Dach1 attenuates airway inflammation in chronic obstructive pulmonary disease by activating Nrf2 signaling

Qian Huang
Huazhong University of Science and Technology

Yiya Gu
Huazhong University of Science and Technology

Jixing Wu
Huazhong University of Science and Technology

Yuan Zhan
Huazhong University of Science and Technology

Zhesong Deng
Huazhong University of Science and Technology

Shanshan Chen
Huazhong University of Science and Technology

Maocuo Peng
Huazhong University of Science and Technology

Ruonan Yang
Huazhong University of Science and Technology

Jinkun Chen
Western University

Jungang Xie (✉ xiejg@hotmail.com)
Huazhong University of Science and Technology

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Abstract

Background: Chronic obstructive pulmonary disease (COPD) is a small airway chronic inflammatory disease with impaired lung function primarily induced by cigarette smoke (CS). Reduced Dach1 expression has a vicious role in numerous disorders, but its role in COPD is rarely known. This study aims to elucidate the role and underlying mechanism of Dach1 in airway inflammation of COPD.

Methods: Dach1 expression in lung tissues of COPD patients has been calculated. Small airway epithelium-specific Dach1 knockdown mice and AAV-transfected Dach1 overexpressed mice were used to explore its role and potential for therapeutic targeting in experimental COPD induced by CS. Furtherly, we uncovered the promising mechanism of Dach1 in inflammation induced by cigarette smoke extract simulation (CSE) in vitro.

Results: The expression of Dach1 decreased in COPD patients compared to non-smokers and smoker without COPD, especially in small airway epithelium. Small airway epithelium-specific Dach1 knockdown aggravated mice airway inflammation and lung function decline caused by CS, while Dach1 overexpression protected mice from airway inflammation and lung function decline. In 16 HBE cells, Dach1 knockdown and overexpression promoted and inhibited the secretion of IL-6 and IL-8 after simulation of CSE, respectively. Nuclear factor erythroid 2-related factor 2 (Nrf2) was identified as novel downstream target of Dach1, which directly binds to its promoter. Induction of Dach1 alleviated inflammation by activating Nrf2 signaling.

Conclusions: Dach1 is decreased in COPD patients. Dach1 has protective effects against inflammation induced by CS by activating Nrf2 signaling pathway. Targeting Dach1 is a potential therapeutic strategy for COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is a small airway chronic inflammatory disease with persistent symptoms and fixed airflow obstruction[1]. With high morbidity and mortality, it has caused a heavy burden on global social economic[2, 3]. There were many risk factors for COPD and cigarette smoke (CS) exposure remains the leading one[4]. Airway inflammation induced by CS is a fundamental pathological change in COPD, which is related to disease progression and mortality[5, 6]. CS exposure activates epithelium to release inflammatory mediators such as IL-6 and IL-8 IL-1β and tumor necrosis factor (TNF)[7], and recruits inflammatory cells including neutrophils, macrophages and lymphocytes[8]. At present, the clinical tests of anti-inflammation therapy in COPD don’t display a desirable affect[7]. Identifying new mechanism of airway inflammation to provide novel insight into therapy targeting of COPD is impending.

Dachshund homolog 1 (Dach1), as a key component of the retinal determination gene network (RDGN), can govern cell fate during development[9, 10]. As a transcription factor or cofactor, Dach1 can regulate genes expression and engage in human diseases. Previous studies reported Dach1 as a new tumor
suppressor in many cancer diseases such as breast, liver and lung cancer\cite{10-14}. Recent studies also showed a protective role of Dach1 on chronic diseases including kidney, pulmonary and cardiovascular disease\cite{9, 15-17}. Dach1 can regulate the expression of inflammatory cytokines\cite{18}. It repressed the secretion of cytokines IL-6 and IL-8 by prostate epithelial cells\cite{19}. It also reduced IL-8 secretion of breast cancer and lung cancer cells. Anyhow, the role of Dach1 on COPD is obscure. We hold the hypothesis that Dach1 may regulate airway inflammation in COPD.

We first calculated the Dach1 expression in COPD patients and experimental COPD model. To further elucidate the role of Dach1 in COPD, we constructed mice with lung Dach1 overexpression and small airway epithelium-special Dach1 deficient. All mice were then exposed to whole body CS exposure, lung function and airway inflammation were assessed after six-month exposure. Moreover, the potential mechanism of Dach1 on inflammation was dissected in vitro.

**Methods**

**Subjects**

Normal lung tissue specimens of 13 non-smokers, 11 smokers and 22 COPD patients were collected from Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China (Fundamental Characteristics of study subjects showed in Table S1 (see Additional file 1). In our study, COPD was diagnosed according to the global initiative for chronic obstructive pulmonary disease (GOLD). Subjects with respiratory infection or other chronic pulmonary diseases (asthmas, bronchiectasis and interstitial lung diseases (ILDs)), having systemic steroid use within the previous 4 weeks, or who had a history of other cancers were excluded. All samples collected were stored in a freezer at a temperature of -80°C until use.

