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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. Boulal,§ and Satish K. Srivastava*a**

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 IkB 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 or Abs against interleukins 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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3 Abbreviations used in this paper: ROS, reactive oxygen species; AR, aldose reductase; GS-HNE, glutathione-4-hydroxynonenal; SAEC, small airway epithelial cell; SARM, 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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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 Research Diagnostics. Nitrite/Nitrate and PGE2 assay kits were obtained from Cayman Chemical. Human IL-6 and IL-8 ELISA kits were from Dianoine and R&D Systems, respectively. Abs against COX2, iNOS, Bcl-XL, Bax, GAPDH, cyclin-D1, and E2F transcription factor-2 (E2F2) were from Santa Cruz Biotechnology. Dihydroethidium (DHE) fluorescent dye was purchased from Molecular Probes, Invitrogen, and polyethylene glycol (E2F2) were from Research Diagnostics. Nitrite/Nitrate and PGE2 assay kits were obtained from Research Diagnostics. Nitrite/Nitrate and PGE2 assay kits were purchased from BioSource International and stored at 80°C. The nuclear extract was centrifuged (12000 rpm; 15 min), and supernatants 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 stored. The protein concentration was measured by the Bradford method. If necessary, nuclear extracts were not used immediately, nuclear extracts were stored at −80°C. The consensus oligonucleotides for NF-κB and AP-1 transcription factors were 5′-end labeled using T4 polynucleotide kinase. EMSA was performed as described (38). The specificity of the

Cell culture
The SAEC were obtained from Lonza (formerly Cambrex BioScience) and cultured according to the supplier’s instructions at 37°C in humidified incubator with 95% O2 and 5% CO2 in SABM supplemented with 52 μg/ml bovine pituitary extract, 0.5 mg/ml human recombinant epidermal growth factor, 0.5 μg/ml epinephrine, 1 μg/ml hydrocortisone, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 mg/ml retinoic acid, 6.5 mg/ml triiodothyronine, 50 μg/ml gentamicin/amphotericin-B (GA-1000), and 50 μg/ml fatty acid-free BSA. Passages 3–7 were used in the experiments.

Cell viability assays
The SAEC were plated at the density of 5000 cells/well in a 96-well plate. After the cells attached, they were growth-arrested for 24 h by replacing complete medium with fresh basal medium containing sorbinil (20 μM) or carrier. The cells were incubated with TNF-α (2 nM), LPS (1 μg/ml), or carrier for an additional 24 h, after which 10 μl of MTT (5 mg/ml) was added to each well and incubated at 37°C for 2 h. The medium was removed and the formazan granules obtained were dissolved in 100 μl DMSO. Absorbance was read at 570 nm using a 96-well ELISA plate reader.

Annexin-V and propidium iodide (PI) staining was used to determine the apoptotic cell death. Approximately 2 × 104 10^6 SAEC per well was plated in 24-well plates overnight. The medium was replaced with serum-free SABM with or without sorbinil (20 μM) and incubated for 24 h. The cells were treated with TNF-α (2 nM) or LPS (1 μg/ml) in a fresh medium containing sorbinil (20 μM) or carrier and incubated for 18 h. Apoptotic cell death was examined using the annexin-V-FITC/PI, (Molecular Probes, Invitrogen) according to the manufacturer’s instructions. Twenty thousand events for each sample were acquired and analyzed by flow cytometry using the LYSIS II software (FACSscan, BD Pharmingen).

Detection of superoxide
Approximately 1 × 105 cells per chamber were seeded on two-chambered slides and incubated overnight at 37°C. The cells were starved in free basal medium containing AR inhibitor, sorbinil, or zopolrestat (20 μM), or carrier for 24 h. The cells were then stimulated with TNF-α (2 nM), LPS (1 μg/ml), or carrier for an additional 16 h. The SAEC were washed with cold PBS twice and incubated with DHE (2.5 μM) in PBS at 37°C in a humidified chamber for 15 min. Cells were washed in PBS twice and mounted with fluorescent mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories). The image of ethidium staining, demonstrated by strong red fluorescent, was obtained with a Nikon epifluorescence microscope with a 585 nm long-pass filter. Additionally, intracellular ROS levels were determined using a fluorescent ROS indicator 5- (and −6)-carboxy-2,7′-dichlorodihydrofluorescin diacetate (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 H2DCF-DA at 37°C for 30 min. Cells were washed 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 prosta glandin E2, IL-6, and IL-8 in cell culture medium

