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Polydatin attenuates reactive oxygen species-induced airway remodeling by promoting Nrf2-mediated antioxidant signaling in asthma mouse model

Haizhu Zeng[a], Yumeng Wang[a], Yuxia Gu[a], Jinrui Wang[a], Huali Zhang[a], Hongchang Gao[a], Qinqin Jin[b,*], Lei Zhao[a,**]

[a] Department of Respiratory Medicine, Shanghai Gongli Hospital, The Second Military Medical University, Shanghai 200135, PR China
[b] Department of Central Laboratory, Shanghai Gongli Hospital, The Second Military Medical University, Shanghai 200135, PR China

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ABSTRACT

Reactive oxygen species (ROS) and epithelial–mesenchymal transition (EMT) play a critical role in transforming growth factor (TGF)-β1-mediated fibrotic airway remodeling in asthma. Polydatin (PD) is a small natural molecule in Chinese medicine; it is isolated from *Polygonum cuspidatum* and has antioxidative properties. In this study, we aimed to determine whether PD was protective against ROS-induced pulmonary fibrosis in asthma. Ovalbumin (OVA) was used to induce asthma in a mouse model that was treated with or without PD. We also created nuclear factor erythroid 2-related factor 2 (Nrf2) knockdown BEAS-2B cells and investigated whether PD reversed TGF-β1-induced pulmonary epithelial cell EMT by promotion of Nrf2-mediated antioxidation. Immunofluorescence showed that ROS and TGF-β1 expression was significantly increased in lung tissue from the OVA-induced asthma model. PD treatment inhibited activity of ROS and TGF-β1. Immunohistochemistry showed that PD treatment decreased OVA-induced lung ROS, TGF-β1 expression and fibroblasts. Western blotting showed that PD treatment reversed OVA-induced NADPH oxidase (NOX)1/4 expression by promoting Nrf2-mediated heme oxygenase-1 and NADPH dehydrogenase (quinone)-1 expression. PD treatment suppressed OVA-induced EMT and lung fibroblast protein expression in lung tissue. Nrf2 downregulation suppressed the protective effect of PD by promoting TGF-β1-induced ROS and EMT and accumulation of extracellular-matrix-related protein. All these data indicate that PD has potential therapeutic effects in asthma by promoting Nrf2-mediated antioxidation.

1. Introduction

An increasing number of studies have found that reactive oxygen species (ROS) production is associated with asthma. Excessive activation of ROS can initiate airway inflammation, which might initiate or promote airway diseases [1,2]. ROS are frequently induced and released from respiratory tract epithelial cells or neutrophils after DNA damage, which results in subepithelial fibrosis and airway remodeling [3]. Transforming growth factor (TGF)-β1 is a central signal in promoting airway fibrosis. When expressed in bronchial epithelium, TGF-β1 leads to the development of several characteristic features of airway remodeling, including subepithelial fibrosis through induced epithelial–mesenchymal transition (EMT) in pulmonary epithelial cells [4–6]. However, the mechanism remains unknown.

Polydatin (PD; 3,4′,5-trihydroxystibene-3-β-mono-D-glucoside) is a major active compound extracted from the traditional Chinese herb *Polygonum cuspidatum* [7]. Increasing evidence indicates that PD has several biological activities including protection against ROS and fibrosis [8–10]. Research has shown that the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) plays an important role in regulating ROS by activating downstream antioxidant proteins including NAD(P)H:quinone oxidoreductase (NQO1) and heme oxygenase (HO-1) [11]. It has been shown that Nrf2 expression alleviates bleomycin-induced pulmonary fibrosis by blocking EMT progression [12].

In the present study, we explored whether NQO1 and HO-1 were involved in regulation of pulmonary fibrosis by activation of Nrf2. Models of ovalbumin (OVA)-induced asthma in mice and TGF-β1-

* Correspondence to: Q. Jin, Department of Central Laboratory, Shanghai Gongli Hospital, Second Military Medical University, 219 Miao-Pu Road, Shanghai 200135, PR China.
** Correspondence to: L. Zhao, Department of Respiratory Medicine, Shanghai Gongli Hospital, Second Military Medical University, 219 Miao-Pu Road, Shanghai 200135, PR China.
E-mail addresses: jqq13866319561@126.com (Q. Jin), zhaolei7012@126.com (L. Zhao).