We obtained approval from the ethics committee of Tongji Hospital, Huazhong University of Science and Technology, Wuhan, Hubei, China (TJ-IRB20210346). Each subject signed an informed consent form before the collection of samples.

**A Mouse Model Of Cigarette Smoke Exposure**

Dach1\textsuperscript{ox/ox} mice were generated via CRISPR–Cas9 system by Nanjing Institute of Biomedicine of Nanjing University (Nanjing, China). Two loxP sites were inserted in upstream and downstream of exon 2 of Dach1, respectively. To generate club cells-specific knockdown Dach1 mice (Scgb1a1-cre\textsuperscript{+}-Dach1\textsuperscript{ox/ox}, hereinafter referred to as Dach1-CKO), the Scgb1a1-Cre transgenic mice were purchased from the Jackson laboratory (Bar Harbor, ME, USA) and then crossed with Dach1\textsuperscript{ox/ox} mice. The littermates (Scgb1a1-Cre-Dach1\textsuperscript{ox/ox}, hereinafter referred to as Dach1-C) of Dach1-CKO were used as controls. Dach1-CKO and Dach1-C mice (6 to 8 weeks) were administered tamoxifen (65 mg/kg, i.p.) for seven days to induce the expression of cre recombinase and the knockdown of Dach1. 14 days after the
last day of tamoxifen injection, Dach1-CKO and Dach1-C mice were exposed to cigarette smoke (CS) or air (exposure twice a day, each exposure for 2 hours) for six months. All mice were housed in a specific pathogen-free (SPF) facility at the Tongji Medical College with a 12-hour light/12-hour dark cycle. All experimental procedures were approved by Huazhong University Animal Experiment Ethics Committee and were conducted in accordance with the animal experimentation guidelines of Huazhong University.

To test the effect of Dach1 on experimental COPD model, mice were intratracheally administered with Dach1-expressing AAV2/9 viral genome particle (total amount of titer \(2 \times 10^{11}\) vg per mouse) 21 days before CS or porcine pancreatic elastase (PPE) exposure.

**A Mouse Model Of Emphysema Induced By Porcine Pancreatic Elastase**

Porcine pancreatic elastase (PPE) was purchased from Sigma (E1250) and then dissolved in phosphate buffer solution (PBS). 2U PPE or PBS were intratracheally injected to AAV-Con or AAV-Dach1 mice. 42 days after injection, mice were tested for lung function and then sacrificed to acquire bronchoalveolar lavage fluid (BALF) and lung tissue.

**Pulmonary Function Test**

Briefly, COPD model mice were first anesthetized with 1% pentobarbital sodium 10 ml/kg body weight intraperitoneally and then were implemented with endotracheal intubation. Subsequently, mice pulmonary function was measured using the FlexiVent system (SCIREQ, Montreal, Quebec, Canada). Lung function parameters \(\text{FEV0.1s/FVC}\) was calculated.

**Reagents And Antibodies**

Cigarette smoke extraction (CSE) were prepared as previously described[20]. Antibodies against β-actin (Anti-β-Actin, 66009-1-Ig), Dach1 (Anti-Dach1, 60082-1-Ig), scgb1a1 (Anti-Scgb1a1, 10490-1-AP), Nrf2 (Anti-Nrf2, 16396-1-ap) were purchased from Proteintech. HO-1 (Anti-HO-1, GB11845) were purchased from Servicebio. Antibodies against Dach1 (Anti-Dach1, ab226176) for immunoprecipitation assay was purchased from Abcam. Antibodies against IgG (Anti-IgG, #2729) for immunoprecipitation assay was purchased from Cell Signaling Technology (CST).

**Cells Culture And Stimulation**

Human airway epithelial cell line (16HBE) was purchased and cultured as previously described[20]. The cells were incubated at 37°C with 5% CO₂. For experimentation, 16HBE were grown in 12-well plates until
70–80% confluence and then exposed to Cigarette smoke extraction (CSE) with different concentrations and stimulation times.

The primary small airway epithelium (SAE, 10th-12th generation bronchi) was collected by fiberoptic bronchoscopy from one healthy nonsmoker (male, 50 years old). Freshly ten brushed cells were washed with cell culture medium (DMEM, Lonza, Walkersville, MD) with 10% fetal bovine serum (FBS, Gibco) and Penicillin-streptomycin (1:100). The collected cells were then centrifuged at 1000 RPM for 5 minutes and suspended with bronchial epithelial basal medium BEBM (Lonza, Walkersville, MD). Suspended cells (Passage zero) were then inoculated into cell culture dishes and fluid was changed every two days. Cell passage was performed when the degree of cell fusion reached 90 to 100%.

**Histopathology, Immunohistochemical And Immunofluorescence Analysis**

Human lung and mice left lung tissues were collected and placed in fresh 4% neutral-buffered paraformaldehyde for 24 hours at room temperature, then embedded in paraffin and subjected to the histological analysis as previously reported. Hematoxylin-eosin (H&E) staining were performed on mice lung sections. Immunofluorescence co-staining of scgb1a1 (Anti-Scgb1a1, 1:400) and Dach1(Anti-Dach1, 1:200) were performed on human and mice lung sections.

