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Original article

Nuclear factor erythroid 2-related factor 2 (Nrf2) regulates airway epithelial barrier integrity

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Abbreviations:
AJ, Adherent junction; AOX1, Aldehyde oxidase 1; ARE, Antioxidant responsive element; DEX, Dexamethasone; EGFR, Epidermal growth factor receptor; ICS, Inhaled corticosteroids; IPA, Ingenuity pathway analysis; Nrf2, Nuclear factor erythroid 2-related factor 2; PCR, Polymerase chain reaction; siRNA, Small interfering RNA; TER, Transepithelial electrical resistance; TJ, Tight junction; ZO-1, Zonula occludens-1

ABSTRACT

Background: Inhaled corticosteroids enhance airway epithelial barrier integrity. However, the mechanism by which they accomplish this is unclear. Therefore, we investigated steroid-inducible genes and signaling pathways that were involved in enhancing airway epithelial barrier integrity.
Methods: A human bronchial epithelial cell line (16HBE cells) was cultured with 10^{-6} M dexamethasone (DEX) for 3 days to enhance epithelial barrier integrity. After measuring transepithelial electrical resistance (TER) and paracellular permeability, we extracted total RNA from 16HBE cells and performed microarray and pathway analysis. After we identified candidate genes and a canonical pathway, we measured TER and immunostained for tight junction (TJ) and adherent junction (AJ) proteins in cells that had been transfected with specific small interfering RNAs (siRNAs) for these genes.
Results: We identified a nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated oxidative stress response pathway which was primarily involved in the steroid-induced enhancement of airway epithelial barrier integrity. Transfecting cells with Nrf2 specific siRNA reduced the steroid-induced enhancement of airway epithelial barrier integrity and the accumulation of TJ and AJ proteins at sites of cell–cell contact. Moreover, based on pathway analysis, aldehyde oxidase 1 (AOX1) was identified as a downstream enzyme of Nrf2. Transfecting cells with *AOX1*-specific siRNA also reduced the steroid-induced enhancement of airway epithelial barrier integrity.
Conclusions: Our results indicated that the Nrf2/AOX1 pathway was important for enhancing airway epithelial barrier integrity. Because the airway epithelium of asthmatics is susceptible to reduced barrier integrity, this pathway might be a new therapeutic target for asthma.

Introduction

Asthma is a highly complex, heterogeneous disease that is characterized by airway inflammation associated with T helper type2 (T_H2) cells, mucus hyperproduction, and airway hyperresponsiveness.[1–3] Inhaled corticosteroids (ICS) have recently been used to treat asthma to control chronic airway inflammation and the associated underlying pathways involved with T_H2 responses.[4] The wider use of ICS has resulted in a reduction of asthma symptoms and airway hypersensitivity, improvements in quality of life, and reduced mortality rates.[5] However, there are a significant number of patients with severe asthma that is resistant to ICS therapy,[6] which suggests that there are other mechanisms underlying asthma in addition to chronic allergic airway inflammation.

It was reported that airway epithelial barrier integrity in patients with asthma was reduced as compared with that of healthy people.[7] We previously reported that steroids could enhance

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airway epithelial barrier integrity by increasing the phosphorylation of epidermal growth factor receptors (EGFR).[8] EGFRs play a major role in the growth and specialization of the airway epithelium.[9] EGFR expression is enhanced not only in the normal airway epithelium but also in the injured airway epithelium of asthma patients.[10]

The airway epithelial barrier is formed by intercellular binding components including adherent junctions (AJ) and tight junctions (TJ). An AJ is comprised of E-cadherin and associates with actin through β-catenin. A TJ is located at the apex of an intercellular adhesion site[11] and is formed by interacting proteins such as zonula occludens (ZO) 1–3, occludin, and claudins 1–5.[12,13] In particular, a TJ is a principle structural component for paracellular permeability.[14] When a TJ is disrupted, environmental factors can readily enter the airway and cause allergic airway inflammation.[7] Enhancing these intercellular binding components could potentially lead to new treatments for asthma.

Thus, in this study, we conducted a global gene expression analysis and attempted to identify steroid-inducible genes and signaling pathways to determine the mechanism by which steroids could enhance airway epithelial barrier integrity.

Methods

Cells and reagents

A differentiated SV40-transformed human bronchial epithelial cell line (16HBE14o−; hereafter referred to as 16HBE cells) was a generous gift from Dr. D.C. Gruenert (University of California, San Francisco, CA, USA). Dexamethasone (DEX) and fluorescein isothiocyanate-labeled dextran (FITC-dextran; 4 and 10 kDa) were from Sigma Chemical Company (St. Louis, MO, USA).

Cell culture

16HBE cells were grown in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and used at passages 10–30. 16HBE cells were seeded on Transwell inserts (Costar, Corning, NY, USA) at a density of 1×10^5 cells/cm^2. 16HBE cells seeded on Transwell inserts were cultured with or without 10^{-6} M DEX for up to 3 days.

Transepithelial electrical resistance measurements

TER measurement using 16HBE cells has been established in previous studies based on paracellular transport.[15–17] These were seeded on Transwell inserts at 1×10^5 cells/cm^2. The integrity of the cell monolayer was evaluated by measuring transepithelial electrical resistance (TER) using a Millicell-ERS system (Millipore Corp., Bedford, MA, USA) according to the manufacture's instruction. TER (ohms × cm^2) was calculated using the equation: (TER sample−TER blank) × surface area (cm^2).[18]

Apparent permeability coefficient

The permeability of a cell monolayer was determined by FITC-dextran fluxes across this layer. A solution that contained 4-kDa FITC-dextran (1 mg/ml) was added to the apical compartment of a Transwell insert. Samples (200 μl) were removed from the basal compartments at 60 min after adding FITC-dextran and FITC-dextran intensity was measured using a PTI fluorometer set for excitation at 492 nm and emission at 520 nm. An apparent permeability coefficient (Papp) was obtained using the equation: Papp (cm/s) = dQ/dt(V/AC $_0$), where dQ/dt was the permeability rate (μg/s), C_0 was the initial concentration in the upper chamber (μg/ml), V was the volume of the upper chamber (cm^3), and A was the membrane surface area (cm^2).[19]

RNA extraction

16HBE cells were cultured for 3 days with or without DEX. Total RNA was isolated on day 0 and day 3 of culture using a QIAGEN RNeasy Minikit (Qiagen, Valencia, CA, USA). RNA samples were adjusted using an Ambion WT Expression Kit (Affymetrix, Santa Clara, CA, USA), according to the manufacturer's protocol.

Microarray analysis, pathways and network analysis

Fragmented second-cycle cDNA was labeled using a GeneChip WT Terminal Labeling Kit (Affymetrix), according to the manufacturer's protocol. We confirmed DNA fragmentation using an Agilent RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA, USA), according to the manufacturer's protocol. Next, we used Gene Chip HU Gene 1.0 ST Arrays (Affymetrix) for microarray analysis. An array was hybridized using Genechip Fluidics Station 450 and a Genechip Scanner 3000 (Affymetrix) to measure fluorescence intensity. The obtained gene data were subjected to statistical analysis using GeneSpring 12.5 software (Agilent Technologies UK Limited, South Queensferry, UK). Candidate genes that were apparently associated with enhanced barrier integrity were identified. We also used gene data to perform pathway analysis using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems Inc., Redwood City, CA, USA).