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induced EMT were constructed to establish whether PD protected against EMT-induced pulmonary fibrosis, and to establish the connection with the Nrf2-dependent antioxidant pathway.

2. Materials and methods

2.1.1. Ethics statement

The study was approved by the Ethics Committee of the Pudong New Area Gongli Hospital, Shanghai, China.

2.1.2. Murine model of OVA-induced asthma

Male BALB/c mice (weighing 18–24 g, aged 6–8 weeks) were purchased from the Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). OVA was used to induce the asthma model. The mice were sensitized by intraperitoneal injection of 0.2 ml OVA (20 μg OVA emulsified in 1 mg aluminum hydroxide) on days 0, 7 and 14. Seven days after the last sensitization, the mice were exposed to 1% OVA aerosol, 1 h a day for 7 consecutive days. The control group received saline instead of OVA. The PD group was treated with 100 mg/kg PD 30 min before OVA stimulation on days 0, 7 and 14. Mice were killed with 30 mg/kg sodium pentobarbital. The lungs and airways were collected for immunohistochemical and western blot analysis after rinsing with 1 ml PBS. Each group had six mice.

2.1.3. Evaluation of oxidative stress levels

2′,7′-Dichlorofluorescin diacetate was used to detect ROS production in fresh lung tissue samples.

2.1.4. Immunohistochemistry and immunofluorescence

After fixation in 10% formalin solution and embedding, lung tissue samples were cut into 5-μm slices. The sections were stained with Masson's trichrome after dewaxing for fibrosis analysis using an Axiophot light microscope (Zeiss, Oberkochen, Germany). TGF-β1 expression in lung tissue was evaluated with TGF-β1 staining and analyzed with a fluorescence microscope (Nikon, Tokyo, Japan).

2.1.5. BEAS-2B cell culture and treatment

The human lung epithelial BEAS-2B cell line was purchased from Procell Life Science Co. Ltd. (Shanghai, China) and cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium/F12 medium (DMEM-F12; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin and 100 ng/ml streptomycin (Invitrogen). To induce airway inflammation and fibrosis, Nrf2 knockdown or wild-type BEAS-2B cells were treated with PD (100 μM) and TGF-β1 (10 ng/ml) alone or in combination for up to 48 h. BEAS-2B cells were collected for the following experiment.

2.1.6. Transfection

siRNA against Nrf2 (siNrf2) and negative control siRNA (siNC) were designed and synthesized by Gene Pharma (Shanghai, China). The primer sequences were: Nrf2, 5′-GAGGAUGGGAAACCUUACUTT-3′ (forward primer) and 5′-AUAUUUGCAGUUGAAGGCCTT-3′ (reverse primer). BEAS-2B cells were transfected with 50 nM siNrf2 or siNC using Lipofectamine 2000 (Invitrogen) for 48 h before Nrf2 expression analysis or other experiments.

2.1.7. miRNA extraction and real-time polymerase chain reaction

Total RNA was isolated from BEAS-2B cells using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized using the PrimeScript™ RT Master Mix Kit (Takara, Dalian, China), which was then used for real-time polymerase chain reaction (Rt-PCR), together with forward and reverse primers and the Power SYBR Green PCR Master Mix (Life Technology, New York. USA). *GAPDH* was used as an internal control. The relative expression of target genes was determined using the $2^{-\Delta\Delta Ct}$ method.

2.1.8. Cell viability assay

Cell Counting Kit-8 (CCK8; Invitrogen) was used to assess cell viability. BEAS-2B cells (1×10^4) were treated with 0, 10, 100, 250 or 1000 μM PD for 48 h before seeding in a 96-well plate and incubation overnight. The medium was removed and washed three times with PBS. DMEM-F12 (90 μl) and 10 μl CCK8 were mixed and added to each well and incubated at 37 °C for 1.5 h; a microplate reader was used to measure optical density (OD) at 450 nm.

2.1.9. Western blotting

Protein from tissues and cells were extracted using RIPA lysis buffer containing proteinase inhibitor (Sigma–Aldrich, St. Louis, MO, USA). The BCA Protein Assay Kit (Vigorous Biotechnology Beijing, Beijing, China) was used for protein concentration detection. Protein lysates (20 μg) were resolved using 10% SDS-PAGE, and then electroblotted onto nitrocellulose membranes (Millipore, Madison, WI, USA). The membranes were blocked with 5% nonfat dry milk for 2 h then incubated with anti-Nrf2, anti-HO-1, anti-NQO1, anti-NOX1, anti-NOX4, anti-E-cadherin, anti-vimentin, anti-α-smooth muscle actin (SMA), anti-collagen I, anti-collagen III and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies overnight at 4 °C. GAPDH was used as an internal control. The membrane was further incubated with horseradish-peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) for 1 h at room temperature. The immune complexes were detected with enhanced chemiluminescence (Cell Signaling Technology, Danvers, MA, USA). The integrated density of the band was quantified by Quantity One software (Bio-Rad, California, USA).

