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ORIGINAL ARTICLE

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CRYAB reduces cigarette smoke-induced inflammation, apoptosis, and oxidative stress by retarding PI3K/Akt and NF-κB signaling pathways in human bronchial epithelial cells

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KEYWORDS
CRYAB;
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Abstract
Background: Chronic obstructive pulmonary disease (COPD) is a familiar airway disease characterized by chronic immune response in the lungs. More and more evidences have assured that cigarette smoking is the primary reason for the progression of COPD, but its related regulatory mechanism requires further clarification. The α-B-crystallin (CRYAB) has been identified to exhibit vital functions in different diseases, and is down-regulated in the alveoli of mice mediated by cigarette smoke extract (CSE).
Methods: The messenger RNA expression of CRYAB was assessed by reverse transcription-quantitative polymerase chain reaction. The proteins' expressions were tested using Western blot method. The cytotoxicity was measured by lactate dehydrogenase assay. The levels of malondialdehyde, superoxide dismutase, catalase, myeloperoxidase, tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 were assessed through enzyme-linked-immunosorbent serologic assay (ELISA).
Results: In this study, it was discovered that the expression of CRYAB was markedly decreased with the increased time of cigarette smoking. Moreover, CRYAB overexpression increased cell viability and decreased cell apoptosis induced by cigarette smoke. In addition, the strengthened oxidative stress and inflammation mediated by CSE treatment was relieved after overexpression of CRYAB. Eventually, results OF Western blot method confirmed that CRYAB retarded the activation of phosphatidylinositol 3-kinase-Ak strain transforming (PI3K-Akt) and nuclear factor kappa B (NF-κB) signaling pathways.
Conclusion: Our results manifested that CRYAB reduced cigarette smoke-induced inflammation, apoptosis, and oxidative stress in normal and diseased bronchial epithelial (NHBE) and human bronchial epithelial (BEAS-2B) cells by suppressing PI3K/Akt and NF-κB signaling pathways, which highlighted the functioning of CRYAB in preventing or treating COPD.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a destructive illness that affects about 300 million people annually worldwide.[1,2] Research has established that COPD is displayed by chronic inflammation and progressive airway obstruction, resulting in chronic bronchiolitis and emphysema.[3] If not treated promptly, the normal breathing of patients gets irreversible and dyspnea occurs simultaneously. Present data indicate that COPD is estimated to kill more than 3 million people and is expected to kill about 8 million people by 2030, thus making it the third leading cause of death globally.[4] Research has confirmed that inflammation in COPD is contributory to major complications, including lung cancer.[5] A growing number of evidences have indicated that smoking affects the immune system and is a major contributor to COPD.[6,7] Some interventions, such as phosphodiesterase 4 inhibitors and surgery, are employed to restrict exacerbation of COPD, lessen airway obstruction, and ameliorate quality of life of COPD patients; however, the results have not been ideal.[8]

Small heat shock proteins (sHSP) are a class of chaperones with low molecular weight (12–43 kDa). HSPB5, also known as Crystallin Alpha B (CRYAB) or αB-crystallin, has N-terminal domain, central domain, and C-terminal domain.[9] It has a key role in regulating cell apoptosis, inflammation, and oxidative stress.[10] CRYAB is an accepted anti-apoptotic protein with a primary characteristic of regulating negatively the pro-apoptotic members of Bax and caspase-3.[11] CRYAB has also been discovered to restrain p53-dependent cell apoptosis triggered by calcium-induced rapidly accelerated fibrosarcoma–mitogen-activated protein kinase kinase–extracellular signal-regulated kinase (Raf/MEK/ERK) signaling pathways by retarding "rat sarcoma virus" (Ras) excitation.[12] Furthermore, CRYAB prevents ventricular arrhythmia by mitigating inflammation and oxidative stress in rats with autoimmune myocarditis.[13] In astrocyte exosomes, CRYAB expression is noticeably increased in response to stress and controlled lipopolysaccharide (LPS)-induced inflammation in microglia and astrocytes.[14] Importantly, CRYAB has been found to be down-regulated in the alveoli of mice treated with cigarette smoke extract (CSE),[15] although the relevant mechanism of action has to be investigated.