Real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) analysis was performed using an Ambion Fast SYBR Green Cells-to-CT Kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. Using the protocols for an Applied Biosystems 7300 Fast Real- Time PCR system and SYBR green PCR master mix (Life Technologies), PCR was performed in 96-well plates with denaturing at 95 °C for 20 s, followed by 40 cycles of annealing at 95 °C for 3 s and amplification at 60 °C for 30 s. We set the initial threshold and determined the Ct value. We evaluated the RNA level of each sample using the comparative determination method (⊿⊿CT method).[20]

The primers sequences were listed below:

BIRC3, 5′-ATGCTTCTGTTGTGGCCTGAT-3′ and 5′-GTTAACGGAATTTAGACTCTGAACGA-3′;

KRT6A, 5′-GGCTGAGGAGCGTGAACAG-3′ and 5′-CCAGGAACCGCACCTTGT-3′;

AOX1, 5′-CAGCGCTGATCACCGAATCT-3′ and 5′-GTACATGTTTATGATTCGCACCTTCT-3′;

LOX, 5′-TGAGTTTAGCCACTATGACCTGCTT-3′ and 5′-AAACTTGCTTTGTGGCCTTCA-3′;

NNMT, 5′-AGAGGCTGGCTACACAATCGA-3′ and 5′-GTTGGCCATGGTGGAAGAATA-3′;

KLK6, 5′-CCTTCGGCAAAGGGAGAGTT-3′ and 5′-GCTGGCGGCATCATAGTCA-3′;

RASGRP1, 5′-TCAATAAGGTTCTCGGTGAGATGA-3′ and 5′-TGAAGTCGGTGCACTCTCCAT-3′;

TFPI2, 5′- CGATGCTTGCTGGAGGATAGA-3′ and 5′-CACTGGTCGTCCACACTCACTT-3′;

LCN2, 5′-TGACTACTGGATCAGGACTTTTGTTC-3′ and 5′-CTTAATGTTGCCCAGCGTGAA-3′;

ID2, 5′-CACGTCATCGACTACATCTTGGA-3′ and 5′-TGGTGATGCAGGCTGACAAT-3′;

GAPDH, 5′-GTCGGAGTCAACGGATTTGG-3′ and 5′-GGCAACAATATCCACTTTACCAGAG-3′

Small interfering RNA preparation and transfection

To determine which candidate gene products might enhance epithelial cell barrier function, we transfected 16HBE cells with small interfering RNAs (siRNAs) of the candidate genes using Lipofectamine RNAiMAX (Life Technologies). After 24 h, transfected cells were transferred to a Transwell chamber, cultured with medium that contained DEX, and assessed for cell monolayer barrier function by measuring TER.

SiRNA used in transfection are listed below:

Stealth RNAi™ siRNA Negative Control Med GC Duplex (Life Technologies)

NFE2L2 siRNA (Sigma): SASI_Hs01_00182394 HUMAN NM_006164 1917 MISSION®

AOX1 siRNA(Sigma): SASI_Hs01_00221863 HUMAN NM_001159 1963 MISSION®

Fluorescent immunostaining

Cell monolayers were fixed with 4% paraformaldehyde at 37 °C for 60 min. Cells were then washed with PBS (Sigma), after which 3% bovine serum albumin (BSA; Sigma) was added to block non-specific binding for 10 min at room temperature. Cells were washed again with PBS, and then reacted with an anti-human E-cadherin rabbit monoclonal antibody (Cell Signaling Technology, Beverly, MA, USA) and an anti-human ZO-1 mouse monoclonal antibody (Zymed Laboratories, Inc., San Francisco, CA, USA) used as primary antibodies. As secondary antibodies, anti-mouse IgG Alexa 488,594, and anti-rabbit IgG Alexa 488,594 (Life Technologies) were used. Staining intensity was assessed using a laser scanning confocal FV1000 microscope (Olympus, Tokyo, Japan) with identical settings used for all conditions.

Western blotting

Stimulated cells were washed with PBS and proteins were extracted using lysis buffer that included RIPA buffer (25 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS), 100 mM PMSF, 10 mg/ml of aprotinin, and 1M dithiothreitol (DTT). Cell lysates were centrifuged for 10 min at 20817 × g. The samples were then boiled, separated by 10% SDS polyacrylamide gel electrophoresis, and transferred to an Immobilin-P membrane (Millipore Corp.). The membrane was incubated with an anti-human E-cadherin rabbit polyclonal

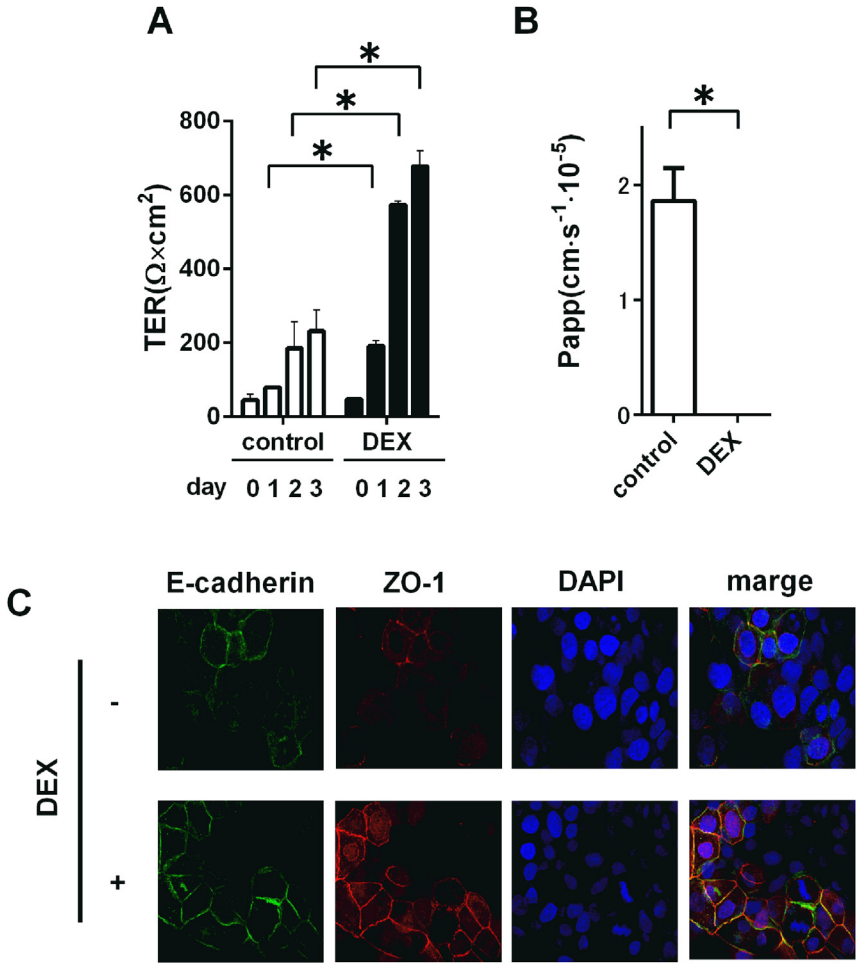

Fig. 1. DEX treatment enhances permeability barrier formation in 16HBE cells. **A**. DEX effects on TER development in 16HBE cells. Cells were seeded on Transwell inserts and cultured with DEX. TER was monitored for 3 days. Results are means ± SDs (n = 3); $^{*}P < 0.05$ vs. cells cultured without DEX. **B**. 16HBE cell monolayers permeability to FITC-dextran. Cells were seeded on Transwell inserts and cultured with DEX for 3 days. Fluorescence intensity in the lower chamber was measured and compared with the amount of FITC-dextran fluorescent signals that crossed the cells that were cultured without DEX used as a control. Results are means ± SDs (n = 3); $^{*}P < 0.05$ vs. cells cultured without DEX. **C**. DEX treatment enhances TJ formation in 16HBE cells. 16HBE cells were cultured with or without DEX for 3 days and were then immunostained with antibodies specific for E-cadherin (upper) and ZO-1 (lower).

