Black Tea Prevents Cigarette Smoke-Induced Oxidative Damage of Proteins in Guinea Pigs[1]

Anup Misra, Ranajay Chattopadhyay, Shuvojit Banerjee, Dhruba J. Chattopadhyay and Indu B. Chatterjee[2]

Dr. B. C. Guha Centre for Genetic Engineering and Biotechnology and the Department of Biochemistry, University College of Science, Kolkata-700019, India

ABSTRACT Cigarette smoke (CS) causes oxidative damage and tea polyphenols have strong antioxidant properties. Therefore, we studied the effect of a black tea (BT) infusion on CS-induced oxidative damage of proteins both in vitro and in vivo. In the in vitro experiment, bovine serum albumin (BSA) or a guinea pig tissue microsomal suspension was incubated with an aqueous extract of CS (CS-solution) in the presence or absence of the BT infusion. Protein oxidation was measured by immunoblotting of the dinitrophenylhydrazone derivatives of the protein carbonyls followed by densitometric scanning. Protein degradation was assessed by SDS-PAGE. BT prevented ($P < 0.05$) CS-induced oxidation of BSA and oxidative degradation of guinea pig lung, liver and heart microsomal proteins. This was also observed when the BT infusion was replaced by its components, i.e, flavonols, theaflavins, thearubigins and catechins. BT prevented microsomal protein degradation by inhibiting oxidative modification of the proteins. The antioxidant effect of BT was similar to that of green tea. In the in vivo experiment, partially ascorbate-deficient guinea pigs were subjected to CS exposure from 5 cigarettes/(guinea pig · d) for 7 d and given water or the BT infusion (20 g/L) to drink. Guinea pigs exposed to CS and given water had extensive oxidation accompanied by 39, 40 and 30% losses ($P < 0.05$) of microsomal proteins of lung, liver and heart, respectively. However, when the CS-exposed guinea pigs consumed the BT infusion instead of water, the oxidation of microsomal proteins was reduced ($P < 0.05$) ~90, 97 and 70% in lung, liver and heart, respectively. Protein loss was reduced ($P < 0.05$) ~92, 98 and 90% in lung, liver and heart, respectively. The results, if extrapolated to humans, would indicate that regular intake of tea may protect smokers from CS-induced oxidative damage and consequent degenerative diseases. J. Nutr. 133: 2622–2628, 2003.

KEY WORDS: • *cigarette smoke* • *black tea* • *bovine serum albumin* • *guinea pigs* • *oxidative protein damage*

Cigarette smoke (CS)[3] causes many life-threatening diseases such as pulmonary and cardiovascular diseases as well as lung cancer and other malignancies (1–5). One of the prominent deleterious effects of CS is the oxidative damage of biological macromolecules including proteins and DNA (6–9). The oxidative damage is caused by stable oxidants present in the aqueous extract of CS (8). The stable oxidants are likely the long-lived free radicals present in tar (8,10,11). We reported previously that the stable oxidants in CS cause oxidation of plasma proteins and extensive oxidative degradation of guinea pig lung tissue microsomal proteins (8). Because tea polyphenols have strong antioxidant properties, tea might prevent CS-induced oxidative damage of biological macromolecules. In fact, there is some evidence to suggest that green tea has chemopreventive effects in cigarette smokers (12,13). Tea (*Camellia sinensis*) is one of the most widely consumed beverages in the world. Although ~80% of the tea produced in the world is consumed as black tea (BT) and only 20% as green tea (GT) (14), research has focused mainly on GT. GT contains ~30% catechins (15). Catechins are monomeric polyphenols, whose principal component is epigallocatechin gallate (EGCG). Catechins are powerful antioxidants for scavenging reactive oxygen species such as superoxide, hydrogen peroxide and hydroxyl radicals (16). On the basis of epidemiologic data and animal experiments, GT has been suggested to prevent CS-induced cancers of the lung, oral cavity and esophagus (17). EGCG and other catechins apparently prevent cancer through inhibition of oxidative reactions (18). It has been shown that GT polyphenols inhibit both CS- and H_2O_2-induced DNA breakdown (19).

In contrast to GT, BT has been assumed to have less antioxidant activity than GT because of its lower monomeric polyphenol content (16,20). As a result, little research has been conducted with BT. During the manufacture of BT, the

[1] Supported by National Tea Research Foundation, Tea Board.
[2] To whom correspondence should be addressed.
E-mail: ibc123@rediffmail.com.
[3] Abbreviations used: BSA, bovine serum albumin; BT, black tea; BT group, guinea pigs exposed to air and given BT to drink; Controls, guinea pigs exposed to air and given water to drink; CS group, guinea pigs exposed to CS and given water to drink; CS + BT group, guinea pigs exposed to CS and given BT to drink; CS, cigarette smoke; CT, catechins; DFO, desferrioxamine; DNP, dinitrophenylhydrazone; DNPH, 2,4-dinitrophenyl-hydrazine; EGCG, epigallocatechin gallate; GT, green tea; PMSF, phenylmethylsulfonyl fluoride; TF, theaflavins; TR, thearubigins.

Manuscript received 1 March 2003. Initial review completed 24 March 2003. Revision accepted 15 May 2003.

green tea catechins undergo oxidation by polyphenol oxidase to form the complex condensation products theaflavins (TF) and thearubigins (TR), by a process commonly known as "fermentation." TF are a mixture of TF and TF-gallates. However, TR are more complex oligomeric flavonols; they vary greatly in molecular weight, ranging from 1 to 40 kDa (15). The contents of TF and TR vary with the species of tea and the process of "fermentation." Nevertheless, TR are the most abundant phenolic fractions in BT. BT contains ~1–4% TF, 10–20% TR and catechins (CT) (15,20). There are contradictory reports concerning the comparative beneficial effects of GT and BT with particular reference to chemoprevention (21,22). Others indicate that TF in BT and CT in GT are equally effective antioxidants (18,23–28). In one study, the extracts from BT scavenged hydrogen peroxide more potently than those from GT (29).

