

Antioxidant Supplementation Prevents Oxidation and Inflammatory Responses Induced by Sidestream Cigarette Smoke in Old Mice

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Sidestream cigarette smoke (SSCS) makes up about 85% of significantly toxic environmental tobacco smoke (ETS). Reactive oxygen species (ROS) in SSCS play an important role in the pathogenesis of a wide range of diseases. Interleukin-6 is a pro-inflammatory cytokine and is closely linked with pathology in cardiovascular disease and conditions that have an inflammatory base. Exposure to SSCS through a burning cigarette for 30 min/day, 5 days a week, for 4 months increased interleukin-6 production in spleen and lipid peroxide level in mouse liver. Our findings suggest that ROS induced by SSCS will promote hepatic lipid peroxidation and may also contribute to an increase in interleukin-6 cytokine production. Multiple antioxidants given as a dietary supplement significantly normalized interleukin-6 cytokine production and prevented hepatic lipid peroxidation. We conclude that the SSCS in moderate intake levels increased oxidation and promoted inflammatory cytokine interleukin-6 production, whereas antioxidants prevented these changes. **Key words:** antioxidants, interleukin-6, lipid peroxidation, sidestream cigarette smoke. *Environ Health Perspect* 109:1007–1009 (2001). [Online 26 September 2001] <http://ehpnet1.niehs.nih.gov/docs/2001/109p1007-1009/zhang/abstract.html>

Environmental tobacco smoke (ETS; i.e., passive or second-hand smoke) is a pervasive contaminant in public places. ETS is a mixture of sidestream and mainstream cigarette smoke (SSCS and MSCS, respectively). SSCS constitutes 85% of ETS. Cigarette smoke is one of the greatest exogenous sources of free radicals (1). More than 4,000 compounds have been identified in cigarette smoke (2), many of which are capable of generating reactive oxygen species (ROS) during metabolism. Major sources of ROS are from both gas and tar phases of cigarette smoke. In the tar phase, at least four different free radical species can be identified. Among them, semiquinone can reduce oxygen to form superoxide ($O_2^{\cdot-}$) in the presence of water. Nitric oxide (NO) exists in cigarette smoke up to 500 ppm, which probably represents one of the greatest exogenous sources of NO to which humans are exposed. The NO reacts quickly with O_2 to form peroxynitrite ($ONOO^-$) and gives rise to alkyl peroxynitrite ($ROONO$) if it reacts with organic peroxy radical (3,4).

ROS such as NO, $ONOO^-$, and $ROONO$ have been recognized as important mediators of damage in biological systems (5). Furthermore, cigarette smoke can also induce endogenous production of ROS such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, which may increase intracellular oxidative stress. Oxidative stress was indicated by an alteration of antioxidant enzymes in heart, liver, and lung tissues (6,7). Exposure to ETS at work increased the level of certain antioxidant enzymes in the blood of employees (8). Oxidative damage to cellular components occurs when the production of ROS overwhelms the cell's antioxidant

defenses and leads to lipid peroxidation and decreased tissue antioxidant levels. ROS also can initiate a series of cellular responses that play an important role in the proinflammatory process. Our previous study indicated that a small amount of SSCS (30 min/day, 5 days/week for 10 days) in immunodeficient mice caused lung injury and lipid peroxidation in the lung and liver (9). To investigate whether moderate intake of SSCS initiates proinflammatory response and promotes oxidative damage in healthy, old mice, we tested hepatic lipid peroxide and vitamin E level and splenocyte interleukin-6 (IL-6) production in both nonsmoking and smoking mice. Our hypothesis is that multiple antioxidants may help to prevent oxidation and proinflammatory response induced by SSCS.

