Environmental Tobacco Smoke Is Just as Damaging to DNA as Mainstream Smoke

Eliezer Bermúdez, Koni Stone, Kristine M. Carter, and William A. Pryor
Biodynamics Institute, Louisiana State University, Baton Rouge, LA 70803-1800 USA

This study demonstrates the ability of tar isolated from environmental tobacco smoke (ETS) to nick DNA in mammalian cells. Solutions of ETS tar behave similarly to aqueous solutions of cigarette tar from mainstream smoke. Both solutions contain the tar semiquinone radical, and this radical associates with the DNA in viable rat alveolar macrophages. Solutions of tar from ETS cause single-strand DNA breaks in rat thymocytes in proportion to the amount of tar present, until a plateau is reached. ETS tar solutions, like mainstream tar solutions, produce hydrogen peroxide. Hydrogen peroxide appears to be an essential component of the mechanism by which both ETS tar and mainstream tar cause DNA damage in rat thymocytes, as catalase substantially protects against DNA damage. Glutathione also protects against DNA nicking by both ETS and mainstream tar solutions by scavenging radicals and/or oxidants. The chelator diethylenetriamine pentacetic acid also provides partial (40%) protection. These studies demonstrate that the water-soluble components of ETS tar can enter cells, associate with, and then nick DNA. Key words: aqueous cigarette tar extract, cancer, catalase, DNA damage, electron paramagnetic resonance, electron spin resonance, glutathione, hydrogen peroxide, superoxide dismutase. Environ Health Perspect 102:870–874

Environmental tobacco smoke (ETS) is a major contributor to indoor air pollution wherever smoking occurs (1–3). Studies have shown that ETS contains a variety of genotoxic and carcinogenic compounds (2,4) that are chemically similar to those in mainstream and sidestream smoke (5). Epidemiological studies indicate an association between environmental tobacco smoke exposure and an increased risk for cancer (6–9). The Environmental Protection Agency (EPA) has classified ETS as a human lung class A carcinogen, on par with radon and asbestos, and concluded that ETS is responsible for approximately 3000 lung cancer deaths per year for non-smokers in the United States (5). ETS is the only agent classified by EPA as a known human carcinogen for which an increased cancer risk has actually been observed at typical environmental levels of exposure (5).

Sidestream smoke, which is emitted from the tobacco products during puff intervals, constitutes at least 85% of ETS (1), contributing nearly all of the vapor-phase constituents and over half of the particulate matter (5). The particles in sidestream smoke are about 10 times smaller (0.01–0.1 μm) than those in mainstream smoke (0.1–1.0 μm) (1,6), but the amount of total particulate matter in sidestream smoke is 1.3–1.9 times greater than in mainstream smoke (10). Thus, sidestream smoke particles reach more distant alveolar spaces of the lung to a larger extent than do the mainstream smoke particles (6). The amount of organic compounds in sidestream smoke is greater than in mainstream smoke (4).

Studies on the mutagenicity of ETS have been reviewed by Claxton et al. (2). Using bacterial mutagenicity as a genotoxic assessment of ETS, they concluded that 70% of the total mutagenicity of ETS is associated with the particle fraction of sidestream or mainstream smoke (2).

Tar from sidestream smoke, like that from mainstream smoke, contains a persistent radical that can be studied directly by electron spin resonance (ESR) (11,12). These radicals can be extracted into aqueous solutions (13). We determined that these aqueous cigarette tar extracts nick plasmid DNA (14) and cause DNA nicking in viable mammalian cells (15), and this nicking follows saturation kinetics (13).

We now report studies that show the tar component in sidestream smoke produces DNA nicks in viable mammalian cells. We also report the effects of reduced glutathione (GSH), catalase, superoxide dismutase (SOD), diethylenetriamine pentacetic acid (DTPA), and deferoxamine on the yield of DNA nicks caused by ETS tar solutions.

Materials and Methods

Chemicals and enzymes were purchased from Sigma Chemical Co. (St. Louis, Missouri) and used without further purification. Solutions of GSH, (800 mM), DTPA (240 mM), catalase (1 mg/ml), SOD (1 mg/ml), and deferoxamine (12 mM), were prepared in distilled water just before use. Inactivated SOD and catalase were prepared by boiling the enzymes for 2 min; catalase was boiled in 1 N NaOH and then the pH was adjusted to 7.4 with HCl. Inactivated enzyme solutions were stored at 4°C.

