Celecoxib mitigates cigarette smoke induced oxidative stress in mice

Ashwani Koul* and Neha Arora
Department of Biophysics, Basic Medical Sciences Block, Panjab University, Chandigarh 160 014 India

Received 16 March 2010; revised 21 September 2010

Cigarette smoke (CS) is rich in radicals, predisposing the cell to oxidative stress resulting in inflammation. Chronic inflammation is a recognized risk factor for carcinogenesis. Cyclooxygenase-2 (COX-2) is a mediator of inflammatory pathway and may, therefore, contribute to carcinogenesis. There are several reports that suggest the association between CS and COX-2 associated risk to cancer. In the present study, we examined the role of celecoxib (a selective COX-2 inhibitor) in modulating the oxidative stress caused by CS inhalation in mice. CS exposure for a period of 10 weeks caused oxidative stress in the pulmonary and hepatic tissues, as evident from the increase in lipid peroxidation levels (LPO) and decrease in reduced glutathione (GSH) levels. Celecoxib (125 mg/kg body weight for 8 weeks) administration to CS inhaling mice reduced the oxidative stress by decreasing the LPO levels and enhancing the GSH levels in comparison to the CS-exposed group. CS exposure repressed the enzymatic antioxidant defense system, as evident from the decrease in catalase (CAT) and superoxide dismutase (SOD) activities. Co-administration of celecoxib considerably reversed the changes in the enzymatic antioxidant defense system. Histopathological studies of lungs showed that CS exposure induced alveolar wall destruction and air space enlargement. In co-treated group, the alveolar septa were thicker than normal with apparent infiltration of inflammatory cells. In CS-exposed group, hepatic tissue exhibited vacuolization and macrophage infiltration. Co-treatment with celecoxib restored the normal histarchitecture in hepatic tissues of CS inhaling mice. Thus, the present study demonstrated that celecoxib administration reduced the oxidative stress-mediated risk to carcinogenesis, due to its ability to boost the antioxidant defense system.

Keywords: Cyclooxygenase, Cigarette smoke, Inflammation, Oxidative stress

Cigarette smoke (CS) is operationally divided into gas phase smoke and particulate matter. Tar, an important constituent of particulate phase is retained on the filter and the gas-phase smoke passes through the filter. Both the tar and gas-phase smoke are rich source of free radicals. About 95% of CS is made up of gases, primarily nitrogen, oxygen and carbon dioxide. CS contains up to 500 ppm nitric oxide (NO) which undergoes oxidation to produce NO₂ free radical¹. Aqueous extract of cigarette tar (ACT) contains a low molecular weight quinone-hydroquinone-semiquinone system (Q-QH₂-QH). The semiquinone radical reduces oxygen to produce superoxide which subsequently leads to the generation of pro-oxidant species such as H₂O₂ and hydroxyl radical. This radical system produces superoxide, H₂O₂ and hydroxyl radical in the vicinity of the DNA². The quinone-hydroquinone-semiquinone system binds to and nicks cellular DNA³. Oxidants are likely to play a significant role in carcinogenesis. Chronic tissue injury by physical and chemical irritants frequently results in inflammation which is accompanied by infiltrating phagocytes⁴. The inflammatory reaction is particularly striking in the bronchial tissue which gets exposed to particulates and irritants in tobacco smoke.

Cyclooxygenase-2 (COX-2) is pro-inflammatory in nature and expressed only in response to certain stimuli, such as mitogens, cytokines, growth factors and carcinogens⁵. The arachidonic acid pathway involving COX-2 generates free radicals, such as peroxyl and hydroxyl radicals and many pro-inflammatory prostaglandins (PGs). Evidences suggest that COX-2 is an important factor in the etiology of CS related lung cancer. Tobacco specific carcinogens, such as 4-(methylnitrosoamine)-1-(3-pyridyl)-1-butanone (NNK) releases arachidonic acid, upregulates COX-2 expression and stimulates proliferation in the lung adenocarcinoma cell lines⁶.
COX-2 pathway is induced by components of CS in normal human lung fibroblasts, a group of cells within the lung that not only confer structural support, but also incite and amplify inflammation. Overexpression of COX-2 ultimately leads to an enhanced oxidative stress, due to an increase in free radical production pre-disposing the cell to carcinogenic risk. Inflammatory-mediated events, such as the production of cytokines, reactive oxygen species (ROS) and mediators of the inflammatory pathway, such as COX-2 may, therefore, contribute to tumor formation.