**Airway And Vascular Inflammation Score**

Airway and vascular inflammation score were done following previous study[21].

**Lentiviral Transfection**

16HBE cells transfected with 60 MOI Lv-con or Lv-Dach1 for 48 hours were treated with 4ug/ml Puromycin to produce cells stably overexpressed Dach1. 4ug/ml polybrene was used to promote transfection efficiency.

**Quantitative Rt-pcr Analysis**

Quantitative RT-PCR analysis

Human lung tissues RNA was extracted by Trizol reagent method (Invitrogen). Total RNA was used for first-strand cDNA synthesis with M-MLV reverse transcriptase (Promega, Madison, WI). qRT-PCR was performed utilizing SYBR Green Master Mix (Takara, Otsu, Shiga, Japan) on the iCycler iQ system (Bio-Rad). PCR conditions included initial denaturation at 95°C for 5 minutes, 95°C for 45 seconds, and 60°C for 1 minute for 45 cycles. Gene expression levels were normalized to β-Actin. The primers for Dach1 showed in Table S2 (see Additional file 1).
ELISA

Cells culture supernatant and bronchoalveolar lavage fluid (BALF) of mice were collected after centrifugation at 1000 RPM for 5 minutes. ELISA kits for human IL8 (DY208), human IL6 (DY206), mouse CXCL1 (DY453) and mouse IL6 (DY406) were purchased from R&D systems.

Western Blotting

Total protein from mice right lung tissue or 16HBE cells homogenate was extracted by RIPA lysis buffer containing a protease inhibitor cocktail and phosphatase inhibitors (Roche, Mannheim, Germany). The proteins were separated by 12% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Germany). The membranes were blocked for 1–2 hours in 5% milk melted in Tris-buffered saline containing 0.05% Tween 20 (TBST) and then incubated with the primary antibody (Anti-β-Actin, 1:4000; Anti-Dach1, 1:2000; Anti-Nrf2, 1:2000; Anti-HO-1, 1:2000)

Reactive Oxygen Species (Ros) Detection

Reactive oxygen species (ROS) detection was performed by ROS assay kit (Servicebio, G1706-100T). 16HBE cells transfected with Lv-con or Lv-Dach1 were seeded in 12-well plates. After treatment of CSE for 12 hours, cells were washed twice with phosphate buffer and then incubated with DCFH-DA probe (diluted in 1:1000 with 1640 medium) at 37°C for 30 minutes. Thereafter, after twice washing with phosphate buffer, the fluorescence intensity of the cell was directly observed with a fluorescence microscope or a cell flow cytometer.

Immunoprecipitation Assay (Ip)

Protein G magnetic beads were purchased from Cell Signaling Technology (CST, #70024s). IP experiments were performed according to the recommended steps on the official website. First, 16HBE cells were seeded in 10-centimeter cell culture dishes until 70–80% confluence and then exposed to 10% CSE for 24 hours. Then, washed cells were harvested and lysed in NP-40 lysis buffer containing a protease inhibitor cocktail and phosphatase inhibitors (Roche, Mannheim, Germany). After centrifugation, the supernatants were collected and pre-cleared with 20ul protein G magnetic beads for 2 hours at 4°C. pre-cleared supernatants then were incubated overnight with rotation at 4°C with Anti-Dach1 (ab226176) or Anti-IgG (#2729) to generate immunocomplex. The next day, immunocomplex solutions and 20ul pre-cleared protein G magnetic beads were incubated with rotation at 4°C with for 3–3 hours. Finally, immunocomplex were eluted from protein G magnetic beads and further analyzed by western blotting analysis.

Chromatin Immunoprecipitation (Chip) Assay
ChIP assays were conducted using the ChIP Assay Kit (CST, #6003). Briefly, $1 \times 10^7$ 16HBE cells transfected with Lv-con or Lv-dach1 were cross-linked with 1% formaldehyde (Sigma-F8775), and chromatin fragmentation was carried out according to the manufacturer's protocol provided. Then, 10ug prepared chromatin fragmentation solution was incubated with an Anti-Flag (1:50), normal rabbit immunoglobulin G (IgG) or antibody against histone H3 (Anti-H3) overnight at 4°C with rotation. The DNA pulled down were subsequently used to performed CHIP-PCR. The PCR-amplified products were simultaneously identified by a 3% agarose gel. Primers to detect DNA enrichment are listed in Table S3 (see Additional file 1). Percent (%) input was analyzed by following standard formula.

**Sirna Transfection**

16HBE cells cultured in 1640 supplemented with 10% FBS were transfected with a Nrf2 siRNA or Scrambled siRNA (Ribobio, Guangzhou, China) using the Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA). 48 hours after tansfection, the transfected cells were exposed to 10%CSE for 24 hours.

