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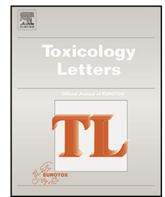

Suppression of nuclear factor erythroid 2-related factor 2 via extracellular signal-regulated kinase contributes to bleomycin-induced oxidative stress and fibrogenesis

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HIGHLIGHTS

- Fibrogenic changes in lung is accompanied by Nrf2 suppression and oxidative stress.
- BLM interferes with Nrf2 activation in lung fibroblasts via ERK.
- Suppressed Nrf2-tuned antioxidant system stimulating cellular ROS promotes fibrogenesis in lung fibroblasts.
- Nrf2 activator attenuates BLM-induced fibrogenesis in lung fibroblasts.

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ABSTRACT

Pulmonary fibrosis is a serious and irreversible lung injury with obscure etiologic mechanisms and no effective treatment to date. This study explored a crucial link between oxidative stress and pulmonary fibrogenesis, focusing on nuclear factor erythroid 2-related factor 2 (Nrf2), a core transcription factor in antioxidative regulation systems. Treatment of C57 BL/6 mice with bleomycin increased fibroblast viability and collagen production and significantly downregulated Nrf2. In addition, prominent oxidative stress was indicated by changes in superoxide dismutase, catalase activity, and glutathione and thiobarbituric acid-reactive substance levels. In a cell-based model, bleomycin suppressed Nrf2 activation via extracellular signal-related kinase phosphorylation, enhancing intracellular reactive oxygen species in lung fibroblasts and stimulating abnormal cell proliferation and collagen secretion. To confirm this novel mechanism of bleomycin-induced fibrogenesis, we attempted to upregulate Nrf2 and related antioxidant proteins in bleomycin-treated fibroblasts using a putative Nrf2 activator, caffeic acid phenethyl ester, and the results showed that bleomycin-induced fibroblast proliferation and collagen content were attenuated through improved redox balance. Collectively, these results disclose a potential regulatory mechanism in pulmonary fibrosis that will aid the development of new therapies.

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1. Introduction

Pulmonary fibrosis (PF) is characterized by altered cellular composition of the alveolar region with excessive deposition of collagen (Thannickal et al., 2004; Wynn, 2008). Its etiology remains obscure. Lung inflammation has long been considered a major underlying component of a wide variety of pulmonary fibroproliferative disorders (Harari and Caminati, 2010; Lasky and Ortiz, 2001). However, anti-inflammatory compounds have produced poor therapeutic results in the treatment of PF. Similarly, corticosteroids combined with immunosuppressants such as cyclophosphamide or azathioprine, colchicine, and other agents have provided no more than 30% improvement in objective indices (Morawiec et al., 2011; Shah et al., 2005). Therefore, new

Abbreviations: ROS, reactive oxygen species; Nrf2, nuclear factor erythroid 2-related factor 2; BLM, bleomycin; PF, pulmonary fibrosis; ARE, antioxidant response element; CAPE, caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester; HE, hematoxylin and eosin; SOD, superoxide dismutase; GSH, glutathione; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DCF-DA, dichlorofluorescein diacetate; NBT, nitroblue tetrazolium; γ-GCS, γ-glutamylcysteine synthetase; DMSO, dimethyl sulfoxide.

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therapeutic agents based on novel mechanisms with improved efficacy are needed to ameliorate or slow the progression of fibrosis.