Inflammation has been involved in the pathogenesis of several cancers, such as cervical, ovary, oesophagus, colon, lung etc. Thus, use of antioxidants, anti-inflammatory agents and COX-2 inhibitors that repress formation of inflammatory PGs and free radicals can be helpful in prevention and treatment of cancer. In the present study, we have investigated the role of Celecoxib (a selective COX-2 inhibitor used widely in the treatment of osteoarthritis, and rheumatoid arthritis) in manipulating the antioxidant defense system which potentially regulates the risk to carcinogenesis during CS inhalation.

Material and Methods

Chemicals

5,5-Dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), bovine serum albumin (BSA), thiobarbituric acid (TBA), NADH and NADPH were obtained Sigma Chemical Co. (St Louis, MO, USA). Capsules containing celecoxib were obtained from a recognized pharmaceutical company Lupin. The contents of capsule were reconstituted in carboxymethyl cellulose (CMC) before oral administration to the animals, in order to attain the required dose. Other chemicals were obtained from local firms (India) and were of analytical grade. Filter tipped cigarettes (Four Square, Godfrey Phillips India, Ltd.) were purchased from the local market.

Animals and treatment

Male Balb/c mice weighing (25-30 g) were procured from the Central Animal House of Panjab University, Chandigarh and housed in polypropylene cages bedded with sterilized rice husk. The animals were given free access to clean drinking water (tap water) and standard animal pellet diet (Ashirwad Industries, Kharar, Punjab, India), throughout the experiment. The temperature of animal room was maintained at 21 ± 1°C, humidity 50-60% and a 12 h dark and light cycle. The experimental protocols were approved by the Institutional Ethics Committee and conducted according to the Indian National Science Academy Guidelines for the use and care of experimental animals.

Prior to the various treatments, the mice were acclimatized to the experimental conditions for 1 week and randomly divided into four groups of 6-8 animals each. Group I served as the control (sham) and was treated with vehicle (CMC). In case of sham-exposed group, mice were exposed to fresh air. Group II was exposed to cigarette smoke (CS) inhalation for a period of 10 weeks. Group III was administered celecoxib orally at a dose of 125 mg/kg body weight every alternate day for a period of 8 weeks. Group IV was exposed to CS inhalation for 10 weeks and co-administered with celecoxib orally at a dose of 125 mg/kg for 8 weeks. Celecoxib treatment was started 2 weeks after the initiation of CS inhalation. Weekly alterations, if any in the body weights, diet and water consumption were observed for the mice in all the groups throughout the experiment. The dose of celecoxib was standardized in our laboratory after extensive literature survey.

Passive cigarette smoke inhalation

Animals (6-8) were exposed to CS from five commercially available filter tipped cigarettes in an inhalation apparatus designed in our laboratory and inhalation continued for 60 min every day. The inhalation apparatus consisted of a Perspex chamber (8.21 liters) with separate inlets for smoke and fresh air. A valve attached at the outlet to a suction pump controlled the air flow (141 cc/min) through the chamber. Each cigarette took approx 6-8 min to burn completely. After exposure to smoke from each cigarette, mice were exposed to fresh air for 5 min. This procedure was standardized in our laboratory in such a manner that animals inhaled CS without any visible respiratory stress as was evident from carboxy-hemoglobin (CO-Hb) levels. It was observed that CO levels in the control and CS exposed were 1.2 ± 0.8 and 12.2 ± 4.9% saturation of hemoglobin, respectively. There was an increase in CO-Hb levels in the smoke-exposed animals as compared to their sham counterparts, however the values were found to be within range that have been normally seen in CS smoke-exposed animals.
Biochemical estimations

After completion of the respective treatments, the mice were sacrificed by cervical dislocation under light ether anesthesia. The pulmonary and hepatic tissues were obtained and perfused with cold normal saline (0.9% NaCl solution), blotted and then weighed carefully. These tissues were then homogenized in 100 mM potassium phosphate buffer (pH 7.4) containing 150 mM KCl to obtain 10% homogenate (w/v). The homogenate was subjected to cold centrifuge at 10,000 x g for 30 min and the supernatant (PMS), thus obtained was used for various biochemical estimations. Aliquots of 10% homogenate were kept for estimation of GSH and LPO levels.