Preparation of ETS. Research-grade cigarettes (2R1) from the Kentucky Tobacco Research Council were smoked to a butt length of 30 mm using a standard puff profile (30 ml puff/30 sec). We collected the sidestream smoke using the apparatus shown in Figure 1 (11,15). The smoke that accumulated inside the threenecked flask was drawn through a Cambridge filter and a water trap by using an electrical pump at a flow of 1500 ml/min. The sidestream smoke from four cigarettes was produced by pulling air through a cigarette, as shown in Figure 1, using a syringe (11,13). The tar was collected on the Cambridge filter (see Fig. 1) and extracted by washing the filter with approximately 15 ml of acetonitrile until the filter was colorless. We then removed the acetonitrile by evaporation with a stream of nitrogen and resuspended the dried residue in 5 ml of phosphate-buffered saline (PBS; 66 mM phosphate, 0.85% sodium chloride, pH 8.0). The solution was then sonicated for 15 min using a Branson 2200 sonicator (Branson Ultrasonics Corporation, Danbury, Connecticut). After standing at 37°C in the dark for 24 hr, the tar solution was filtered through a 0.2-μm filter (25 mm, polypyrrole, Whatman International Ltd, Mainstone, England). This method produced solutions that contain 6.5 ± 1.5 mg of tar/ml.

Isolation of Thymocytes and Alveolar Macrophages. Male Sprague-Dawley specific pathogen-free rats 90–92 days old (360–400 g) (Harlan Sprague-Dawley, Houston, Texas) were used. Rats were acclimated in standard cages with access to food (Purina Mills, Inc.) and water for at least 5 days before being used. We anesthetized rats with an intraperitoneal injection of sodium pentobarbital (40 mg/kg), sacrificed them by exsanguination, and performed a thoracotomy to expose the lungs and thymus. The thymus was removed, placed in a normal saline solution (0.9% NaCl), and then mashed with tweezers to release the thymocytes. The cells were centrifuged at 400 g for 10 min and resuspended in 0.87% NH₄Cl/10 mM Tris-HCl (pH 7.3)/10 mM NaHCO₃ to lyse any red blood cells. After 20 min the cells were centrifuged as above and resuspended in a buffered isotonic solution (pH 7.2) containing 0.25 M myo-inositol-10 mM sodium phosphate-1 mM magnesium chloride. We counted the thymocytes using a standard hemocytometer and determined their viability using the trypan blue dye exclusion technique. This procedure yields 1.5 to 1.8 × 10⁶ cells per animal with 90–95% viability.

Address correspondence to W.A. Pryor, Biodynamics Institute, 711 Choppin Hall, Louisiana State University, Baton Rouge, LA 70803-1800 USA.

This work was supported by National Institutes of Health grant HL 25920. We are grateful to H. C. Birnboim for helpful discussions. Received 28 April 1994; accepted 25 July 1994.
We performed bronchoalveolar lavage (BAL) to harvest macrophages. A cannula was introduced into the trachea, and three 10-ml aliquots of isotonic saline (0.9%) at 37°C were injected into the lung sequentially via a syringe and the tracheal cannula. Each infusion was allowed to remain for 1 min and then was recovered. An average of 9 ± 1 ml was recovered. We centrifuged the lavage fluid at 400 g for 15 min at 4°C. The total cell count and viability was obtained in a 1:10 dilution of the cells using trypan blue and a standard hemocytometer. Typically, 5–6 × 10⁶ alveolar macrophages with 85–92% viability were harvested.

**Association of Cigarette Tar Radical with DNA in Rat Alveolar Macrophages.** Alveolar macrophages, isolated as described above, were placed in 2.0 ml of a solution containing 24 mM EDTA and 75 mM NaCl, pH 7.5. The cells were then incubated with 1.0 ml of ETS tar solution or 1.0 ml of pH 8.0 PBS on ice for 90 min. The cell viability decreased markedly for incubations longer than 90 min. After incubation, cell viability was 85–90% by trypan blue dye exclusion. To remove any unbound ETS material, the cells were pelleted by centrifugation at 400 g (4°C) and resuspended in PBS, pH 7.4. We used a modification of the alkaline elution method of Kohn et al. (16) to trap double-stranded DNA on polycarbonate filters (PC filters). A Micro/Por polycarbonate membrane (25 mm, 0.2 μm, Spectrum Medical Industries, Inc., Los Angeles, California) was soaked in ice-cold PBS, pH 7.4, for 20 min and then placed in an alkaline elution funnel (Millipore, Bedford, Massachusetts). A small volume of PBS was run through the filter to check for leaks, then the alveolar macrophages were carefully deposited on the filter. We used a peristaltic pump to pull fluid through the filter at a flow of 1 ml/min. After loading the cells on the filter, 10 ml of a lysis solution [2% sodium dodecyl sulfate (SDS), 0.025 M EDTA, pH 9.7] was added and allowed to remain at room temperature for 60 min. This solution was then pulled through the filter and 4 ml of the lysis solution with added protease K (0.5 mg/ml) was carefully added to the funnel. After 30 min, the protease K solution was pulled through the filter, and the filter was washed with 10 ml PBS and then air dried. These steps lyse the cells and remove protein, RNA, and any single-stranded DNA. The filter retains large pieces of double-stranded DNA. Control experiments included incubations of cells with no ETS tar solutions and incubations of ETS tar solutions and no cells. We shedded the dried filters with scissors and packed them into quartz tubes for examination by ESR spectroscopy.