2.2. Statistical analysis

The data were expressed as the mean ± standard deviation (SD). One-way analysis of variance was performed for multiple comparisons using GraphPad Prism version 5.0 (GraphPad, La Jolla, CA, USA). $P \leq 0.05$ indicated a significant difference.

3. Results

3.1.1. PD attenuates OVA-induced ROS and pulmonary fibrosis

ROS are a major cause of airway remodeling in asthma [13,14]. To investigate the protective effects of PD on pulmonary bronchial epithelial fibrosis in OVA-induced asthma, BALB/c mice were used to construct an asthma model, with or without PD treatment (Fig. 1A) before OVA stimulation. After 28 days treatment, mice were killed and lung tissue was removed for immunohistochemistry and western blotting. ROS level in lung tissues was increased, and PD treatment significantly suppressed OVA-induced ROS (Fig. 1B). Previous studies have found an interaction between ROS and TGF-β1. TGF-β1 expression plays an important role in promotion of fibrosis [15,16]. We also found that TGF-β1 expression was induced by OVA. PD treatment suppressed TGF-β1 expression (Fig. 1C). Immunohistochemistry found that PD treatment reversed OVA-induced pulmonary fibrosis (Fig. 1D).

Western blotting showed that PD treatment reversed OVA-induced Nrf2 inhibition. There is increasing evidence that Nrf2 expression suppresses ROS and we also identified that PD treatment promotes HO-1 and NQO-1 expression, but inhibits NOX1 and NOX4 expression (Fig. 2A–F). It has been shown that EMT plays an important role in OVA-induced pulmonary fibrosis [17]. We found that PD treatment reversed OVA-induced EMT by promotion of vimentin expression and suppression of E-cadherin expression. We showed that PD treatment suppressed OVA-induced extracellular matrix protein α-SMA, collagen I and collagen III expression (Fig. 2G–L).