antibody (Cell Signaling Technology) and an anti-human ZO-1 mouse monoclonal antibody (Zymed Laboratories) used as primary antibodies to assess junctional proteins' expression. An appropriate secondary horseradish peroxidase-conjugated antibody was also used. Western blots were visualized using an enhanced chemiluminescence system (GE Healthcare, Little Chalfont, UK). Bands on Western blots were quantified using Image J software and staining intensity was determined as a percentage of the control.

Statistical analysis

Results are given as means ± standard deviations (SDs). Results for different experimental groups were compared using Student's t-tests. $P < 0.05$ was considered significant. Statistical analyses were performed using GraphPad Prism Software (La Jolla, CA, USA). To determine P-values for pathway analysis, we used Fisher's exact test to estimate how many molecules were non-coincidentally included in the list of our gene data as compared with molecules included in the respective pathways. $P < 0.05$ was considered significant.

Results

Steroid treatment enhances airway epithelial barrier integrity

We measured TER and FITC-dextran permeability for 16HBE cells to confirm that dexamethasone (DEX) treatment had enhanced airway epithelial barrier integrity. Compared to cells without DEX treatment, TER was significantly increased from day 1 to day 3 in cells that were treated with DEX (Fig. 1A). FITC-dextran permeability was significantly reduced in cells treated with DEX as compared to cells not treated with DEX (Fig. 1B). During DEX treatment, cells did not proliferate and their viability was not affected by DEX treatment as determined by Trypan blue exclusion (data not shown).

Next, to evaluate DEX effects on airway epithelial barrier formation and enhancement, we assessed for any morphological changes in AJs and TJs by confocal microscopy. We evaluated the distributions of ZO-1 and E-cadherin in cells with and without DEX treatment. In sites of epithelial cell–cell adhesions, ZO-1, and E-cadherin were strongly expressed in cell monolayers after DEX

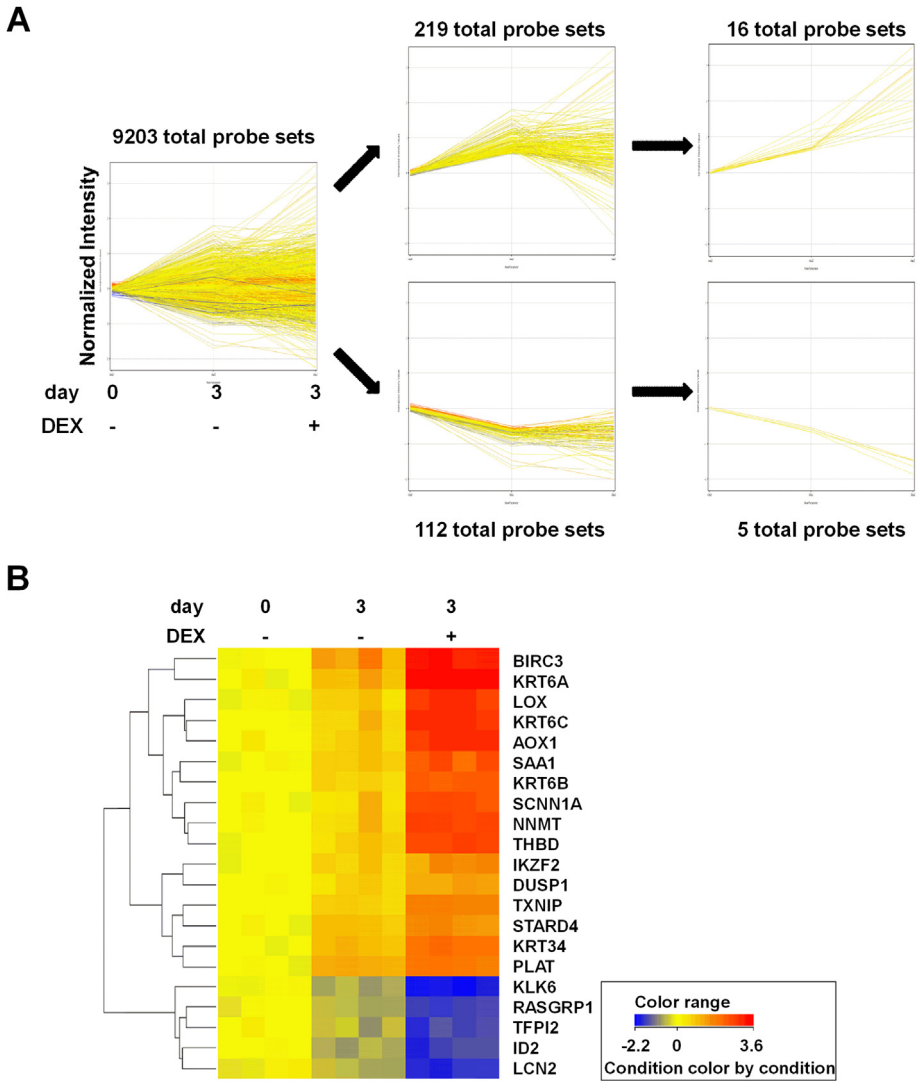

Fig. 2. Global gene expression profiles to identify steroid-inducible genes involved in enhancing airway epithelial barrier integrity. **A**. Microarray data analysis to identify steroid-iexpressions. Expression profiles were normalized to control levels (day 0 of culture without DEX). Data were first filtered by percentiles (20–100%) and CV of <50.0% (CV: coefficient of variation). Fold-change analysis was performed using a cut-off of ≥1.5, assumed as significant. A total of 16 up-regulated genes and five down-regulated genes were identified. **B**. Heat map illustrating differential gene mRNA expression patterns for cells cultured with or without DEX for 3 days and a control. Cluster plot. Probe sets that were expressed above the average are shown in red, below the average in blue, and at the average in yellow.

