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Original Article

Lyciumbarbarum polysaccharide pretreatment mitigates cigarette smoke extract -induced oxidative stress in normal human bronchial epithelial cells through regulating Nrf2/Bach1 pathway

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Abstract: Free radical injury and oxidant/antioxidant imbalance are two mainstream pathological theories of asthma. The study was aimed to explore whether *Lyciumbarbarum* polysaccharide (LBP), which is one of active constituents of Chinese traditional medicine *Lyciumbarbarum,* can rescue human bronchial epithelial (NHBE) cells from cigarette smoke extract (CSE)-induced oxidative stress, and probe into the possibility of LBP as a prophylactic agent for asthma. Cell viability was detected using CCK-8 assay. ROS level and apoptosis rate were determined by the means of flow cytometry (FCM). Detection of mRNA and protein expression was carried out using real-time quantitive PCR (RT-qPCR) and Western blot. Our study found an CSE-induced elevation of ROS level in NHBE cells accompanied by inhibition of cell viablity and increase of apoptosis rate. In injured cells, oxidative stress increased the expression of Krüppel-like Factor 2 (KLF2), which results in the up-regulation of its downstream genes, including γ-glutamyl cysteine synthetase (γ-GCS) and Nuclear factor (erythroid-derived 2)-like 2 (Nrf2). KLF2 and Nrf2/Bach1 pathway interacted to increase compensatory expression of antioxidant γ-GCS in oxidative stress. As a result, we detected that LBP pretreatment was able to mitigate ROS increase and apoptosis abnormality in CSE-induced damage, and narrowed the differences in related gene expression between CSE injury model and normal NHBE cells through regulating KLF2 and Nrf2/Bach1 expression. The results suggested that LBP has great potential to be a new natural prophylactic agent for asthma patients.

Keywords: Asthma, *Lyciumbarbarum*polysaccharide, oxidative stress, CSE, γ-glutamyl cysteine synthetase (γ-GCS), Nrf2, Bach1

Introduction

Asthma is a serious global health problem affecting people of all ages, in recent decades, its prevalence is increasing worldwide [1]. The pathogenesis of asthma has been an area of intense investigation recently; however, its complete mechanism still remains unclear. Free radical injury and oxidant/antioxidant imbalance are demonstrated as two mostly related pathological mechanisms [2, 3]. Based on previous researches, antioxidant function of lung tissue in asthma patient was usually disordered, oxidaizing agent induced oxidant/antioxidant imbalance promoted development and deterioration of asthma [4, 5]. Oxygen radicals, which are inhaled from air or generated by inflammatory cells, can directly damage intracellualr biomacromolecules including proteins, nucleic acids, lipids and so on, resulting in dysfunction of cells, tissues and even organs [6].

Antioxidant system in airway epithelial cells is essential to maintain homeostasis. Poisonous components of cigarette are common stimulating factors of oxidative stress in daily life which stimulate key processes of airway inflammation and trigger oxidative stress activities [7]. Interactions between inflammation and oxidative stress further damage cellular structure and function, consequently leading cells to apoptosis abnormality. As the primary line of defense

from airway injury factors, airway epithelial cells are not only the key role in homeostasis maintenance, but also the first to be affected [8, 9].

The fruits of *Lyciumbarbarum* (family *Solanaceae*), commercially named goji berry, are characterized by various biological effects and pharmacological functions [10]. For years in China, *Lyciumbarbarum* fruits have been consumed to prevent and treat a number of chronic diseases, such as diabetes, hyperlipidemia, hepatitis, hypo-immunity function, thrombosis and male infertility. *Lyciumbarbarum* polysaccharide (LBP), a soluble polysaccharide extracted from the fruits, is the primary active ingredients contriubuting to those biological benefits [11]. Therefore, the present study was aimed to investigate the anti-oxidation effect of LBP on cigarette smoke extract (CSE)-induced oxidative stress in human bronchial epithelial (NHBE) cells, and explore thepotential of LBP as an effective prophylactic agent for asthma patients.

Materials and methods

Cells and CSE preparation

NHBE cells were purchased from Wuhan Procell Biotechnology Co. Ltd. (Wuhan, Hubei, China). CSE was prepared from ChungHwa cigarette (Shanghai Cigarette Factory, Shanghai, China) followed the protocol of Nakamura [12]. Smoke of one cigarette was collected by a 60 ml syringe, injected into a flask containing polyphenylene sulfite (PPS), and then sufficiently dissolved in PPS with shaking. Solution was suspended in 25 ml serum free DMEM in sealed bottle with shaking to be sufficiently dissolved. The pH value was adjusted to 7.3. After filtration sterilization, a part of CSE solution was diluted to 5%, 10%, 20%, 40%, and 80% for cell viability detection.