**Statistical analysis**

Data from n independent experiment were presented as means ± SEM. Normality analysis was performed via the Shapiro-Wilk test. Differences were evaluated using unpaired Student's t test between two groups before any testing. One-way ANOVA was performed followed by Bonferroni post hoc test for comparisons between > 2 groups. The non-normal distributed data were analyzed using non-parametric testing (Mann-Whitney U test for two groups and Krushal-Wallis H test for > 2 groups). P values less than 0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism 8.0.1 (GraphPad Software Inc., San Diego, CA).

**Results**

**Decreasing expression of Dach1 in COPD**

A decrease of mRNA expression of pulmonary Dach1 in chronic obstructive pulmonary disease (COPD) patients comparing with non-smokers was found in an existing dataset (GSE76925) (Fig. 1A). To further evaluate the expression of Dach1, mRNA of 13 non-smokers,11 smokers and 22 COPD patients have been collected. Comparing with non-smokers, decreasing expression of Dach1 in smokers and COPD patients has been detected (Fig. 1B). Notably, the mRNA expression of Dach1 have a positive correlation with lung function (FEV1% predicted) (Fig. 1C). Immunofluorescence staining showed that Dach1 protein expressed in many cells of lung tissues, and downregulated in small airway epithelium of COPD patients (Fig. 1D). Furthermore, exposed primary small airway epithelium of non-smoker to cigarette smoke extract (CSE) also reduced Dach1 expression (Fig. 1E).
Dach1 Expression Reduced By Cigarette Smoke Exposure

To dissect the influence of cigarette smoke (CS) on Dach1 expression in vivo and vitro, we exposed mice and 16 HBE cells to CS. After whole body exposure of CS, the expression of Dach1 in mice lung have been reduced, especially in small airway epithelium (Fig. 2A-B). In vitro, exposing 16 HBE cells to 10% CSE also downregulated Dach1 expression with a time dependence (Fig. 2C-D).

Generation of mice with small airway epithelium-special deficiency of Dach1 and mice with lung overexpression of Dach1

To elucidate the effect of Dach1 on COPD, we explored the function of Dach1 in animal COPD models. We first established mice with small airway epithelium-special deficiency of Dach1 using the CRISPR-Cas9 system (Fig. 3A-B). Two weeks after seven days injected of tamoxifen, a significant reduction of Dach1 in small airway epithelium has been calculated in Dach1-CKO mice comparing with Dach1-C (Fig. 3C-D). The flox allele form Dach1\textsuperscript{flox/flox}, WT and Dach1\textsuperscript{flox/-} and the Scgb1a1-Cre allele have also been showed (Fig. 3E). Furthermore, we also overexpressed lung Dach1 in mice, three weeks after intratracheal administration of AAV2/9 virus (AAV-Dach1 and AAV-con), the lung Dach1 increased significantly in AAV-Dach1 mice contrasting to AAV-Con mice (Fig. 3F).

Deficiency Of Dach1 Aggravated Airway Inflammation Induced By Cigarette Smoke

After six months exposure of cigarette smoke (CS) (Fig. 4A), there were significant decline in mice lung function, accumulation of inflammatory cells and increasing secretion of IL-6 and CXCL1 in bronchoalveolar lavage fluid (BALF). In contrast, Dach1-CKO mice exhibited more significant decline of lung function (Fig. 4B), worse infiltration of inflammatory cells in peri-airway and peri-vascular and destruction of the alveolar septum (Fig. 4C-G), and more increasing secretion of IL-6 and CXCL1 than Dach1-C mice (Fig. 4H-I).

Intratracheal Administration Of Aav2/9-dach1 Alleviated Airway Inflammation Induced By Cigarette Smoke

To probe for a potential therapeutic effect of Dach1 inhibition in COPD, mice with intratracheal administration of AAV-Dach1 or AAV-Con have also been exposed to CS for six months (Fig. 5A). CS exposure also caused significant lung function decline in AAV-Con mice but not in AAV-Dach1 mice (Fig. 5B). After cigarette smoke exposure, in contrast to AAV-Con mice, AAV-Dach1 mice displayed alleviated airway and vascular inflammation and emphysema (Fig. 5C-G). Furthermore, AAV-Dach1 mice also have fewer increasing secretion of inflammatory factors IL-6 and CXCL1 after CS simulation (Fig. 5H-I).
Furthermore, we also calculated the impact of Dach1 on emphysema induced by porcine pancreatic elastase (PPE) (Fig. 6A). Decreasing Dach1 had also been detected in lung of PPE-exposed mice (Fig. 6B). PPE intratracheal injection destructed mice lung function and caused significant emphysema. AAV-Dach1 protected mice from lung function decline and destruction of the alveolar septum but had no influence on inflammatory cells in BALF (Fig. 6C-F).