GSH levels

GSH was estimated as the total non-protein sulphydryl group by the method described previously\textsuperscript{15}. Homogenates were immediately precipitated with 0.1 mL of 25% trichloroacetic acid and the precipitate was removed after centrifugation at 1500 x g for 10 min. The free -SH groups were assayed in a total 3 mL volume by adding 2 mL of 0.6 mM DTNB prepared in 0.2 M sodium phosphate buffer (pH 8.0), to 0.1 mL of the supernatant and the absorbance was read at 412 nm using a Shimadzu UV-160 spectrometer. GSH was used as a standard to calculate micromole of GSH contents/mg protein.

LPO levels

The assay for lipid peroxidation (LPO) was performed according to the method of Wills\textsuperscript{16}. Briefly, 1 ml of the reaction mixture contained 500 µl of phosphate buffer (100 mM, pH 7.6), 200 µl of DDW, 50 µl of EDTA (60 mM), 50 µl of BSA (20 mg/ml), 50 µl of NADPH (2 mM), 50 µl of GSSG (60 mM) and 100 µl of PMS. The appropriate blanks lacking GSSG were carried out simultaneously. The enzyme activity was measured indirectly by monitoring the oxidation of NADPH, following the decrease in OD/min for a minimum of 3 min. One unit of enzyme activity was defined as nM of NADPH consumed/min/mg of protein using an extinction coefficient of 6.22 mM\textsuperscript{-1} cm\textsuperscript{-1}.

Histopathology

Formalin fixed tissues were processed for hematoxylin and eosin staining using the conventional laboratory procedure. Briefly, the tissues were dehydrated through ascending grades of alcohol, cleared in benzene and embedded in low melting point paraffin wax. Sections of 5-7 micrometer thick were cut, placed serially on clean glass slides and then de-paraffinised through descending grades of alcohol. The sections obtained were stained with hematoxylin and eosin for evaluation under light microscope.

Protein estimation

The protein content of various samples were estimated by the method of Lowry et al.\textsuperscript{20} using BSA as a standard.

Statistical analysis

The data were expressed as mean ± SD. Statistical significance was analyzed by one-way ANOVA, followed by Student’s Newman Keul Test.
Results and Discussion

It is well recognized that oxidants play a well defined role in carcinogenesis. CS is a very rich source of free radicals which are responsible for its cancer causing action. ROS serve as ‘Oxidant carcinogens’ and interact with multiple cellular targets including membranes, proteins, and nucleic acids. They have the potential to mutate cancer-related genes, manipulate signal transduction pathways and alter the expression of growth- and differentiation-related genes. CS also induces the expression of pro-inflammatory COX-2 [21] which aggravates the oxidative stress emanating from CS. CS exposure has been reported to upregulate COX-2 and prostaglandin E2 which is further responsible for lung inflammation and cancer [22]. Evidences suggest that ROS and COX-2 produced in response to CS exposure initiates chronic inflammation, leading to increased likelihood of lung cancer. Earlier [23], we have demonstrated that celecoxib administration to CS inhaling mice modulates the carcinogen biotransformation in pulmonary and hepatic tissues, consequently altering the carcinogen-DNA adduct formation.

In the present study, the 10 weeks CS exposure caused a significant decrease in the pulmonary and hepatic GSH levels when compared with the control group. No significant alterations were observed in the pulmonary and hepatic GSH levels of the celecoxib per se group when compared with the control group. A significant increase in the pulmonary and hepatic GSH levels was observed in the co-treatment group when compared with the group exposed to CS only (Table 1). Earlier [13], we have also reported an association between CS and GSH levels in heart of CS inhaling mice and a decrease in GSH content in the pulmonary tissue [9] after CS exposure to mice. An increase in intestinal GSH content is also reported, following celecoxib administration [24]. Increase in GSH levels in the lungs and liver of animals receiving diclofenac and naproxen is also reported [25].

CS exposure caused a significant increase in the pulmonary MDA levels (a marker of LPO) when compared with the control group. However, CS exposure was unable to significantly alter the hepatic LPO levels when compared with the control group. Celecoxib administration to CS inhaling mice caused a significant decrease in the pulmonary and hepatic LPO levels when compared with the group exposed to CS only. Pulmonary LPO levels increased significantly and hepatic LPO levels decreased significantly in celecoxib per se group when compared with the control group (Table 1). The decrease in MDA levels, following celecoxib

Table 1—Effect of cigarette smoke, celecoxib and co-treatment of cigarette smoke and celecoxib on hepatic and pulmonary oxidative stress markers and antioxidant enzymes