It has been demonstrated in the present study that CSE induced down-regulation of CRYAB, and the expression of CRYAB was reduced with increase of treatment time. Overexpression of CRYAB enhanced the cell viability induced by CSE, reduced cell apoptosis, oxidative stress, and inflammation as well as retarded the stimulation of phosphatidylinositol 3-kinase–Ak strain transforming (PI3K/Akt) and nuclear factor kappa B (NF-κB) signaling pathway. This discovery could offer a novel and useful biomarker for COPD treatment.

Materials and methods

Cell line and culture

All experiments were conducted using human bronchial epithelial cell lines (normal and diseased bronchial epithelial [NHBE] and human bronchial epithelial [BEAS-2B]) which were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were put in Dulbecco's Modified Eagle Medium (DMEM; Gibco™, Thermo Fisher Scientific Inc., MA, USA) with 10% fetal bovine serum (FBS; Hyclone, Logan City, UT, USA). They were kept in a humid incubator at 37°C with 5% carbon dioxide (CO_2). CSE (0, 6, 12, 24, and 36 h) was treated to above-mentioned cells.

Preparation of CSE

Preparation of CSE was in line with the method described in previous reports.[16,17] Cigarette smoke (400 mL) from commercial cigarettes (per cigarette with 2.5-mg nicotine and 12-mg tar; Shanghai, China) was put into modified 50-mL syringes. Smoke and serum-free DMEM (20 mL) were mixed by vigorous shaking, and this solution was deemed as 100% CSE. The solution was filtered using a 0.22-μm filter membrane (Millipore, MA, USA). CSE was used when its value at OD320–OD540 nm was 0.9–1.2. CSE solution was diluted by DMEM to 5% concentration and used in experiments within 15 min of preparation.

Cell transfection

The plasmid cloning DNA (pcDNA) 3.1 acquired from GenePharma (Shanghai, China) were intended to overexpress CRYAB (pc-CRYAB) and the empty pcDNA3.1 as negative control (NC). NHBE and BEAS-2B cells were transfected with these plasmids through Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The RNAs extracted from NHBE and BEAS-2B cell lines were carried through TRIzol reagents (Invitrogen). The synthesis of complementary DNA (cDNA) was exercised using the Prime-Script One Step RT-PCR kit (Takara Biotechnology Co. Ltd., Shanghai, China). RT-qPCR was performed by SYBR Premix Ex Taq™ (Takara, Shanghai, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was recognized as an internal reference. The $2^{-\Delta\Delta Ct}$ method was employed for data quantification.

The respective primers presented were as follows:
CRYAB
F: 5′-GAGTCCCTTCTACCTTCGG-3′,
R: 5′-CCATGCACCTCAATCACA-3′;
GAPDH
F: 5′-ATGTTCGTCATGGGTGTGAAC-3′,
R: 5′-ATGGACTGTGGTCATGAGTCC-3′.

Western blot method

The proteins extracted through radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific Inc.) were separated with 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on polyvinylidene fluoride (PVDF) membranes (Millipore). Then

the membranes were mixed with primary antibodies at 4°C overnight against CRYAB (1:1000; ab13497; Abcam, Shanghai, China), B-cell lymphoma 2 (Bcl-2) (1:1000; ab196495), Bax (1:1000; ab53154), cleaved caspase-3 (1:500; ab2302), phospho (p)-PI3K (1:1000; ab182651), PI3K (1:1000; ab191606), p-Akt (1:1000; ab38449), Akt (1:1000; ab8805), p-IκBα (1:10,000; ab133462), IκBα (1:1000; ab32518), p-p65 (1:1000; ab76302), p65 (1:1000; ab16502), nuclear factor erythroid 2-related factor 2 (Nrf2; 1:1000; ab137550), heme oxygenase-1 (HO-1; 1:2000; ab13243), and GAPDH (1:1000; ab8245). Next, the membranes were mixed with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000; ab7090; Abcam) for 2 h at room temperature. Finally, the bands were examined with chemiluminescence detection kit (Thermo Fisher Scientific Inc.). GAPDH served as an internal reference.