**DNA Nicking in Rat Thymocytes.** Rat thymocytes (18 million cells/tube, 1.2 ml final volume) were incubated on ice with aliquots of an ETS tar solution for 90 min. The final tar concentrations ranged from 0.10 to 4.0 mg tar/ml. For experiments involving inhibitors, we added the inhibitor to the cells before the ETS tar solution before incubation. ETS concentration for the experiments involving inhibitors was 1.5 mg tar/ml. After incubation, the cells were centrifuged for 10 min at 400 g (4°C) and resuspended in a buffered isotonic solution, pH 7.2, containing 0.25 M myoinositol, 10 mM sodium phosphate, and 1 mM magnesium chloride. We used the fluorescence analysis of DNA unwinding (FADU) assay, described by Birnboim (17, 18), to determine DNA damage. The principle of the FADU assay is that the concentration of double-stranded DNA can be measured using ethidium bromide, with relatively little interference from single-stranded DNA and RNA. Under alkaline conditions, nicked DNA unwinds faster than undamaged DNA. After exposure of the cells to ETS tar, the cells were lysed and then exposed to alkaline denaturing conditions for a short time. Then the pH was lowered to stop further unwinding, and the amount of double-stranded DNA remaining was determined using the fluorescence of ethidium bromide (17). The cells were divided into three types of tubes: T-tubes (total double-stranded DNA); P-tubes (partially unwound DNA); and B-tubes (blank, no double-stranded DNA). The DNA unwinding time at 15°C was 45 min. As a final step, 1.5 ml of 6.67 μg/ml ethidium bromide was added to each tube. After this, all tubes were put in water bath at 26°C for 10 min. The amount of double-stranded DNA in each tube is determined by measuring the fluorescence of ethidium bromide at 520 nm excitation and 590 nm emission. We used three of each type of tube, T, P, and B, for each determination and averaged the results. The amount of double-stranded DNA remaining was calculated from Equation 1:

\[ D = \frac{(P - B)}{(T - B)} \times 100 \quad (1) \]

The amount of DNA damage was determined by calculating the damage quotient, Qd, as shown in Equation 2:

\[ Qd = \frac{(\log D_c - \log D_{ETS})}{\log D_{ETS}} \times 100 \quad (2) \]

where \( D_c \) is double-stranded DNA in control cells and \( D_{ETS} \) is double-stranded DNA in ETS-treated cells (17, 19). The amount of protection provided by the various inhibitors was calculated as shown in Equation 3:

\[ % \text{ Protection} = \left[ 1 - \left( \frac{Qd_{\text{Inhibitor}}}{Qd_{ETS}} \right) \right] \times 100 \quad (3) \]

**Determination of Hydrogen Peroxide in ETS Tar Solutions.** We determined hydrogen peroxide in ETS tar solutions as described for mainstream tar solutions (20). Briefly, the ETS tar solution was passed through a solid-phase extraction column (Supelclean LC-18, Supelco, Inc., Bellefonte, PA) and its \( \text{H}_2\text{O}_2 \) concentration determined by differential pulse polargraphy using an automatic reference subtraction system. The differential pulse polargraphy with a static mercury drop electrode was performed using a Model...
Results

The production of hydrogen peroxide by ETS tar solutions was measured as described in Materials and Methods. Figure 2 shows that the production of \( \text{H}_2\text{O}_2 \) by ETS solutions is time dependent. These ETS tar solutions produce approximately 1.5 \( \mu \text{mol} \) \( \text{H}_2\text{O}_2 \)/mg tar per 24 hr. The amount of \( \text{H}_2\text{O}_2 \) detected increased linearly with time and reached a value of 4.0 \( \mu \text{mol} \)/mg after 6 days.