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (Group I)</th>
<th>Smoke exposed (Group II)</th>
<th>Celecoxib treated (Group III)</th>
<th>Smoke + Celecoxib treated (Group IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pulmonary GSH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.56 ± 0.03</td>
<td>0.49 ± 0.02ᵇ</td>
<td>0.60 ± 0.03</td>
<td>0.58 ± 0.003ᵇ</td>
</tr>
<tr>
<td></td>
<td>0.83 ± 0.06</td>
<td>0.56 ± 0.09ᵃᵃ</td>
<td>0.85 ± 0.007</td>
<td>0.87 ± 0.07ᵇᵇ</td>
</tr>
<tr>
<td></td>
<td>Hepatic GSH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.73 ± 0.16</td>
<td>21.2 ± 0.16ᵃᵃᵃ</td>
<td>14.5 ± 1.10ᵃᵃ</td>
<td>13.0 ± 0.12ᵃᵃᵇᵇ</td>
</tr>
<tr>
<td></td>
<td>4.66 ± 0.26</td>
<td>4.33 ± 0.12</td>
<td>2.43 ± 0.18ᵃᵃ</td>
<td>3.10 ± 0.58ᵃᵃᵇᵇ</td>
</tr>
<tr>
<td></td>
<td>Pulmonary LPO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.39 ± 0.06</td>
<td>4.86 ± 0.44ᵃ</td>
<td>4.36 ± 0.24</td>
<td>5.70 ± 0.19ᵃᵃᵇᵇ</td>
</tr>
<tr>
<td></td>
<td>22.2 ± 1.10</td>
<td>21.5 ± 0.27</td>
<td>20.2 ± 0.57</td>
<td>32.5 ± 0.90ᵇᵇ</td>
</tr>
<tr>
<td></td>
<td>Hepatic LPO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.07 ± 0.66</td>
<td>0.71 ± 0.12ᵃᵃᵃ</td>
<td>5.78 ± 0.21</td>
<td>5.64 ± 0.23ᵇᵇᵇᵇ</td>
</tr>
<tr>
<td></td>
<td>7.89 ± 0.64</td>
<td>1.08 ± 0.10ᵃᵃ</td>
<td>13.8 ± 1.45ᵃᵃ</td>
<td>10.2 ± 0.39ᵇᵇᵇᵇ</td>
</tr>
<tr>
<td></td>
<td>Pulmonary SOD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.87 ± 0.24</td>
<td>0.72 ± 0.08ᵃᵃ</td>
<td>0.68 ± 0.51ᵃᵃ</td>
<td>6.35 ± 0.48ᵃᵃᵇᵇᵇᵇ</td>
</tr>
<tr>
<td></td>
<td>4.66 ± 0.26</td>
<td>4.43 ± 0.12ᵃ</td>
<td>2.43 ± 0.18</td>
<td>3.10 ± 0.58ᵇᵇ</td>
</tr>
<tr>
<td></td>
<td>Hepatic SOD</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data were analyzed using one-way ANOVA, followed by Students-Newman Keul Test

aaa: p<0.001; aa: p<0.01; a: p<0.05 significant as compared to control group
bb: p<0.001; bb: p<0.01; b: p<0.05 significant as compared to smoked group

Units: LPO: nanomoles of MDA-TBA chromophore/mg of protein; GSH: nanomoles of GSH/mg of protein; CAT: milli moles of H2O2 decomposed/mg of protein; SOD: IU/mg of protein; GR: nano moles of NADPH consumed/min/per mg of protein
administration can be attributed to its high COX-2 selectivity, as the latter is pro-inflammatory in nature and induces LPO in cellular systems. A decrease in MDA levels is also reported in the small intestine of rats administered with celecoxib. Earlier, we have also established relationship between GSH depletion and LPO in lung of CS inhaling mice. The decrease in pulmonary GSH level in response to CS could be responsible for the observed increase in LPO levels in the pulmonary tissue. Perhaps, the GSH depletion in hepatic tissue of CS inhaling mice was not sufficient enough to affect the MDA levels. The decrease in the hepatic and pulmonary MDA levels in the co-treated group was supported by the observation that an increase in GSH levels in the conjunctive treated group was observed up to levels comparable to the control group. Therefore, celecoxib administration to CS inhaling mice caused restoration of GSH levels which prevented an increase in MDA levels in response to CS exposure.