Lactate dehydrogenase (LDH) cytotoxicity assay

The levels of LDH were measured using commercial LDH kit (Jiancheng Institute of Bioengineering, Nanjing, China). The LDH activity of cell supernatant was detected at 450 nm.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed through commercial ELISA kits (R&D, Minneapolis, MN, USA) to determine the levels of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO), tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β in NHBE and BEAS-2B cell lines.

Statistical analysis

Statistical analysis was performed with THE SPSS 20.0 software. The data were presented as mean ± standard deviation (SD) of three independent experiments. Comparison in groups was done using Student's *t*-test or one-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

Results

Cigarette smoke-induced down-regulation of CRYAB expression

CRYAB has been investigated to play a vital function in numerous diseases, but its particular activity in human bronchial epithelial cells was triggered by CSE. First, the decreased messenger RNA (mRNA) expression of CRYAB was markedly discovered with the increased time of CSE (Figure 1A). In addition, the protein expression of CRYAB in NHBE and BEAS-2B cell lines indicated the same changes (Figure 1B). Moreover, the LDH levels were elevated with the increased time of CSE (Figure 1C). Hence, 24-h treat time was selected for additional experiments.

CRYAB decreased cell apoptosis and increased cell viability induced by cigarette smoke

CRYAB is an accepted anti-apoptotic protein. Its primary function is the negative regulation of pro-apoptotic members of Bax and caspase-3. As observed in Figure 2A, the protein expressions of Bcl-2 and CRYAB were down-regulated with CSE treatment, but this effect was reversed by overexpression of CRYAB. The protein expressions of Bax and cleaved caspase-3 were up-regulated with CSE treatment, but this change was carried through by overexpression of CRYAB. Furthermore, the enhanced LDH level mediated by CSE treatment was attenuated with overexpression of CRYAB (Figure 2B). These data revealed that CRYAB increased cell viability and decreased cell apoptosis induced by CSE.

CRYAB reduced oxidative stress induced by cigarette smoke

Additionally, it was revealed that the levels of oxidative stress of relevant proteins were also affected. Results demonstrated that the decreased levels of SOD

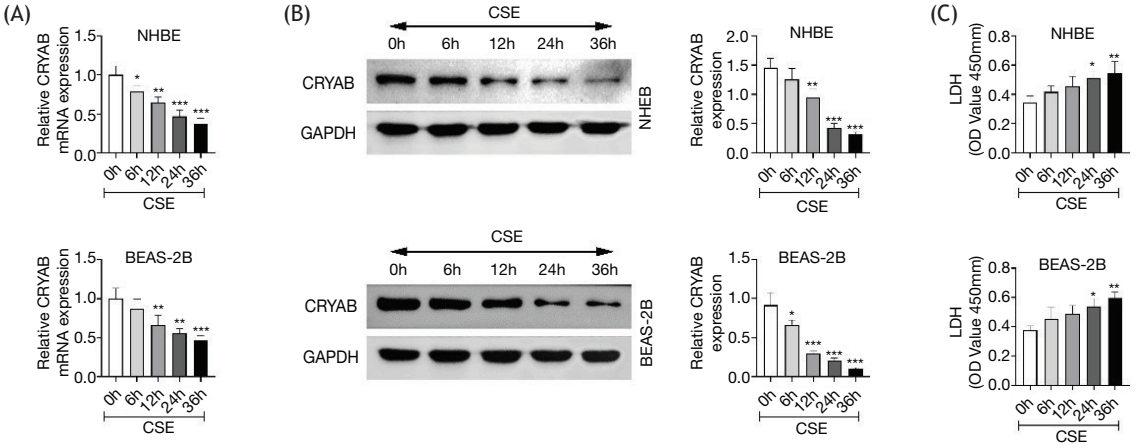

Figure 1 Cigarette smoke induced down-regulation of CRYAB expression. (A) The mRNA expression of CRYAB was examined in NHBE and BEAS-2B cell lines by RT-qPCR. (B) The protein expression of CRYAB was detected in NHBE and BEAS-2B cell lines by Western blot method. (C) The LDH levels were measured in NHBE and BEAS-2B cell lines using LDH detection kit. *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