Approximately 2 × 104 10^6 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 nM), 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 PGE2 (Cayman Chemical); IL-6 (Diacalone); 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 × 10^4 10^6 SAEC were plated per well in 6-well plates. After ~80% confluence, cells were serum-starved in SABM containing sorbinil (20 μM) or carrier for 24 h which they were washed twice 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) were reverse transcribed with Omniscript and Sensiscript reverse transcriptase one-step RT-PCR system with HotStar Taq DNApolymerase (Qiagen) at 55°C for 30 min followed by PCR amplification. The oligonucleotide primer sequences were as follows: 5′- ATGACTTTCCTTTACCCAAGCCG-3′ (sense) and 5′-GAAGACGGCTCTA AGTTTCTG-3′ (antisense) for IL-6; 5′-ATGACTTCACAGGCTG-3′ (sense) and 5′-GGCTGGACTG-3′ (antisense) for IL-6; 5′-ATGACTTTCCTTTACCCAAGCCG-3′ (sense) and 5′-TCTTAGGCTCTTAAATGCTTCTC-3′ (antisense) for IL-8; 5′-TGAACACACTCTCAACAGACAG-3′ (sense) and 5′-CTTACGAGGAAAGGAA-3′ (antisense) for COX-2; and 5′- ATGTCGACGACGCTCACTGCAG-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, 72°C 1 min, and then 72°C 10 min for final extension. PCR products were electrophoresed with 1.5% agarose-1% Tris-ace late-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 (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 stored. The protein concentration was measured by the Bradford method. If necessary, nuclear extracts were not used immediately, nuclear extracts were stored at −80°C. The consensus oligonucleotides for NF-κB and AP-1 transcription factors were 5′-end labeled using T4 polynucleotide kinase. EMSA was performed as described (38).
assay was examined by competition with an excess of unlabeled oligonucleotide and supershift assays with Abs to p65.

**NF-kB-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 pNF-kB-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 and analyzed 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 AGA ACT TTA) or scrambled siRNA (AAC AGC GTG 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. The lysate was cleared by centrifugation and 40 μg of cytoplasmic proteins were resolved on 10% SDS-PAGE gel. After electrophoresis, the proteins were electro-transferred to a nitrocellulose membrane, blocked with 5% nonfat milk in TBST, and probed with Abs against COX-2, iNOS, Bcl-XL, and GAPDH (1/1,000 dilution), and cyclin D1, E2F2 (1/1,000 dilution), and GAPDH (1/10,000 dilution) in TBST, and probed with Abs against COX-2, iNOS, Bcl-XL, were resolved on 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, blocked with 5% nonfat milk in TBST, and probed with Abs against COX-2, iNOS, Bcl-XL, and GAPDH (1/10,000 dilution).

**Animals**

Mice (C57BL/6; wild-type) were bred in a specific-pathogen free facility at Louisiana State University Health Sciences Center, New Orleans, LA, and allowed unlimited access to sterilized chow and water. Maintenance, experimental protocols, and procedures were all approved by the Louisiana State University Health Sciences Center Animal Care and Use Committee.

**Sensitization, challenge, and administration of drugs**

Six- to eight-week-old C57BL/6 wild-type mice were sensitized with inhaled OVA (3% OVA in saline). The OVA-aerosol was generated by a Bennett Superfrost (2 nM) nebulizer. The mice in the experimental group were challenged by placing them in a Plexiglas chamber and exposed for 30 min to aerosolized OVA (3% OVA in saline). Sensitization, challenge, and administration of drugs were essentially as described by the manufacturer (Clontech), using a 96-well chemiluminescence plate reader.

**Cytokine and IgE assessment in BAL fluid**

The cytokine assessment in the BAL fluid was conducted using the Bio-Rad Bioplex System (Bio-Rad Laboratories) for mouse cytokines namely IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, and chemokines such as G-CSF, keratinocyte-derived chemokine (KC) and MCP-1 according to the manufacturer’s instructions and specifications. Quantitative assessment of OVA-IgE in the BAL fluid was conducted using OVA-specific mouse-IgE ELISA kit from MD Biosciences.

**Statistical analysis**

For the cell culture experiments data presented are mean ± SD and p values were determined by unpaired 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. p < 0.05 was considered as statistically significant.

**Results**

**AR inhibition prevents TNF-α- and LPS-induced SAEC apoptosis**

To determine the effects of AR inhibition on TNF-α or LPS-induced apoptosis in the SAEC, we measured cell viability by MTT assay. Both TNF-α and LPS caused loss of viability in SAEC by ~42% and 26%, respectively, and inhibition of AR prevented it (Fig. 1A). We also used Annexin-V staining to ascertain these findings. As shown in Fig. 1B, both TNF-α and LPS caused 75% and 55% more cell death (including both apoptotic and dead cells), respectively, as compared with control cells in 18 h of treatment (quantified and tabulated in Fig. 1Bb). Preincubation of the cells with AR inhibitors significantly reduced cell death by TNF-α and LPS (Fig. 1). Under similar conditions, AR inhibition alone did not cause apoptosis of SAEC.