Cell grouping and treatment

NHBE cells were randomly distributed into normal control group, CSE group, LBP1 + CSE group and LBP2 + CSE group. Cells in LBP1 + CSE and LBP2 CSE groups were pretreated with 100 μg/mL and 300 μg/mL LBP, respectively, and then incubated for 4 h. After incubation, cells in these experimental groups and CSE group were treated with appropriate concentration of CSE.

CCK-8 assay

Cell viabilities with different CSE contents were determined using CCK-8 kit (Dojindo Molecular Technologies, Inc., Japan). NHBE cells were seeded into 96-well plates, grouped and respectively treated with 5%, 10%, 20%, 40%, 80% and 100% prepared CSE. Cells were incubated at 37°C in a 5% CO_2 incubator; 4 h after incubation, added with 10 μL CCK reagent in each well, and then putted into incubator for another 1-4 h. Optical density (OD) values of groups at each time point of 6 h, 12 h, 24 h and 48 h were read at 450 nm by microplate reader (Bio-Rad Laboratories, Inc., USA). Cell viability was calculated according to OD values. Appropriate treat time and concentration of CSE were selected based on the results.

Flow cytometry (FCM)

Intercellular ROS levels and apoptotic rates were detected by the means of FCM. Cells were seeded in 6-well plates at density of 2×10^4 cells/well, digested by EDTA free trypsin (Shanghai SangonBIotech Co. Ltd., Shanghai, China), stained by Annexin V-FITC and propidium iodide (Shanghai BestBio Science Co. Ltd., Shanghai, China), then incubated in dark place at room temperature for 15 min. Apoptosis rates in all groups were analyzed by EPICS XL-MCL FCM (Beckman Coulter, Inc., USA) with 488 nm of excitation wavelength and 530 nm of emission wavelength.

After washed with PBS, the cells collected from each groups were stained with DCFH-DA probe (Sigma-Aldrich, USA) (10 uM) in dark space for 30 min at 37°C. After that, cells were rewashed with pre-cold PBS, and the ROS generation was detected according to the fluorescence signals by flow cytometer assay (Calibur, Becton Dickinson, USA).

RT-PCR

Detection of mRNA expression in control, CSE, LBP1 + CSE and LBP2 + CSE group was carried out using RT-PCR and SYBR Green I chemistry (TransStart Top Green qPCR, SuperMix, TransGen Biotech Co., Ltd., Beijing, China). Cells were seeded into 6-well plates at a density of 2×10^6 cells/well. Total RNA were extracted by Trizol (Thermo Fisher Scientific Inc, New York, USA). Purity and concentration of extracted RNA were read through a UV spectrophotometer (Thermo