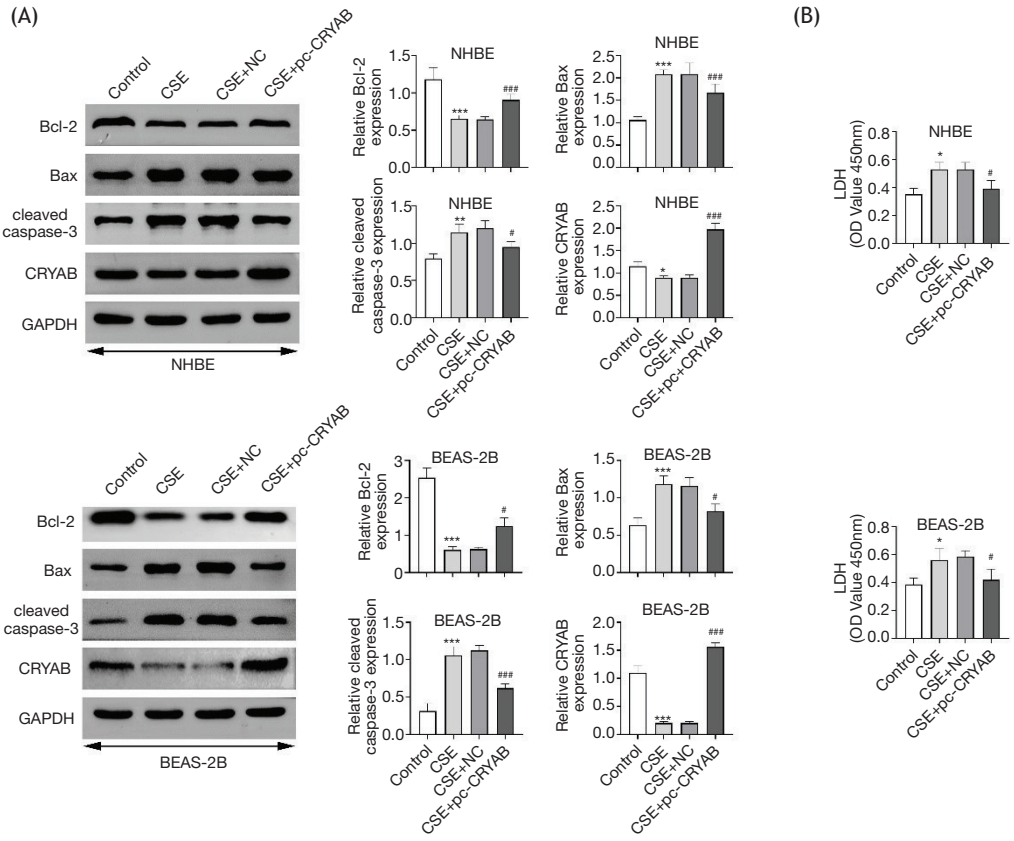

Figure 2 CRYAB increased cell viability and decreased cell apoptosis induced by cigarette smoke. Groups were divided as control, CSE, CSE+NC, and CSE+pc-CRYAB groups. (A) The protein expressions of Bcl-2, Bax, cleaved caspase-3, and CRYAB were tested in NHBE and BEAS-2B cell lines by Western blot method. (B) The LDH levels were examined in NHBE and BEAS-2B cell lines by LDH detection kit. *P<0.05, **P<0.01, and ***P<0.001 vs. the control group; #P<0.05 and ###P<0.001 vs. the CSE+NC group.

and CAT mediated by CSE were counterbalanced by up-regulation of CRYAB, and the increased levels of MDA and MPO induced by CSE were reversed by overexpression of CRYAB (Figure 3A). Next, the Nrf2/HO-1 pathway related to oxidative stress was assessed by Western blot method. The down-regulated levels of Nrf2 and HO-1 mediated by CSE treatment were reversed by overexpression of CRYAB (Figure 3B). These results proved that CRYAB reduced the oxidative stress triggered by CSE treatment.

