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Phenylbutyric acid inhibits epithelial-mesenchymal transition during bleomycin-induced lung fibrosis

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HIGHLIGHTS

- UPR signaling is activated in the pathogenesis of BLM-induced pulmonary fibrosis.
- PBA, an ER chemical chaperone, inhibits BLM-evoked UPR activation in the lungs.
- PBA inhibits BLM-induced pulmonary NF-κB activation in mice.
- PBA alleviates BLM-induced epithelial mesenchymal transition and lung fibrosis.

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ABSTRACT

A recent report showed that unfolded protein response (UPR) signaling was activated during bleomycin (BLM)-induced pulmonary fibrosis. Phenylbutyric acid (PBA) is an endoplasmic reticulum (ER) chemical chaperone that inhibits the UPR signaling. The present study investigated the effects of PBA on BLM-induced epithelial-mesenchymal transition (EMT) and pulmonary fibrosis. For induction of pulmonary fibrosis, all mice except controls were intratracheally injected with a single dose of BLM (3.0 mg/kg). In PBA + BLM group, mice were intraperitoneally injected with PBA (150 mg/kg) daily. Three weeks after BLM injection, EMT was measured and pulmonary fibrosis was evaluated. BLM-induced pulmonary UPR activation was inhibited by PBA. Moreover, BLM-induced pulmonary nuclear factor kappa B (NF-κB) p65 activation was blocked by PBA. In addition, BLM-induced up-regulation of pulmonary inflammatory cytokines was repressed by PBA. Further analysis showed that BLM-induced α-smooth muscle actin (α-SMA), a marker for EMT, was significantly attenuated by PBA. Moreover, BLM-induced pulmonary collagen (Col1α1 and Col1α2) was obviously inhibited by PBA. Importantly, BLM-induced pulmonary fibrosis, as determined using Sirius red staining, was obviously alleviated by PBA. Taken together, these results suggest that PBA alleviates ER stress-mediated EMT in the pathogenesis of BLM-induced pulmonary fibrosis.

1. Introduction

Idiopathic pulmonary fibrosis, characterized by fibroblast proliferation and extracellular matrix remodeling, is a chronic pulmonary disease of unknown origin ultimately leading to death (Borchers et al., 2011; King et al., 2011). Bleomycin (BLM), a widely used antineoplastic drug, causes a dose-dependent interstitial pulmonary fibrosis (Adamson and Bowden, 1974). Intratracheal instillation of BLM into the lungs of rodent animals causes alveolar cell damage, an inflammatory response, epithelial-mesenchymal transition (EMT) and subsequent extracellular matrix deposition, resembling human interstitial pulmonary fibrosis (Moore and Hogaboam, 2008). BLM-induced pulmonary fibrosis has been the most commonly used model for idiopathic pulmonary fibrosis (Moeller et al., 2008). Nevertheless, the mechanisms of BLM-induced pulmonary fibrosis are not completely understood.

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Endoplasmic reticulum (ER) is an important organelle required for normal cellular function. In the ER, nascent proteins are folded with the assistance of ER chaperones. Accumulation of unfolded and misfolded proteins aggregated in the ER lumen causes ER stress and activation of a signal response termed unfolded protein response (UPR) (Wu and Kaufman, 2006). The UPR signaling is mediated by three transmembrane ER proteins: inositol requiring ER-to-nucleus signal kinase (IRE) 1, activating transcription factor (ATF) 6 and double-stranded RNA-activated kinase (PKR)-like ER kinase (PERK) (Kohno, 2007). An earlier report showed that the level of processed p50 ATF6 and spliced x-box binding protein (sXBP)-1, a downstream molecule of IRE1 pathway, was significantly increased in the lungs of IPF patients (Lawson et al., 2008). Two recent studies found that pulmonary UPR signaling was activated in the pathogenesis of BLM-induced pulmonary fibrosis (Lawson et al., 2011; Zhong et al., 2011).

Phenylbutyric acid (PBA) is a low molecular weight fatty acid that has been used for treatment of urea cycle disorders in children and sickle cell disease (Burlina et al., 2001). Numerous studies have demonstrated that PBA acts as a chemical chaperone that inhibits ER stress and UPR signaling activation (Basseri et al., 2009). Indeed, PBA could attenuate ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes (Ozcan et al., 2006). Moreover, PBA inhibits UPR signaling activation and protects against leptin resistance in the hypothalamus of obese mice (Ozcan et al., 2009). Recently, we showed that PBA not only significantly attenuated ER stress but also inhibited fructose-evoked hepatic SREBP-1c activation and lipid accumulation (Zhang et al., 2012). In humans, PBA partially alleviated lipid-induced insulin resistance and β-cell dysfunction (Xiao et al., 2011). According to a recent report, pretreatment with PBA significantly reduced ischemia–reperfusion-induced inflammation, apoptosis and necrosis, and improved liver regeneration through inhibiting UPR signaling activation (Ben Mosbah et al., 2010). Nevertheless, whether PBA protects against BLM-induced pulmonary fibrosis remains to be determined.