Materials and Methods

Experimental design. We purchased 13-month-old C57BL/6 female mice from Charles River Laboratories (Wilmington, DE). They were housed in transparent plastic cages with stainless wire lids (four mice per cage) in the animal facility of the Arizona Health Science Center. The housing facility was maintained at 20–22°C and 60–80% relative humidity, with a 12-hr light–dark cycle. The mice had free access to water and a semi-purified diet (4% mouse diet, #7001; Teklad, Madison, WI). After 2 weeks of housing, the mice were randomly divided into four groups with 12 mice in each group: group I, nonsmoking group fed control diet; group II, nonsmoking group fed multiple antioxidants diet; group III, 30-min SSCS exposure fed control diet; group IV, and 30-min SSCS exposure fed multiple antioxidants diet. The control diet was AIN 93 M, synthetic pelleted

diet (Dyets Inc., Bethlehem, PA). It was supplemented with placebo beadlets. The supplemented diet was AIN 93 M synthetic, supplemented with β -carotene (15 mg/g diet; Hoffmann-la Roche, Nutley, NJ), bioflavonoids (300 μ g/g diet), coenzyme Q10 (300 μ g/g diet), D- α -tocopherol (10-fold increase over control diet, 1.5 mg/g diet), L-ascorbic acid (300 μ g/g diet), L-carnitine (300 μ g/g diet), magnesium (5-fold increase over control diet, 4.2 mg/g diet), N-acetylcysteine (300 μ g/g diet), retinol (80 μ g/g diet), selenium (1.8 μ g/g diet), and zinc (289 μ g/g diet; 3-fold increase over control diet).

Sidestream cigarette exposures. Standard research cigarettes (1R4; University of Kentucky Smoking & Health Effects Laboratory, Lexington, KY) were used in this study. The mice were exposed to SSCS for 30 min/day, two cigarettes every 10 min, 5 days/week using an IN-TOX vacuum-drawn (15 L/min) exposure system (Albuquerque, NM) modified for cigarette smoke exposure. The methodology for SSCS exposure and aerosol characterization has been previously reported (9). The SSCS-exposure mice were placed in the IN-TOX exposure system for a 30-min exposure period. SSCS was generated in the following pattern: the first two lit cigarettes were placed upright in a clamp 2.5 cm below the bottom edge of an inverted 220-cm³ funnel and allowed to burn for 10 min. The second two cigarettes were lit at the 10-min time point of the exposure period and replaced the first two cigarettes. Then the second two were replaced by the third until 30-min exposure was completed. The control group was treated in a similar manner except that the cigarettes were not lit. The SSCS treatment lasted for 2 months. The animals were killed the day after the last exposure. Spleen and liver tissues were then removed for cytokine, lipid peroxidation, and vitamin E assays.

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Lipid peroxidation assay. Quantitative determination of lipid peroxides in liver was done by using a quick LPO-CC K-ASSAY (Hamiya Biomedical Company, Seattle, WA). The range of the method is approximately 2–300 nmol/mL. Briefly, 0.2 mg mouse liver was homogenized in 1 mL chloroform (CHCl₃)/methanol (2:1 v/v). Next, 0.3 mL 0.9% NaCl was added to clarify, and the mixture was centrifuged at 3,000 × *g* for 10 min. The supernatant was collected and evaporated under nitrogen gas. We then added 100 μL of isopropanol to dissolve the lipid residue. Test sample, standard, and control were added 20 μL in triplicate in 96-well microplate. Lipid peroxides were quantitated by colorimetrically measuring methylene blue at 675 nm. Lipid peroxide values were calculated following the manufacturer's instructions.

Vitamin E determination. We measured vitamin E by HPLC as described previously (9). Briefly, about 0.2 g liver tissue was homogenized in 1.0 mL water. Butylated hydroxytoluene was added to prevent oxidation of α-tocopherol from the homogenate. Extracts were evaporated under a steady flow of nitrogen gas at 20°C and then redissolved in 0.5 mL of methanol injection onto a C18 column (3.9 × 150 mm NovaPak; Millipore, Bedford, MA). The mobile phase was composed of methanol: distilled water in the ratio of 98:2 (by volume) with a flow rate of 1.2 mL/min. α-Tocopherol, eluting at 6.5 min, was monitored by a fluorescence detector at 290 nm excitation and 340 nm emission wavelengths. A set of α-tocopherol standard concentrations was analyzed to make a standard curve and to verify calibration.