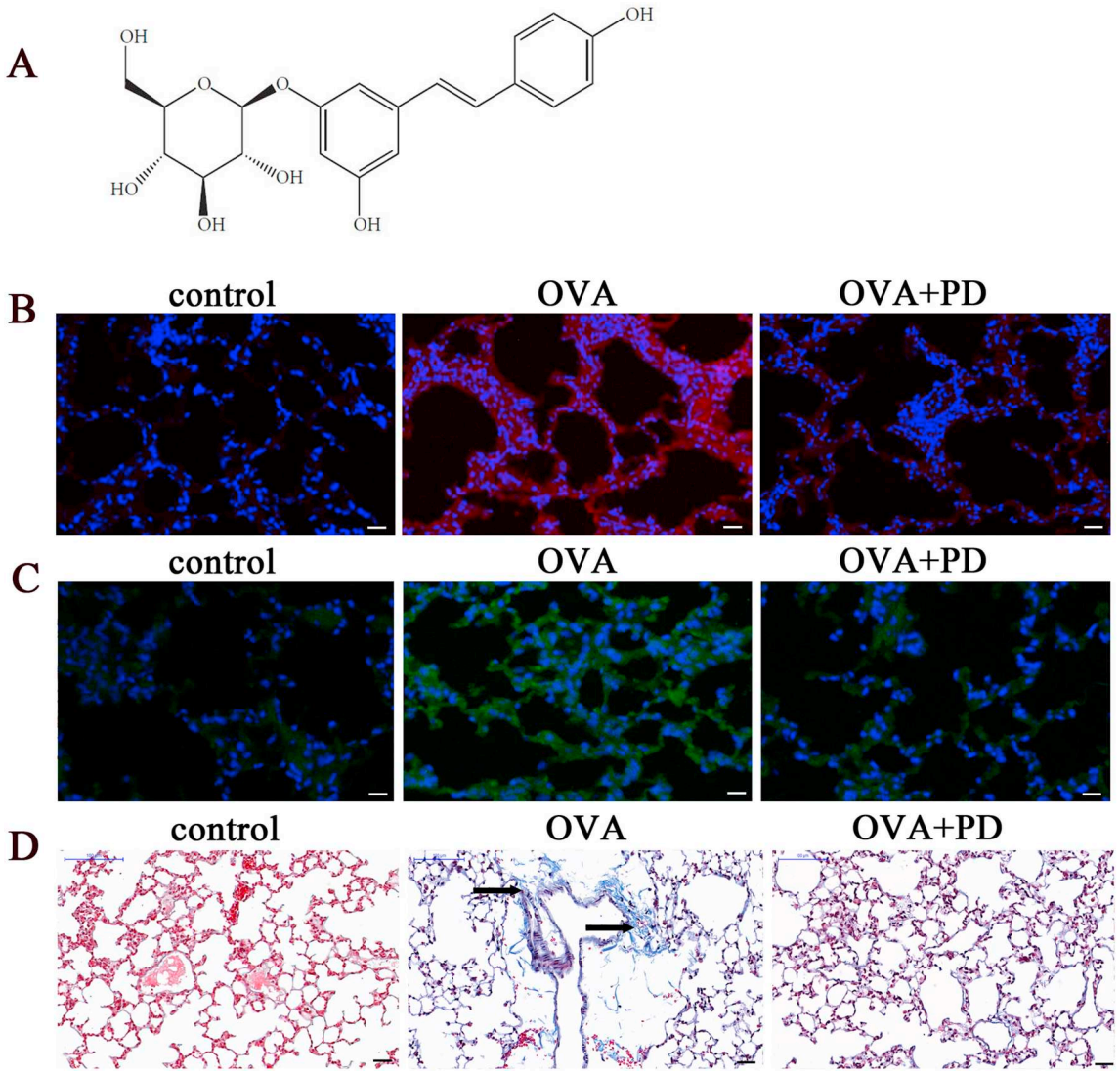

Fig. 1. Polydatin (PD) treatment attenuated reactive oxygen species (ROS) and pulmonary fibrosis in ovalbumin-induced mouse asthma model. (A) Chemical structure of PD. (B) Immunofluorescence of ROS level in OVA-induced mouse asthma model with or without PD treatment. Images of ROS formation (red) and DAPI (blue) (Magnification 200×), scale bars: 30 μM. (C) Immunofluorescence of TGF-β1 expression in lung tissues. Nuclei were stained with DAPI (blue) (magnification 100×), scale bars: 50 μM. (D) Analysis of pulmonary fibrosis by Masson's trichrome staining of lung tissues, (magnification 200×), scale bars: 30 μM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.1.2. PD treatment decreased TGF-β1-induced ROS by promotion of Nrf2 antioxidant pathway in lung epithelial BEAS-2B cell line

The proliferation and viability of BEAS-2B cells were determined with CCK8. There was no significant effect of PD on cell proliferation and viability until the concentration reached 1000 μM for 48 h (Fig. 3A). So, we selected 100 μM PD for the following experiment. To establish whether Nrf2 played an important role in suppression of TGF-β1-induced ROS and EMT, siNrf2 was constructed. Expression of Nrf2 protein (Fig. 3B) and mRNA (Fig. 3C) was significantly decreased compared with the siNC group. Wild-type or siNrf2 BEAS-2B cells were used and treatment with or without 100 μM PD in the presence of 10 ng/ml TGF-β1. PD treatment significantly downregulated TGF-β1-induced ROS-related NOX1 and NOX4 expression, but increased antioxidant proteins NQO1 and HO-1. Nrf2 downregulation significantly decreased antioxidant proteins NQO1 and HO-1 expression, but increased ROS-related proteins NOX1 and NOX4 in the presence of TGF-β1. Nrf2 knockdown suppressed the antioxidant effect of PD (Fig. 3D–H).

3.1.3. PD treatment decreases TGF-β1-induced EMT by promoting Nrf2 antioxidant pathway in lung epithelial BEAS-2B cell line

Western blotting showed that PD treatment significantly suppressed TGF-β1-induced BEAS-2B cell EMT by inhibition of E-cadherin and promotion of vimentin expression. PD also promoted expression of fibrosis-related proteins collagen I, collagen III, and α-SMA. Nrf2 downregulation significantly increased EMT by promotion of vimentin and decreased E-cadherin expression in the presence of TGF-β1. Nrf2 knockdown suppressed expression of fibrosis-related proteins collagen I, collagen III, and α-SMA (Fig. 4).