Previously we and others reported that oxidative modification of proteins by stable oxidants of CS introduces carbonyl groups into the side-chain amino acids (6,8,9,11). The formation of the carbonyl groups can be measured spectrophotometrically after they are converted into dinitrophenylhydrazone (DNP) derivatives by reaction with 2,4-dinitrophenylhydrazine (DNPH) (6,30). However, the quantification of the preventive effect of BT on CS-induced oxidation of proteins by spectrophotometric assay of the DNP derivative was incorrect because BT interacts with proteins and the product of that interaction absorbs at 360–390 nm, the wavelength at which the DNP derivative is measured. In this paper we used immunoblotting and densitometric scanning to measure the preventive effect of black tea and its components on the CS-induced oxidative damage of proteins.

MATERIALS AND METHODS

Reagents. Unless otherwise specified, CTC black tea was used. The source of both black and green teas was West Bengal Tea Development, Kolkata, India. CT, EGCG, bovine serum albumin (BSA) and tea extract were purchased from Sigma Chemical, St. Louis, MO. A tea extract containing ~80% TF was used as the source of TF. TR was prepared by the method described below. All other chemicals were of analytical grade.

Preparation of the tea infusion. Black or green tea (1 g) was added to 10 mL of boiling water, brewed for 5 min, cooled to room temperature and filtered. The filtrate was designated as the BT or GT infusion.

Measurement of theaflavins and catechins in BT. Diluted samples of the BT infusion were analyzed by HPLC in a Shimadzu instrument (Kyoto, Japan) consisting of a SPD-10A VP UV-Vis detector, LC-10A VP dual pump systems, attached to a chromatopac C-R6A. Individual polyphenols were used as standards. A Lichrospher 100 RP-18 encapped (5 μm; 250 mm × 4 mm) column (Merck, Darmstadt, Germany) was used. CT and TF were detected at 270 nm using a binary gradient cycle as described by Lee et al. (15).

Preparation of the polyphenol-free BT infusion. The BT infusion as prepared above (100 g/L) was treated twice with polyvinylpolypyrolidone (30 g/L of infusion) until the filtrate was a faint yellow color. The filtrate was then passed through a bed of activated charcoal to remove the residual polyphenols as well as other low-molecular-weight components. The effluent from the charcoal bed was adjusted to the original volume. It was practically colorless and did not absorb at 270 nm.

Preparation of TR. TR were isolated from BT following a combination of methods of Subramanian et al. (31) and Hara (32). BT (6 g) was boiled in 50 mL sodium acetate (10 mmol/L, pH 5.0) for 10 min, cooled and filtered. The filtrate was extracted successively with equal volumes of chloroform, methyl isobutyl ketone and ethyl acetate. The organic layers were discarded and the aqueous layer was extracted with butanol followed by lyophilization. The residual dark orange powder constituted the TR; its content was measured spectrophotometrically at 270 nm (31) as described below.

Measurement of TR in BT. Spectroscopic analysis showed that the BT infusion and all the individual polyphenols, TF, TR and CT had λ_{max} at 270 nm. We calculated the TR content of BT by subtracting the combined A270 due to TF and CT from the A270 of the BT infusion. As stated above, TF and CT in BT were estimated separately by HPLC.

Preparation of the cigarette smoke solution (CS-solution). Smoke from an Indian commercial filter-tipped cigarette (74 mm) with a tar content of 22 mg was dissolved in 1 mL phosphate buffer (50 mmol/L), pH 7.4, as described (8). The pH of the yellow-colored solution was adjusted to 7.4 by the addition of sodium hydroxide solution (80 g/L) followed by filtration through a 0.22-μm Millipore filter.

Experimental designs. In the in vitro experiment, BSA or guinea pig tissue microsomes were incubated with CS-solution in the presence or absence of the BT infusion. In the in vivo experiment, partially ascorbate-deficient pair-fed guinea pigs were exposed to air or CS and given water or the BT infusion to drink.

Exposure of guinea pigs to cigarette smoke (CS). Male shorthair guinea pigs weighing 400–500 g were obtained from a local animal supplier (M/S S. C. Ghosh, Kolkata, India). All animal care procedures met NIH guidelines (33). The guinea pigs were fed an ascorbate-free diet for 7 d to minimize the ascorbate level in plasma and tissues; ascorbate is a potential inhibitor of CS-induced oxidative damage of proteins, which would otherwise counteract the damaging effect of CS (8,9). The diet contained in g/kg: wheat flour, 700; casein, 200; sucrose, 80; USP XVII salt mixture, 10; AOAC vitamin mixture, 10 (34). The guinea pigs were divided into the following experimental groups (n = 4/group). Control: exposed to air instead of CS and given water to drink; CS: exposed to CS and given water to drink; CS + BT: exposed to CS and given the BT infusion (20 g/L), to drink instead of water; BT: exposed to air and given the BT infusion (20 g/L) to drink. The tea infusion was freshly prepared and replaced every morning and evening;

All guinea pigs were pair-fed individually with respect to a guinea pig in the CS group. The pairs were set up by initial weight. The amounts of food and fluid consumed by the CS group on the previous day were given to the individual guinea pigs of the other groups.

After consuming the ascorbate-free diet for 7 d, the guinea pigs were subjected to air or CS exposure from 5 cigarettes/(guinea pig · d) for another 7 d in a smoke chamber as described (9). The duration of exposure was 1 min for each puff. In the CS-exposed guinea pigs, each guinea pig was given two puffs per cigarette, allowing them a 1-min rest in a smoke-free atmosphere. The gap between exposures was 1 h. This was done to simulate human smoking.

After 7 d of CS/air exposure, both the controls and the CS-exposed guinea pigs were deprived of food overnight and killed the next day by diethyl ether asphyxiation. The lung, liver and heart tissues were excised immediately and processed without delay. Oxidative damage of the tissue microsomal proteins, without any further treatment, was assayed by immunoblotting (described below) and SDS-PAGE (8). Protein was estimated by the method of Lowry et al. (35) as described (8).

Preparation of microsomes. Portions of the lung, liver and heart from the guinea pigs of each group were pooled, homogenized and microsomes prepared and washed as described (8). The washed microsomes were dispersed in potassium phosphate buffer (50 mmol/L), pH 7.4, so that 1 mL of the dispersion was equivalent to 1 g tissue.