Cytokine ELISA assay. Mitogen-stimulated splenocytes were cultured in triplicate in 96-well microtiter plates as described previously (10). Briefly, the spleen was gently teased with forceps in a culture medium (RPMI 1640 containing 10% fetal bovine serum, 2 nmol/L glutamine, 1 × 10⁵ U/L penicillin and streptomycin), producing suspension of spleen cells. Red blood cells

were lysed by the addition of a lysis buffer (0.16 mol/L ammonia chloride Tris buffer, pH 7.2) at 37°C for 3 min. The cells were then washed twice with culture medium. Cell concentration was adjusted to 1 × 10⁷ cells/mL (splenocyte viability was determined by trypan blue exclusion). Splenocytes were cultured in triplicate on 96-well, flat-bottom culture plates (Falcon 3072; Becton Dickinson, Lincoln Park, NJ) with culture medium. Splenocytes were incubated 24 hr after the addition of lipopolysaccharide 1 × 10⁻² g/L (Gibco, Grand Island, NY) to induce IL-6 production in a 37°C, 5% carbon dioxide incubator. After 24 hr of incubation, supernatants were collected and stored at -70°C until analysis. To measure the quantity of murine IL-6 in supernatants of splenocyte cultures, we used a specific solid-phase ELISA assay, using the multiple antibody sandwich principle. An IL-6 kit was obtained from Pharmingen (Endogen, MA). All tests were performed according to the manufacturer's instructions and were done in triplicate in 96-well microtiter plates.

Statistical analyses. Statistical analyses were carried out using an SPSS 10.0 Windows statistical package (SPSS Inc., Chicago, IL). Values are expressed as the mean ± SD. Values were compared using a one-way analysis of variance (ANOVA), followed by a two-tailed Student's *t*-test for comparison between any two groups. Values with *p* < 0.05 were considered significant.

Results

Figure 1 shows the lipid peroxide production in mouse liver. The liver is the major organ that has been studied for tissue lipid peroxidation. The production of hepatic lipid peroxides was significantly increased by SSCS exposure (*p* < 0.05). However, the production of hepatic lipid peroxides was significantly decreased in both nonsmoking and smoking mice with antioxidant supplementation (*p* < 0.05).

Hepatic vitamin E levels in each group are shown in Figure 2. Hepatic vitamin E in

smoke-exposed mice was significantly decreased compared to mice not exposed to smoke (*p* < 0.05). Hepatic vitamin E in both exposed and control groups fed multiple antioxidants was significantly higher than in smoke-exposed mice fed a control diet (*p* < 0.05).

We determined the production of IL-6 in splenocytes in each group (Figure 3). Overproduction of IL-6 in the spleen was shown in smoke-exposed mice compared to control mice (*p* < 0.05). IL-6 production in spleen was significantly reduced in smoke-exposed mice with antioxidant supplementation compared to exposed mice on the control diet (*p* < 0.05).

Discussion

Cigarette smoke contains large amounts of both carbon- and oxygen-centered free radicals, which can directly or indirectly initiate and propagate the process of lipid peroxidation. The results of the present study suggest that SSCS-induced lipid peroxidation was increased in the liver. A cell may defend itself against oxidative stress through the use of antioxidants. These antioxidants are consumed in the process of scavenging free radicals before more important structures are damaged. Antioxidants can be conveniently divided into water soluble and lipid soluble, and exist in environments such as lipoproteins and cell membranes to prevent the propagation phase of lipid peroxidation (chain-breaking antioxidants). Of the aqueous molecules, the best known is vitamin C (ascorbate), which is the most powerful electron donor and the first plasma antioxidant to be sacrificed upon exposure to oxidative stress (11). There is evidence that ascorbate contributes up to 24% of the total peroxy radical-trapping antioxidant capacity in the human plasma (12–14). Evidence exists that ascorbic acid inhibits both phagocyte-induced and endothelial cell-induced and lipid peroxidation processes (15). Vitamin E is a lipid-soluble, powerful chain-breaking antioxidant (16). It has a protective role

