OXIDATIVE STRESS AND CYTOTOXICITY IN MOUSE LUNG INDUCED BY CIGARETTE SMOKE; A MAJOR ANTHROPOGENIC POLLUTANT IN AIR

Pankaj Mehta and Sushma Sharma

KEYWORDS

Cigarette smoke (CS)
Cytotoxicity
Oxidative stress
Antioxidant enzymes
ABSTRACT

Cigarette smoke (CS) is a major anthropogenic pollutant and contributor to the permanent load of ambient particulate matter i.e. environmental tobacco smoke (ETS). The lung, given direct exposure to oxygen in the ambient air, experiences enhanced oxidant stress compared with other organs. Thus, the present study was an attempt to examine the histopathological status and antioxidant enzyme activities in lungs of mice exposed to cigarette smoke. Mice were exposed to the smoke of five whole commercial cigarettes/day for 20 minutes upto 8 weeks to whole body exposure in a chamber. Animals were sacrificed after 2nd, 4th, 6th and 8th week respectively. Lung tissue was used for histologic and biochemical studies. The results of the present study showed that alteration in glutathione requiring enzymes mainly glutathione peroxidase (GPO), glutathione reductase (GR) and other antioxidant enzymes occurs in cigarette smoke induced oxidative stress which may contribute to lung cytotoxicity. Activities of antioxidant enzymes increased significantly after 4, 6 and 8 week of CS exposure. This enhanced antioxidant activity sufficed to protect the lungs of the mice from smoke-induced free radical injury. Histology profiles showed that when the mice exposed to CS for 2-8 weeks at an exposure rate of 5 cigarette/day, there was abnormal permanent enlargement of the air spaces distal to the terminal bronchioles, accompanied by destruction of their walls. The results of present study suggested that alterations in antioxidant enzymes may contribute to enhanced oxidative stress in the lungs of CS treated mice and hence lung cytotoxicity.

INTRODUCTION

Humans have always polluted their environment and, to an extent, the associated adverse consequences have increased in parallel with the global population. These damaging pollutants interfere with fundamental physiological processes in all animal species, disrupting respiratory and other functions. Air pollution is reported to be associated with increased rates of mortality and cardio-respiratory disease. Cigarette smoke is a major anthropogenic pollutant and contributor to the permanent load of ambient particulate matter i.e. environmental cigarette smoke (Chung and Cherukupalli, 1993). Environmental tobacco smoke (ETS) is an important source of anthropogenic air pollution in indoor environment. It has become obvious that exposure to ETS i.e. ‘passive’ or involuntary smoking, increases the risk of even non-smokers to develop various lung diseases. Both active and passive cigarette smoke exposure is linked in adults to an increased incidence and severity of asthma, impaired lung function and airway inflammation. Exposure to cigarette smoke has similar adverse effects to active smoking on lung infections, with young children at higher risk (Chan-Yeung and Dimich-Ward, 2003; DiFranza et al., 2004).

Cigarette smoking is one of the most prevalent social habits practiced worldwide today. Each year, over five million people throughout the world die from smoke related illness. According to the USA Environmental Protection Agency, the burden of particulate matter in ambient air is increasing worldwide. Many types of ingested particles have the ability to generate free radicals in biological systems and to activate oxidative stress- responsive signaling pathways in cells. Cigarette smoke (CS) is a mixture of chemicals having direct and/or indirect toxic effects on different lung cells. Cigarette smoke contains more than 4000 compounds (Stewart and Kleihues, 2003). Among these, nicotine is the primary source of tobacco dependence. Most of these chemicals are considered to be causative agents for CS - induced life - threatening diseases, particularly cancer of the lungs and other organs, cardiovascular diseases, myeloid leukaemia and chronic obstructive pulmonary disease (COPD), including bronchitis and emphysema (Wald and Hackshaw, 1996; Harris et al., 2004).

All cells are exposed to oxidative stress during the course of normal metabolism. The respiratory tract as the main entrance for various inhalative substances has great potential to generate reactive species directly or indirectly in excess. Cigarette smoke is known to induce oxidative stress and inflammation in pulmonary tissues and cells, both in vitro and in vivo. It contains high concentrations of oxidants (Church and Pryor, 1985). The respiratory system has several antioxidant factors that protect tissue from injury by oxidants. Some enzymes with antioxidant properties are superoxide dismutase, glutathione reductase, glutathione peroxidase and catalase that accounts for most of the observed anti-H2O2 properties (Cantin et al., 1990). The imbalance between oxidants and antioxidants is involved in development of pulmonary diseases (Snider, 1992). Keeping in view the role of anthropogenic pollutants in various lung diseases, the present study was designed to evaluate the effects of cigarette smoke on antioxidant defense system and lipid peroxidation (LPO) levels in mice inhaling CS for different time interval.
MATERIALS AND METHODS

Male Balb/c mice weighing 20-25g were procured from Central Research Institute, Kasauli (H.P.), India. All the methodologies opted for the whole investigation had prior approval of Institutional Animals Ethics Committee of the University (IAEC/BIO/4/2008).