4. Discussion

Pulmonary fibrosis is a serious lung disease characterized by extracellular matrix over-deposition and airway remodeling of lung tissue. However, the specific etiology of pulmonary fibrosis formation in asthma is still unclear. The mechanisms associated with pulmonary fibrosis are not well understood, which results in a lack of an effective

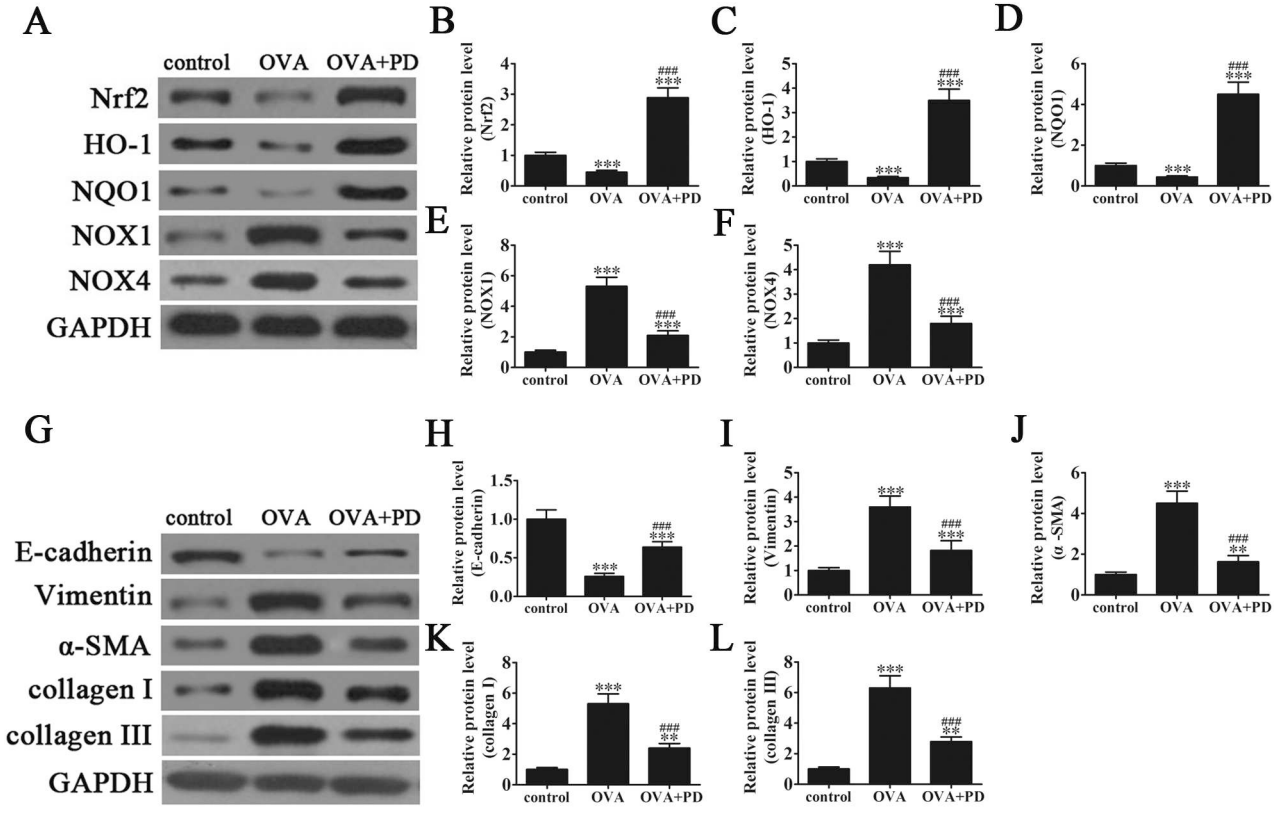

Fig. 2. Polydatin treatment increased the Nrf2 antioxidant pathway and suppressed epithelial–mesenchymal transition in ovalbumin (OVA)-induced mouse asthma model. (A–F) Western blotting of Nrf2, HO-1, NQO1, NOX1 and NOX4 expression. Values represent the mean ± SEM ($n = 3$), $^{***}P < 0.001$ versus control. $^{###}P < 0.001$ versus OVA group. (G–L) Western blotting of E-cadherin, vimentin, α-SMA, collagen I and collagen III. Values represent the mean ± SEM ($n = 3$), $^{**}P < 0.01$, $^{***}P < 0.001$ versus control. $^{###}P < 0.001$ versus OVA group.