CRYAB relieved inflammation induced by cigarette smoke

Next, the inflammation related proteins (TNF-α, IL-1β, and IL-6) were enhanced after CSE treatment, but these effects were neutralized by overexpression of CRYAB (Figure 4).

CRYAB retarded the activation of PI3K/Akt and NF-κB signaling pathways

Finally, it was confirmed that the protein levels of p-PI3K/PI3K, p-Akt/Akt, p-IκBα/IκBα, and p-P65/P65 were enhanced by CSE treatment, but these changes were reversed by overexpression of CRYAB (Figure 5). Overall, CRYAB retarded the stimulation of PI3K/Akt and NF-κB signaling pathways.

Discussion

Chronic obstructive pulmonary disease is a chronic illness with a variable range of survival and prognosis, which gravely threatens human health.[18] Evidence demonstrated that cigarette smoking is primary peril in COPD progression, and it could modulate cell apoptosis, inflammation, and oxidative stress of lung epithelial, vascular endothelial, and other cell types.[19,20] Therefore, in our experiments, CSE treatment was used to induce COPD cell model. Data established that the cytotoxicity (LDH level) was elevated with the increased time of CSE. CRYAB has been manifested to exhibit vital functioning in different diseases,[10,12-14] and has also been discovered as down-regulated in the alveoli of mice mediated by CSE.[15] In our work, we observed that the expression of CRYAB was markedly decreased with the increased time of CSE. Moreover, overexpression of CRYAB reduced cell apoptosis and enhanced cell viability induced by CSE.

The oxidative stress and inflammation exhibit important functioning in the progression of lung diseases. For instance, Zingerone modulates transforming growth factor beta 1 (TGF-β1) and inducible nitric oxide synthase (iNOS) expressions in pulmonary fibrosis triggered by bleomycin to