Incubation system. The incubation mixture contained BSA (1 mg) or tissue microsomal suspension (equivalent to 1 mg of protein) with or without 50 μL of CS-solution (equivalent to one twentieth of a cigarette) in the presence or absence of 50 μL of tea infusion (equivalent to 5 mg tea) or its components in a final volume of 200 μL potassium phosphate buffer (50 mmol/L), pH 7.4. Incubation was carried out at 37°C with shaking for 1 h or as described. In studies on the interaction of BSA with whole-phase CS, instead of CS-solution, smoke from one cigarette was released directly into 1 mL of potassium phosphate buffer (50 mmol/L), pH 7.4, containing 20 mg BSA as described (8). Fifty microliters of this solution containing 1 mg of the smoke-treated protein was added to 150 μL potassium phosphate buffer (50 mmol/L), pH 7.4, to adjust the final incubation volume to 200 μL.

In experiments to measure CS-induced oxidation of guinea pig tissue microsomal proteins, phenylmethylsulfonyl fluoride (PMSF)

and EDTA were added to the incubation mixture to prevent proteolytic degradation of the oxidized proteins (8).

Production of the DNP derivative of oxidized protein. After incubation as described above for 1 h, 5 μL of the incubation mixture was mixed with 5 μL SDS (120 g/L) solution. After 5 min, 10 μL of the DNPH solution (10 mmol/L) in HCl (2 mol/L) was added and the reaction was allowed to continue for 25 min followed by the addition of 5 μL of neutralizing solution according to the Oxyblot protein oxidation detection kit described below.

Measurement of protein carbonyl by immunoblotting of the DNP-protein. Twenty-five microliters of the DNP derivative of BSA or microsomal proteins containing 25 μg of protein as described above were separated by SDS-PAGE followed by Western blotting using a polyvinylidene difluoride membrane according to the protocol for the Oxyblot protein oxidation detection kit (Catalog #S7150, Intergen company, New York, NY). The membrane was then incubated with primary antibody, specific to the DNP-moiety. This step was followed by incubation with a secondary antibody conjugated with horseradish peroxidase (goat anti-rabbit IgG) directed against the primary antibody. The membrane was treated with chemiluminescent reagents (luminol and enhancer) and the chemiluminescence was detected by autoradiography. The intensity of the blots produced by autoradiography was quantified by densitometric scanning.

Measurement of ascorbic acid in BT. Ascorbic acid was measured by HPLC of samples of the tea infusion in a Shimadzu instrument as described above. The column used was a Lichro CART 250–4 NH2 (Merck). The mobile solvent was a mixture of acetonitrile and potassium dihydrogen phosphate solution (50 mmol/L) at a ratio of 75:25, v/v. The flow rate was 1.5 mL/min. Ascorbic acid was detected at 254 nm with a retention time was 6.1 min and a limit of detection of 500 pg.

Statistical analysis. Both in vitro and in vivo experiments were analyzed by two-way ANOVA (CS × BT). The Bonferroni post-hoc test was used when the *F*-values were significant. Differences with *P*-values <0.05 were considered significant.

RESULTS

Prevention of CS-induced BSA oxidation by BT and its constituent polyphenols. Incubation of BSA with CS-solution followed by reaction with DNPH and immunoblotting resulted in extensive formation of the DNP derivative (**Fig. 1**, lane 2), which was a measure of the extent of carbonyl groups produced by oxidation of the side-chain amino acids of BSA. Under similar conditions, the amount of protein carbonyl formed was 10 ± 0.5 nmol, as measured by the formation of the DNP derivative (8). When BSA was incubated with CS-solution in the presence of 50 μL of BT, the oxidation of protein was inhibited ~70% (Fig. 1, lane 3), as noted by comparative scanning of the blots. Incubation of BSA with BT alone did not produce any oxidation of protein. The inhibition by BT infusion was not affected by the addition of desferrioxamine (DFO, 20 μmol/L) to the incubation mixture, indicating that the inhibition was independent of free iron. TF, TR and CT, the principal components of BT, also inhibited CS-induced BSA oxidation. HPLC analysis indicated that 50 μL of the BT infusion contained 50 μg of TF, which equals ~1 g/100 g of TF in the dry sample of BT used. When instead of 50 μL of BT, 50 μg of TF was added to the incubation mixture containing 1 mg of BSA and 50 μL of CS-solution, the inhibition of BSA oxidation was 50% (Fig. 1, lane 4). The amount of TR in the BT used was 890 μg/50 μL of the BT infusion (~17.8 g/100 g). This amount of TR inhibited CS-induced BSA oxidation ~72% (Fig. 1, lane 5). HPLC analysis revealed that in addition to TF, 50 μL of the BT infusion used contained 300 μg of CT (~6%). When used separately, 300 μg of CT produced 85% inhibition (Fig. 1, lane 6). Among the polyphenols, CT had the maximum antioxidant effect when used at the level present in BT. However, when used at that same level, TF was the most potential antioxidant for inhibiting CS-induced BSA oxidation. Although 50 μg of TF inhibited 50%, 50 μg of CT inhibited only 20% and 50 μg of TR had little effect. When instead of the BT infusion, a mixture of 50 μg of TF, 890 μg of TR and 300 μg of CT was used, the inhibition of BSA oxidation was almost 100% (Fig. 1, lane 7), which was more than that obtained with the BT infusion. The data indicate that the additive effect of the constituent polyphenols is greater than that present in the BT infusion. The preventive effects of the polyphenols were confirmed by adding the individual polyphenols to a polyphenol-free BT infusion. This infusion alone did not inhibit CS-induced BSA oxidation. When TF, TR and CT, in amounts proportional to their levels in BT as stated above, were added separately and conjointly to the polyphenol-free BT infusion, the polyphenols inhibited CS-induced BSA oxidation similarly to the level of inhibition with individual polyphenols in the absence of the BT infusion. The addition of 50 μg of TF, 890 μg of TR and 300 μg of CT to 50 μL polyphenol-free BT infusion produced 47, 70 and 80% inhibition, respectively. When TF, TR and CT were used together, the inhibition was almost 100%. When instead of BT, 50 μL of GT infusion was used, the inhibition of BSA oxidation was 72% (Fig. 1, lane 8). These results demonstrate that although BT contains mostly oxidized and complex condensation products of CT, both BT and GT have similar antioxidant activity against CS-induced BSA oxidation. The amount of EGCG present in 50 μL of GT was 870 μg. When 870 μg of EGCG was added to the incubation mixture, the inhibition of CS-induced BSA oxidation was 98%, which was more than that obtained with the GT infusion. This again indicates that the antioxidant effect of individual polyphenols is greater than that when they are present in the tea infusion. Similar preventive results of BT and GT were obtained when BSA was oxidized by whole-phase cigarette smoke instead of CS-solution (data not shown).