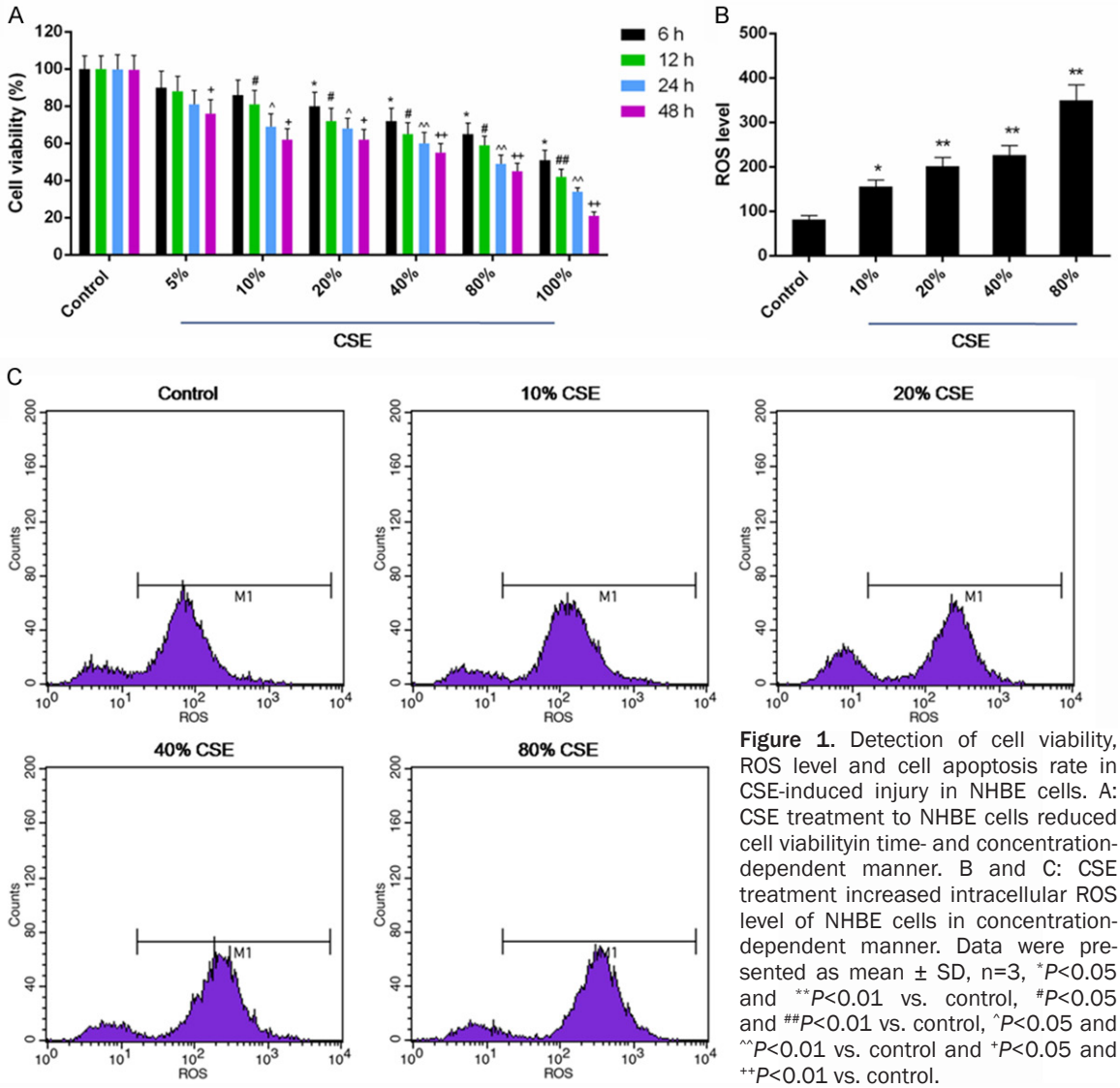

Figure 1. Detection of cell viability, ROS level and cell apoptosis rate in CSE-induced injury in NHBE cells. A: CSE treatment to NHBE cells reduced cell viabilityin time- and concentration-dependent manner. B and C: CSE treatment increased intracellular ROS level of NHBE cells in concentration-dependent manner. Data were presented as mean ± SD, n=3, $^{*}P<0.05$ and $^{**}P<0.01$ vs. control, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. control, $^{\wedge}P<0.05$ and $^{\wedge\wedge}P<0.01$ vs. control and $^{+}P<0.05$ and $^{++}P<0.01$ vs. control.

Fisher Scientific Inc, New York, USA). cDNA was synthesized by reverse transcription, and then fluorescence quantitative detection of target genes was performed. Reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) was applied as the internal control to monitor the efficiency of RT-PCR. Each reaction was run in triplicate. All primers in the study were designed by Shanghai Sangon Biotech Co. Ltd. (Shanghai, China). The specific primer sequences for each gene were listed as follows: 5' TGTTCCATGAAGGCAGAGCCA 3' and 5' CCTGGCAGCATCATCCACAC 3' for Caspase-3 (product: 91 bp); 5' AGGATCGAGCAGGGCGAATG 3' and 5' TCAGCTTCTTGGTGGACGCA 3' for Bax (product: 84 bp); 5' CCACCTGTGGTCCACCTGAC 3' and 5' GGCTGGACATCTCGGCGAA 3' for Bcl-2 (product: 85 bp); 5' GCACGCACACAGGTGAGAAG 3' and 5' AGAAGGCACGATCGCACAGA' for KLF2 (product: 144 bp); 5' GTCCGGTTGGTCCTGTCTGG 3' and 5' CGCATGTTGGCCTCAACTGT ' for γ-GCS (product: 182 bp); 5' GAGGGCAGTGGACTCTGAGG 3' and 5' AAAGCAGGAAAGGGCCAACC' for Nrf2 (product: 157 bp), 5' GCAGGAGTCCCAGCAGATGT 3' and 5' GGCTTTCAAGACGCTGCCAA' for Bach1 (product: 192 bp) and 5' TGGCCTTCCGTGTTCCTACC 3' and 5' TTCAGTGGGCCCTCAGATGC 3' for GAPDH (product: 121 bp).