**AR inhibition prevents TNF-α- and LPS-induced ROS generation in SAEC**

Because it is known that both TNF-α and LPS cause oxidative stress, which could cause reduction in SAEC viability, we measured the levels of ROS in SAEC by two different methods; 1, qualitatively by DHE staining followed by acquiring fluorescent photomicrographs (Fig. 2A) and 2, quantitatively by H2DCF-DA staining followed by fluorometric analysis (Fig. 2B). As shown in Fig. 2, there was a significant increase in the ROS levels in TNF-α- and LPS-treated cells, suggesting increased oxidative stress in SAEC. Preincubation of the cells with different AR inhibitors sorbinil or zopolrestat significantly prevented these changes. AR inhibition alone caused no significant changes in the ROS level in SAEC.

**AR inhibition prevents TNF-α- and LPS-induced production of inflammatory markers in SAEC**

Both TNF-α and LPS are known to elevate the levels of inflammatory markers that could cause inflammation and aggravate the
allergic reaction (43, 44). We therefore examined the effect of AR inhibition on TNF-α/H9251- or LPS-induced increase in the levels of various inflammatory markers in the SAEC culture medium. As shown in Fig. 3A, 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- and 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 genes 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...
Therefore, we examined the effect of AR inhibition on TNF-α and iNOS, respectively. Both TNF-α and LPS caused ~2-fold increased expression of proinflammatory 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. 4Ba, 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. 5A 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 ($53 \pm 14.8$ ng/ml) increased OVA-specific IgE. Interestingly, AR inhibitor decreased the IgE levels by $\sim 50\%$ ($24 \pm 13.9$ 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 world-wide, 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 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

FIGURE 4. AR inhibition prevents TNF-$\alpha$- and LPS-induced (A) expression of inflammatory, apoptosis, and cell cycle proteins, and (B) activation of redox-sensitive transcription factors NF-$\kappa$B and AP-1 in SAEC. A. Approximately $2 \times 10^5$ SAEC were seeded in six-well plates and incubated until 80% confluence. The cells were growth-arrested by replacing medium with basal medium without or with AR inhibitor for overnight. The cells were treated with TNF-$\alpha$ (2 nM) or LPS (1 $\mu$g/ml) for further 24 h. Western blotting was performed to determine the expression of various proteins. GAPDH was used as loading control. Representative blots are shown ($n = 3$), numbers below the blots represent fold-changes. Lanes: 1, Control; 2, TNF-$\alpha$; 3, LPS; 4, Sorbinil+Control; 5, Sorbinil+TNF-$\alpha$; 6, Sorbinil+LPS. Bb. Approximately $2 \times 10^6$ SAEC were seeded in T-150 cm$^2$ flasks and incubated till >90% confluence. Medium was replaced with basal (serum-free) medium containing AR inhibitor or carrier for 24 h. The cells were treated with TNF-$\alpha$ (2 nM) for 1 h or LPS (1 $\mu$g/ml) for 2 h. nuclear extracts were prepared and EMSA was performed. The numbers below the blots represent fold-changes. Lanes: 1, Control; 2, TNF-$\alpha$; 3, LPS; 4, Sorbinil+Control; 5, Sorbinil+TNF-$\alpha$; 6, Sorbinil+LPS. Bb. For SEAP assay, SAEC were growth-arrested by preincubating with basal medium containing AR inhibitors, sorbinil or zopolrestat, or carrier for 24 h followed by transfection with NF-$\kappa$B-pSEAP vector or control (pTAL) vector and after 6 h the cells were treated with TNF-$\alpha$ (2 nM) or LPS (1 $\mu$g/ml) and incubated for 48 h. Media were collected and NF-$\kappa$B-dependent SEAP activity was determined by chemiluminescence’s method essentially as described by the manufacturer. Bars represent mean ± SD ($n = 6$). *, $p < 0.001$ vs control; **, $p < 0.001$ vs TNF-$\alpha$; #, $p < 0.01$ vs control; ##, $p < 0.01$ vs LPS.
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 (Aa). 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. (B). 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. (Ba). 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 SAEC 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 and leukotriene (33–35, 57–59), or inhibitors of enzymes such as COX-2 (60) or pharmacological agents that inhibit signaling kinases such as 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.

References
SUPPRESSION OF ASTHMA BY ALDOSE REDUCTASE INHIBITION