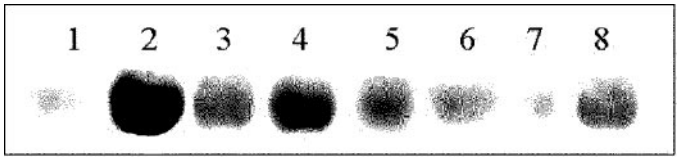

FIGURE 1 Immunoblot of the dinitrophenylhydrazone (DNP)-derivatives of bovine serum albumin (BSA) incubated with or without cigarette smoke (CS) in the presence and absence of black tea (BT), its constituent polyphenols and green tea (GT). Lane 1, BSA; lane 2, BSA + CS; lane 3, BSA + CS + BT; lane 4, BSA + CS + TF; lane 5, BSA + CS + TR; lane 6, BSA + CS + CT; lane 7, BSA + CS + TF + TR + CT; Lane 8, BSA + CS + GT. Compared with lane 2, the percentage inhibition by BT was 70 (lane 3); TF, 50 (lane 4); TR, 72 (lane 5); CT, 85 (lane 6); TF + TR + CT,100 (lane 7); GT,72 (lane 8). *P*-values estimated by two-way ANOVA were <0.0001 for control and CS-treatment (lanes 1 and 2) and <0.05 for CS-treatment and CS-treatment in the presence of BT (lane 3), TF (lane 4), TR (lane 5), CT (lane 6), TF + TR + CT (lane 7) and GT (lane 8); $n = 4$. TF, theaflavins; TR, thearubigins; CT, catechins.

The antioxidant effect of BT was similar whether the tea was harvested in summer or autumn. In a separate experiment, tea infusion prepared from orthodox Darjeeling or Assam tea had the same antioxidant effects as those of CTC tea. The inhibition of BSA oxidation by BT was a function of the concentration of the BT infusion. Although 25 μL of tea gave 43% protection, 50 μL of tea inhibited ~70%, 75 μL, 90% and 100 μL gave full protection (**Fig. 2**). With 50 μL of tea, the percentage inhibition of BSA oxidation increased with

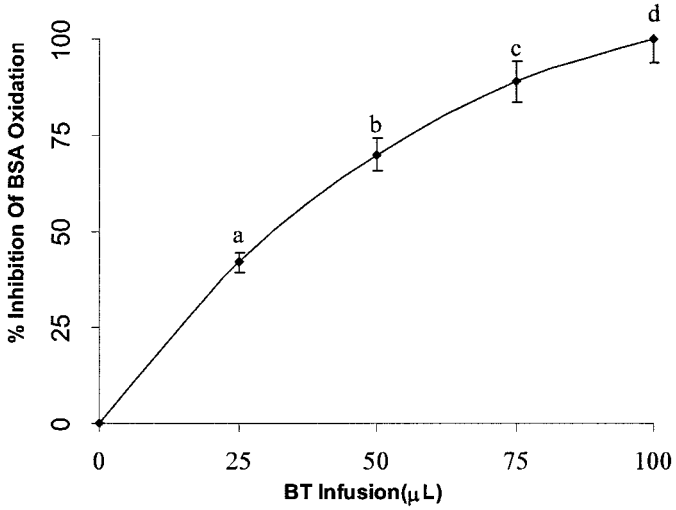

FIGURE 2 Protection of cigarette smoke (CS)-induced bovine serum albumin (BSA) oxidation as a function of concentration of black tea (BT) infusion in vitro. Values are means ± SD; $n = 4$. Means not sharing a common letter differ, $P < 0.05$.

increased time of incubation and attained a maximum value after 1 h (**Fig. 3**).

In addition to the CS-solution, a smoke solution prepared from bidi extensively oxidized BSA and guinea pig tissue microsomal proteins. This was prevented (70%) by the black tea infusion, as demonstrated by immunoblotting (not shown).

The inhibition of CS-induced oxidation of BSA and guinea pig tissue microsomal proteins by tea infusion was not due to the presence of ascorbic acid. HPLC analyses of both BT and GT infusions revealed little ascorbic acid in the tea infusion.

Prevention of CS-induced oxidation of guinea pig lung microsomal proteins by BT and GT in vitro. Incubation of guinea pig lung microsomes with CS-solution, followed by reaction with DNPH and immunoblotting, oxidized the microsomal proteins (**Fig. 4**, lane 2). Protein oxidation was inhibited by ~97% by the BT infusion (Fig. 4, lane 3). Incubation of the microsomes with only the BT infusion did not produce any oxidation (Fig. 4, lane 4). GT gave 93% protection (Fig. 4, lane 5). This indicates that the antioxidant effects of BT and GT are similar.

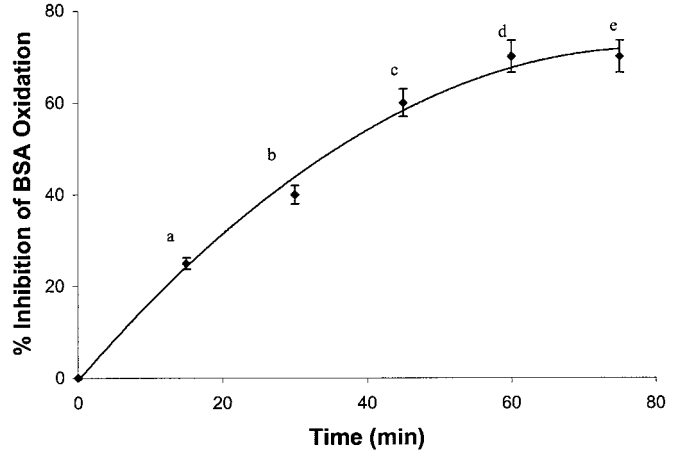

FIGURE 3 Inhibition of cigarette smoke (CS)-induced bovine serum albumin (BSA) oxidation by black tea (BT) infusion as a function of time in vitro. Values are means ± SD; $n = 4$. Means not sharing a common letter differ, $P < 0.05$.

