Therefore, we tested the effects of AR inhibition in vivo by using a well-established murine model of OVA-induced allergic airway disease. When challenged with an immunogen, mice show a typical airway hyperresponsiveness, mucous secretion, inflammation, and tissue remodeling similar to that observed in allergic asthma pathogenesis in humans (61). AR inhibitor significantly reduced the infiltration of eosinophils in perivascular and peribronchial spaces as well as significantly prevented the airway hyperresponsiveness in the methacholine-challenged mice. Th2 cytokines, which mediate asthma pathogenesis (62, 63), significantly increased in the BAL fluid of OVA-challenged mice and AR inhibition significantly prevented the increase. Specifically in the OVA-challenged mice, there was a remarkable increase in cytokines such as IL-4, IL-5, IL-6, IL-10, and IL-13 and chemokines such as KC and G-CSF compared with control (where they were not or barely detectable) and AR inhibitor treatment significantly decreased (70–90%). MCP-1 also significantly increased in OVA-challenged mice, but AR inhibitor treatment decreased it by only ~30%. It was interesting to note that in OVA-challenged mice, besides a significant increase in the proinflammatory Th2 cytokines, the anti-inflammatory cytokine IL-10 and Th1 specific cytokine IFN-γ also increased. Though the exact function of IL-10 and IFN-γ in asthma is contentious, they are known to play immunomodulatory role (64, 65). In contrast, IL-10 is known to down-regulate both Th1- and Th2-cytokine-induced inflammation (64) and IFN-γ has been shown to suppress the Th2 response by driving the Th1 commitment of naive Th cells and by inhibiting Th2 cytokine production (65, 66). Therefore, the observed simultaneous increase in IL-10 and IFN-γ along with Th2 cytokines in the BAL fluid of OVA-challenged mice (Fig. 6) could be body’s defense against inflammation. This is further substantiated by a significant decrease in the inflammatory Th2 cytokine and a proportionate decrease in IL-10 and IFN-γ upon AR inhibition. Further, in AR inhibitor-treated mice, the decrease in the levels of inflammatory cytokines and chemokines may be responsible for the decrease in eosinophils infiltration in the BAL fluid and lung tissue. In summary, we have demonstrated that AR inhibition could significantly block the increase in inflammatory cytokines and chemokines induced by TNF-α and bacterial toxin, LPS, in human small airway epithelial cell culture model as well as in OVA-challenged mice. Furthermore, the asthma pathogenesis in OVA-challenged mice as determined by the levels of OVA-specific IgE, cytokines, and chemokines and eosinophils infiltration in the BAL fluid and airway hyperresponsiveness were significantly prevented by AR inhibition. Our results thus indicate that AR could be a novel therapeutic target of asthma pathogenesis in humans.

Disclosures
The authors have no financial conflict of interest.

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