Western blot

The protein level was determined using Western blot. Cells in each group were seeded into 6-well plates at a density of 2×10^6 cells/well, harvested and washed twice with PBS, protein lysed in ice-cold radio immunoprecipitation

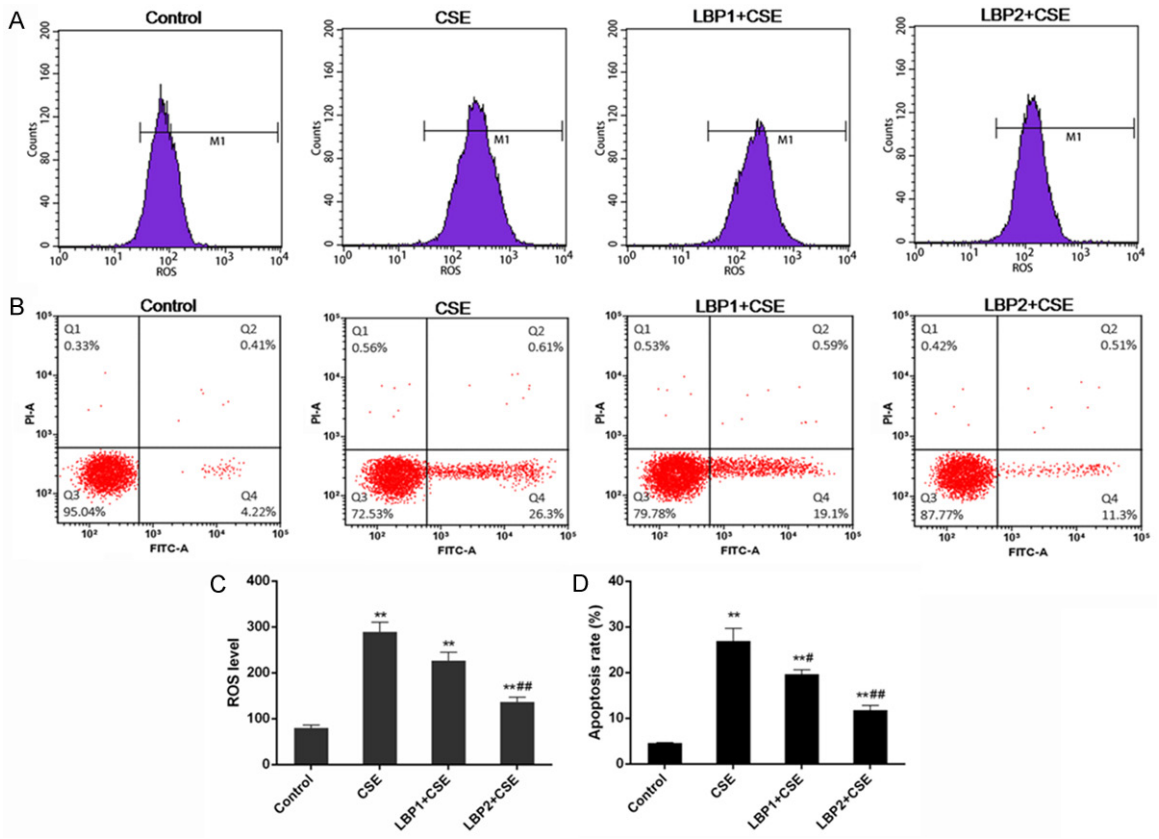

Figure 2. ROS level and apoptosis incontrol, CSE (40%) and LBP (100 and 300 μg/mL) + CSE (40%) groups were determined by FCM. A and C: LBP pretreatment in LBP (100 and 300 μg/mL) + CSE (40%) groups decreased CSE (40%)-induced increase in ROS level. B and D: LBP pretreatment mitigated CSE (40%)-induced cell apoptosis in LBP (100 and 300 μg/mL) + CSE (40%) groups. Data were presented as mean ± SD, n=3, $^{*}P<0.05$ and $^{**}P<0.01$ vs. control, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. CSE model (40%).