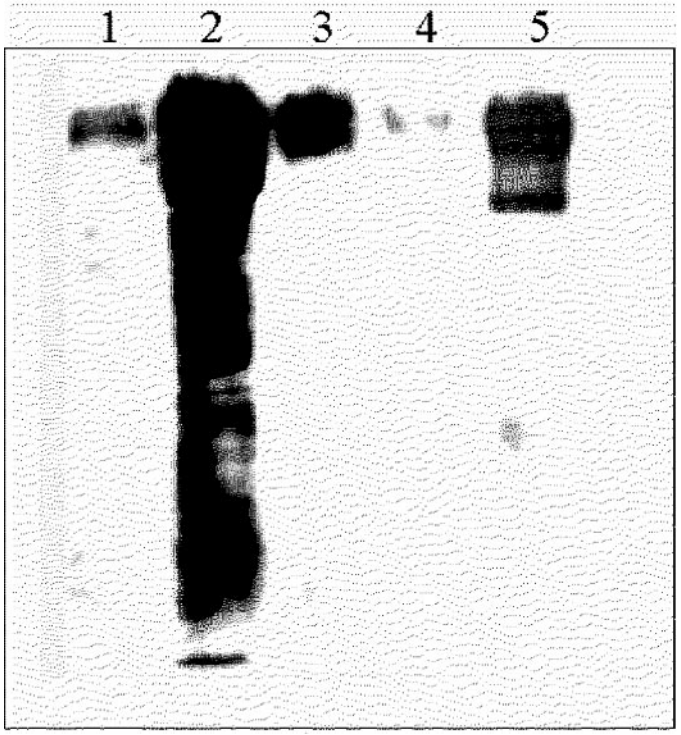

FIGURE 4 Immunoblot of the dinitrophenylhydrazone (DNP)-derivatives of guinea pig lung microsomal proteins incubated with or without cigarette smoke (CS) in the presence and absence of black tea (BT) and green tea (GT) in vitro. Lane 1, microsomes; lane 2, microsomes + CS; lane 3, microsomes + CS + BT; lane 4, microsomes + BT; lane 5, microsomes + CS + GT. CS-induced protein oxidation was inhibited ~97% by BT and 93% by GT (lanes 3 and 5). *P*-values estimated by two-way ANOVA were <0.0001 for control (lane 1) and CS treatment (lane 2) and <0.05 for CS treatment (lane2) and CS treatment in the presence of BT and GT (lanes 3 and 5). Microsomes treated with BT alone did not undergo protein oxidation (lane 4); $n = 4$.

CS-induced degradation of microsomal proteins and protection by BT in vitro. Incubation of guinea pig lung microsomes with the CS-solution degraded the microsomal proteins 90%, as shown by densitometric scanning of the protein bands (**Fig. 5**, lanes 1 and 2). In this case, the protease inhibitors PMSF and EDTA were not included in the incubation medium. The CS-induced protein degradation was inhibited 80% by the BT infusion (Fig. 5, lane 3). The inhibition of microsomal protein degradation by BT was apparently due to prevention of CS-induced protein oxidation, as reported earlier (8).

CS-induced oxidation of tissue microsomal proteins of guinea pigs in vivo and protection by BT. In each panel of immunoblots of lung, liver and heart microsomal proteins of guinea pigs (**Fig. 6**), lane 1 represents air-exposed guinea pigs (controls); lane 2, those exposed to CS and given water to drink (CS group); and lane 3, those exposed to CS and given the BT infusion to drink (CS + BT group). When guinea pigs were exposed to CS for 7 d at an exposure rate of 5 cigarettes/(guinea pig · d) and given water to drink, extensive oxidation of the microsomal proteins of lung, liver and heart occurred (Fig. 6, lane 2). There was practically no oxidation in the tissues of air-exposed guinea pigs (Fig. 6, lane 1; controls). When the guinea pigs were given the BT infusion to drink instead of water, CS-induced protein oxidation was reduced

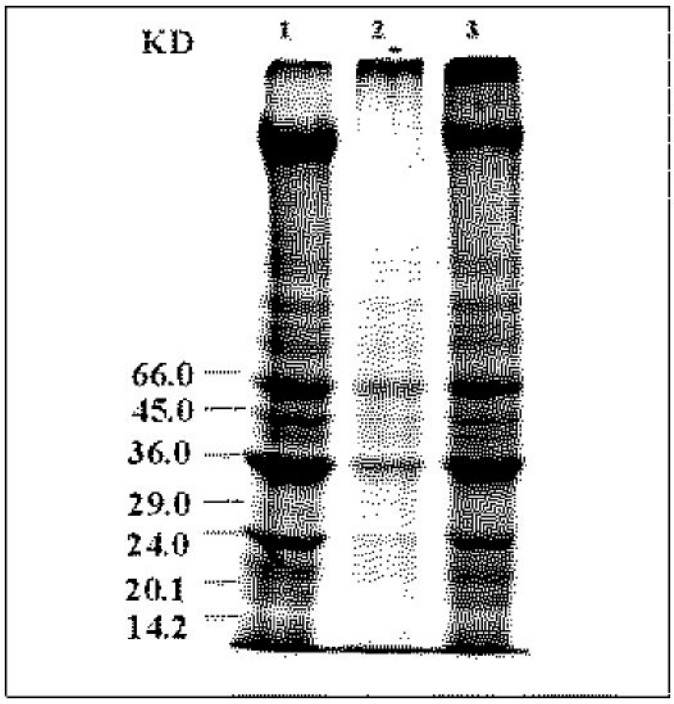

FIGURE 5 SDS-PAGE of guinea pig lung microsomal proteins incubated with or without cigarette smoke (CS) in the presence and absence of black tea (BT) in vitro. The gels (75 g/L) were stained with Coomassie brilliant Blue-250. Lane 1, microsomes incubated without CS; lane 2, microsomes incubated with 50 μL CS-solution in the absence of BT; lane 3, microsomes incubated with 50 μL CS-solution in the presence of 50 μL BT. BT inhibited CS-induced protein oxidation by 80%. *P*-values estimated by two-way ANOVA were <0.0001 for control (lane 1) and CS treatment (lane 2) and <0.05 for CS treatment (lane 2) and CS treatment in the presence of BT (lane 3); $n = 4$.