Smoke-exposure protocol: Cigarette smoking experiment was performed in a closed chamber as described by Santiago et al. (2009). Mice were exposed to the smoke of five cigarettes smoke in the specially designed chamber for 20 min/day. The animals were exposed 6 days per week for up to 8 weeks. After their respective treatments, mice from each group were sacrificed.

Experimental design: Mice were divided into two groups and treated as G1 – untreated (control) which received saline only; G2 - exposed to cigarette smoke. The animals (n = 5) from each group were sacrificed at 2, 4, 6 and 8th week after treatment respectively. The excised lungs were used for biochemical analyses as well as for histological assessment.

Histology: Paraffin- embedded lung tissues were prepared, and sections (3-4μm) mounted onto slides were stained with hematoxylin and eosin (H and E) for histologic analysis.

Biochemical study: 100 mg of lung tissue was homogenized in 1.15% KCl. Homogenates were centrifuged at 3000 rpm for 30 minutes at 4°C. The supernatant was used for different biochemical assays. Protein concentration of the tissue was determined as per the method of Lowry et al. (1951). Levels of malondialdehyde (index of lipid peroxidation) will be estimated according to the method of Dhindsa et al. (1981). Assays of superoxide dismutase (SOD) activity in tissue was determined by the method of Mishra and Fridovich (1972). Catalase activity was estimated by measuring the change in absorption at 240 nm using H₂O₂, as substrate (Aebi, 1984). Glutathione reductase (GR) activity was estimated in lung according to the method of Racker (1955) with slight modifications. Glutathione peroxidase (GPO) was determined by following the oxidation of NADPH at 340 nm using hydrogen peroxide as described by Nakamura and Hosada (1974).

RESULTS AND DISCUSSION

Histopathological findings clearly showed the presence of inflammatory infiltrate cells and destruction of alveolar walls with the consequent formation of large cyst like sacs. There was marked emphysematous damage of the lung as compared with control. The lung damage progressed and became severe at 8 weeks (Figs. 1 - 2). Figure 1a shows lung sections from normal mice. Alveoli that form lung parenchyma giving appearance of fine lace. The next sections (Fig. 1b-1d) are showing considerable inflammation, with prominent inflammatory cells cuffing in the lung parenchyma after exposed to cigarette smoke. Fibroblasts (F) and nuclei of endothelial cells (NEC) were undergoing pyknosis. It seems smoke alone caused a mild inflammatory reaction comprising alveolar infiltrates of dust cells/macrophages and neutrophils. In normal lung, two alveoli are separated from each other by an interalvolar septum (IS) or alveolar wall which is composed of capillary (C). The nucleus of whose endothelial lining bulges into the lumen containing RBCs (Fig. 2a). The lungs of the mice exposed to cigarette smoke revealed parenchymal lung cells (fibroblasts/endothelial cells) death and marked air space

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time interval</th>
<th>LPO</th>
<th>Catalase</th>
<th>SOD</th>
<th>GPO</th>
<th>GR</th>
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<tr>
<td>Lung</td>
<td>2nd week</td>
<td>N</td>
<td>10.61±0.76</td>
<td>3.52±0.59</td>
<td>6.53±0.24</td>
<td>9.52±0.02</td>
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<tr>
<td></td>
<td></td>
<td>CS</td>
<td>13.19±0.70</td>
<td>4.19±0.07</td>
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<td>10.53±0.24</td>
</tr>
<tr>
<td></td>
<td>4th week</td>
<td>N</td>
<td>11.45±0.83</td>
<td>3.4±0.28</td>
<td>5.93±0.12</td>
<td>9.92±0.03</td>
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<td>CS</td>
<td>16.78±0.74</td>
<td>6.72±0.32</td>
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<td>11.94±0.36</td>
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<td></td>
<td>6th week</td>
<td>N</td>
<td>11.60±1.92</td>
<td>3.72±0.26</td>
<td>6.76±0.28</td>
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<td></td>
<td>8th week</td>
<td>N</td>
<td>12.19±0.92</td>
<td>4.19±0.07</td>
<td>6.94±0.31</td>
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<td>CS</td>
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<td>12.03±0.62</td>
<td>13.93±0.29</td>
<td>16.6±0.31</td>
</tr>
</tbody>
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All values are significant at p < 0.005; SOD/ Catalase (Units/mg protein); GPO/GR (mU/mg protein); LPO (micromoles of MDA/mg protein)

Figure 1: (1a): Photomicrograph of H and E stained lung tissue control. Normal lung showing alveoli (A), alveolar sac (AS), alveolar wall (AW) and capillary (C) containing red blood cells (RBCs). 200x; (1b). Further inflammation in lung tissue (F) was observed at 6th week; (1c): Disappearance of interalveolar septa (IS) and presence of cyst like sacs clearly revealed on 8th week; (1d). 200x; Photomicrographs of H and E stained lung tissue of smoke-exposed mice. Lung tissue showing recruitment of inflammatory cells, macrophages (M) and dust cells (DC) on 4th week
Figure 2: (2a): Photomicrograph of normal lung showing adjacent alveoli (A) sharing a common alveolar wall (AW). Presence of capillary in the wall supported by fibroblasts, endothelial and other cells in connective tissue visualized. 400x. (2b-2d): Photomicrographs of smoke exposed sections clearly showing enlargement of airspaces, increased number of dust cells (DC) in mice on 4th and 6th week (Fig 2b-2c). Parenchymal lung cells death followed by alveolar wall destruction observed on 8th week (Fig 2d). 400x

enlargement followed by alveolar wall destruction (Fig. 2b-2d).