Examples from in vitro studies have implied that lung fibrosis can be attenuated through regulation of fibroblast proliferation and collagen production, the main processes of fibrogenesis (Chen et al., 2008; Lu et al., 2010). Fibroblasts are important cells in lung interstitium and are responsible for the majority of the collagen protein expressed therein (Scotton and Chambers, 2007). The activation, differentiation, proliferation, and apoptosis of fibroblasts are closely related to their cellular redox states and the oxidant/antioxidant balance (Amara et al., 2010; Bocchino et al., 2010; Ramirez et al., 2007). Additionally, abnormal signaling of reactive oxygen species (ROS), the production of which occurs under normal physiological conditions in the human body, and the accompanying oxidative stress are believed to be important factors in lung fibrosis (Bargagli et al., 2009; Kliment and Oury, 2010). Several studies have focused on the inflammatory effects induced by excess ROS or oxidative stress (Albrecht et al., 2005; Hosakote et al., 2009; MacNee, 2001); however, few reports have demonstrated the direct effects of cellular redox disequilibrium on fibrogenesis, a late and key stage of PF (Walters et al., 2008).

To maintain proper redox balance, the respiratory system is endowed with an antioxidant defense mechanism consisting of endogenous antioxidant enzymes. Expression of most antioxidant enzymes is tightly controlled by the antioxidant response element and activated by nuclear factor erythroid 2-related factor 2 (Nrf2), a redox-sensitive transcription factor (Singh et al., 2010). Previous reports have highlighted the protective effects of Nrf2 activation in reducing oxidative stress in many pulmonary disorders (Kode et al., 2008; He et al., 2008). In the lung, the dysfunction of cellular defense systems resulting from Nrf2 deletion has been proven to increase animal's susceptibility to acute lung injury (Wu et al., 2012) or pulmonary emphysema (Boutten et al., 2010). Importantly, a series of paper from Kleeberger's Lab reported Nrf2 deficiency in lung exacerbated toxicity caused by multiple oxidative insults including hyperoxia (Cho et al., 2012), virus (Cho et al., 2009) or bleomycin (Cho et al., 2004). Thus, there is a clear demonstration of the critical role of Nrf2 in regulating pulmonary disease. However, direct evidence of its role in fibrotic process and the mechanism through which it is regulated during this process remain to be uncovered. Mitogen-activated protein kinases (MAPKs) are well-conserved enzymes connecting cell surface receptors to intracellular regulatory targets; they are activated in response to a wide variety of stimuli. Recent evidence has shown that extracellular signal-related kinase (ERK) activation increases in the bleomycin hydrochloride (BLM) model of lung fibrosis (Galuppo et al., 2011). In the present study, we showed that BLM directly induces fibrogenesis in lung fibroblasts by exaggerating ERK activity in vitro. In the lungs of BLM-induced PF mice, the expression of Nrf2 was significantly depressed in late-stage fibrosis. This depressed expression was associated with oxidative stress. Induction of Nrf2 overexpression by caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester (CAPE), an activator of Nrf2 (Balogun et al., 2003; Lee and Surh, 2005), reduced BLM-induced ROS production, inhibited cell proliferation and collagen secretion, and upregulated the expressions of catalase (CAT), superoxide dismutase (SOD), and gamma-glutamylcysteine synthetase (γ-GCS) in lung fibroblasts. However, knockdown of Nrf2 expression by Nrf2-specific small interfering RNA (siRNA) had reverse effects in cells. These results indicate that Nrf2 and its downstream antioxidants are significant regulators in BLM-induced oxidative stress and fibrogenesis. Furthermore, we propose a new strategy to prevent fibrogenesis and PF based on the modulation of the redox status of lung fibroblasts.

2. Materials and methods

2.1. Reagents and cell culture

BLM (12 U/8 mg, >98% pure) was provided by Taihe Biotechnology. CAPE was purchased from Sigma Aldrich. Antibodies against Nrf2, collagen I and III, and β-actin were obtained from Santa Cruz Biotechnology; anti-SOD2, catalase, and γ-GCS were from Abcam; and antibodies against ERK1/2 and phosphorylated ERK1/2 were from Bioworld. RPMI 1640 medium and fetal bovine serum were obtained from Hyclone. Other chemical reagents were purchased from Sigma Aldrich.