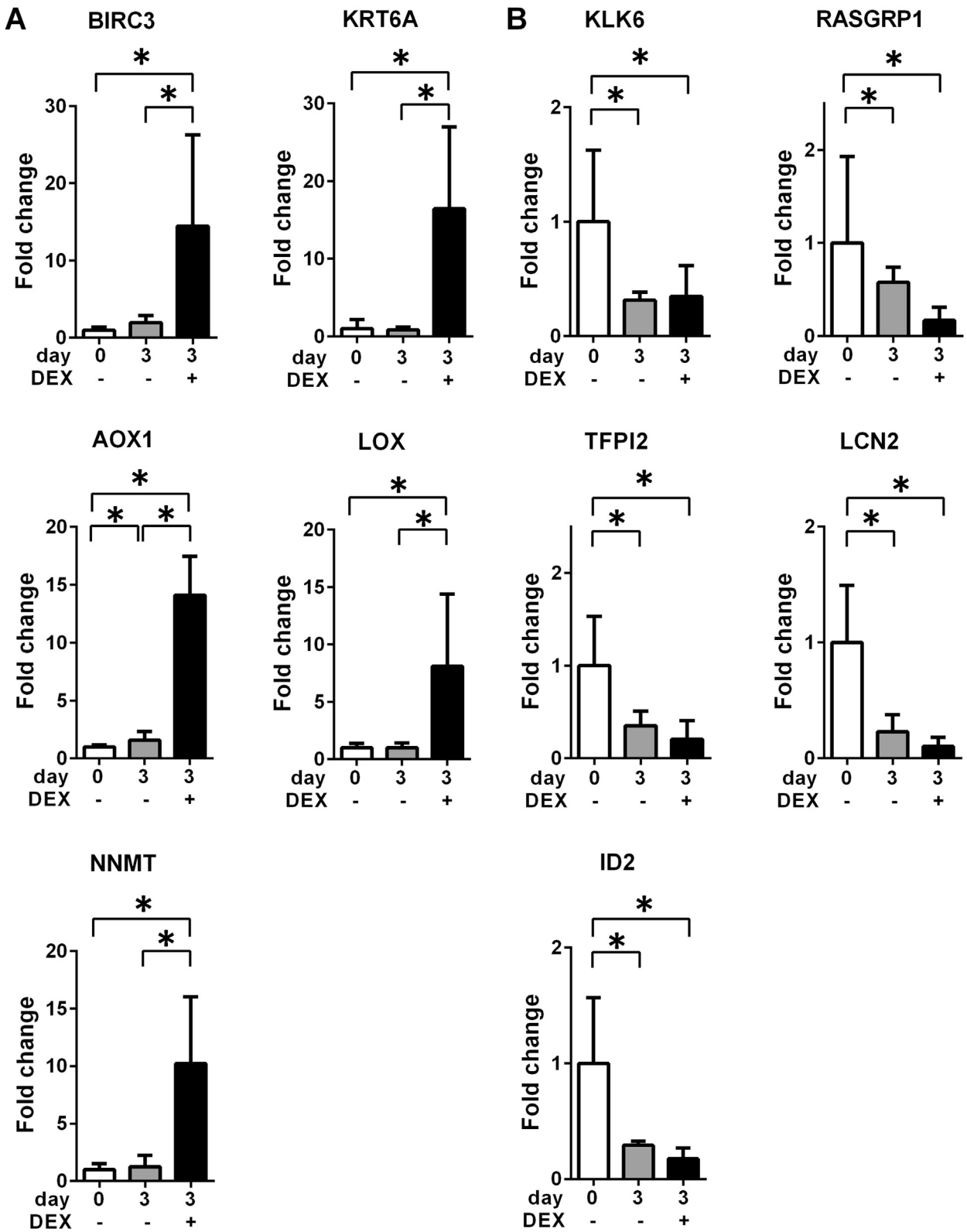

Fig. 3. Validation of expression data for steroid-inducible genes by real-time RT-PCR. **A.** Expression levels of up-regulated genes. **B.** Expression levels of down-regulated genes. Expression levels in all PCR samples were normalized against GAPDH mRNA, used as a housekeeping gene. Normalized intensity equals the fold-change relative to that of day 0 of culture without DEX.

treatment as compared with cells without DEX treatment (Fig. 1C). Thus, this confirmed that DEX treatment had enhanced airway epithelial barrier integrity.

Global gene expression analysis using DNA microarrays

Next, we assessed global gene expression profiles to identify steroid inducible genes related to the formation and enhancement of airway epithelial barrier integrity. We extracted total RNA from epithelial cells that had been cultured with or without DEX at day 0 and day 3 and used these for microarray analysis. Expression profiles were normalized to the control (day 0 of culture without DEX). We performed a fold-change analysis using a cut-off value of a 1.5-fold increase.

First, 9,203 probe sets were identified (Fig. 2A, left). These data were analyzed for significant variations between day 0 and day 3 of culture without DEX. These genes were filtered to select those genes whose expressions were either up-regulated (219 genes) or down-regulated (112 genes) (Fig. 2A, middle) and further filtered to select those genes whose expressions were either up-regulated (16 genes) or down-regulated (five genes) after DEX treatment (Fig. 2A, right). We identified 16 up-regulated genes and five down-regulated genes as steroid-inducible genes that were involved in the formation and enhancement of airway epithelial barrier integrity.

Next, we used heat map analysis to determine differential gene mRNA expression patterns for three conditions: cells cultured with and without DEX for 3 days and a control. Because the errors between samples were less in the heat map, the 21 DEX-induced up- and down-regulated genes we identified by microarray analysis might have been involved in the formation and enhancement of airway epithelial barrier integrity (Fig. 2B).

Validation of steroid-inducible genes expression

We next validated the expressions of steroid-inducible genes that were identified by microarray analysis using real-time PCR. We confirmed that the expressions of five up-regulated (Fig. 3A) and down-regulated (Fig. 3B) genes were increased and decreased in our DNA microarray analysis.

Pathway analysis

Because the 21 genes we identified had not been previously reported to be associated with airway epithelial barrier, we next performed pathway analysis using IPA software (Ingenuity Systems Inc.) to identify those pathways that were most likely involved in

