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Celastrol enhances Nrf2 mediated antioxidant enzymes and exhibits anti-fibrotic effect through regulation of collagen production against bleomycin-induced pulmonary fibrosis

Thomas Divya [a], Vadivel Dineshbabu [a], Syamala Soumyakrishnan [a], Anandasadagopan Sureshkumar [b], Ganapasam Sudhandiran [a, *]

[a] Cell Biology Laboratory, Department of Biochemistry, University of Madras, Guindy Campus, Chennai 600 025, India
[b] Department of Biochemistry, Central Leather Research Institute, Chennai 600 020, India

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ABSTRACT

Pulmonary fibrosis (PF) is characterized by excessive accumulation of extracellular matrix components in the alveolar region which distorts the normal lung architecture and impairs the respiratory function. The aim of this study is to evaluate the anti-fibrotic effect of celastrol, a quinine-methide tri-terpenoid mainly found in Thunder God Vine root extracts against bleomycin (BLM)-induced PF through the enhancement of antioxidant defense system. A single intratracheal instillation of BLM (3 U/kg.bw) was administered in rats to induce PF. Celastrol (5 mg/kg) was given intraperitoneally, twice a week for a period of 28 days. BLM-induced rats exhibits declined activities of enzymatic and non-enzymatic antioxidants which were restored upon treatment with celastrol. BLM-induced rats show increased total and differential cell counts as compared to control and celastrol treated rats. Histopathological analysis shows increased inflammation and alveolar damage; while assay of hydroxyproline and Masson's trichrome staining shows an increased collagen deposition in BLM-challenged rats that were decreased upon celastrol treatment. Celastrol also reduces inflammation in BLM-induced rats as evidenced by decrease in the expressions of mast cells, Tumor necrosis factor-alpha (TNF- α) and matrix metalloproteinases (MMPs) 2 and 9. Further, Western blot analysis shows that celastrol is a potent inducer of NF-E2-related factor 2 (Nrf2) and it restores the activities of Phase II enzymes such as hemoxygenase-1 (HO-1), glutathione-S-transferase (GSTs) and NADP(H): quinine oxidoreductase (NQO1) which were declined upon BLM administration. The results of this study show evidence on the protective effect of celastrol against BLM-induced PF through its antioxidant and anti-fibrotic effects.

1. Introduction

Pulmonary fibrosis (PF) is a devastating and ir-reversible human interstitial lung disease characterized by chronic interstitial inflammation, deposition of excessive amounts of collagen resulting in impaired lung function and respiratory failure [1,2]. Though the precise pathologic mechanisms of PF are not fully understood, fibrosis is thought to be the result of an aberrant wound-healing response to sequential lung injury [3]. The development of PF is associated with influx of activated inflammatory cells like neutrophils, eosinophils and mast cells in lung parenchyma which are likely to be among the first immune cells exposed to pro-inflammatory and toxic agents [4]. Once activated, mast cells can rapidly release a wide range of preformed mediators including histamine and serotonin. These vasoactive chemotactic mediators sequestered in the lung during inflammatory process mediate chain of events that leads to fibrosis [4]. When inflammation persists, a chronic fibrotic process develops, which is characterized by replacement of extracellular matrix (ECM) by abnormal fibrillar collagen and collagen producing fibroblast and myofibroblast. The infiltrating inflammatory cells induce the secretion of matrix remodeling proteinases, predominantly the matrix metalloproteinases (MMPs). MMPs are a family of ECM degrading, zinc-

* Corresponding author.
E-mail addresses: sudhandiran@yahoo.com, sudhandiran@unom.ac.in (G. Sudhandiran).

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dependent enzymes comprising over 18 members. Among these MMPs, two collagenolytic MMPs (MMP 2 and MMP 9) have been reported to possess substrate specificity to type IV collagen which occupies a significant portion of the basement membrane structure in the lung and can degrade basement membrane structures via collagenolytic actions [5,6].

The etiology of PF is not completely elucidated till now. However, there are reports that oxidative stress and cellular redox status contribute to its progression [7,8]. During inflammation, several inflammatory mediators and cytokines release large amounts of reactive oxygen species (ROS), which may be involved in tissue injury and in impeding tissue repair, which leads to PF [9]. There is evidence of disruption of the normal oxidant/antioxidant balance in the lungs of PF patients [10]. To diminish the potential toxicity of ROS, animals possess several cellular and extracellular enzymatic and non-enzymatic antioxidant systems. Among these, classic antioxidant enzymes, including superoxide dismutases (SOD), catalase, and glutathione peroxidases (GPx), directly inactivate ROS and prevent ROS-initiated reactions [11]. Phase II detoxifying enzymes, including glutathione-S-transferases (GSTs) and NADP(H): quinine oxidoreductase (NQO1), also indirectly act as antioxidant enzymes by controlling biosynthesis of thiols or facilitating excretion of oxidized reactive secondary metabolites [12]. Heme oxygenase-1 (HO-1) is a rate-limiting enzyme that catalyzes the degradation of heme to biliverdin, carbon monoxide and iron. It is one of the antioxidant response element (ARE) - regulated phase II detoxifying enzymes and antioxidants, which are regulated by the redox-sensitive transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2) [13]. Nrf2 is a member of Cap-N-collar transcription factor family and recognizes the AREs in the promoter of target genes [14]. Under normal condition, Nrf2 is held with Keap1 (Kelch like ECH associated protein) in the cytoplasm due to an association between a single Nrf2 protein and a Keap1 dimer, which, in turn, enhances the ubiquitination and subsequent proteolysis of Nrf2 [15]. Oxidative stress or treatment of cells with ARE inducers causes dissociation of Nrf2 from Keap1, allowing Nrf2 to travels to the nucleus and binds to a DNA promoter and initiates transcription of anti-oxidative genes and their proteins. Based on the observations of our own laboratory as well as from other investigators, Nrf2 has been reported as an important therapeutic target in the treatment of PF [16,17].

