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Betulin inhibited cigarette smoke-induced COPD in mice

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ABSTRACT

The purpose of the present study was to evaluate the protective effect of betulin (BE) on CS (cigarette smoke)-induced COPD in mice and explore its underlying mechanisms. 60 male ICR mice were randomly assigned to five groups: control group, model group, dexamethasone (2 mg/kg) group, BE (20 mg/kg) group and BE (40 mg/kg) group. The COPD mice were induced by cigarette smoke exposure for 8 weeks. The result of H&E staining demonstrated that BE inhibited CS-induced pathological injury in lung tissue. Besides, BE could restore the activities of superoxide dismutase (SOD) in serum and in lung, catalase (CAT) in serum and reduce the content of malondialdehyde (MDA) in serum and in lung. BE also inhibited the overproductions of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β). Furthermore, the administration of BE significantly inhibited the protein expression of ROCK/NF-κB pathway in CS-induced mice. Our findings suggested that BE might effectively ameliorate the progression of COPD via ROCK/NF-κB pathway in mice.

1. Introduction

Cigarette smoke (CS)-induced chronic obstructive pulmonary disease (COPD), a common chronic respiratory disease, is the third leading cause of death in China. COPD has a serious impact on quality of life and produces enormous costs to the health system [1], while its pathogenesis has not been fully elucidated. The doctrines of pulmonary inflammation, oxidative imbalance and protease-antiprotease imbalance are considered to be responsible for the progression of COPD [2]. Despite numerous achievement has been made before, there are no effective disease-modifying drugs for COPD. Thus, there is an urgent need to search for the efficient medicine for the intervention of COPD.

As a serine/threonine kinase, Rho-kinase has been identified as one of the downstream effector proteins of Rho. The Rho/ROCK signaling pathway is involved in pro-inflammatory molecule generations [3], ainflammatory [4], apoptotic [5], depression [6], aging [7], neuroprotective [8,9] and cardioprotective effects [10,11]. Rho protein is also implicated in the regulation of NF-κB signal transduction cascades related to inflammation [12,13]. Betulin (lup-20(29)-ene-3β, 28-diol), a pentacyclic triterpene alcohol with a lupane skeleton, is extracted from birch tree bark and exhibits diverse pharmacological activities, such as anti-tumor, anti-HIV, anti-viral, anti-bacterial anti-inflammatory activities [14,15]. This experiment aims at studying the effect of BE on CS-induced COPD mice and investigating its potential mechanism.

2. Materials and methods

2.1. Main reagents and kits

BE (purity 98%) was purchased from National Institutes for Food and Drug Control (Beijing, China). Dexamethasone (Dex) was provided by Simcere Drug Store (Nanjing, China). Cigarettes were purchased from Hongta Tobacco Group Company Limited. TNF-α, IL-6 and IL-1β enzyme-linked immuno-sorbent assay (ELISA) kits were supplied by Nanjing KeyGEN Biotech. Co., Ltd. (Nanjing, China). MDA and SOD commercial kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Antibodies against Rho, ROCK1, ROCK2, IκBα, p-IκBα, NF-κBp65 and p-NF-κBp65 were produced by Cell Signaling Technology (Danvers, USA).

2.2. Animals

60 male ICR mice (weighing 20–22 g) were purchased from Comparative Medicine Centre of Yangzhou University. All animals were housed in a specific pathogen-free (SPF) laboratory in the Animal Center of China Pharmaceutical University at 22 °C ± 1 °C temperature and 40–50% humidity under a 12 h light/dark cycle with free access to water and standard laboratory chow. All

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procedures were approved by the Institutional Ethics Committee for Animal Experimentation and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.3. Experimental protocol

60 male ICR mice were randomly assigned to five groups: control group, model group, dexamethasone (2 mg/kg) group, BE (20 mg/kg) group and BE (40 mg/kg) group. All mice (except those in control group) were exposed 5 days a week to the mainstream cigarette smoke of 5 cigarettes (Reference cigarette3R4F without filter, University of Kentucky, Lexington, KY, USA), 4 times a day with a 10 min smoke free interval between exposures. A standard smoking apparatus was used with the smoking chamber adapted for a group of mice. A smoke/air ratio of 1/6 was obtained. Control mice were exposed to room air simultaneously. CS exposure started at the weight of 20–22 g and the exposure period was 8 weeks. The mice in group of dexamethasone (Dex) were treated with 2 mg/kg/d dexamethasone by intragastric administration, followed by 4 weeks of smoke exposure. The mice in low and high dose group of BE were treated with 20 mg/kg/d and 40 mg/kg/d BE by intragastric administration, followed by 4 weeks of smoke exposure. The mice in group of control and model were treated with the same amount of distilled water in the same way.