The aim of the present study was to investigate the effects of PBA on BLM-induced EMT and pulmonary fibrosis in mice. We demonstrate for the first time that PBA inhibits not only BLM-induced pulmonary ER stress but also pulmonary inflammation and subsequent EMT in the pathogenesis of BLM-induced lung fibrosis. Moreover, PBA effectively protects against BLM-induced pulmonary fibrosis in mice.

2. Materials and methods

2.1. Chemicals and reagents

BLM and PBA were purchased from Sigma Chemical Co., (St. Louis, MO). GRP78, IRE1α, phosphor-IRE1α, C/EBP homologous protein (CHOP), and phosphor-eukaryotic initiation factor 2α (eIF2α) antibodies were from Cell Signaling Technology (Beverley, MA). NF-κB p65, α-smooth muscle actin (α-SMA) and β-actin antibodies, horseradish peroxidase-conjugated goat anti-rabbit, goat anti-mouse and anti-donkey anti-goat IgGs were from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA). TRI reagent was purchased from Molecular Research Center, Inc., (Cincinnati, OH), RNase-free DNase, AMV and GoTaq® qPCR master mix were from Promega Corporation (Madison, WI). All specific primers were synthesized by Life Technologies Corporation (Carlsbad, CA). Chemiluminescence (ECL) detection kits were obtained from Thermo Fisher Scientific Inc., (Rockford, IL). Polyvinylidene fluoride (PVDF) membrane was from Milipore Corporation (Belford, MA). All other reagents were purchased from Sigma Chemical Co., (St. Louis, MO) if not otherwise stated.

2.2. Animals and treatments

Adult female CD-1 mice (8 week-old, 28–32 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc. The animals were allowed free access to food and water at all times and maintained on a 12 h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50 ± 5%) environment. For the induction of pulmonary fibrosis, mice were intratracheally injected with a single dose of BLM (3.0 mg/kg body weight in 50 μL phosphate buffered saline). The doses of BLM used in the present study referred to others (Zhao et al., 2014). To investigate the effects of PBA on BLM-induced pulmonary fibrosis, mice were intraperitoneally (i.p.) injected with PBA (150 mg/kg) daily, beginning at 30 min before BLM. The doses of PBA used in the present study referred to others (Zhang et al., 2012). Control mice received an i.p. injection of NS daily. All mice were euthanized by exsanguination during pentobarbital anesthesia (75 mg/kg, i.p.) three weeks after BLM injection. Lung fibrosis was assessed by Sirius red staining as well as lung histology. Some lung samples were collected and kept at −80 °C for subsequent immunoblots and real-time RT-PCR. This study was approved by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University (Permit Number: 13-0016). All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

2.3. Pulmonary histology

Lung tissues were fixed in 4% formalin and embedded in paraffin according to the standard procedure. Paraffin-embedded lung tissues were serially sectioned. At least five consecutive longitudinal sections were stained with hematoxylin and eosin (H and E) and scored for the extent of pathology on a scale of 0–5, where 0 was defined as no lung abnormality, and 1, 2, 3, 4, and 5 were defined as the presence of inflammation involving 10%, 10–30%, 30–50%, 50–80%, or >80% of the lungs, respectively. Lung fibrosis was evaluated by Sirius red staining for collagen accumulation. The percentages of collagen deposition areas were quantified using NIH ImageJ software (http://rsb.info.nih.gov/ij/).

2.4. Immunoblots

Total pulmonary lysate was prepared by homogenizing 50 mg lung tissue in 300 μL lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsylphate, 1 mM phenylmethylsulfonyl fluoride) supplemented with a cocktail of protease inhibitors (Roche). For nuclear protein extraction, total pulmonary lysate was suspended in hypotonic buffer and then kept on ice for 15 min. The suspension was then mixed with detergent and centrifuged for 30 s at 14,000 × *g*. Protein concentrations were determined with bicinchoninic acid (BCA) protein assay reagents (Pierce, Rockford, IL) according to manufacturer's instructions. For immunoblots, same amount of protein (30–60 μg) was separated electrophoretically by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membranes were incubated for 2 h with the following antibodies: α-SMA, CHOP, GRP78, phosphor-IRE1α, IRE1α and phosphor-eIF2α. For total proteins, β-actin was used as a loading control. After washes in DPBS containing 0.05% Tween-20 four times for 10 min each, the membranes were incubated with goat anti-rabbit IgG or goat anti-mouse antibody for 2 h. The membranes were then washed for four times in DPBS containing 0.05% Tween-20 for 10 min each, followed by signal development using an ECL