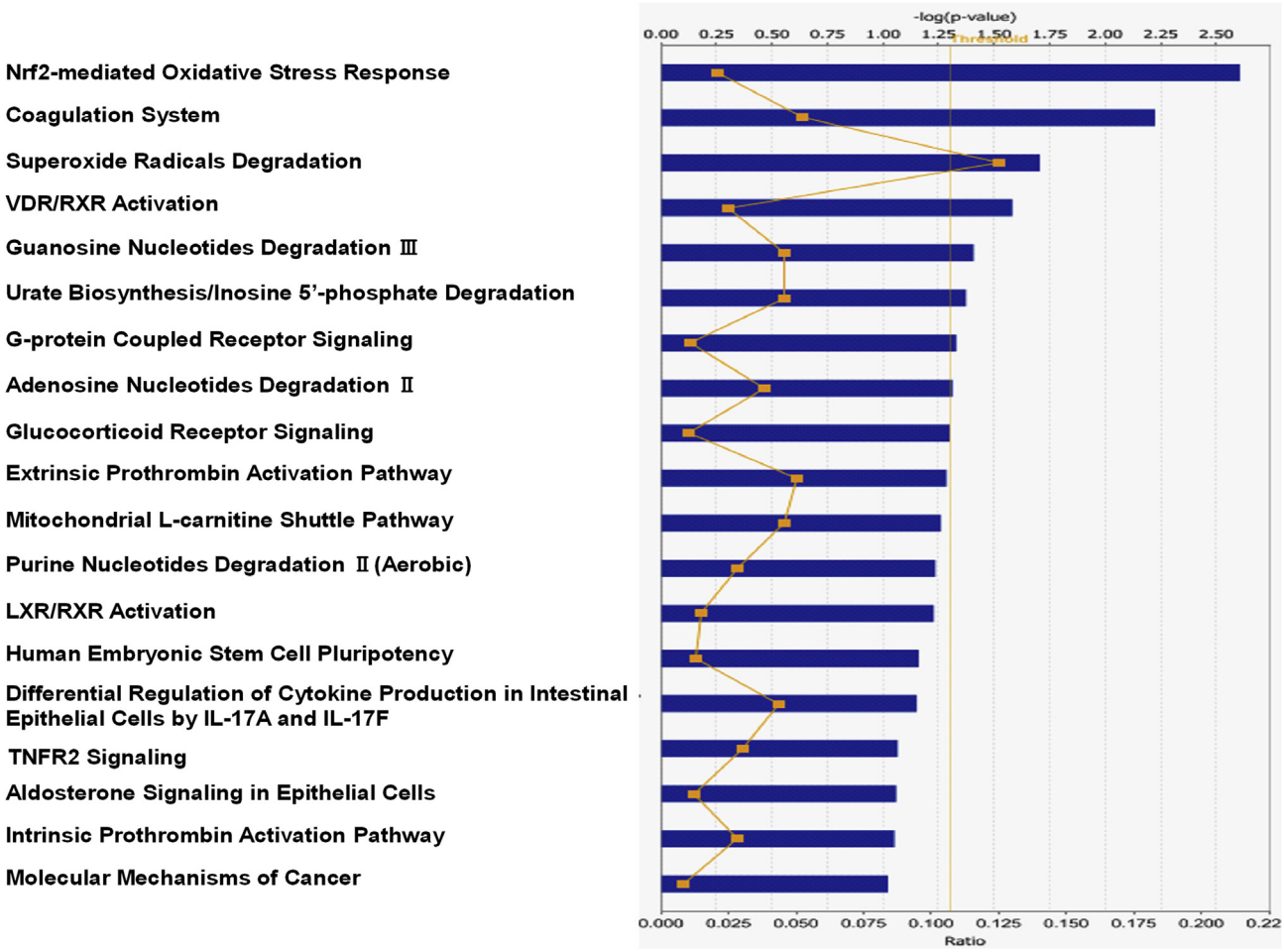

Fig. 4. Top 19 canonical pathways associated with the formation and enhancement of airway epithelial barrier function by DEX. Pathway analysis of gene sets that were differentially expressed in cells cultured for 3 days with DEX vs. cells cultured for 3 days without DEX or vs. control cells. Significance is expressed as the negative exponent of the P-value calculated for each function on the x-axis, with increasing expression indicated by bar height. Functional categories are represented on the y-axis. Threshold P-values provided information regarding how many "false positives" (i.e., functions falsely identified as significant) that we would maximally expect among the significant functions.

Table 1
Genes that are up- or down-regulated by steroid in Nrf2 pathway.

Symbol	Entrez gene name	Fold change	Location	Type(s)
AOX1	Aldehyde oxidase 1	4.713	Cytoplasm	Enzyme
EPHX1	Epoxide hydrolase 1, microsomal (xenobiotic)	−1.174	Cytoplasm	Peptidase
FTL	Ferritin, light polypeptide	−1.108	Cytoplasm	Enzyme
NQO1	NAD(P)H dehydrogenase, quinone 1	−1.091	Cytoplasm	Enzyme

the formation and enhancement of airway epithelial barrier integrity. The top canonical pathway was the nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated oxidative stress response pathway (Fig. 4). The Nrf2 pathway included 192 total genes in IPA software, of which four genes were detected in our data. The only up-regulated gene was that for aldehyde oxidase 1 (*AOX1*) (Table 1).

Nrf2 is involved in enhancing airway epithelial barrier integrity

To determine whether Nrf2 was involved in enhancing airway epithelial barrier integrity even if Nrf2 was not up-regulated by DEX treatment, we transfected Nrf2 specific siRNA into 16HBE cells and measured these cells' TER and paracellular permeability. TER was significantly reduced in Nrf2 knockdown cells as compared with cells transfected with control siRNA (Fig. 5A). FITC-dextran permeability was also significantly increased in Nrf2 knockdown cells as compared with cells transfected with control siRNA (Fig. 5B). These data suggested that Nrf2 was involved in regulating airway epithelial barrier integrity.

Next, we examined the accumulation of AJ and TJ proteins in epithelial cell–cell junctions by confocal microscopy to evaluate the role of Nrf2 for airway epithelial barrier formation and enhancement using cells that were transfected with Nrf2-specific siRNA or control siRNA and cultured with DEX. At sites of cell–cell junctions, E-cadherin and ZO-1 were strongly expressed in control cell monolayers, whereas their expressions were reduced in Nrf2 knockdown cell monolayers (Fig. 5C). To confirm that these proteins' expressions were not increased or decreased in whole cells including cytosol, we performed Western blot analysis to assess total E-cadherin and ZO-1 protein expressions. E-cadherin and ZO-1 protein expression did not change in 16HBE cells that were transfected with Nrf2 siRNA or control siRNA (Fig. 5D, E). Thus, Nrf2 knockdown had suppressed the accumulation of TJ and AJ proteins to reduce the formation and enhancement of airway epithelial barrier integrity induced by DEX treatment.

Nrf2/AOX1 pathways is involved in airway epithelial barrier integrity

Next, we explored for downstream molecules of Nrf2 that might be associated with enhanced airway epithelial barrier integrity induced by DEX treatment. Based on our pathway analysis data, only *AOX1* gene expression in the Nrf2 pathway was increased by DEX (Table 1). Up-regulated *AOX1* mRNA expression induced by DEX was significantly reduced in Nrf2 knockdown cells as compared with that in cells transfected with control siRNA (Fig. 6A), which suggested that AOX1 was a downstream molecule of Nrf2. Then, we assessed whether AOX1 actually participated in enhancing airway barrier integrity.