Celastrol, a quinine-methide tri-terpenoid derived from the traditional Chinese medicine 'God of Thunder Vine' is reported to have anti-cancer and anti-oxidative properties [18,19]. It suppresses the production of inflammatory cytokines such as interleukin-1 (IL-1), TNF-α, IL-6, and IL-8, induces the heat shock response, and disrupts heat shock protein 90 (Hsp90), possibly through its interaction with cdc37 and co-chaperone p23 [20]. Celastrol also inhibits nuclear factor-kappa B (NF-κB) activation and arrests the cell cycle [21]. In this study, we have elucidated the anti-fibrotic effect of celastrol in reducing inflammation and collagen production through the enhancement of Nrf2 mediated antioxidants against BLM-induced experimental PF.

2. Materials and methods

2.1. Chemicals

Primary antibodies were purchased from Santa Cruz biotechnology, USA. BLM was procured from Sigma–Aldrich Co. (St. Louis, MO, USA). Celastrol was purchased from Cayman Chemicals, USA. All other chemicals and reagents used in this study were of analytical grade.

2.2. Animals

Male Wistar albino rats weighing between 200 g and 240 g were purchased from Central Animal House, King Institute of preventive medicine and research center, Chennai, India. The animals were acclimatized to the laboratory conditions for a period of 2 weeks. The animals were housed under standard conditions of 12 h light/dark cycles and were given a standard rat feed (Hindustan Lever Ltd., Bangalore) and water *ad libitum*. The experiments were conducted according to ethical norms approved by the Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethical Committee Guidelines (IAEC No.12/01/2014). Care was taken to minimize animal suffering.

2.3. Bleomycin-induced pulmonary fibrosis

The mode of induction of PF in animals has been well established in our laboratory [22,23]. Briefly, after the weight was recorded, the rats were anesthetized via i.p. injection of 0.5 mg/kg of ketamine and 1 mg/kg of xylazine. A tracheal cannula (i.d. = 2 mm, length = 2.5 cm) was inserted into the trachea under direct visualization. Rats (n = 6) received a single dose of 3 U/kg (based on body weight) bleomycin sulfate (Sigma, St. Louis, USA) dissolved in 0.3 ml of 0.9% NaCl solution by intratracheal instillation on day 1 of the experimental period. Control rats were given a single intratracheal dose of saline alone.

2.4. Experimental design

Initially, a preliminary study was conducted with five different doses of celastrol (1, 2, 5, 7 and 10 mg/kg body weight) to determine the dose dependent effect of celastrol in BLM-induced rats. Animals were administered with different doses of celastrol in BLM-induced rats for 28 days. It was observed that celastrol treatment at a dose of 5 mg/kg b.w resulted in modulation of the abnormalities in the lung histopathology near to normal. At this dose, celastrol significantly ($p < 0.05$) altered the lung hydroxyproline and lactate dehydrogenase (LDH) closer to normal level. Further, it was also observed that the levels of lipid peroxidation and myeloperoxidase (MPO) in bronchoalveolar lavage fluid (BALF) were altered to near normal level in BLM-induced rats after the experimental study. Increased concentration of celastrol (7 and 10 mg/kg b.w) also renders protection to BLM-induced rats, but there is no significant difference as compared with 5 mg/kg b.w. Hence, the dose of 5 mg/kg b.w was chosen for this study.

The animals were divided in the following four groups (six rats in each group). Group I served as normal control rats; Group II served as BLM-induced rats; and Group III served as treatment group where celastrol (dissolved in 0.01% DMSO) at a dosage of 5 mg/kg b.w. was administered intraperitoneally twice a week in BLM-induced rats, throughout the experimental period. Group IV rats served as normal rats treated with celastrol alone at a dosage of 5 mg/kg b.w.

At the end of the experimental period of 28 days, animals were sacrificed by decapitation. Both lungs were quickly excised, washed with ice-cold physiological saline, and homogenized in 0.1 M Tris–HCl buffer (pH 7.4) using a tissue homogenizer with a teflon pestle at 4 °C. The resultant tissue homogenate was used for further experiments.

2.5. Isolation of bronchoalveolar lavage fluid (BALF)

BALF was obtained by washing the lungs four times with 5 ml