**Dach1 Mitigated Inflammation Induced By Cse In 16hbe Cells**

To further calculate the influence of Dach1 on inflammation in vitro, Dach1 overexpression in 16 HBE cells have been implemented by lentiviral transfection (Lv-Dach1, Fig. 7A). 16HBE cells stimulated with CSE exhibited increasing secretion of IL-6 and IL-8. After stimulation of CSE, Lv-Dach1 16HBE cells have fewer secretion of IL-6 and IL-8 in comparation with 16 HBE cells transfected with control lentiviral (Lv-Con) (Fig. 7B-C). What’s more, the silence of Dach1 have also been carried out by small interference RNA (siRNA), and the #3siRNA can significantly reduce Dach1 expression. (Fig. 7D). Consistently, Dach1 silencing in 16HBE cells exacerbated the increasing secretion of IL-6 and IL-8 induced by CSE (Fig. 7E-F).

**Dach1 Regulated Nrf2/ho-1 Signal Pathway**

Dach1 have been found to evolve in many diseases as a transcription factor or transcription cofactor. To elucidate the underlying mechanism of Dach1 on alleviating inflammation, Dach1-targeted genes have been predicted (http://gtrd.biouml.org/). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a potential target gene of Dach1, which also belongs to transcription factor (Figure S1A). Nrf2 signal pathway plays an essential role in inflammation and has also been reported to be related to COPD[22, 23]. Nrf2 targeting is a promising therapeutic strategy in COPD[24]. We speculated that Dach1 inhibited inflammation through regulating Nrf2 signal pathway. Nrf2 is a regulator of cellular resistance to oxidants by controlling antioxidant response element-dependent genes including heme oxygenase-1 (HO-1), glutathione peroxidase 2 (GPX2), NAD(P)H: quinone oxidoreductase 1 (NQO1) and PRDX1 [25, 26]. HO-1, as a cytoprotective protein with antioxidant properties, takes part in many chronic diseases by regulating inflammation and cell death[27–29]. Activating Nrf2/HO-1 signal pathway might be a beneficial factor for COPD[30, 31].

To clarify the influence of Dach1 on Nrf2/HO-1 signal pathway, we first monitored the expression of HO-1. We found that CSE induced a significant upregulation of HO-1, which can be enhanced by Dach1 overexpression and be inhibited by Dach1 silence in 16 HBE cells (Fig. 8A-B). Simultaneously, CS exposure also increased HO-1 expression in mice lung. In contrast with AAV-dach1 mice, AAV-Con mice had a more increased HO-1 expression, however, Dach1-CKO mice showed a lesser HO-1 expression comparing to Dach1-C mice (Fig. 8C-D). Besides, Dach1 also promoted Nrf2 expression (Fig. 8E). Cytoplasmic Nrf2 can transport to the nucleus and then regulate HO-1 expression by binding the promoter of HO-1. We further found Dach1 increased nuclear Nrf2 but had no significant impact on cytoplasm Nrf2 (Fig. 8F-H). Silencing Nrf2 significantly reduced HO-1 expression and offset increased HO-1 induced by
Dach1 overexpression (Fig. 8I-J). Reactive oxide species (ROS) were downstream products of Nrf2/HO-1 signaling, we found Dach1-overexpressed also can reduced ROS after CSE stimulation, which can be neutralized by Nrf2 silence (Fig. 8K-M). All those results prompted Dach1 can regulate Nrf2/HO-1 signaling.

**Dach1 Regulated Inflammation Through Transcriptional Regulation Of Nrf2**

To further elucidate the impact of Dach1 on Nrf2/HO-1 signal pathway and inflammation, we first tested whether silencing of Nrf2 may break the protective role of Dach1 in inflammation. We found the silence of Nrf2 obviously upregulated IL-6, IL-8 induced by CSE in 16HBE cells (Fig. 9A-B). What's more, we also calculated the underlying mechanism of Dach1 on Nrf2/HO-1 signal pathway. Nrf2 had been predicted as a targeted gene as before, so we first calculated if Dach1 can bind to the promotor of Nrf2. The promotor sequence of Nrf2 were acquired from NCBI. Seven pairs of primers (P1-P7) that cover binding sites for ChIP-PCR assay (Table S3, see Additional file 1). PCR analysis using Dach1-conjugated DNA samples showed amplifications with P5 primers (Fig. 9C-E) but not others (Figure S1B-G, see Additional file 2), suggesting it as candidate binding site of Dach1 on Nrf2. In addition, as a cofactor, we hypothesized that Dach1 regulated Nrf2 by protein-protein interaction. This notion was supported by Immunofluorescence staining showing colocalization of Dach1 and Nrf2 in 16 HBE cells and Immunoprecipitation assay identifying an interaction between Dach1 and Nrf2 (Fig. 9F-G)

**Discussion**

In this study, we confirmed the decreased Dach1 level in COPD patients and experimental COPD model. We found Dach1 played a protective role in CS-induced inflammation in COPD. Dach1 silencing aggravated lung function decline and airway inflammation, while Dach1 overexpression protected mice from lung function decline and airway inflammation induced by cigarette smoke (CS). In vitro, Dach1 inhibited the secretion of inflammatory cytokines IL-6 and IL-8 by activating Nrf2/HO-1 signaling through enhancing the expression and nuclear translocation of Nrf2 in 16 HBE cells.