90% in the lung (Fig. 6, lane 3), 97% in the liver and 70% in the heart. Each guinea pig consumed ~25 mL of the BT infusion, which was equivalent to 0.5 g BT/d. Tissue micro-

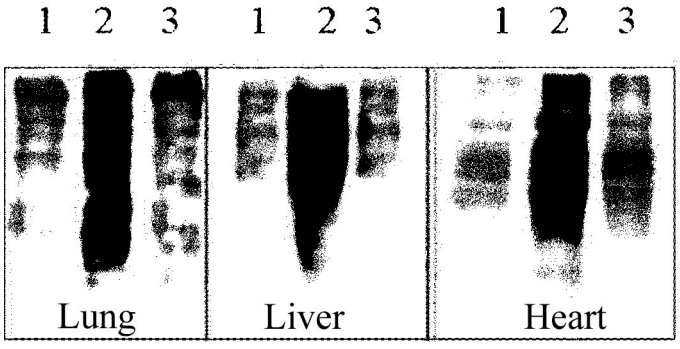

FIGURE 6 Immunoblot of the dinitrophenylhydrazone (DNP)-derivatives of lung, liver and heart microsomal proteins of guinea pigs exposed to air or cigarette smoke (CS) (in vivo) with or without black tea (BT) infusion. In each panel, lane 1, microsomes from guinea pigs exposed to air instead of CS (control) and given water to drink; lane 2, microsomes from guinea pigs exposed to CS (CS group) and given water to drink; lane 3, microsomes from guinea pigs exposed to CS and given the BT infusion to drink (CS + BT group). CS-induced protein oxidation was reduced 90% in the lung, 97% in the liver and 70% in the heart (lane 3 in each panel). *P*-values estimated by two-way ANOVA were <0.05 for CS, BT and CS $\times$ BT.

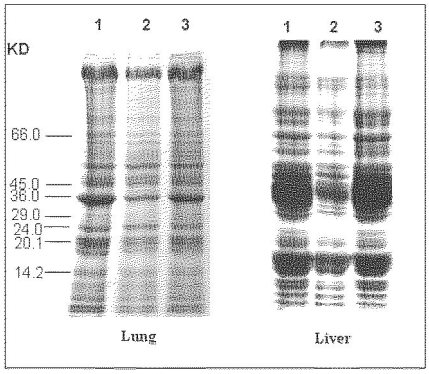

FIGURE 7 SDS-PAGE of lung and liver microsomal proteins of guinea pigs exposed to air or cigarette smoke (CS) (in vivo) with or without black tea (BT) infusion. The gels (100 g/L) were stained with Coomassie Brilliant Blue-250. Lane 1, guinea pigs exposed to air (control) and given water to drink; lane 2, guinea pigs exposed to CS and given water to drink (CS group); lane 3, guinea pigs exposed to CS and given the BT infusion to drink (CS + BT group). There was ~39% loss of protein bands in the lung and 40% in the liver (lane 2 in each panel). BT reduced the protein loss ~92% in the lung and 98% in the liver (lane 3 in each panel). *P*-values estimated by two-way ANOVA were <0.0001 for CS and <0.05 for BT.

somal proteins were not oxidized in the guinea pigs given BT infusion to drink without CS-exposure (BT only group, not shown).

CS-induced degradation of tissue microsomal proteins of guinea pigs in vivo and protection by BT. Protein profiles obtained by SDS-PAGE (**Fig. 7**, lane 2) showed that compared with air-exposed controls (control group, lane 1), there was an ~39% loss of protein bands in the lung microsomes and 40% loss in the liver microsomes of guinea pigs exposed to CS (CS group) for 7 d at an exposure rate of 5 cigarettes/(guinea pig · d). The food intake of the guinea pigs (~65 ± 5 g/d) did not differ among the groups. Thus, the microsomal protein loss in the guinea pigs of the CS group was not due to inanition but apparently to oxidative degradation of proteins as a result of CS exposure. When the CS-exposed guinea pigs were given the BT infusion to drink instead of water, the microsomal protein loss was inhibited by 92% in the lung (Fig. 7, lane 3) and 98% in the liver, as shown by comparative densitometric scanning. There was also a discernible loss of protein bands (~30%) of the heart microsomes of guinea pigs after exposure to CS, which was again prevented ~90% by drinking the BT (not shown).

DISCUSSION

Oxidative damage is one of the deleterious effects of CS (6–9,11,36–39). There have been many attempts to find suitable antioxidants that can prevent CS-induced oxidative damage and the consequent degenerative diseases. We reported before that CS-induced oxidative damage of proteins is markedly inhibited by ascorbic acid both in vitro and in vivo (8,9,11). Here, we demonstrated that CS-induced protein oxidation is almost completely prevented by a BT infusion. The antioxidant effect of BT did not differ from that of GT. This indicates that although BT contains oxidized and complex condensation products of CT, the antioxidant properties are not diminished. HPLC and spectrophotometric analyses showed that the sample of BT (CTC) used contained ~1% of TF, 17.8% of TR and 6% of CT. The antioxidant property of BT was a combined effect of the flavonols. When used in

relative proportions of their occurrences in BT, CT and TR had greater antioxidant effects than TF. But when used in identical amounts, TF and its gallate were better antioxidants than were TR and CT. HPLC analysis also revealed that the percentage distribution of TF and its gallates in the sample of BT used was TF, 27%; TF-3-monogallate, 18%; TF-3′-monogallate, 23% and TF 3,3′-digallate, 32%. The mechanism of the preventive action of the BT flavonols on CS-induced oxidation of protein is not clear at present. Polyphenols are reducing agents that function as antioxidants by virtue of their hydrogen-donating properties of the polyphenolic hydroxyl groups as well as their transition metal–chelating abilities (20). However, the addition of DFO to the incubation system did not alter the preventive effect of BT on CS-induced BSA oxidation. This showed that under the conditions present, TF, TR and CT of BT did not act as chelating agents and possibly reduced the CS-oxidants. The CS-oxidants are long-lived free radicals present in aqueous solutions of CS (10). It is possible that the antioxidant flavonols in BT quenched the free radicals and inactivated them. Recently, several studies have found that BT and GT offer protection against oxidative damage of RBC caused by a variety of inducers, e.g., H_2O_2, primaquine, phenyl hydrazine, Cu^{2+}-ascorbic acid and the xanthine/xanthine oxidase system (26–28). Oral administration of GT or BT inhibited lipid peroxidation of liver induced by *tert*-butylhydroperoxide in rats (40). Seraphine et al. (41) demonstrated that ingestion of GT and BT significantly increased human plasma antioxidant capacity. Catechin polyphenols also decreased peroxynitrite-induced nitration of tyrosine and protected apolipoprotein of LDL from modification of critical amino acids (42).