Rat alveolar macrophages were incubated with ETS tar solutions, and the DNA was immobilized on a polycarbonate filter using a modification of the alkaline elution technique (16). Control incubations of ETS tar solutions alone or alveolar macrophages alone show no ESR signal remaining on the filter. The filter containing DNA from alveolar macrophages exposed to ETS tar solutions has an ESR spectrum with a signal at \( g = 2.0047 \) (Fig. 3). The same radical is present in the ETS tar solutions (\( g = 2.003 \)) (11). These \( g \)-values are within the range for the semiquinone radical signal (23).

The DNA nicking caused by ETS tar solutions using rat thymocytes is shown in Figure 4. The amount of DNA damage produced by ETS tar solutions was expressed as \( Qd \). The data show DNA damage increases with increasing ETS tar concentration, but the damage plateaus at an ETS tar concentration of 1.5 mg/ml. The maximum \( Qd \) value was 204 \pm 10.5 for tar concentrations of 1.5 mg/ml or greater. This is the amount of DNA produced by approximately one 2R1 cigarette.

Catalase, SOD, GSH, and DTPA were tested to determine if they protect DNA against nicking caused by ETS tar solutions, as shown in Table 1. Catalase provides a 96% protection against DNA damage, whereas SOD, inactivated catalase, and inactivated SOD do not provide any protection. When catalase and SOD are used together, the protection is equivalent to the protection due to catalase alone. DTPA provided only partial protection against DNA nicking caused by ETS tar solutions.

Figure 5 is a plot of GSH concentration versus protection against DNA damage, and a dose dependency is observed. The highest GSH concentration used (200 mM) gives 87% protection against DNA damage caused by ETS tar solutions.

Discussion

For this study, tar from the sidestream smoke of 2R1 research-grade cigarettes was collected on a Cambridge filter and extracted into aqueous buffers. These solutions were used as a model of ETS tar because sidestream smoke makes up at least 85% of the ETS. Our results show that ETS tar solutions can cause DNA nicking in viable mammalian cells; to our knowledge, this is the first report of the DNA nicking capability of tar from ETS.

Hydrogen Peroxide Production. Both mainstream tar solutions (20) and ETS tar solutions produce hydrogen peroxide with a time dependency (Fig. 2). Mainstream tar solutions produce 15 times more \( \text{H}_2\text{O}_2 \) than ETS tar solutions (see Table 2). The generation of hydrogen peroxide may arise from the dismutation of superoxide formed during autoxidation of polyphenols, \( \text{QH}_2 \), present in the tar solutions, as shown in Equations 4 and 5 (20,24–26):

\[
\text{QH}_2 + \text{O}_2^- \rightarrow \text{QH} + \text{O}_2^- + \text{H}^+ \tag{4}
\]

\[
2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \tag{5}
\]
DNA Association. Alveolar macrophages are target cells for cigarette smoke damage (27–36). Using a modification of the alkaline elution method of Kohn et al. (13,16), DNA from alveolar macrophages that had been exposed to ETS tar solutions was trapped on polycarbonate filters. Our data show that the tar radical becomes associated with the DNA in these cells (Fig. 3). The polycarbonate filters with the trapped DNA contain the tar radical as observed by ESR. Control exposure with cells alone or ETS tar solution alone does not result in a radical being trapped on the filter. Similar results were obtained when tar from mainstream smoke was used (13). Thus, tar from either mainstream smoke or ETS can enter a cell and associate with DNA. Since alveolar macrophages were used and these cells are not associated with lung cancer, we cannot infer a relationship between the DNA association observed in these experiments and the carcinogenicity of cigarette tar.

DNA Nicking. The DNA nicking studies were done using the FADU assay of Birnboim (17). Rat thymocytes were used. Although these cells are not a primary target for DNA damage in smokers and non-smokers exposed to ETS, they have been used in the FADU assay for DNA damage (18), and they can be isolated and obtained as a homogeneous cell population. Also, enough thymocytes for a complete dose–response curve can be obtained from a single rat.

Environmental tobacco smoke tar solutions cause DNA nicking in these viable rat thymocytes. Previous tests of DNA damage were done using cigarette smoke or tar solutions from mainstream smoke (13,37–40). There are a number of reports of an association between ETS exposure and cancer (41–44). Most of the reports are epidemiological studies (6–9,41,42). One of the studies shows a direct relationship between ETS exposure and carcinogen–hemoglobin adducts in non-smokers (41), while another shows that sidestream smoke condensate causes more skin cancer in mice than mainstream smoke condensate (43). Even though these studies do not assess direct damage to DNA caused by ETS, it is clear that components of ETS are genotoxic. As mentioned before, to our knowledge, this is the first report of the DNA-damaging capability of tar from ETS.