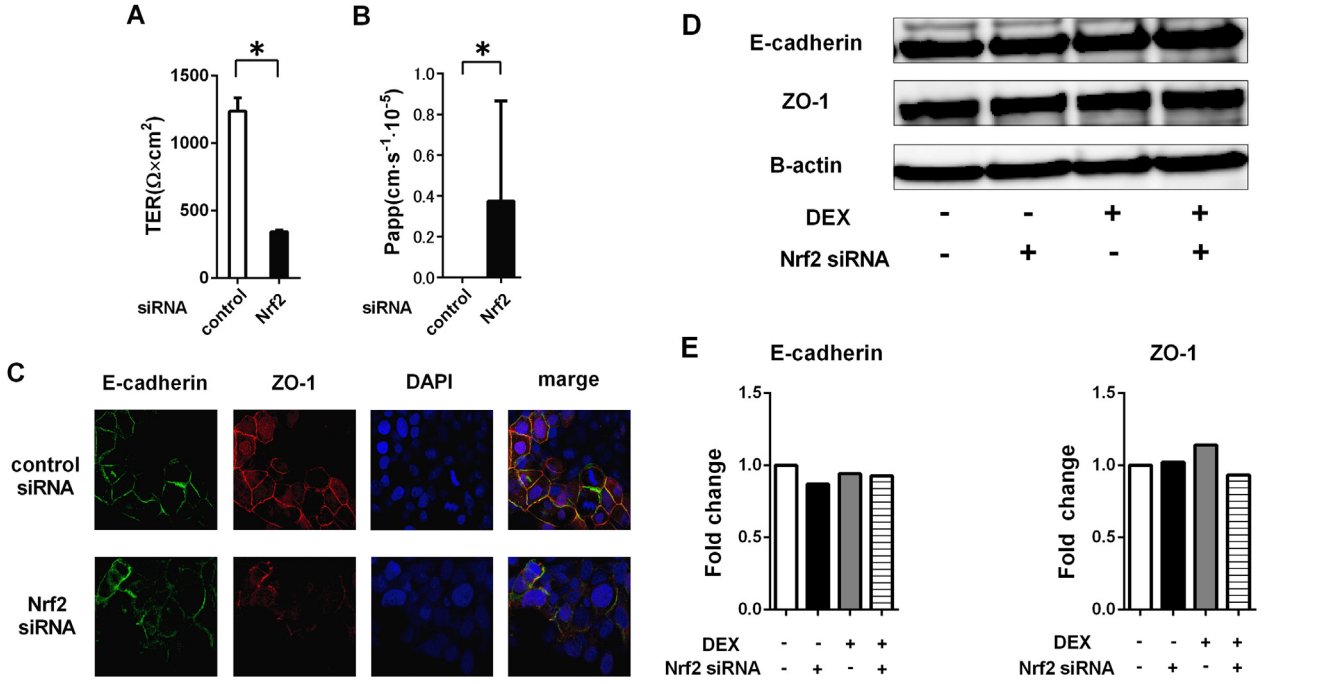

Fig. 5. Nrf2 knockdown effects on TER development and TJ organization. **A.** Nrf2 knockdown effects on TER development. 16HBE cells were transfected with control or Nrf2-specific siRNAs. After 24 h, cells were cultured with or without DEX for 2 days. TER was monitored for 2 days. Results are means ± SDs (n = 3); $^*P < 0.05$ vs. control siRNA. **B.** 16HBE cell monolayers permeability to FITC-dextran. After measuring TER, FITC-dextran (4 kDa) was added to the upper chamber of a Transwell insert and allowed to cross the cell monolayer for 1 h. Fluorescence intensity in the lower chamber was measured and compared to the amount of FITC-dextran fluorescent signals that crossed the cells cultured with or without DEX and transfected with control or Nrf2-specific siRNAs. Results are means ± SDs (n = 3); $^*P < 0.05$ vs. control siRNA. **C.** Immunocytochemistry analysis of E-cadherin and ZO-1 expression in Nrf2 knockdown cells. 16HBE cells were subjected to immunocytochemistry with antibodies specific for E-cadherin (top) and ZO-1 (bottom). **D.** Western blotting analysis for E-cadherin and ZO-1 proteins in 16HBE cells after treatment with Nrf2 siRNA. **E.** Densitometric analysis of E-cadherin and ZO-1 is represented as fold change, normalized by β-actin. Western blots were prepared for three independent experiments. Results from one typical experiment are shown.

Cells that were transfected with *AOX1*-specific siRNA were cultured with DEX, after which we measured TER and evaluated paracellular permeability using FITC-dextran. TER was significantly reduced in *AOX1* knockdown cells as compared to cells that were transfected with control siRNA (Fig. 6B). FITC-dextran permeability was also significantly increased in *AOX1* knockdown cells as compared with cells transfected with control siRNA (Fig. 6C). Together, these data suggested that the Nrf2/AOX1 pathway was involved in the formation and enhancement of airway epithelial barrier integrity.

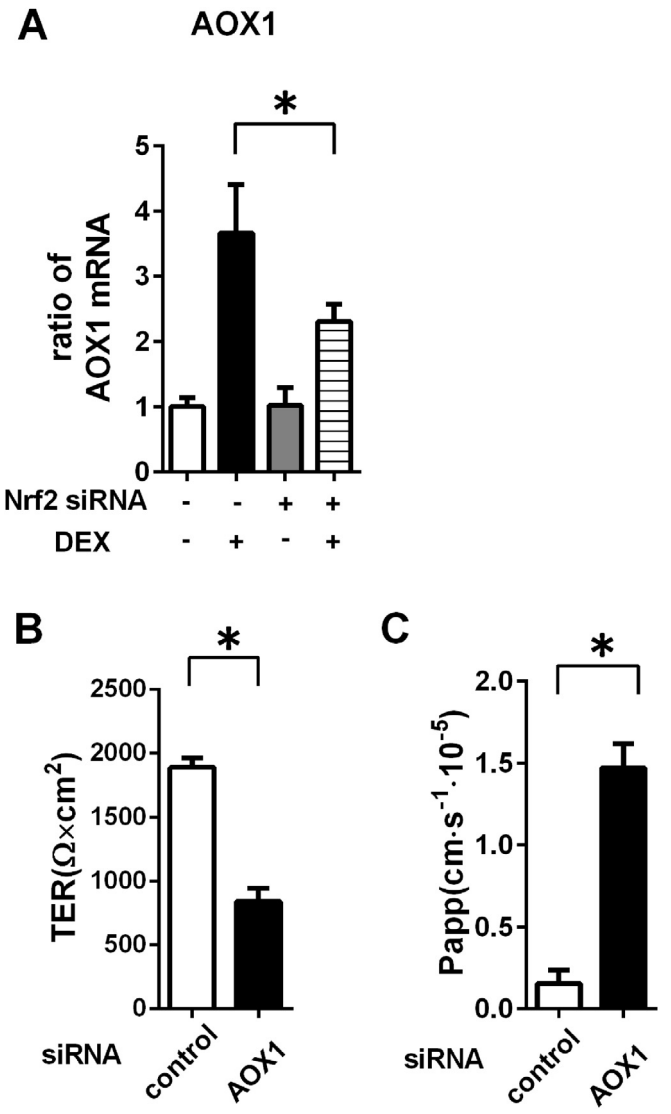

Fig. 6. Effects of suppressing the expression of oxidative stress-responsive genes by Nrf2 gene silencing. **A**. 16HBE cells were transfected with control or Nrf2-specific siRNAs. After 72 h, RNA was extracted. The differences in *AOX1* gene expression levels were compared for cells with or without DEX treatment using real-time PCR. mRNA expression levels in all PCR samples were normalized against GAPDH mRNA, used as a housekeeping gene. Normalized intensity equals the fold-change relative to cells transfected with control siRNA and without DEX treatment. Results are means ± SDs (n = 3); $^*P < 0.05$ vs. control siRNA. **B**. AOX1 knockdown effects on TER development. 16HBE cells were transfected with control or *AOX1*-specific siRNAs. After 24 h, cells were cultured with or without DEX for 2 days. TER was monitored for 2 days. Results are means ± SDs (n = 3); $^*P < 0.05$ vs. control siRNA. **C**. AOX1 knockdown effects on paracellular permeability of 16HBE cell monolayers to FITC-dextran. After measuring TER, FITC-dextran (4 kDa) was added to the upper chamber of a Transwell insert and allowed to cross a cell monolayer for 1 h. Fluorescence intensity in the lower chamber was measured and compared to the amount of FITC-dextran fluorescent signals that crossed the cells cultured with or without DEX and transfected with control or *AOX1*-specific siRNAs. Results are means ± SDs (n = 3); $^*P < 0.05$ vs. control siRNA.