Primary fibroblasts were isolated from the lungs of mice as previously described (Phan et al., 1985). Cells from the murine fibroblast L929 cell line were obtained from the animal laboratory of the Fourth Military Medical University (Xi'an, China). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin and cultured in an incubator at 37 °C with 5% CO_2. The cells were passaged at preconfluent densities using a solution containing 0.05% trypsin and 0.5 mM ethylenediaminetetraacetic acid.

2.2. Animal model of BLM-induced PF

Eight-week-old male C57 BL/6 mice weighing 18.5 ± 2.2 g were obtained from the animal laboratory of Fourth Military Medical University. Experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*, Department of Health, Education and Welfare Publication No. (National Institutes of Health) 85–23, 1985. All procedures involving animals were approved by the institutional review committee. A 12-h light/dark cycle was maintained, and the mice had free access to water and food.

An animal model of PF was induced in mice as previously described (Liu et al., 2007) with minor modification. Briefly, after recording the weight of each mouse, we anesthetized them with an intraperitoneal injection of 100 mg/kg phenobarbital sodium. A midline incision was made in the neck and the trachea was exposed via blunt dissection. A tracheal cannula (i.d., 2 mm; length, 2.5 cm) was inserted into the trachea under direct visualization. BLM dissolved in 250 μL phosphate-buffered saline was instilled into the lungs via the tracheal cannula at a dose of 7.5 U/kg body weight. Sterile saline was administered to animals in a sham operation group. The animals were shaken to facilitate distribution of BLM or saline throughout the lung. Mice were killed on day 21 (as well as on days 7 and 14) after treatment. PF was determined through histological check.

2.3. Tissue preparation for biochemical analysis

Lung tissues were obtained from mice for biochemical analysis, and contaminating blood was washed out with ice-cold buffered saline. The specimens were weighed and cut thinly with a clean scalpel blade and then homogenized in 0.15 M ice-cold KCl for 3 min at 16,000 rpm with a homogenizer (Ultra Turrax Type T-25-B; Labortechnic, Staufen, Germany). The homogenates were then centrifuged for 1 h at 4 °C and 5000 × *g*. All measurements were performed using the supernatant.

2.4. Hydroxyproline (OH-proline) content

Tissue samples used for OH-proline determination were washed with physiological saline and dried at 100 °C over 72 h. OH-Proline levels were determined spectrophotometrically using the method reported by Woessner (1961) after the samples were weighed and hydrolyzed in concentrated HCl (12 mol/L) at 130 °C for 3 h. After each sample was adjusted to a final volume of 1 mL with deionized water, the samples were centrifuged at 3000 × *g* for 15 min to obtain supernatant. A second centrifugation at 2500 × *g* for 10 min was performed after the addition of isopropanol to produce an equal volume of supernatant. Serial dilutions of commercially purified OH-proline (Sigma) were used as a standard. The OH-proline concentrations of the samples were calculated using the absorbance–concentration curve derived from the standard OH-proline solutions. Results were expressed in milligrams per gram of dry tissue.

2.5. Redox markers

The extent of lipid peroxidation in the lungs was determined by measuring the thiobarbituric acid-reactive substance (TBARS) level (Yagi, 1976). Total SOD activity was determined according to the method reported by Kono (1978). SOD activity was also expressed in units per milligram of protein. CAT activity was determined according to the method of Pang et al. (2000). Results were expressed as K (rate constant) per gram of protein. Reduced glutathione (GSH) in samples was measured fluorometrically as described previously (Senft et al., 2000). Total protein concentration was determined using the Lowry et al. (1951) method.

2.6. Western blotting analysis

The lung tissues or harvested cells were lysed on ice for 30 min in 1 mL/100 mg or 1 mL/well lysis buffer (150 mM NaCl, 50 mM Tris [pH 8], 1% Nonidet P 40, and 100 mM sodium orthovanadate, protease inhibitor cocktail [Sigma]). Extracts were clarified via centrifugation at 20,000 × *g* for 20 min. The supernatant was collected

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