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Temporal analysis of oxidative effects on the pulmonary inflammatory response in mice exposed to cigarette smoke

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ABSTRACT

The most common factor related to the chronic obstructive pulmonary disease (COPD) development is the chronic smoking habit. Our study describes the temporal kinesis of pulmonary cellular influx through BALF analyses of mice acutely exposed to cigarette smoke (CS), the oxidative damage and antioxidative enzyme activities. Thirty-six mice (C57BL/6, 8 weeks old, male) were divided in 6 groups: the control group (CG), exposed to ambient air, and the other 30 mice were exposed to CS. Mice exposed to CS presented, especially after the third day of exposure, different cellular subpopulations in BALF. The oxidative damage was significantly higher in CS exposed groups compared to CG. Our data showed that the evaluated inflammatory cells, observed after three days of CS exposure, indicate that this time point could be relevant to studies focusing on these cellular subpopulation activities and confirm the oxidative stress even in a short term CS exposure.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by chronic airway inflammation, irreversible airflow limitation and emphysema [1,2]. The risk factors of COPD include indoor air pollution from biomass fuel, pulmonary tuberculosis, chronic asthma, socioeconomic status, genetic background and environmental factors [3], however, the disease occurs predominantly in adult cigarette smokers [4]. Despite this, over 1 billion people continue to smoke and half of them are likely to develop a serious smoking-related disease. Although the efforts to reduce smoking prevalence has to be brought into focus, understanding the processes that contribute to the inception and progression of smoking-related illnesses are of equal importance, given the highly addictive nature of cigarette smoke [5].

The lungs are an important way of exposure to environmental pathogens and antigens; nonspecific and specific defense mechanisms are involved in cleaning up these foreign substances from the lungs. Protection against the foreign material reaching the lung alveoli involves innate and adaptive immune responses.

The innate defense system of the lung is provided by the epithelial barrier and the acute inflammatory response which follows tissue injury, including the recruitment and activation of neutrophils, eosinophils and macrophages [7]. Resident and inflammatory lung macrophages exhibit different origins and lifespans in lungs and have been identified as key regulators of pathological and reparative processes. Alveolar macrophages, which are considered tissue-resident macrophages, populate lung tissue during early embryogenesis and remain viable for prolonged periods with minimal replenishment from bone marrow-derived monocytes. In contrast, inflammatory macrophages originate from bone marrow-derived monocytes and have a shorter half-life [7]. Macrophages are activated by CS extract and secrete not only elastolytic enzymes, but also many inflammatory chemokines (e.g., interleukin-8 and CXCR3-ligands), attracting neutrophils and cells from acquired immunity [6].

The adaptive immune response is dependent upon B- and T-lymphocytes ($CD4^+$ and $CD8^+$), and has a longstanding memory for previous damage [6]. The acquired immunity involves specific immune responses that are elicited by antigens of various origins and that are executed primarily by T and B cells. Acute smoke

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exposure resulted in significant increases in neutrophils and mononuclear cells within the lung [5], suggesting that all the different inflammatory cells together are responsible for lung injury caused by cigarette smoke [6].

Oxidative stress has been implicated as a strong factor favoring the pathogenesis and progression of COPD [9]. Cigarette smoke (CS) is associated to the oxidative stress in several organs because it contains high concentrations of free radicals and reactive oxygen species (ROS) [10]. Oxidants present in cigarette smoke can stimulate alveolar macrophages to produce ROS and to release mediators, some of which attract neutrophils and other inflammatory cells into the lungs. [11]. In *in vitro* studies using alveolar macrophages and bronchial epithelial cells, ROS have been shown to induce gene expression of inflammatory mediators, such as IL-1 and TNF alpha. The direct or indirect oxidant stress to the airway epithelium and alveolar macrophages may also generate cytokines, such as TNF alpha and IL-1beta, which in turn can activate airway epithelial cells to induce pro-inflammatory genes, such as TNF alpha, IL-8, IL-1, iNOS, COX-2, ICAM-1, VCAM-1, IL-6, MMP-9, MIP-1alpha, GM-CSF, stress response genes and antioxidative enzymes (such as MnSOD and thioredoxin) [12].

Evidences support an imbalance between oxidant and antioxidant agents in the lungs and bloodstream of cigarette smokers and COPD patients [13]. Structural changes to essential components of the lung are caused by oxidative stress, contributing to irreversible damage of both parenchyma and airway walls [14]. These changes result in inflammatory cells influx followed by the increase of lipid peroxidation products, pro-inflammatory cytokines and altered antioxidative capability [15]. In order to minimize the oxidative damage, mammalian lungs present an integrated antioxidative enzymatic system [16,17]. The main components of this antioxidative enzymatic system are superoxide dismutase (SOD), catalase (CAT) and glutatione peroxidase (GPx). SOD is the primary enzymatic defense in the lungs against the damaging effects of O_2^- and H_2O_2 by converting O_2^- into H_2O_2, which is a substrate for CAT and GPx [18]. The antioxidative enzymes constitute a critical mechanism to protect the lung parenchyma from damage caused by free radicals [19].