Discussion

It was recently proposed that the susceptibility to reduced airway epithelial barrier integrity may be involved in the pathogenesis and development of bronchial asthma.[21] We previously reported that the promoting effect of phosphorylation to EGFRs by DEX was involved in enhancing airway epithelial barrier integrity.[8] However, the mechanism by which DEX enhances airway epithelial barrier integrity is not well understood.

In this study, we performed a global gene expression analysis to determine the mechanism by which DEX could enhance airway epithelial barrier integrity and to identify steroid-inducible genes that were involved in enhancing airway epithelial barrier integrity. Based on this analysis, we identified 16 genes as steroid-inducible genes. However, there is no report that the top five up-regulated genes, including *BIRC3*, *KRT6A*, *AOX1*, *LOX*, and *NNMT*, alone are involved in the airway epithelial barrier integrity. Thus, we performed a pathway analysis to identify the signaling pathways that were upstream or downstream of these genes with altered DEX-induced expression. Based on our pathway analysis, Nrf2-mediated oxidative stress response was the most important canonical pathway involved in enhancing airway epithelial barrier integrity by steroid treatment. We confirmed that Nrf2 mRNA expression after DEX treatment was not increased using real-time PCR (data not shown).

Nrf2 is a transcription factor that has an antioxidant effect and has a basic leucine zipper (b-Zip) structure.[22] Nrf2 binds to Kelch-like ECH-associated protein1 (Keap1) under non-oxidative stress conditions. Nrf2 is inhibited its activation when it is degraded by the proteasome.[22] When the cells are exposed to oxidative stress, Nrf2 binding site of Keap1 is saturated and the newly synthesized Nrf2 migrates into the nucleus.[23] Then, Nrf2 binds to an electrophile/antioxidant responsive element (EpRE/ARE) that is in the expression control region of a target gene, Nrf2 can enhance the expression of antioxidant genes.[24] Our data consistent with this mechanism that Nrf2 pathway may be activated by DEX without increasing gene expression of Nrf2. Reactive oxygen species levels that are increased by various environmental factors induce oxidative stress, which has been shown to one component in the pathogenesis of asthma.[25] It has been shown that human immunodeficiency virus (HIV)-1-related proteins inhibit Nrf2-mediated antioxidant defenses and thereby disrupt the normally tight alveolar epithelial barrier.[26] We hypothesized that a steroid-activated Nrf2 would regulate the expression of antioxidant molecules and, subsequently, epithelial barrier integrity. Unexpectedly, the gene expressions for antioxidant molecules, such as UGT1a6 and NQO1, were decreased in cells that were cultured with DEX based on our microarray analysis data (data not shown). This suggested that steroid-enhanced airway epithelial barrier integrity might be regulated by an antioxidant effect independent pathway. Because Nrf2 knockdown attenuated airway epithelial barrier integrity, Nrf2 might still be involved in airway epithelial barrier integrity even if Nrf2 expression was not up-regulated by DEX.

Next, we focused on the Nrf2 pathway to determine the role of Nrf2 for airway epithelial barrier integrity. In our pathway analysis, four genes were detected in the Nrf2 pathway. Only *AOX1* gene expression was increased during formation and enhancement of airway epithelial barrier integrity. AOX1 is a molybdenum flavin enzyme, which is particularly strongly expressed in the liver and catalyzes the oxidation of various aldehyde compounds with broad substrate specificity.[27] AOX1 is important as a xenobiotic

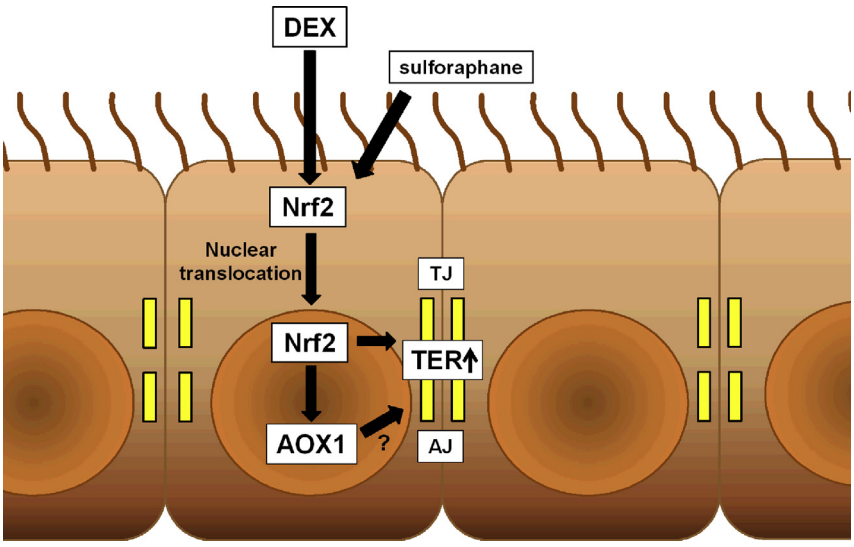

Fig. 7. Schematic diagram showing the relationship between the Nrf2 pathway and airway epithelial barrier integrity. Scheme for a possible connection with Nrf2 and molecules involved in airway epithelial barrier integrity.

metabolizing enzyme that affects the metabolism of anticancer drugs, immunosuppressive agents, and antiviral agents.[28]

The *AOX1* gene expression level after steroid treatment was significantly reduced in those cells that were transfected with Nrf2 siRNA as compared with control cells, which suggested that AOX1 was one of the downstream molecules regulated by Nrf2. We measured TER and membrane permeability for cells that were transfected with *AOX1*-specific siRNA to determine whether AOX1 was involved in the formation and enhancement of airway epithelial barrier integrity after DEX treatment. This showed that AOX1 was involved in the formation and enhancement of airway epithelial barrier function by steroids in the downstream pathway of Nrf2.

Nrf2 enhances the expression of target genes after it translocates into the cell nucleus and binds to an ARE. Maeda et al. reported that an ARE was in the 5′ upstream region of the *AOX1* gene and that *AOX1* gene expression was enhanced when Nrf2 bound to this ARE.[29] It is possible that Nrf2 binding to this ARE in the *AOX1* gene is involved in the formation and enhancement of airway epithelial barrier function. Further studies will be needed to elucidate the mechanism underlying the formation and enhancement of airway epithelial barrier integrity via the Nrf2/AOX1 pathway.