COPD is a small airway chronic inflammatory disease with heterogeneity and cigarette smoke (CS) remains a common risk factor. Inflammation induced by CS is a consistent feature of COPD and contributes to the pathogenesis and progression of COPD. Inflammation in COPD has many guises, neutrophil-associated COPD with Th1, Th17 immunity and the activation of inflammasome is the most usual phenotype, while eosinophil-associated COPD with Th2 immunity is not common[7]. Airway epithelium is the first barrier against outside stimulus, CS can stimulate epithelium release inflammatory mediators IL-6, IL-8 and IL-1β, and then recruit inflammatory cells, such as neutrophils, macrophages, lymphocytes, eosinophils and so on, to airway[8]. Those inflammatory exudates result in airway damage, remodeling, occlusion and even the emphysema[7]. Small airway obliteration and emphysema are main causing of decline in lung function[32]. For neutrophil-associated COPD, clinical trails showed that
directly targeting IL-8, IL-6, the inflammasome, TNF and IL17 have no effect in COPD but increased risk of infection in some cases[7, 33, 34]. Those indicate that finding new indirect targets for anti-inflammatory therapy is necessary.

Endogenous Dach1 is widely expressed in many tissues containing breast, heart, kidney, lung. Dach1 expressed in the nucleus and takes part in cell cycle, proliferation, invasion and cytokines secretion. As a tumor suppressor, downregulated Dach1 accelerated cancer cells proliferation and invasion via repression cytokines secretion of IL-6 and IL-8[18]. As a co-integrator or transcription factor, Dach1 can also control gene expression including cytokines[16, 35, 36]. Decreasing Dach1 expression has been reported promotes the occurrence and progression of diseases[9, 15, 16, 37]. However, the role of Dach1 on COPD has not been reported up to now.

In our study, the decreasing expression of Dach1 in COPD patients have been found, which displayed a positive correlation with forced expiratory volume (FEV1 actual value). Cigarette smoke (CS) exposure, as a primary risk factor for COPD, induced the decreasing of Dach1 expression in airway epithelium. Persistent airflow obstruction is a feature for COPD patients, which is not reversible[3]. Airway inflammation can result in airway obliteration and then lung function decline. Up to know, the influence of decreased Dach1 on airway inflammation and lung function decline of COPD have not known. We hypothesized decreasing Dach1 might trigger airway inflammation and lung function decline in COPD by regulating the cytokines expression.

To further validate, Dach1 overexpressed and silencing mice were generated to establish experimental COPD model. We found declined lung function induced by CS can be aggravated by Dach1 silencing while be improved by Dach1 overexpression. Moreover, Decreased Dach1 was found to augment the accumulation of inflammatory cells around airway and the secretion of cytokines IL-6 and CXCL1/IL-8 in mice and 16 HBE cells, while overexpressed Dach1 protected them from inflammatory cells accumulation and IL-6 and IL8 secretion induced by CS. Those indicated Dach1 may protect lung function decline and airway inflammation from CS exposure. At present, many strategies targeting innate inflammation in COPD are not sufficient[34]. IL-8 is a key cytokine involved in neutrophil recruitment[38]. However, the clinical effect of biologic directly targeted to IL-8 don't have been impressive[39]. Our study found Dach1 induction can inhibit IL-8, which may be a promising therapeutic targeting of COPD.

Dach1 perform a protective role on many diseases as a transcription factor or transcription cofactor. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a predicted target gene of Dach1. Nrf2 plays a critical role in keeping cellular redox homeostasis and regulating inflammation response[40]. As a transcription factor, Nrf2 can regulate the expression of downstream genes and heme oxygenase-1 (HO-1) is a Nrf2-derived gene. Both Nrf2 activation and HO-1 expression provide cytoprotection against numerous pathologies including cancer initiation, neurodegenerative and metabolic disorders, autoimmune, and chronic diseases of the lung and liver[41]. In COPD, Nrf2 targeting is a promising therapeutic strategy[24]. Nrf2-targeted HO-1 also plays a crucial role. HO-1 is a common cytoprotective protein with anti-inflammatory and antioxidant effects. Repression of Nrf2/HO-1 signaling aggravated inflammation and
emphysema induced by CS or elastase[42, 43]. We found HO-1 expression can be induced by CSE stimulation, which can be promoted and inhibited by Dach1 overexpression and silencing in 16 HBE cells, respectively. Silencing Nrf2 eliminated HO-1 expression and destroyed increased HO-1 level and decreased reactive oxygen species (ROS) caused by Dach1 overexpression, which hinted that Dach1 might regulate HO-1 expression by targeting Nrf2 signal.

