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Bilirubin treatment suppresses pulmonary inflammation in a rat model of smoke-induced emphysema

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ABSTRACT

Background: Cigarette smoking is a significant risk factor for emphysema, which is characterized by airway inflammation and oxidative damage.
Objectives: To assess the capacity of bilirubin to protect against smoke-induced emphysema.
Methods: Smoking status and bilirubin levels were recorded in 58 patients with chronic obstructive pulmonary diseases (COPD) and 71 non-COPD participants. The impact of smoking on serum bilirubin levels and exogenous bilirubin (20 mg/kg/day) on pulmonary injury was assessed in a rat model of smoking-induced emphysema. At sacrifice lung histology, airway leukocyte accumulation and cytokine and chemokine levels in serum, bronchoalveolar lavage fluid (BALF) and lung were analyzed. Oxidative lipid damage and anti-oxidative components was assessed by measuring malondialdehyde, superoxide dismutase (SOD) activity and glutathione.
Results: Total serum bilirubin levels were lower in smokers with or without COPD than non-smoking patients without COPD ($P < 0.05$). Indirect serum bilirubin levels were lower in COPD patients than patients without COPD ($P < 0.05$). In rats, cigarette smoke reduced serum total and indirect bilirubin levels. Administration of bilirubin reduced mean linear intercept and mean alveoli area, increased mean alveoli number, reduced macrophage, neutrophil and TNF-α content of BALF, and increased BALF and serum IL-10 level, but lowered local and systemic CCL2, CXCL2, CXCL8 and IL-17 levels. Bilirubin suppressed the smoke-induced systemic and regional oxidative lipid damage associated with increased SOD activity.
Conclusion: Bilirubin attenuated smoking-induced pulmonary injury by suppressing inflammatory cell recruitment and pro-inflammatory cytokine secretion, increasing anti-inflammatory cytokine levels, and anti-oxidant SOD activity in a rat model of smoke-induced emphysema.

1. Introduction

Chronic obstructive pulmonary disease (COPD) has become a major public health problem with high morbidity and mortality, affecting millions of people worldwide [1]. COPD is characterized by irreversible chronic inflammation and progressive airflow limitation and emphysema. Cigarette smoke (CS) is a significant risk factor for COPD, and about 80–90% of COPD patients have a current or past history of cigarette smoking. CS contains toxic components which may provoke inflammatory responses and oxidative stress [2].

Neutrophils, macrophages and lymphocytes are involved in the development of COPD. These cells generate reactive oxygen species, which in turn activate inflammatory cells to release inflammatory mediators, including LTB4, TNF-α, IL-17 and chemokines [1,3–6]. IL-17 promotes the secretion of CXCL-8 and TNF-α, and also recruits neutrophils, thus prolonging their activity in the lungs, and causing localized inflammation [6–9]. CXCL8, CXCL2, and CCL2 recruit neutrophils and monocytes to inflammatory sites [10–14]. These chemokines play an important role in COPD pathogenesis by promoting infiltration of inflammatory cells.

Bilirubin, a heme metabolic end product containing conjugated (direct) and unconjugated (indirect) bilirubin, has long been considered to be toxic. Bilirubin has been shown to exhibit anti-inflammatory and anti-oxidative activities in various pathologies

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including septicemia, endotoxemia, transplantation and ischemia-reperfusion injury by reducing inflammatory factors products and oxidative stress, inhibiting inflammatory cell infiltration, and down-regulating adhesion molecule expression [15]. Bilirubin has also been reported to be beneficial in various clinical conditions [16–18], indicating that bilirubin protects against smoke-induced pulmonary inflammation and oxidative damage in emphysema.

We hypothesized that bilirubin treatment could attenuate the development of emphysema by modulating inflammatory cell infiltration, the secretion of inflammatory cytokines and chemokines, and oxidative stress. A rat model of smoke-induced emphysema was used to test our hypothesis.

2. Materials and methods

2.1. Patient data collection

Male patients diagnosed with COPD at the Department of Pulmonary Medicine, Shanxi Medical University Second Hospital between May 1, 2011 and May 31, 2014 were enrolled in a retrospective study. COPD diagnosis was based on the 2014 GOLD Guidelines [1], FEV1/FVC% of every COPD patient was lower than 70% (the standard in GOLD) and the mean of FEV1/FVC% of all COPD patients was 57.38 ± 13.43%. The Patients with hepatobiliary, pancreatic or hemolytic diseases that could affect serum bilirubin levels were excluded. Non-COPD control patients were recruited from the orthopedic, ENT, ophthalmology, and dentistry departments and had no digestive, hemolytic or respiratory diseases. Data extracted from medical records for both groups included age, diagnosis, smoking history, and serum total bilirubin (Tbil), direct (conjugated) and indirect (unconjugated) bilirubin levels (Dbil and Ibil, respectively). Patients were divided into five groups: non-smoking/non-COPD group, smoking/non-COPD group, non-smoking/COPD group, smoking/COPD group, smoked/COPD group (Table 1). Patients categorized as “smoking” were those that had smoked 10–30 cigarettes every day for 10–50 years. Participants categorized as “smoked” had previously qualified as smokers, but had ceased to smoke within the last year. Bilirubin level was defined as “low” if it was below the mean minus standard deviation of all control patients. This study was reviewed and approved by the Board for Human Subject Research, Shanxi Medical University. All patients provided written informed consent.