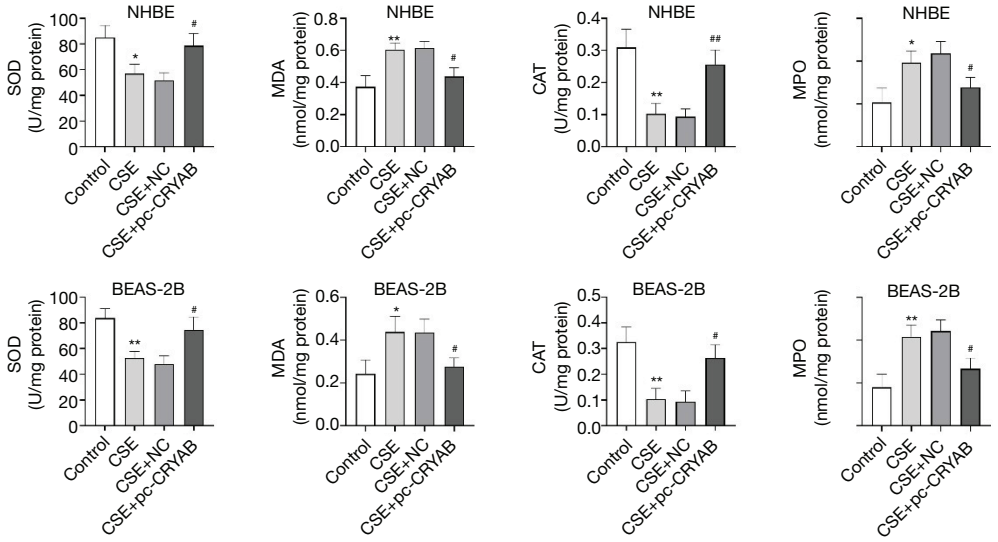

Figure 3 CRYAB reduced oxidative stress induced by cigarette smoke. Groups were divided as control, CSE, CSE+NC, and CSE+pc-CRYAB groups. (A) The levels of MDA, SOD, CAT, and MPO were assessed in NHBE and BEAS-2B cell lines by ELISA. (B) The protein levels of Nrf2 and HO-1 were evaluated by Western blot method. *P<0.05 and **P<0.01 vs. the control group; #P<0.05 and ##P<0.01 vs. the CSE+NC group.

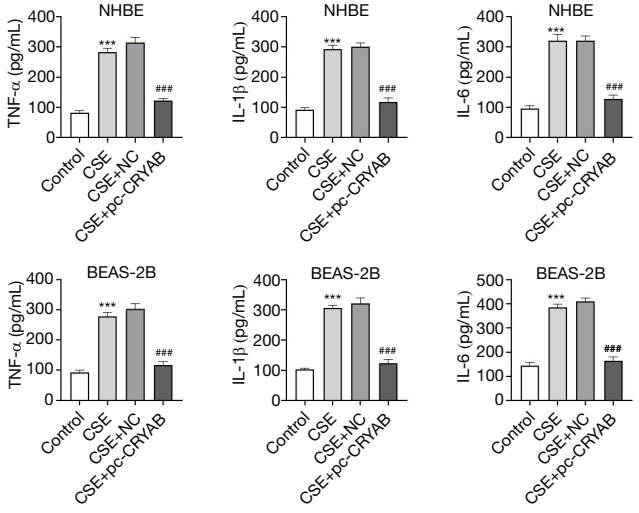

Figure 4 CRYAB relieved inflammation induced by cigarette smoke. Groups were divided as control, CSE, CSE+NC, and CSE+pc-CRYAB groups. TNF-α, IL-1β, and IL-6 levels were evaluated in NHBE and BEAS-2B cell lines by ELISA. ***P<0.001 vs. the control group; ###P<0.001 vs. the CSE+NC group.

attenuate oxidative stress and inflammation.[21] In addition, miR-223 targets high-mobility group protein B2 (HMGB2) in acute lung injury expression to affect oxidative stress.[22] Trilobatin exhibits anti-inflammatory functioning on LPS-triggered acute lung injury by affecting the AMPK/GSK3β-Nrf2 pathway.[23] Moreover, XIST/miR-370-3p/TLR4 axis contributes to LPS-mediated cell apoptosis and inflammation in acute pneumonia.[24] NLRP9b facilitates inflammation and oxidative stress to aggravate acute lung injury.[25] Importantly, oxidative stress and inflammation are now recognized as dominant predisposing factors in the development of COPD. For example, LINC00987 targets Let-7b-5p/SIRT1 axis to modulate LPS-triggered inflammation, oxidative stress, and autophagy in COPD.[26] Hsa_circ_0006872-miR-145-5p/NF-κB axis in human pulmonary microvascular endothelial cells (HPMECs) and BEAS-2B cells contributes to CSE-mediated oxidative stress and inflammatory response.[27] Resveratrol exhibits therapeutic effects to ameliorate inflammation and oxidative stress in COPD.[28] Alantolactone activates Nrf2/HO-1 and retards NF-κB pathways to reduce oxidative stress and inflammation in human bronchial epithelial cells mediated by CSE.[17] Ergosterol affects inflammation and oxidative stress to improve CSE-mediated COPD.[29] In our work, it was demonstrated that the strengthened oxidative stress and inflammation mediated by CSE treatment was relieved by overexpression of CRYAB.