However, whether and how Dach1 regulates Nrf2 expression is not clear. In our study, we found Dach1 facilitated Nrf2 expression, especial in the nucleus. To further explore the relationship between Dach1, Nrf2 and inflammation, we silenced Nrf2 in Dach1-overexpressed 16HBE cells. Nrf2 silencing counteracted the protective role of Dach1 on alleviating inflammation. Dach1 regulated inflammation by activating Nrf2 signaling. As a transcription factor or co-factor, Dach1 could directly regulate Nrf2 signaling or enhance the transcriptional regulation of Nrf2 on downstream genes. Subsequently, we found Dach1 bound to the promotor of Nrf2, we also found the colocalization of Dach1 and Nrf2 in the nucleus and the protein-protein interaction between them. We hypothesized Dach1 not only facilitated Nrf2 expression but also enhancing the transcriptional regulation of on downstream gene HO-1 by Nrf2.

There are many limitations in this study. We elucidated the protective role of Dach1 on neutrophil-associated inflammation by regulating IL-6 and IL-8. The inflammation in COPD is heterogenous[7]. The influence of Dach1 on the inflammasome, Th17 immunity and even the eosinophil-associated inflammation is not clear. In addition, both airway inflammation and emphysema are common features of COPD and contribute to lung function decline, though inflammation can contribute to emphysema, the direct mechanism of Dach1 on emphysema is not clear and desire to further explore.

**Conclusions**

In summary, our study uncovered the protective role of Dach1 on airway inflammation through inhibited secretion of inflammatory cytokines IL-6 and IL-8. Dach1 can activate Nrf2 signal, whose activating considers a therapy targeting of COPD. Dach1 alleviated inflammation by activating Nrf2 signaling and its downstream gene HO-1. Those findings provide a novel insight into inflammation therapy of COPD.

**Abbreviations**

COPD: Chronic obstructive pulmonary disease; Dach1: Dachshund homolog 1; CS: Cigarette smoke; CSE: Cigarette smoke extraction; AAV2/9: adeno-associated virus 2/9; Lv: lentivirus; NC: negative control.

**Declarations**

*Ethics approval and consent to participate*

The study protocol was conducted with approval from the ethics committee of Tongji Hospital, Huazhong University of Science and Technology, Wuhan, Hubei, China (TJ-IRB20210346). Each subject signed an informed consent form before the collection of samples.
Consent for publication

Availability of data and materials

All data may be available from the corresponding author upon reasonable request.

Not Applicable.

Competing interests

The authors have no conflict of interest.

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Authors' contributions

Jungang Xie conceived the research and revised the manuscript. Qian Huang drafted the manuscript, performed experiments and analyzed data. Yiya Gu, Jixing Wu, Yuan Zhan, Zhesong Deng, Shanshan Chen, Maocuo Peng, Ruonan Yang and Jinkun Chen contributed to patient recruitment, data collection, and part of the animal experiments.

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References


Figures
Dach1 expression in non-smokers, smokers and COPD patients. (A) Dach1 mRNA level in lung tissues from COPD patients and non-COPD patients in an existing microarray dataset (GSE76925). (B) Dach1 mRNA level in lung tissues from 13 non-smokers, 11 smokers and 22 COPD patients. (C) The correlation between FEV1% predicted and Dach1 mRNA level of 22 COPD patients. (D) Representative Co-immunostainings of Dach1 and Scgb1a1 in non-smokers, smokers and COPD patients. (E) Dach1 expression in primary airway epithelium after cigarette smoke extract exposure (CSE) (n=4). *P<0.05, **P<0.01.
Figure 2

Dach1 expression after cigarette smoke (CS) exposure. (A) Representative Co-immunostainings of Dach1 and Scgb1a1 in mice lung with/without CS exposure. (B) Western blot analysis and quantification of Dach1 proteins in mice lung after six-month CS exposure (n=4). (C-D) Western blot analysis and quantification of Dach1 proteins in 16 HBE cells after smoke extract exposure (CSE) simulation with different concentrations for different times (n=6). *P<0.05, **P<0.01, ***P<0.001.
Figure 3

Generation of Dach1 knockdown and overexpression mice. (A-B) Dach1\textsuperscript{flox/flox} mice were generated by inserting two loxP sequences in the same direction into the introns flanked with the exon 2 of Dach1 based on the CRISPR-Cas9 system, which could produce a nonfunctional Dach1 protein by generating a stop codon in exon 3 after Cre-mediated gene deletion. Dach1\textsuperscript{flox/flox} was then crossed with the Scgb1a1-Cre transgenic mice to get the small airway epithelium Dach1-knockout mice, which were named as Scgb1a1-Cre\textsuperscript{+}-Dach1\textsuperscript{flox/flox} (Dach1-CKO). (C) Representative Co-immunostainings of Dach1 and Scgb1a1.
in Dach1-CKO mice and Dach1-C mice. (D) Western blot analysis and quantification of Dach1 proteins in Dach1-CKO mice and Dach1-C mice (n=4). (E) Southern blot to analysis the flox allele form Dach1\(^{flox/flox}\), WT and Dach1\(^{flox/-}\) and the Scgb1a1-Cre allele. (F) Western blot analysis and quantification of Dach1 proteins in AAV-Dach1 and AAV-Con mice (n=4). *P<0.05, **P<0.01.