Sulforaphane is a naturally occurring chemical that is found in plants, particularly in large amounts in broccoli and sprouts,[30] and exhibits antioxidant activity by activating Nrf2.[28] Sulforaphane suppressed T_H2 responses when it was administered intraperitoneally along with ovalbumin in an animal model of asthma.[31] Sulforaphane, an Nrf2 activator, has been reported to enhance the epithelial barrier *in vitro*.[32] Indeed, we confirmed that sulforaphane enhanced airway epithelial barrier integrity in our model system (data not shown). Nrf2 activators, such as sulforaphane, might have potential for suppressing asthma pathogenesis by activating the Nrf2 pathway and enhancing barrier integrity.

In conclusion, the Nrf2/AOX1 pathway that we identified in airway epithelial cells might be a new therapeutic target for asthma patients by enhancing airway epithelial barrier integrity (Fig. 7). Clinically, the use of an ICS has dramatically improved the long-term control of bronchial asthma[4]; however, there are patients with severe asthma who are steroid-resistant.[6] It is possible that Nrf2 activators might be a new treatment strategy for asthma by enhancing airway epithelial barrier integrity in place of an ICS.

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Conflicts of interest

The authors have no conflict of interest to declare.

Authors' contributions

YS, SM, YG, and SH conducted majority of the experiments and designed experiments. YS and SM wrote the manuscript. DK, AY, YK, IT, and ET performed experiments based on barrier integrity, western blotting, and siRNA transfection. KK and ET performed confocal microscopy. KS performed microarray analysis. All authors discussed the results and commented on the manuscript.

References

1. Bousquet J, Chanez P, Lacoste JY, Barnéon G, Ghavanian N, Enander I, et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990;**323**:1033–9.
2. Ohta K, Ichinose M, Nagase H, Yamaguchi M, Sugiura H, Tohda Y, et al. Japanese guideline for adult asthma 2014. *Allergol Int* 2014;**63**:293–333.
3. Holgate ST. Innate and adaptive immune responses in asthma. *Nat Med* 2012;**18**:673–83.
4. Barnes PJ. Efficacy of inhaled corticosteroids in asthma. *J Allergy Clin Immunol* 1998;**102**:531–8.
5. Suissa S, Ernst P, Benayoun S, Baltzan M, Cai B. Low-dose inhaled corticosteroids and the prevention of death from asthma. *N Engl J Med* 2000;**343**:332–6.
6. Trevor JL, Deshane JS. Refractory asthma: mechanisms, targets, and therapy. *Allergy* 2014;**69**:817–27.
7. Holgate ST. Epithelium dysfunction in asthma. *J Allergy Clin Immunol* 2007;**120**:1233–44.
8. Sekiyama A, Gon Y, Terakado M, Takeshita I, Kozu Y, Maruoka S, et al. Glucocorticoids enhance airway epithelial barrier integrity. *Int Immunopharmacol* 2012;**12**:350–7.
9. Terakado M, Gon Y, Sekiyama A, Takeshita I, Kozu Y, Matsumoto K, et al. The Rac1/JNK pathway is critical for EGFR-dependent barrier formation in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2011;**300**:L56–63.
10. Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST, et al. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB J* 2000;**14**:1362–74.

11. Georas SN, Rezaee F. Epithelial barrier function: at the front line of asthma immunology and allergic airway inflammation. *J Allergy Clin Immunol* 2014;**134**:509–20.
12. Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J Cell Biol* 1986;**103**:755–66.
13. Claude P. Morphological factors influencing transepithelial permeability: a model for the resistance of the zonula occludens. *J Membr Bio* 1978;**39**:219–32.
14. Tsukita S, Yamazaki Y, Katsuno T, Tamura A, Tsukita S. Tight junction-based epithelial microenvironment and cell proliferation. *Oncogene* 2008;**27**:6930–8.
15. Forbes B, Ehrhardt C. Human respiratory epithelial cell culture for drug delivery applications. *Eur J Pharm Biopharm* 2005;**60**:193–205.
16. Geys J, Nemery B, Hoet PH. Optimisation of culture conditions to develop an in vitro pulmonary permeability model. *Toxicol In Vitro* 2007;**21**:1215–9.
17. Grainger CI, Greenwell LL, Lockley DJ, Martin GP, Forbes B. Culture of Calu-3 cells at the air interface provides a representative model of the airway epithelial barrier. *Pharm Res* 2006;**23**:1482–90.
18. Ferrell N, Desai RR, Fleischman AJ, Roy S, Humes HD, Fissell WH. A microfluidic bioreactor with integrated transepithelial electrical resistance (TEER) measurement electrodes for evaluation of renal epithelial cells. *Biotechnol Bioeng* 2010;**107**:707–16.
19. Artursson P, Lindmark T, Davis SS, Illum L. Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). *Pharm Res* 1994;**11**:1358–61.
20. McGarvey LP, Butler CA, Stokesberry S, Polley L, McQuaid S, Abdullah H, et al. Increased expression of bronchial epithelial transient receptor potential vanilloid 1 channels in patients with severe asthma. *J Allergy Clin Immunol* 2014;**133**:704–12.
21. Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, Puxeddu I, et al. Defective epithelial barrier function in asthma. *J Allergy Clin Immunol* 2011;**128**:549–56.
22. Motohashi H, Yamamoto M. Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol Med* 2004;**10**:549–57.
23. Itoh K, Wakabayashi N, Katoh Y. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev* 1999;**13**:76–86.
24. Ganan-Gomez I, Wei Y, Yang H, Boyano-Adanez MC, Garcia-Manero G. Oncogenic functions of the transcription factor Nrf2. *Free Radic Biol Med* 2013;**65**:750–64.
25. Andreadis AA, Hazen SL, Comhair SA, Erzurum SC. Oxidative and nitrosative events in asthma. *Free Radic Biol Med* 2003;**35**:213–25.
26. Fan X, Staitieh BS, Jensen JS, Mould KJ, Greenberg JA, Joshi PC, et al. Activating the Nrf2-mediated antioxidant response element restores barrier function in the alveolar epithelium of HIV-1 transgenic rats. *Am J Physiol Lung Cell Mol Physiol* 2013;**305**:L267–77.
27. Kitamura S, Sugihara K, Ohta S. Drug-metabolizing ability of molybdenum hydroxylases. *Drug Metab Pharmacokinet* 2006;**21**:83–98.
28. Garattini E, Fratelli M, Terao M. Mammalian aldehyde oxidases: genetics, evolution and biochemistry. *Cell Mol Life Sci* 2008;**65**:1019–48.
29. Maeda K, Ohno T, Igarashi S, Yoshimura T, Yamashiro K, Sakai M. Aldehyde oxidase 1 gene is regulated by Nrf2 pathway. *Gene* 2012;**505**:374–8.
30. Fahey JW, Zhang Y, Talalay P. Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proc Natl Acad Sci U S A* 1997;**94**:10367–72.
31. Fahey JW, Talalay P. Antioxidant functions of sulforaphane: a potent inducer of phase II detoxication enzymes. *Food Chem Toxico* 1999;**37**:973–9.
32. Park JH, Kim JW, Lee CM, Kim YD, Chung SW, Jung ID, et al. Sulforaphane inhibits the Th2 immune response in ovalbumin-induced asthma. *BMB Rep* 2012;**45**:311–6.