The PI3K/Akt and NF-κB signaling pathways manifested key modulators related to cell survival, cell apoptosis, inflammation, and oxidative stress.[30,31] In addition, the PI3K/Akt and NF-κB signaling pathways demonstrated important functions in progression of COPD. For example, Scutellaria baicalensis ameliorates CSE-mediated COPD by regulating airway remodeling through PI3K/Akt/NF-κB pathway.[32] Bu-Shen-Fang-Chuan formula regulates the PI3K/Akt-Nrf2 and NF-κB pathways to reduce CSE-mediated inflammation in COPD.[33] Crocin suppresses inflammation to attenuate COPD-stimulated depression through PI3K/Akt pathway.[34] In this work, results of Western blot test revealed that CRYAB retarded the stimulation of PI3K/Akt and NF-κB signaling pathways.

In conclusion, it was evidenced for the first time that CRYAB reduced CSE-induced inflammation, apoptosis, and oxidative stress in NHBE and BEAS-2B cell lines by retarding PI3K/Akt and NF-κB signaling pathways. However, our findings about the affect of CRYAB on progression of COPD were limited. Additional experiments are required to investigate the functioning of CRYAB on COPD progression.

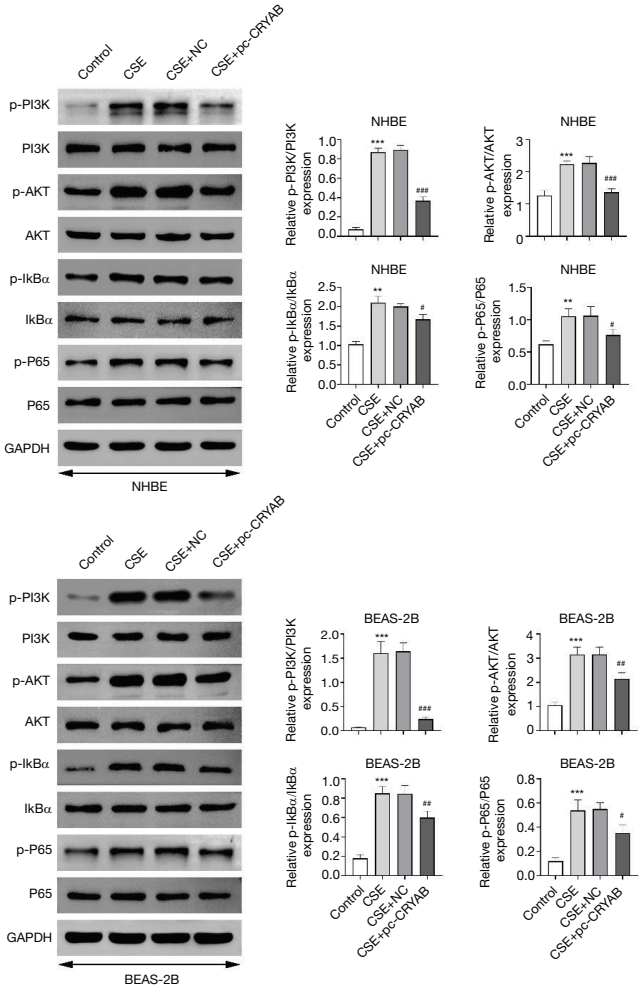

Figure 5 CRYAB retarded the activation of PI3K/Akt and NF-κB signaling pathways. Groups were divided as control, CSE, CSE+NC, and CSE+pc-CRYAB groups. The protein expressions of p-PI3K, PI3K, p-Akt, Akt, p-IκBα, IκBα, p-P65, and P65 were examined in NHBE and BEAS-2B cell lines by Western blot method. **P<0.01 and ***P<0.001 vs. the control group; #P<0.05, ##P<0.01, and ###P<0.001 vs. the CSE+NC group.

Competing Interests

The authors stated that there were no conflicts of interest to disclose.

Ethics Approval

This research was conducted by authors without human or animal participation.

Data Availability

The authors declare that all data pertaining to the findings of this study are available in the paper, and any raw data could be obtained upon request from the corresponding author.

Author Contributions

Shiliang Xie and Xiaofeng Wang designed and executed the experiments. Shiliang Xie analyzed and interpreted the data, and Xiaofeng Wang prepared the manuscript with contributions of both authors.

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