The in vitro observations were corroborated by the results obtained in vivo. For in vivo experiments, partially ascorbate-deficient guinea pigs were used because ascorbate prevents CS-induced protein damage (8,9). When guinea pigs were exposed to CS, there was extensive oxidation of protein accompanied by protein loss of tissue microsomes. The oxidation and protein loss were prevented when the guinea pigs were given the BT infusion to drink instead of water.

The degenerative diseases that have been linked to oxidative damage caused by CS are emphysema (43–45), atherosclerosis (38,46–48), lung cancer and other malignancies (49–52). Epidemiologic reports and other experimental studies suggest that tea has chemopreventive effects against cigarette smoking and tobacco use (12,13,17,25). Virtually one third of the world's population are direct or indirect smokers (53) and the hazardous effect of smoking is a global public health problem of great concern. Discouraging smoking would definitely be the best measure to eradicate this deleterious practice. However, in spite of the warnings and anti-smoking campaigns, smoking continues. Thus, a practicable approach is to find a way to prevent the deleterious effects of CS. The results presented in this paper, if extrapolated to humans, indicate that regular intake of tea may protect smokers from CS-induced oxidative damage and the consequent degenerative diseases.

ACKNOWLEDGMENTS

We thank K. Panda, Cleveland Clinic Foundation, OH, for his interest in this work and A. K. Dasgupta, Department of Biochemistry, Calcutta University, for assisting in the densitometric scanning. We also thank S. Ganguly and HILLA tea estate, West Bengal Tea Development, for the supply of BOP (CTC) tea.

LITERATURE CITED

1. Bartecchi, C. E., Mackenzie, T. D. & Schier, R. W. (1994) The human costs of tobacco use. N. Engl. J. Med. 330: 907–912.
2. Frank, E. (1993) Benefits of stopping smoking. West. J. Med. 159: 83–87.
3. U. S. Surgeon General's Report (1985) Department of Health Education and Welfare, Department of Health and Human Services, Washington, DC.
4. Shah, P. K. & Helfant, R. H. (1998) Smoking and coronary heart disease. Chest 94: 449–452.
5. Sherman, P. K. (1991) Health effect of cigarette smoking. Clin. Chest Med. 12: 643.
6. Reznick, A. Z., Cross, C. E., Hu, M. L., Suzuki, Y. J., Khwaja S., Safadi, A., Mottchnick, P. A., Packer, L. & Halliwell, B. (1992) Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation. Biochem J. 286: 607–611.
7. Kiyosawa, H., Suko, M ., Okudaira, H., Murata, K. & Nishimura, S. (1990) Cigarette smoking induces formation of 8 hydroxyguanosine, one of oxidative DNA damages in human peripheral leukocytes. Free Radic. Res. Commun. 11: 27–29.
8. Panda, K., Chattopadhyay, R., Ghosh, M. K., Chattopadhyay, D. J. & Chatterjee, I. B. (1999) Vitamin C prevents cigarette smoke induced oxidative damage of proteins and increased proteolysis. Free Radic. Biol. Med. 27: 1064–1079.
9. Panda, K., Chattopadhyay, R., Ghosh, M. K., Chattopadhyay, D. J. & Chatterjee, I. B. (2000) Vitamin C prevents cigarette smoke induced oxidative damage *in vivo*. Free Radic. Biol. Med. 29: 115–124.
10. Pryor, W. A., Prier, D. G. & Church, D. F. (1983) Electron spin resonance study of mainstream and side stream cigarette smoke: nature of the free radicals in gas phase smoke and cigarette tar. Environ. Health Perspect. 47: 345–355.
11. Panda, K., Chattopadhyay, R., Ghosh, M. K., Chattopadhyay, D. J. & Chatterjee, I. B. (2001) Cigarette smoke-induced protein oxidation and proteolysis is exclusively caused by its tar phase: prevention by vitamin C. Toxicol. Lett. 123: 21–23.
12. Shim, J., Kang, M., Kim, Y., Roh, J., Roberts, C. & Lee, I. (1995) Chemo preventive effect of green tea among smokers. Cancer Epidemiol. Biomark. Prev. 4: 387–391.
13. Lee, I. P., Kim, Y. Hi. & Kang, M. H. (1997) Chemo preventive effect of green tea against cigarette smoke-induced mutations in humans. J. Cell. Biochem. 27: S68–S75.
14. Krisnamoorthy, K. K. (1991) The nutritional and therapeutic value of tea. In: Proceedings of the International Symposium on Tea Science. Organizing Committee of ISTS, Shizuoka, Japan.
15. Lee, M.-J., Prabhu, S., Meng, X., Li, C. & Yang, C. S. (2000) An improved method for the determination of green and black tea polyphenols in biomatrices by high-performance liquid chromatography with colorimetric array detection. Anal. Biochem. 279: 164–169.
16. Katiyar, S. K. & Mukhtar, H. (1996) Tea and chemoprevention of cancer. Int. J. Oncol. 8: 221–238.
17. Weisburger, J. H. (1999) Tea and health: the underlying mechanisms. Proc. Soc. Exp. Biol. Med. 220: 271–275.
18. Lin, J.-K. & Liang, Y.-C. (2000) Cancer chemoprevention by tea polyphenols. Proc. Natl. Sci. Counc. Repub. China B. 24: 1–13.
19. Leanderson, P., Faresjo, A. O. & Tagesson, C. (1997) Green tea polyphenols inhibit oxidant-induced DNA strand breakage in cultured lung cells. Free Radic. Biol. Med. 23: 235–242.
20. Yang, C. S. & Wang, Z. Y. (1993) Tea and cancer. J. Natl. Cancer Inst. 85: 1038–1049.
21. Jankun, J., Selman, S. H. & Swiercz, R. (1997) Why drinking green tea could prevent cancer. Nature (Lond.) 387: 561.
22. Yang, C. S. (1997) Inhibition of carcinogenesis by tea. Nature (Lond.) 389: 134.
23. Leung, L. K., Su, Y., Chen, R., Zhang, Z., Huang, Y. & Chen, Z. Y. (2001) Theaflavins in black tea and catechins in green tea are equally effective antioxidants. J. Nutr. 131: 2248–2251.
24. Wiseman, S. A., Balentine, D. A. & Frie B. (1997) Antioxidant in tea. Crit. Rev. Food Sci. Nutr. 37: 705–718.
25. Weisburger, J. H. (1997) Tea and health: a historical perspective. Cancer Lett. 114: 315–317.
26. Grinberg, L. N., Newmark, H., Kitrossky, N., Rahamim, E., Chevion, M, & Rachmilewitz, E. A. (1997) Protective effects of tea polyphenols against oxidative damage of human red blood cells. Biochem. Pharmacol. 54: 973–978.
27. Zhang, A., Zhu, Q. Y., Luk, Y. S., Ho, K. Y., Fung, K. P. & Chen, Z. Y. (1997) Inhibitory effects of jasmine green tea epicatechins isomers on free radical-induced lysis of red blood cells. Life Sci. 61: 383–394.
28. Haldar, J. & Bhaduri, A. N. (1998) Protective role of black tea against oxidative damage of human red blood cell. Biochem. Biophys. Res. Commun. 244: 903–907.
29. Wei, H., Zhang, X., Zhao, J. F., Wanh, Z. Y., Bickers, D. & Lebwohl, M. (1999) Scavenging of hydrogen peroxide and inhibition of ultraviolet light-induced oxidative DNA damage by aqueous extracts from green ad black tea. Free Radic. Biol. Med. 26: 1427–1435.
30. Levin, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, L., Lenz, A., Ahn, B., Shaltiel, S. & Stadtman, E. R. (1990) Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186: 464–478.