assay buffer with freshly mixed 0.01% protease inhibitor phenylmethanesulfonyl fluoride (Beijing O'BioLab Technology Co. Ltd., Beijing, China), then incubated for 30 min on ice. Cell lysis was centrifuged at 10,000×g for 5 min at 4°C, collected supernatants containing 20-30 μg of protein were run on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and electrophoretically transferred to a nitrocellulose membrane (Millipore, Shanghai, China). Protein levels of Caspase-3, Bax, Bcl-2, KLF2 and γ-GCS were detected. To research for protein level and nuclear translocation of Nrf2 and Bach1, cytoplasm and cell nucleus were separated by using CelLytic™ NuCLEAR™ Extraction Kit (Sigma-Aldrich Co. LLC, Darmstadt, Germany) according to instruction. Anti-glyceraldehyde 3-phosphate dehydrogenase (antiGAPDH) monoclonal antibody and Lamins-B2 monoclonal antibody were applied to estimate protein loading in respective cytoplasm and cell nucleus. Blots were visualized through an enhanced chemiluminescence (Thermo Fisher Scientific Inc., NY, USA).

Statistical analysis

Statistical analyses were performed using SPSS software, version 22.0 (SPSS, Inc., Chicago, IL, USA). Each experiment was repeated three times, with all the data presented as mean ± standard deviation. Differences between groups were assessed by means of two tailed student's t-test or one-way ANOVA followed by turkey's post hoc tests considering treatment time as variables. Statistical significance was defined as $P<0.05$ or $P<0.01$.

Results

CSE treatment reduced viability of NHBE cells

CCK-8 results illustrated that cell viability of NHBE cells was affected by CSE treatment with the increase of CSE concentration and treating

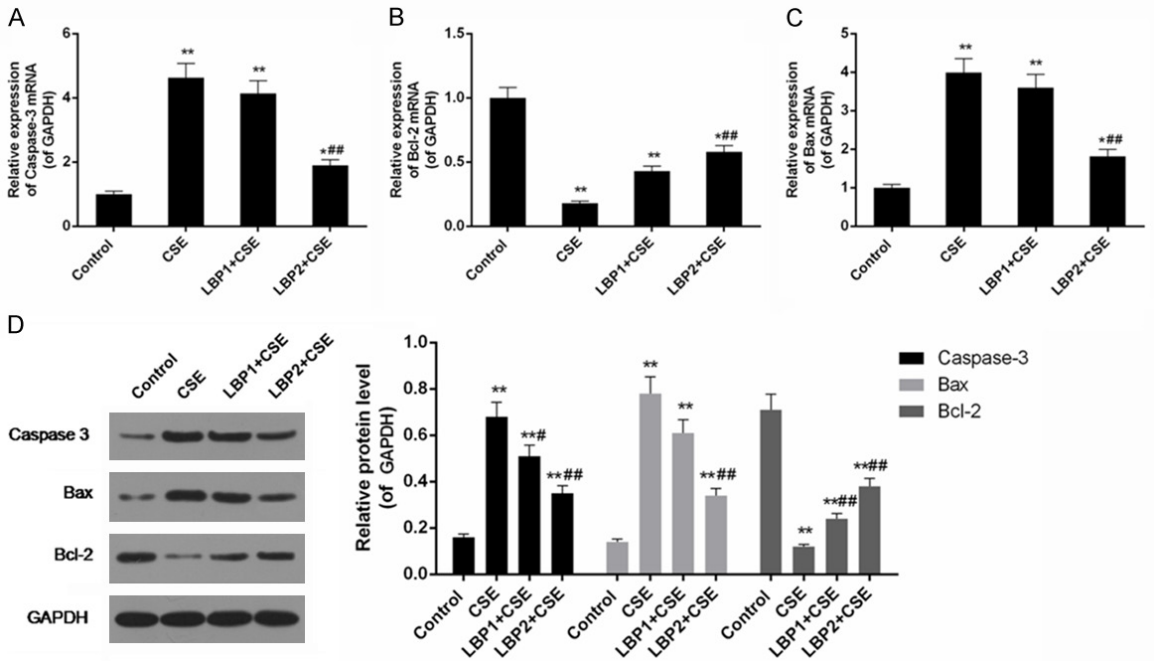

Figure 3. Detection of expression level of Caspase-3, Bax and Bcl-2 in control, CSE (40%) and LBP (100 and 300 μg/mL) + CSE (40%) groups. A: LBP pretreatment down-regulated the expression of Caspase-3 mRNA in CSE-induced oxidative stress. B: LBP pretreatment down-regulated the expression of Bax mRNA in CSE-induced oxidative stress. C: LBP pretreatment up-regulated the expression of Bcl-2 mRNA in CSE-induced oxidative stress. D: LBP pretreatment down-regulated protein level of Caspase-3 and Bax, and up-regulated protein level of Bcl-2 in CSE-induced oxidative stress. Data were presented as mean ± SD, n=3, $^{*}P<0.05$ and $^{**}P<0.01$ vs. control, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. CSE model (40%).