The present work aimed to evaluate, phenotypically, the temporal cellular influx, the oxidative damage and antioxidative enzymatic system activities in the lung of mice acutely exposed to cigarette smoke.

2. Methods

2.1. Animals

Male C57BL/6 mice, 8 weeks old (Laboratory of Experimental Nutrition, Department of Food – School of Nutrition, Federal University of Ouro Preto) were housed under controlled conditions in standard laboratory cages. They were provided free access to water and food. All in vivo experimental protocols in animals at the Federal University of Ouro Preto were approved by the Ethics Committee.

2.2. Reagents

Coomassie blue, bovine serum albumin (BSA), thiobarbituric acid (TBA), trichloroacetic acid, tetramethoxypropane (TMP), adrenaline, glycine buffer, catalase, nicotinamide adenine dinucleotide phosphate (NADPH), ethylenediaminetetraacetic acid (EDTA), hydrogen peroxide, phosphate buffered saline (PBS), glutathione reductase, $NaHCO_3$, sodium azide, monoclonal antibodies (anti-CD4-PE, CD8-PerCP, GR1-FITC, F4/80$^-$ APC and CD11b$^-$PE).

2.3. Cigarette smoke exposure protocol

C57BL6 male mice ($n = 36$) were exposed to 6 commercial full-flavor filtered Virginia cigarettes (10 mg of tar, 0.9 mg of nicotine and 10 mg of carbon monoxide) per day for 5 days by using a smoking chamber previously described [20] [21]. The groups exposed to CS for 1, 2, 3, 4 or 5 days were called CSD1, CSD2, CSD3, CSD4, and CSD5, respectively. Briefly, each group of mice was placed in the inhalation chamber (40 cm long, 30 cm wide and 25 cm high), inside an exhaustion chapel. A cigarette was coupled to a plastic 60 mL syringe so that puffs could be drawn in and subsequently expelled into the exposure chamber. One liter of smoke from one cigarette was aspirated with this syringe (20 puffs of 50 mL) and the puff was immediately injected into the chamber. The 6 animals of each group were maintained in this smoke-air condition (∼3%) for 6 min, then the cover was removed from the inhalation chamber and by turning on the exhaust fan of the chamber of the chapel, the smoke was evacuated within 1 min. The mice were then immediately exposed to CS from a second cigarette for 6 min. The treatment was performed three times per day (morning, noon and afternoon), being two cigarettes per inhalation. The mice exposed to ambient air were used as the control group (CG; $n = 6$) [22].

2.4. Bronchoalveolar lavage fluid (BALF), cell staining and flow cytometry

The animals were killed by cervical displacement. Airspaces were washed with buffered saline solution (0,5 mL) for three consecutive times in the lung (final volume 1.2–1.5 mL). The fluid was withdrawn and stored on ice. Cells from BALF samples were counted using standard morphologic criteria and used to flow cytometry analyses.

BALF cells were incubated with the different antibody solutions for 30 min at 4 °C, washed with phosphate-buffered saline (PBS, pH 7.2) and fixed in a formaldehyde-containing solution. The expression of surface molecules was investigated combining differentially labeled anti-CD4 and anti-CD8 or anti-F4/80, anti-CD11b and anti-GR1. All the antibodies were from BD-Pharmingen, San Jose, CA. Fixed samples were maintained in the dark at 4 °C until the acquisition of FACSCalibur (Becton–Dickinson). The data analyses were performed using the software FlowJo (Tree Star).

2.5. Processing and homogenized tissue

After performing BALF, the right ventricle was perfused with saline to remove blood from the lungs. The right lung was clamped so that just the left lung could be perfused with 4% buffered formalin (pH 7.2) at a pressure of 25 cm H_2O for 2 min, via trachea. The left lung was removed and then immersed in a fixative solution for 48 h. Then, the material was processed as follows: bath with tap water for 30 min.

Bath in 70% alcohol and 90% for 1 h each step, two baths in 100% ethanol for 1 h each and embedded in paraffin. The serial 5 μm sagittal sections with hematoxylin and eosin were obtained from the left lung for histologic analyses. Following the removal of the left lung for histology, the right lung was immediately removed and stored on crushed ice in tubes duly labeled. Then, the organ was homogenized in 1 ml potassium phosphate buffer pH 7.5 and centrifuged at 1500*g* for 10 min. The supernatant was collected and the final volume of all samples adjusted to 1.5 mL with phosphate buffer. The samples were stored in a freezer for a biochemical analysis.