2.2. Induction of experimental emphysema and its intervention

Male Wistar rats at 8 weeks of age were purchased from the Experimental Animal Center, Chinese Academy of Military Science and randomly divided into three groups (n = 10 rats per group). Two groups were exposed to the smoke of 20 unfiltered commercial cigarettes (Houwang, 11 mg tar and 1.1 mg nicotine/cigarette, Baoji Cigarette Factory, Baoji, Shaanxi, China) in a closed 0.54 m^3 space once per day (5 min per cigarette with 10 min smoke free intervals) 6 days a week for 12 week in a COPD modeling smoke exposure device (PAB-S200, Beijing Biolaunching Technologies Co. Ltd, Beijing, China) [19]. An optimal ratio of smoke to air (1:6) was established, and the oxygen concentration in the chamber was maintained at a 21 ± 1%. The remaining group of rats was exposed to flash air (sham smoking). One group of smoke-exposed rats was pre-treated with gavage of indirect bilirubin (0.3 ml, 20 mg/kg in saline, Frontier scientific, USA) (bilirubin/smoke group), and rats in the two other groups (sham group and smoke group) received an equal volume of normal saline. All experimental procedures and animal care were reviewed and approved by the Board for Biomedical Research, Shanxi Medical University.

2.3. Measurements of rat serum bilirubin

Animals were euthanized by carbon oxide inhalation. Peripheral blood specimens were collected from the abdominal aorta and serum was prepared. The levels of total and direct bilirubin were measured by Diazo kit (Beijing LABO Biotech, CO., LTD) using an automatic biochemical analyzer (AU2700, Olympus, Japan). Indirect bilirubin level was calculated by subtracting direct bilirubin from total bilirubin. In all cases, bilirubin levels were expressed as μmol/L.

2.4. Collection of bronchoalveolar lavage fluid (BALF), cell counting, specimen preparation and histology

Right lung was ligated for histology and homogenate preparation. The left lung was lavaged via trachea with 2 ml cold saline three times. Cells in bronchoalveolar lavage fluid (BALF) were counted. Differential leukocyte counts were performed using cytocentrifuged preparation stained with Wright. The upper lobe of the right lung was fixed with formalin, embedded in paraffin, sectioned in 5 μm slices and stained with hematoxylin & eosin (HE). A portion of each right lung was homogenized in a nine-fold excess of saline on ice using an ultrasonic homogenizer. Homogenate was centrifuged and the supernatant was collected. Serum, BALF and lung homogenate supernatants were stored at −80 °C.

2.5. Morphological quantification of emphysema

Emphysema was assessed by measuring the mean linear intercept (MLI), mean alveoli number (MAN), and mean alveoli area (MAA) in HE-stained sections using NIH ImageJ 1.32 as previously described [20]. Analyses were in blind performed by two independent investigators. MLI was obtained by dividing the total length of a line drawn cross the center of lung section by the total number of intercepts encountering the line. MAN was calculated by dividing the number of alveoli by the area in a given field. MAA was calculated by subtracting the area of HE-stained parenchyma area from the total field area, and dividing the result by the number of alveoli in each filed.

Table 1
Patient characteristics.

Group	N	Mean age (year)	Age range (year)	Low TBil (n, %)	Low DBil (n, %)	Low IBil (n, %)
No smoking/no COPD	37	60.97	40–96	6/37, 16.2%	0/37, 0%	8/37, 21.6%
Smoking/no COPD	34	55.37	40–86	17/34, 50%	8/34, 23.5%	16/34, 47.1%
No smoking/COPD	9	67.78	45–85	3/9, 33.3%	0/9, 0%	4/9, 44.4%
Smoking/COPD	20	62.40	45–85	10/20, 50%	0/20, 0%	12/20, 60%
Smoked/COPD	29	71.79	52–86	7/29, 24.1%	1/29, 3.4%	14/29, 48.3%
P				0.01055	0.001107	0.03654

TBil: total Bilirubin; DBil: direct Bilirubin; IBil: indirect Bilirubin.
Bilirubin level was categorized as “low” when below the mean minus standard deviation of all control patients.