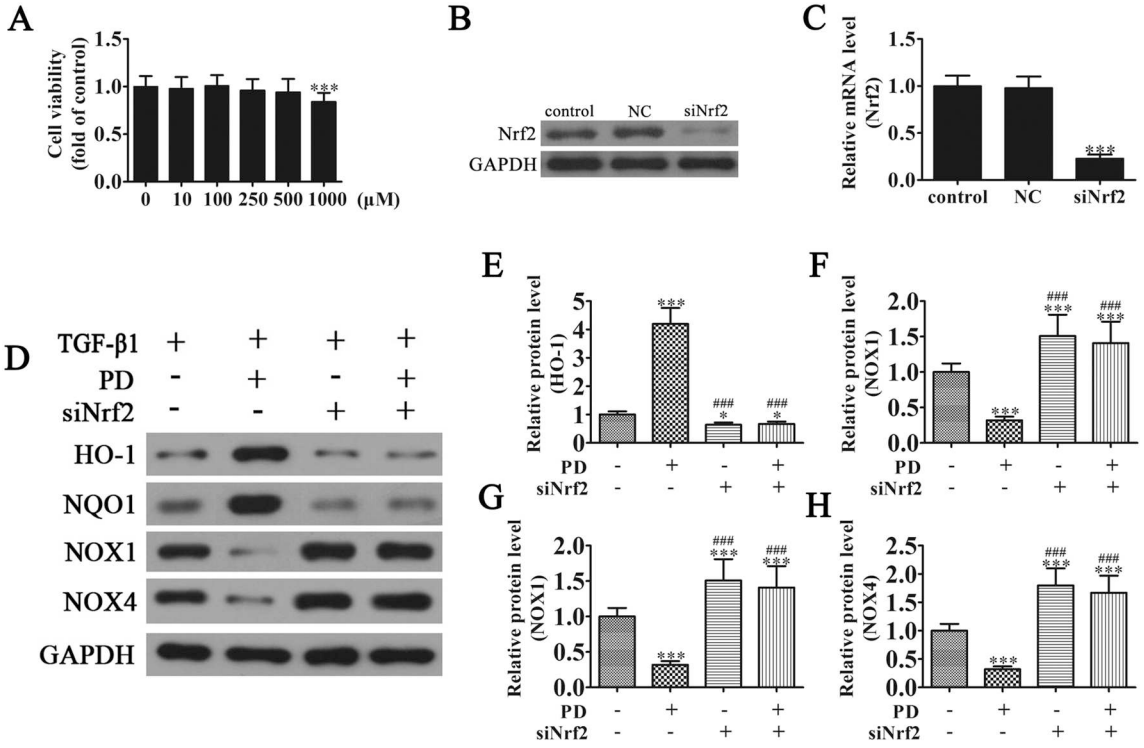

Fig. 3. Polydatin (PD) treatment decreased TGF-β1-induced reactive oxygen species by promoting Nrf2 antioxidant pathway in lung epithelial BEAS-2B cell line. (A) Effect of PD on cell viability. Cells were treated with 0, 10, 100, 250 or 1000 μM PD for 48 h, and cell viability was detected with cell counting kits. Western blotting (B) and PCR (C) detection of Nrf2 expression after transfection with siNrf2 or siNC for 48 h. (D–H) Western blotting of HO-1, NQO1, NOX1 and NOX4 expression in the presence of TGF-β1 (10 ng/ml) with or without 100 μM PD treatment in wild-type or Nrf2-downregulated BEAS-2B cells cultured for 48 h. Values represent the mean ± SEM ($n = 3$), $^{*}P < 0.05$, $^{***}P < 0.001$ versus control. $^{###}P < 0.001$ versus PD treatment group.