time (**Figure 1A**). With different concentrations of CSE from 10% to 100%, cell viability was significantly decreased in comparison to control group ($P<0.05$). In the study, treat time of 24 h was applied for further experiments. As 100% CSE is a scarcely possible condition in daily life, we selected 10%, 20%, 40% and 80% of CSE for next-step detection for determination of an appropriate concentration of ROS level.

CSE increased ROS level in NHBE cells

Evident increases of ROS level in cells treated with 10%, 20%, 40% and 80% CSE were detected using FCM ($P<0.01$ or $P<0.05$). Compared to control group, the influence of CSE on intracellular ROS level was positively correlated with CSE concentration (**Figure 1B** and **1C**). Concentration of 40% CSE was selected to construct CSE-induced injury model for following experiments.

LBP pretreatment reduced ROS level and mitigated apoptosis in CSE injured cells

By the means of FCM, protective effects of LBP from ROS enhancement and apoptosis abnormality were determined. In CSE model group, which was treated with 40% for 24 h, ROS level and apoptosis rate were distinctly increased in comparison with control group ($P<0.01$). With LBP pretreatment, obvious mitigation of the increase induced by CSE injury was detected. The ROS level in LBP2 + CSE group was reduced by nearly a half ($P<0.01$; **Figure 2A**, **2C**). The apoptosis rate was reduced from 26.91% ± 2.78% in CSE group to 19.69% ± 1.87% and 11.81% ± 1.02% in respective LBP1 + CSE and LBP2 + CSE group ($P<0.01$; **Figure 2B**, **2D**). The results implied that higher concentration of LBP in LBP2 + CSE had more effective protective effect.

LBP pretreatment down-regulated expression of Caspase-3 and Bax while up-regulated expression of Bcl-2 in CSE-induced injury

Expression level of apoptosis-related proteins, including Caspase-3, Bax and Bcl-2, were detected using RT-PCR and Western blot. CSE treatment to cells substantially up-regulated the expression of mRNA and protein level of Caspase-3 in comparison with control group