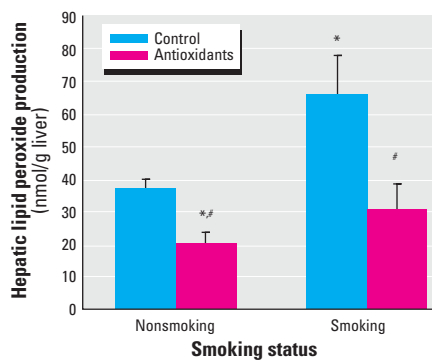


Figure 1. Hepatic lipid peroxide production.

*Compared to nonsmoking control (*p* < 0.05). #Compared to control diet (*p* < 0.05).

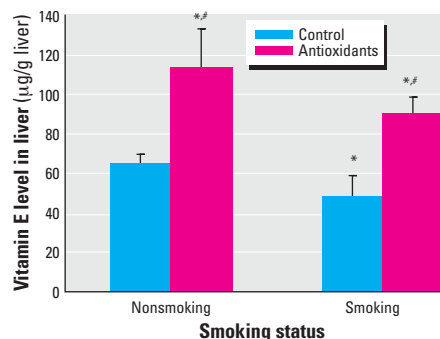


Figure 2. Hepatic vitamin E level.

*Compared to nonsmoking control (*p* < 0.05). #Compared to control diet (*p* < 0.05).

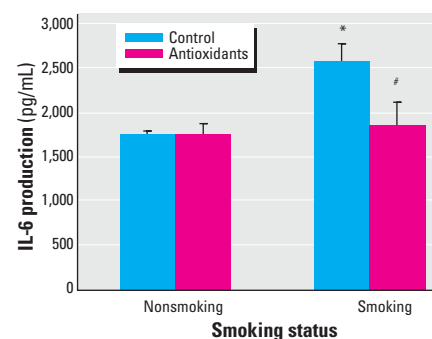


Figure 3. Splenocyte IL-6 production in mouse splenocytes.

*Compared to nonsmoking control (*p* < 0.05). #Compared to control diet (*p* < 0.05).

against the damaging effects of smoke, including in the immune cells that produced large amounts of oxidants (17–19). Our data indicated that the tissue antioxidant (e.g., vitamin E in SSCS-exposed mouse liver) was depleted. Tissue vitamin C level was not determined in this experiment. It has been reported that plasma levels of ascorbic acid, the reduced form of vitamin C, were significantly lower in active smokers (20). These results suggest that the cell antioxidant defense system is affected by SSCS exposure.

Proinflammatory cytokine IL-6 is closely linked with pathology in a wide range of diseases and conditions that have an inflammatory basis. Our data suggest that SSCS stimulated IL-6 production in the spleen, while antioxidant vitamins reduced it. Oxidants stimulate proinflammatory cytokine production through activation of nuclear factor κ B (NF- κ B). Antioxidant nutrients have the potential to modulate inflammatory aspects of immune function by disrupting the NF κ B pathway (21). Another study shows that nicotine stimulates the release of norepinephrine, which can induce IL-6 synthesis in the liver and spleen (22). There is also evidence that tobacco glycoprotein stimulates the immune system and induces IL-6 mRNA synthesis in spleen cells (23). Serum levels of nicotine and tobacco glycoprotein were not determined in the present study, so we cannot exclude the possibility that nicotine or tobacco glycoprotein induced IL-6 production in the spleen.

Overall, we observed that moderate levels of SSCS exposure induced oxidation and

promoted inflammatory cytokine production. Multiple antioxidant supplementations could attenuate lipid peroxidation and proinflammatory response. Nevertheless, further studies need to be conducted to explore the most plausible pathway of inflammatory immune response upon exposure to SSCS. The antioxidant enzyme activities and other antioxidant levels of tissue or serum should be tested as well.

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