Fibroblasts are the main cell type in connective tissue, and they play a major role in the repair of pulmonary tissue. In previous studies, Carnevali et al. (2003) showed that fibroblasts were targets for a wide variety of stimuli including cigarette smoke. CS was able to inhibit fibroblast recruitment and proliferation (Nakamura et al., 1995). In the present study, we demonstrated that CS induces fibroblast apoptosis possibly because direct cigarette smoke oxidants and/or intracellular reactive oxygen species generated by CS can switch on apoptotic pathway(s) in fibroblasts.

Inflammation of the airways and lung parenchyma plays a major role in the pathogenesis of pulmonary disease. In the present study, inflammation was investigated in the lungs of mice during 2 - 8 weeks of exposure to CS. In response to cigarette smoke, inflammatory cells (i.e. neutrophils, macrophages and lymphocytes) progressively accumulated in the lung parenchyma of mice. Similar observations were noticed by those Vlahos et al. (2006), where smoke exposure protocol causes lung inflammation, but not acute lung injury. Microscopic analysis of lung tissue sections revealed an abnormal enlargement of the airspaces accompanied by destruction of their wall after 8 weeks of smoke exposure. These symptoms may cause significant degree of emphysema. Emphysema is a structural disorder characterised by damage to the lung parenchyma. The destruction of the alveolar walls will lead to enlargement of the alveolar spaces. The destruction and the enlargement processes may proceed chronologically or simultaneously (Saetta et al., 1985; Saito et al., 1989).

Previously, Banerjee et al. (2008) had also shown that exposure of guinea pigs to smoke from cigarette causes emphysematous lung damage.

The primary host defense mechanisms of the lungs against exposure to toxic gases and particles are the innate and adaptive inflammatory 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 (Zhang et al., 2000). In present investigation, after CS exposure dark blotches appear to be scattered throughout the lung tissue. These represent dust cells, a type of macrophages that probably phagocytosed particulate matter. These results support recent findings on the importance of resident lung parenchymal cells in the development of emphysema. In a previous study (Kasahara et al., 2001), cigarette smoke has been reported to alter maintenance of pulmonary endothelial cells, and this may result in emphysema due to pulmonary endothelial cells followed by progressive disappearance of the alveolar septa by apoptosis.

Currently, oxidative stress is seen to be at least partly responsible for several lung diseases. An increase in lipid peroxidation were noticed in the present investigation. As the exposure of CS to the mice was extended from 2 to 4, 6 and 8 weeks, a significant increase in the LPO levels were observed (Table 1). Lipid peroxidation products were in high concentration in the lungs of cigarette smokers, correlating to the duration of smoking. These findings are suggestive of the fact that the increase in the duration of exposure to cigarette smoke, enhances the oxidative stress and there by the LPO levels. Lipid peroxidation products such as thiobarbituric acid reactive substances (TBARS) measured in body fluids were significantly increased in the plasma of healthy smokers compared to healthy nonsmokers were also reported by Rahman et al. (1997). Currently, there is striking evidence for oxidative stress and imbalance between oxidants and antioxidants in smokers (Rahman et al., 1996).

In the present investigation, where mice were subjected to CS inhalation for 2 weeks, no significant change in pulmonary enzymatic antioxidants was observed, except for GR. It may be possible that 2 weeks of CS was not enough to tilt the balance between the oxidative stress due to CS and the endogeneous antioxidants. We also observed that the other antioxidant enzymes like catalase, GPO, SOD and GR increased significantly in the smoked group after 4, 6 and 8 weeks of CS exposure but still could not prevent the increase in LPO levels (Table 1). Changes in antioxidant enzymes in response to cigarette smoke varied. Increased activity of antioxidant enzymes such as superoxide dismutase and catalase has also been reported in alveolar macrophages from young smokers (Mc Cusker and Hoidal, 1990). It seems that this increase in the enzyme level perhaps was a nonspecific response to the treatment or could be that this increase was not significant to combat the oxidative stress posed by CS exposure.

In this study, we showed that parenchymal lung cells may undergo apoptosis, which parallels the increase in oxidative stress. We speculate that the development of pulmonary...
emphysema might be not only the result of an imbalance between oxidants and antioxidants but also the consequences of parenchymal cells damage caused by cigarette smoke. In conclusion, it seems likely that all the different inflammatory cells together were responsible for lung cytotoxicity caused by cigarette smoke. However, whereas the role of neutrophils and macrophages in the pathogenesis of respiratory diseases has been clearly highlighted, the marked increase of dust cells in the smoke induced lung inflammation prompts further investigation into the potential role of these cells in the pathophysiology of lungs. It’s clear from our studies that anthropogenic pollutants plays an important role in oxidative stress and hence resulted in lung cytotoxicity.

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