CS exposure caused a drastic decrease in the pulmonary and hepatic CAT activity when compared with the control group. Celecoxib administration to CS inhaling mice restored the CAT activity in both hepatic and pulmonary tissues. No significant alterations were observed in the pulmonary CAT activity of the celecoxib per se and control group. A significant increase in the hepatic CAT activity was observed in the celecoxib per se group when compared with the control group (Table 1).

A significant decrease was also observed in the pulmonary and hepatic SOD activity, following CS exposure when compared with the control group. A significant increase in the pulmonary SOD activity and a significant decrease in the hepatic SOD activity were observed in the co-treated group when compared with the group exposed to CS only. No changes were observed in the pulmonary and hepatic SOD activity when compared with the group exposed to CS only. No changes were observed in the hepatic and pulmonary GR activity of the celecoxib per se group when compared with the control group (Table 1). Earlier, we have also reported an increase in pulmonary GR activity in mice exposed to CS. Possibly, the increase in GSH dependent antioxidant enzymes is an adaptive response to increased oxidative stress due to CS.

The lung of mice from control group showed normal histarchitecture with no abnormality (Fig. 1a). Smoke exposure clearly induced alveolar wall destruction and air space enlargement. The alveolar wall was thinner than normal with some emphysematous areas (Fig. 1b). No changes were observed in the lung tissue of mice administered with celecoxib only (Fig. 1c). In co-treatment group, there was enlargement of air spaces (Fig. 1d). Emphysema is a destructive disease of the pulmonary parenchyma characterized by large air spaces in the lungs, which are associated with a smaller number of alveoli with thin walls. Cigarette smoking is the leading cause of emphysema. One of the hypotheses that explain the CS induced emphysema is that the oxidants in the CS deplete the antioxidant supply in the lung and cause oxidative injury to the tissues, leading to emphysema. During exposure to CS, large amounts of oxygen free radicals are generated which could damage the lipid components of the cell membranes as well as the matrix components of the lung. Destruction of lung matrix, especially elastin may lead to emphysema. The pro-oxidant milieu created in the lungs due to CS exposure might be responsible for the observed changes. No changes were observed in the lung tissue of mice administered with celecoxib only. In the co-treatment group, enlargement of air spaces was observed. The alveolar septa were thickened significantly with infiltration of inflammatory cells.
Histological studies of liver of control animals revealed normal histoarchitecture, clear cut hepatic lobules, separated by interlobular septa, traversed by portal veins. The hepatocytes were almost polyhedral in shape (Fig. 2a). Liver of animals exposed to CS exhibited a slightly altered histoarchitecture. A few of the hepatocytes were vacuolated and not having a usual polyhedral shape. Vacuolization was apparently more near the portal tracts. Hyperchromatia was also evident in the liver of CS exposed animals (Fig. 2b). In the liver of celecoxib-treated animals, vacuolization was a prominent feature. In general, some degree of hepatic hypertrophy was also evident (Fig. 2c). Slight restoration of the normal histoarchitecture of the liver was observed in the co-treatment group. Here, also vacuolization was evident, however, to a lesser degree (Fig. 2d). Moderate vacuolization of hepatocytes in liver of rats exposed to tobacco smoke has been reported earlier 31.

The cellular antioxidant defenses play a significant role in oxidant carcinogenesis. Epidemiologic studies on serum antioxidants and diet suggest that elevated levels of vitamin E and β-carotene reduce mortality in patients affected with lung and colon cancer 32,33. The antioxidant system protects DNA from oxidative damage and mutagenesis and also protects “initiated” cells from excessive oxidant toxicity and their clonal expansion in tumor promotion 34-36. Therefore, scavenging the free radicals and boosting the antioxidant defense system can act as an effective strategy in reducing the risk to carcinogenesis.

In the present study, celecoxib reduced the oxidative stress produced in response to CS exposure. Therefore, suppression of CS induced ROS and COX-2 by selective COX-2 inhibitors like celecoxib could be beneficial in counteracting the risk to ‘oxidant carcinogenesis’ posed by CS inhalation.

References
1 Cueto R & Pyror W A (1994) Vib Spectrosc 7, 97-111
5 Mazhar D, Gillmore R & Waxman J (2005) Quan J Med 98, 711-718
8 Balkwill F & Mantovani A (2001) Lancet 357, 539-545
18 Kono Y (1978) *Arch Biochem Biophys* 186, 189-195
19 Williams C M & Arscott I D (1971) *Meth Enzymol* 17, 503-509