31. Subramanian, N., Venkatesh, P., Ganguli, S. & Sinkar, V. P. (1999) Role of polyphenol oxidase and peroxidase in the generation of black tea theaflavins J. Agric. Food Chem. 47: 2571–2578.

32. Hara, Y. (1995) Action of tea on cardiovascular disease. In: Proceedings of the International Tea Quality Human Health Symposium, pp. 16–31. Sanghai, China.

33. National Research Council (1985) Guide for the Care and Use of Laboratory Animals. Publication no. 85–23 (rev.) National Institutes of Health, Bethesda, MD.

34. Nandi, B. K., Majumder, A. K., Subramanian, N. & Chatterjee, I. B. (1973) Effects of large doses of vitamin C in guinea pigs and rats. J. Nutr. 103: 1688–1695.

35. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. & Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.

36. Shalini, V. K., Luthra, M., Srinivas, L., Rao, S. H. & Balasubhramaniam, D. (1994) Oxidative damage to the eye lens caused by cigarette smoke and fuel smoke condensate. Indian J. Biochem. Biophys. 314: 261–266.

37. Hulea, S. A., Olinesku, K., Crocnan, D. C. & Kummerow, F. A. (1995) Cigarette smoking causes biochemical changes in blood that are suggestive of oxidative stress: a case control study. J. Environ Pathol. Toxicol. Oncol. 143- 144: 173–180.

38. Veyssier, B. C. (1997) Tobacco smoking and cardiovascular risk. Rev. Med. Interne. 189: 702–708.

39. Kleages, L. M., Murray, D. M., Brown, J. E., Cliver. S. P. & Goldenberg, R. L. (1998) Relation of cigarette smoking and dietary antioxidants with placental calcification. Am. J. Epidemiol. 1472: 127–135.

40. Sano, M., Takahashi, Y., Yoshino, K., Shimoi, K., Nakamura, Y., Tomita, I., Oguni, I. & Konomoto, H. (1995) Effect of tea (*Camellia sinensis* L.) on lipid peroxidation in rat liver and kidney: a comparison of green and black tea feeding. Biol. Pharm. Bull. 18: 1006–1008.

41. Serafini, M., Ghisellii, A. & Ferro-Luzzi, A. (1996) *In vivo* antioxidant effect of green and black tea in man. Eur. J. Clin. Nutr. 50: 28–32.

42. Pannala, A. S., Rice-Evans, C. A., Halliwell, B. & Singh, S. (1997) Inhibition of peroxynitrite-mediated tyrosine nitration by catechin polyphenols. Biochem. Biophys. Res. Commun. 232: 164–168.

43. Schraufstatter, I. U., Revak, S. D. & Cochrane, C. G. (1984) Protease and oxidants in experimental pulmonary inflammatory injury. J. Clin. Investig. 73: 1175–1184.

44. Janoff, A. (1986) Investigations into the biochemical mechanisms of pulmonary emphysema: effects of cigarette smoke on enzymes and anti-enzymes in the lung. Respiration 50 (suppl.): 13–25.

45. Pryor, W. A., Deoley, M. M. & Chrch, D. F. (1986) The inactivation of α1-proteinase inhibitor by gas-phase cigarette smoke: Protection by antioxidants and reducing species. Chem.-Biol. Interact. 57: 271–183.

46. Ueyama, K., Yokode, M., Arai, H., Nagano, Y., Li, Z. X., Cho, M. & Kita, T. (1998) Cholesterol efflux effect of high-density lipoproteins is impaired by whole phase cigarette smoke extracts through lipid peroxidation. Free Radic. Biol. Med. 24: 182–90.

47. Yokode, M., Ueyama, K. & Arai, N. H. (1996) Modification of high and low-density lipoproteins by cigarette smoke oxidants. Ann. N.Y. Acad. Sci. 786: 245–251.

48. Mahhfouz, M. M., Hulea, S. A. & Kummerow, F. A. (1995) Cigarette smoke increases cholesterol oxidation and lipid peroxidation of low-density lipoproteins and decreases its binding to hepatic receptors in vitro. J. Environ. Pathol. Toxicol. Oncol. 14: 181–192.

49. Leob, L. A., Ernster, V. L., Warner, K. E. & Abbots, J. (1984) Cigarette smoke and lung cancer. Cancer Res. 44: 5940–5958.

50. Asami, S., Manabe, H., Miyake, J. & Itoh, H. (1997) Cigarette smoking induces an increase in oxidative DNA damage, 8-hydroxyguanosine in central site of the human lung. Carcinogenesis. 189: 1763–1766.

51. Kasai, H. (1989) DNA damage by oxygen radicals and carcinogenesis. Gan to Kahaku Ryoho (Japan). 163: 459–465.

52. Cross, C. E. (1987) Moderator. Oxygen radicals and human disease. Ann. Intern. Med. 107: 526–545.

53. Pianezza, M. L., Sellers, E. M. & Tyndale, R. F. (1998) Nicotine metabolism defect reduces smoking. Nature (Lond.) 393: 750.