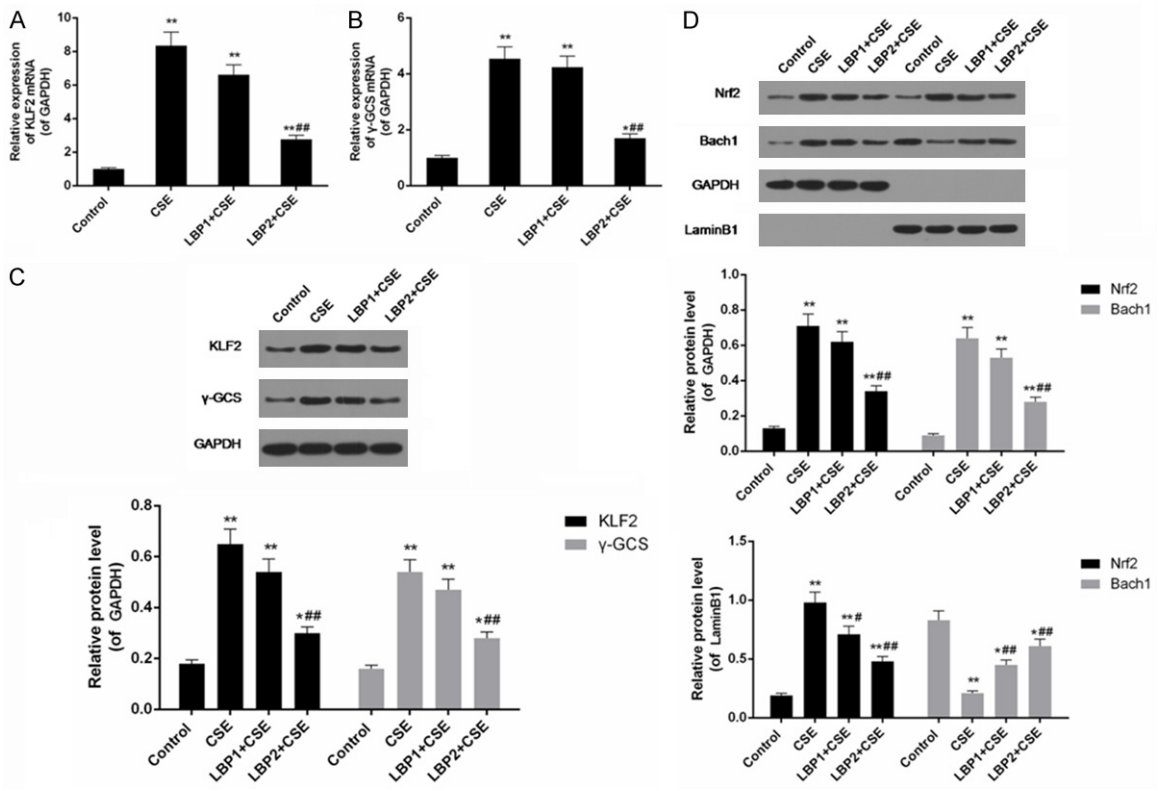

Figure 4. Expression of KLF2, γ-GCS, Nrf2 and Bach1 in control, CSE (40%) and LBP (100 and 300 μg/mL) + CSE (40%) groups. A: LBP pretreatment decreased the expression of KLF2 mRNA in CSE-induced injury. B: LBP pretreatment decreased the expression of γ-GCS mRNA in CSE-induced injury. C: LBP pretreatment reduced protein level of KLF2 and γ-GCS in CSE-induced injury. D: LBP pretreatment down-regulated the expression of Nrf2, and mitigated nuclear translocation of Nrf2 and Bach1 in CSE-induced injury. Data were presented as mean ± SD, n=3, $^{*}P<0.05$ and $^{**}P<0.01$ vs. control, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. CSE model (40%).

($P<0.01$), LBP pretreatment in LBP1 + CSE and LBP2 + CSE groups down-regulated the increasing expression of Caspase-3 compared with CSE group, particularly in LBP2 + CSE group ($P<0.01$). Moreover, in CSE modeling group, Bax expression was markedly up-regulated while Bcl-2 level was down-regulated compared to control group ($P<0.01$). With LBP pretreatment, increased expression of Bax and decreased level of Bcl-2 induced by CSE were significantly attenuated. However, there was significant difference between LBP2 + CSE and control group ($P<0.01$; **Figure 3A-D**).

LBP pretreatment decreased expression of KLF2 and γ-GCS in CSE-induced injury

It was showed that mRNA and protein levels of KLF2 and γ-GCS were obviously increased by CSE treatment ($P<0.01$). LBP pretreatment, especially in higher concentration, was detected to significantly reduce the levels of KLF2 and γ-GCS in CSE-induced injury ($P<0.01$) (**Figure 4A-C**).

LBP pretreatment mitigated changes of protein level of Nrf2 and Bach1 in CSE-induced injury

The protein levels of Nrf2 and Bach1 were determined using Western blot. The results showed a significant elevation of Nrf2 and Bach1 protein levels in cytoplasm in CSE-induced oxidative stress ($P<0.01$). By contrary, in cell nucleus, expression of Nrf2 was increased while Bach1 was reduced ($P<0.01$), which implied the nucleus translocation of Nrf2 and Bach1. With LBP pretreatment, the changes of protein level induced by CSE were mitigated, particularly in high concentration of LBP pretreated group ($P<0.01$; **Figure 4D**).

Discussion

Dynamic equilibrium of generating and clearing ROS in body is mostly dependent on natural oxidative and anti-oxidative systems. Imbalance of oxidants and antioxidants caused by biological or pathological factors including CSE can

induce oxidative stress, and then damage lung cells in asthma patients [13]. In the present study, we confirmed that CSE decreased viability of NHBE cells in a concentration-dependent manner, and meanwhile increased ROS level and apoptosis rate. NHBE cells injured by CSE can be rescued by LBP pretreatment. The study found that with LBP pretreatment, ROS increase and aberrant apoptosis were significantly attenuated, particularly in high LBP group.

The reduction of apoptosis abnormality accompanied with corresponding changes in apoptosis-related gene expressions such as Caspase-3, Bax and Bcl-2. In the process of cell apoptosis, Caspase-3, the primary cleavage enzyme, is activated through regulating Caspase-9 and/or Caspase-8 in mitochondrial pathway and/or death receptor-mediated pathway to induce cell apoptosis [14]. Interaction of anti-apoptotic gene Bcl-2 and pro-apoptotic gene Bax plays a significant role in influence of cell survive or perish. As the center of apoptosis regulation in Bcl-2 family, the expression of Bcl-2 and Bax proteins is commonly used as the marker for apoptosis in researches [15]. In CSE injured NHBE cells, Caspase-3 and Bax levels were markedly up-regulated while Bcl-2 expression was apparently reduced, leading to aberrant apoptosis. With LBP pretreatment, the differences in apoptosis-related gene expression between CSE injured and normal cells were diminished.