Figure 4

Dach1 knockdown alleviates airway inflammation after cigarette smoke (CS) treatment. (A) Dach1-CKO and Dach1-C mice were exposed to CS for six months (n=4-6). (B) Lung function of Dach1-CKO and Dach1-C mice after CS treatment. (C) Total inflammatory cells in BALF. (D) Representative images of H&E staining of mouse lung sections. (E) Quantitative analysis of pulmonary mean linear intercept (MLI). (F-G) Airway and vascular inflammation score in Dach1-CKO and Dach1-C mice after CS exposure. (H-I) ELISA to calculate IL-6 and CXCL1 in bronchoalveolar lavage fluid (BALF) of Dach1-CKO and Dach1-C mice after CS exposure. *P<0.05, **P<0.01, ***P<0.001.
Dach1 overexpression protects mice from airway inflammation caused by cigarette smoke (CS) treatment (n=4-6). (A) AAV-Dach1 and AAV-C mice were exposed to CS for six months. (B) Lung function of AAV-Dach1 and AAV-C mice after CS treatment. (C) Total inflammatory cells in BALF. (D) Representative images of H&E staining of mouse lung sections. (E) Quantitative analysis of pulmonary mean linear intercept (MLI). (F-G) Representative images of H&E staining of mouse lung sections. (F-G) Airway and vascular inflammation score in AAV-Dach1 and AAV-C mice after CS exposure. (H-I) ELISA to calculate IL-6 and CXCL1 in bronchoalveolar lavage fluid (BALF) of AAV-Dach1 and AAV-C mice after CS exposure. *P<0.05, **P<0.01, ***P<0.001.
Figure 6

Dach1 overexpression protects mice from emphysema induced by porcine pancreatic elastase (PPE) treatment (n=4-6). (A) AAV-Dach1 and AAV-C mice were exposed to PPE for 42 days. (B) Western blot analysis and quantification of Dach1 proteins in mice with PPE treatment. (C) Lung function of AAV-Dach1 and AAV-C mice after PPE treatment. (D-E) Representative images of H&E staining of mouse lung...
sections and pulmonary mean linear intercept (MLI) to calculate emphysema. (F) Total inflammatory cells in BALF of AAV-Dach1 and AAV-C mice. *P<0.05, **P<0.01, ***P<0.001.

Figure 7

Dach1 regulates inflammation induced by smoke extract exposure (CSE) in 16 HBE cells (n=4). (A) Western blot analysis and quantification of Dach1 proteins in 16 HBE cells after transfection of Lv-Dach1 and Lv-Con. (B-C) ELISA to calculate IL-6 and IL-8 in 16 HBE cells after transfection of Lv-Dach1 and Lv-Con with/without CSE stimulation. (D) Western blot analysis and quantification of Dach1 proteins in 16 HBE cells transfected with small interference RNA of Dach1. (E-F) ELISA to calculate IL-6 and IL-8 in 16 HBE cells after transfection of Dach1 siRNA and negative control (NC) with/without CSE stimulation. *P<0.05, **P<0.01, ***P<0.001.
Figure 8

Regulation of Dach1 on Nrf2/HO-1 signal pathway. (A-B) Western blot analysis and quantification of HO-1 proteins in 16 HBE cells after transfection of Lv-Dach1 and Lv-Con (A), and in 16 HBE cells transfected with small interference RNA of Dach1 or negative control (B) with/without CSE stimulation (n=4). (C-D) Western blot analysis and quantification of HO-1 proteins in AAV-Dach1 and AAV-Con mice (C), and in AAV-Dach1 and AAV-Con mice after six-months CS exposure (D) (n=4). (E) Western blot analysis and
quantification of Nrf2 proteins in 16 HBE cells transfected with small interference RNA of Nrf2 or negative control (n=4). (F-H) Western blot to analysis and quantify Nrf2 proteins in cytoplasm and nuclear, respectively (n=4). (I) Western blot analysis and quantification of Nrf2 proteins in 16 HBE cells transfected with small interference RNA of Nrf2 (n=4). (J-M) Western blot analysis and quantification of HO-1 proteins (J) (n=5), flow cytology analysis and immunostainings analysis of ROS (K-M) in Lv-Dach1 16 HBE cells transfected with small interference RNA of Nrf2 or negative control with/without CSE stimulation (n=5). *P<0.05, **P<0.01, ***P<0.001.

Figure 9

Dach1 inhibits inflammation induced by CSE by transcriptional regulation of Nrf2. (A-B) ELISA to calculate IL-6 and IL-8 in Lv-Dach1 16 HBE cells transfected with small interference RNA of Nrf2 or negative control with/without CSE stimulation (n=5). (C-E) CHIP-PCR showed Dach1 bound the P5 region of Nrf2 promotor. (E) CHIP-qPCR assay demonstrated a significant increase in the amplification of the fragments in P5 binding sites (n=3). (F) Immunofluorescence staining of Dach1 and Nrf2 in 16 HBE cells
with or without CSE stimulation. (G) Immunoprecipitation assay to identify an interaction between Dach1 and Nrf2. *P<0.05, **P<0.01, ***P<0.001.

Supplementary Files

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