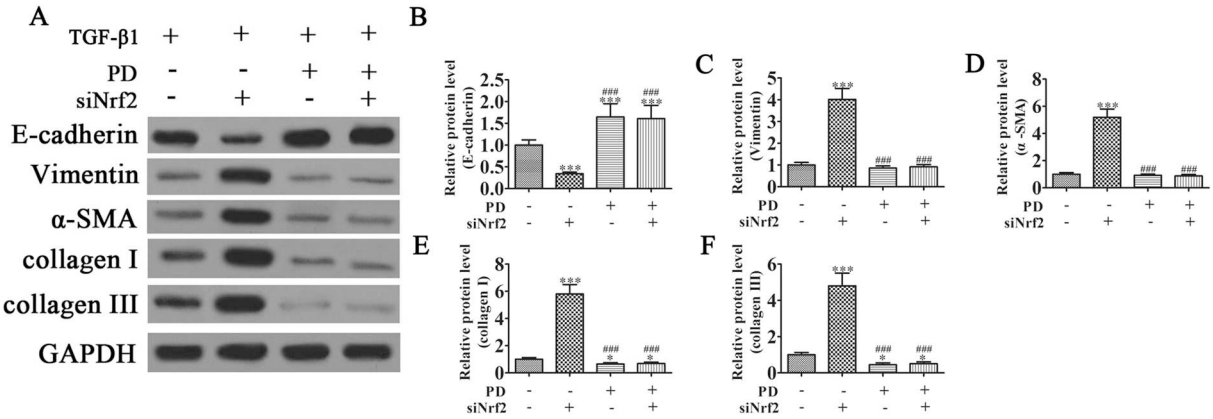

Fig. 4. Polydatin (PD) treatment decreased TGF-β1-induced epithelial–mesenchymal transition by promoting Nrf2 antioxidant pathway in lung epithelial BEAS-2B cell line. (A–E) Western blotting of expression of E-cadherin, vimentin, α-SMA, collagen I and collagen III in the presence of 10 ng/ml TGF-β1 with or without 100 μM PD treatment in wild-type or Nrf2-downregulated BEAS-2B cells cultured for 48 h. Values represent the mean ± SEM ($n = 3$), $^{*}P < 0.05$, $^{***}P < 0.001$ versus control. $^{\#\#\#}P < 0.001$ versus PD treatment group.

therapy. Increasing evidence has found that ROS play an important role in inducing pulmonary inflammation and fibrosis [18–20]. Chronic inflammation can promote TGF-β expression, which plays a central role in the development and progression of chronic respiratory diseases, including pulmonary fibrosis [21]. In the present study, we found that ROS and TGF-β1 were increased, but Nrf2 level was downregulated in OVA-induced asthma. We also showed that pulmonary fibrosis significantly increased in OVA-induced asthma. We suggest that ROS play an important role in pulmonary fibrosis, and suppression of ROS has potential for asthma therapy.

Previous reports show that PD acts as a new mitochondrial protector against sepsis [22], shock [23–25], and ischemia/reperfusion injury [26,27]. The present work revealed that treatment with PD reversed the reduction in the activity of antioxidant enzyme NQO1. Moreover, we investigated Nrf-2 and HO-1 expression to establish the mechanisms underlying the dramatic enhancement of oxidant defense systems by PD treatment. PD treatment promoted Nrf2 and HO-1 expression. HO-1, the downstream protein of Nrf-2, plays a key role in strengthening resistance capacity and restraining redox disorder, such as suppressed NOX1 and NOX4 expression.

Previous studies have found that Nrf2-mediated antioxidants have a protective effect of inhibiting EMT [12,28]. We also found that Nrf2 is involved in EMT-induced pulmonary fibrosis. Downregulation of Nrf2 expression by siRNA promotes TGF-β1-induced EMT by suppression of E-cadherin and increased vimentin expression. We found that Nrf2 knockdown reversed PD-induced EMT inhibition in the presence of TGF-β1 induction. Our study confirmed that PD suppressed pulmonary fibrosis by increasing expression of Nrf2.

Taken together, our data found that PD treatment alleviated asthma-induced ROS and fibrosis in lung tissues. The mechanism might be related to EMT inhibition and Nrf2 activation. Our data suggest that PD is a potential clinical treatment for asthma.

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Authors' contributions

Qinqin Jin and Lei Zhao conceived and designed the study, and participated in drafting of the manuscript. Haizhu Zeng, Yumeng Wang and Yuxia Gu revised the manuscript, Jinrui Wang and Huali Zhang performed and analyzed the experiments. Hongchang Gao helped with data analysis and statistics. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All human tissue samples were collected and the protocol was approved by the bioethics committee of the Pudong New Area Gongli Hospital, Shanghai, China.

Conflict of interests

All authors declare they have no conflicts of interest.

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