2.4. Tissue preparation

Blood samples were collected from the orbit and centrifuged at 4500 rpm for 15 min. The supernatant was collected and set aside at −80 °C for the evaluations of antioxidant and anti-inflammation activities of BE. The lungs were harvested subsequently and stored at −80 °C for histological observation and protein quantification.

2.5. Cytokine measurements

The levels of IL-6, IL-1β and TNF-α in serum were measured using an ELISA assay as described in the manufacturer's protocol.

2.6. Measurement of CAT in serum, SOD and MDA levels in serum and lung

The activities of CAT *in serum*, SOD and the content of MDA in serum *and lung* were determined using the kits according to the manufacturer's instruction.

2.7. Histopathology examination

For the histological analysis, mice were sacrificed after the collection of orbital blood. After gross examination, the extracted lungs were fixed in 10% buffered formalin and further fixed for at least 24 h. Sections (4 μm thick) were cut from paraffin-embedded tissues, placed on poly-L-lysine-coated slides, and then incubated overnight at 55–60 °C. Deparaffinized sections were stained with hematoxylin and eosin (H & E). After that, pathological conditions in the lung tissues were visualized under a light microscope.

2.8. Western blot

For the examination of the proteins related to the ROS-MAPK/NF-κB signaling pathways in mice and HBE cells, western blot was conducted. Firstly, the lung tissues were cut to smash the tissues, homogenated with RIPA buffer and proteases inhibitor for 30 min on ices. Then the tissue homogenates was centrifuged at 12000 rpm for 15 min and the supernatant was collected and stored at −20 °C. The total protein in the lung tissues was determined by the BCA protein assay kit (Beyotime, Nanjing, China). The supernatant was added the SDS-PAGE loading buffer at the ratio of 4:1, mercaptoethanol at ratio of 20:1, and then boiled in boiling water for 5 min. After the protein separation by SDS-PAGE electrophoresis, the protein was transferred to the nitrocellulose membrane (NC) membrane. Afterwards, the membranes were block with 5% skim milk in TBST buffer at room temperature for 2 h. Membranes containing target proteins were incubated with primary antibodies diluted in TBST buffer at 4 °C overnight. After washed in TBST buffer for 3 times, the membranes were incubated with second antibodies for 2 h. The the proteins were visualized using an ECL Key-GEN system (KeyGEN Biotechnology, Nanjing, China) and detected with a Clinx ChemiScope chemiluminescence imaging system (Gel Catcher 2850, China)

2.9. Statistical analysis

Experimental data were expressed as means ± standards deviation (SDs). Differences between groups were determined by one-way ANOVA with the Tukey multiple comparison test. P-values < 0.05 were considered to be significant. Calculations were made using Graphpad Prism.

3. Results

3.1. Effects of BE on antioxidant activity

Superoxide dismutase (SOD) and catalase (CAT) exert the protective effect against oxidative stress, which keep a balance between the producting and scavenging of ROS. As depicted in Fig. 1, the CS-induced COPD model group significantly reduced the levels of SOD in serum and lung and CAT in serum, when compared with those in control group. However, animals with the treatments of BE (20, 40 mg/kg) and dexamethasone (2 mg/kg) significantly increased the levels of SOD in serum and lung and CAT in serum compared with those in model group. The results demonstrated BE could increase the antioxidant capacity of SOD and CAT in COPD mice.

3.2. Effect of BE on lipid peroxidation

Malondialdehyde (MDA) concentration has been frequently employed as an index of lipid peroxidation, indicating the oxidative injury of the cell plasma membrane. The CS-induced COPD model group significantly increased the level of MDA in serum and in lung compared with that in control group. In contrast, the BE (20 and 40 mg/kg) group both significantly suppressed the elevation of MDA content in serum and in lung, revealing the ameliorative effect of BE on lipid peroxidation (Fig. 2).

3.3. Effects of BE on pro-inflammatory factors

As shown in Fig. 3, the CS-induced COPD mice model group displayed a marked increase in the levels of TNF-α, IL-6 and IL-1β in lung tissue and serum. Compared with those in model group, BE (20 and 40 mg/kg) groups apparently reduced the contents of TNF-α, IL-6 and IL-1β in lung tissue and serum, indicating that BE prevented COPD progression via the reductions of pro-inflammatory factors. The finding demonstrated that BE could suppress CS-stimulated inflammation by inhibiting the generations of pro-inflammatory cytokines in COPD mice.

3.4. Histopathological examination of lung tissues

To evaluate the protective role of BE on pulmonary physiological impairment, hematoxylin and eosin (H&E) staining was performed. As observed in Fig. 4, in control group, scarce obvious