Rahman I. and MacNee W. detected that over one hundred times more content of reduced glutathione (GSH)-the important antioxidant in cell-was existed in airway epithelial cells than in plasma [16]. Apart from distinct antioxygenation property, GSH plays a major part in keeping the integrity of airway epithelial cells and relieving airway inflammatory, and is involved in numerous physiological processes, such as synthesis of proteins and nucleic acids, regulation of genes, and so on [17]. γ-GCS is a rate-limiting enzyme of GSH which is able to catalyze holoenzyme but is suppressed by GSH feedback [18, 19]. KLF2 is one of the members in Krüppel transcription factor family. Based on previous studies, KLF2 targets γ-GCS, and is also able to up-regulate expression of antioxidant genes, such as heme oxygenase-1 (HO-1) and NAD (P) H: quinineoxidoreductase (NQO1) through promoting nuclear localization of Nrf2 [20, 21]. γ-GCS and KLF2 expression reflects the antioxidantive ability of cells [22]. In CSE induced oxidative stress with high ROS level, it was detected that not only KLF2 but also γ-GCS were highly expressed in the both mRNA and protein levels. High level of KLF2 in injured cells promoted the expression of γ-GCS, compensatory up-regulation of γ-GCS was to resist of oxidative stress. Furthermore, we found that LBP pretreatment apparently decreased the abundance of KLF2 and γ-GCS in CSE injured NHBE cells, which reflected the protective function of LBP from oxidative stress.

Nrf2, belonging to CNC transcription factor family, is also one of the target genes of KLF2. Nrf2 is a primary transcription factor to regulate expression of antioxidant genes including γ-GCS in response to oxidative stress [23]. In normal physiological condition, inactive Nrf2 combines with Kelch-like ECH-associated protein 1 (KEAP1) in cytoplasm while active Nrf2 exists in cell nucleus to take part in gene mediation. Under the condition of oxidative stress, inactive Nrf2 begins to dissociate from KEAP1-Nrf2, and then translocates into cell nucleus. Nrf2 accumulated in cell nucleus combines with specific ARE sequence of γ-GCS to regulate gene transcription [23]. Bach1, one of members in CNC transcription factor family, is able to down-regulate the expression of antioxidant genes by competitively combining with MAF protein identification element to Nrf2. Most of Bach1 proteins gather in cell nucleus in normal condition, but translocate into cytoplasm to combine with hyaluronan-binding proteins to regulate gene expression in respond to oxidative stress [24, 25]. In the condition of oxidative stress, kfl2 as the important anti-oxidative regulating gene of Nrf2 in upstream up-regulates Nrf2 expression and promotes nuclear translocation to improve nuclear stability. Moreover, klf2 is also likely to down-regulate Bach1 expression in mRNA level in cell nucleus and stimulate its transposition. The combined action of KFL2, Nrf2 and Bach1 results in abundance of anti-oxidative gene γ-GCS. In our present study, wedetected nuclear translocation of transcription factors Nrf2 and Bach1 in CSE model group, and provided evidence ofCSE-induced oxidative stress. In injured NHBE cells, Nrf2 was increasingly expressed and translocated into cell nucleus while Bach1 was translocated into cytoplasm to up-regulate the

expression of antioxidant gene γ-GCS. With LBP pretreatment, abundance of Nrf2 and Bach1 in cytoplasm was reduced, and nuclear translocation was also decreasd, which was possibly resulted from protective effect of LBP on CSE-induced oxidative stress.

Conclusion

CSE induced oxidative stress in NHBE cells with increasing ROS level, resulting in cell viability inhibition and apoptosis abnormality. In respond to the injury, KLF2 was up-regulated to enhance the level of anti-oxidative gene γ-GCS. Nrf2 and Bach1 were highly likely to participate in the regulation of γ-GCS expression. LBP pretreatment was able to rescue NHBE cells from CSE-induced increases of ROS level and apoptosis rate, and markedly narrowed the differences in related gene expression between CSE model and normal NHBE cells.

Disclosure of conflict of interest

None.

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