Our results with ETS tar solutions are similar to those obtained with aqueous cigarette tar from mainstream smoke. The mainstream smoke data were obtained during the same experiments as the ETS data but were published separately (13). There is an increase in the amount of DNA damage detected in mammalian cells as the concentration of tar from ETS increases up to a maximum (saturation) point (see Fig. 4). This effect also occurs when mainstream tar solutions are used (13).

To include (0.0) as the intercept in Figure 4, an S-shaped curve appears to be required, although the confidence in this conclusion is masked by a substantial error in the points with low amounts of DNA nicking. Cells exposed to low concentrations of tar (0.1–0.2 mg tar/ml) give Qf values that are close to zero but do not appear to lie on a straight-line extrapolation of data at higher yields of DNA damage. If the S-shaped curve shown in Figure 4 is correct, this might imply there is threshold for ETS-induced DNA damage. However, results with low yields of DNA nicking may be due to limitations of the FADU assay.

The maximum DNA nicking occurs at a concentration of tar that is equivalent to 1.0 cigarette for ETS tar solutions or 0.24 cigarette for mainstream smoke tar solutions (Table 2). Thus, ETS tar solutions behave similarly to mainstream smoke tar solutions but are less potent on a per-cigarette basis. These results (Figs. 3 and 4) show that tar components in both ETS tar solutions and mainstream smoke tar are capable of entering a cell, penetrating the nucleus, associating with DNA, and causing nicks (45).

Effect of Inhibitors on DNA Damage. Table 1 is a summary of the effects of inhibitors for protection against DNA nicking by ETS tar solutions and mainstream tar solutions. Of all the DNA damage inhibitors used in our experiments, catalase gave the greatest protection. Because catalase confers at least 90% protection against DNA nicking caused by ETS tar extracts or mainstream tar solutions, we can conclude that hydrogen peroxide is an essential component of the ETS-mediated damage to DNA.

Hydroxyl radicals have been detected in aqueous cigarette tar solutions (24) and have been proposed to contribute to DNA damage (14). High concentrations of DTPA (30 mM) abolish the signal of the hydroxyl radical spin-adduct in aqueous cigarette tar from mainstream smoke (24). DTPA also provides partial protection (40%) against DNA damage caused by ETS tar solutions. This partial protection may arise from inhibition of metal-catalyzed hydroxyl radical production (24).

Superoxide dismutase does not protect more than boiled SOD against DNA damage caused by ETS tar solutions or mainstream tar solutions (see Table 1). As SOD catalyzes the dismutation of superoxide to hydrogen peroxide and hydrogen peroxide is involved in nicking DNA, we do not expect SOD to be protective.

Glutathione protects against DNA nicking by ETS tar solutions, probably by scavenging oxidants. The same results were observed when mainstream tar solutions were used (13). Glutathione is known to form covalent adducts with quinones and hydroquinones (46–48). Thus, the GSH protection of DNA may be related to the ability of GSH to add to quinones and hydroquinones and perhaps prevent the addition to and nicking of DNA, or to the well-known ability of GSH to quench radical signals (49).

Conclusions
Both ETS tar solutions and mainstream smoke tar solutions bind to and damage DNA by similar mechanisms. Mainstream tar solutions produce more hydrogen peroxide than ETS tar solutions, and hydrogen peroxide is an important contributor to the tar-mediated DNA nicking. Environmental tobacco smoke tar solutions are less effective than mainstream tar solutions, requiring four times more cigarettes to cause the same amount of DNA damage (Table 2). However, ETS particles, which are smaller than mainstream particles (1,6), can reach more distal alveolar spaces of the lung, where the ETS tar par-
articles could release reactive species into the aqueous medium of the lung, and cause DNA damage. Clearly, ETS is involved in nicking DNA, and this damage may account for carcinogenic effect of ETS tar.

References
1. Baker RR, Proctor CJ. The origins and properties of environmental tobacco smoke. Environ Int 16:231–245 (1990).
2. Claxton LD, Morin RS, Hughes TJ, Lewtas J. A genotoxic assessment of environmental tobacco smoke using bacterial bioassays. Mutat Res 222:81–99 (1989).
3. Löfroth G, Burton RM, Forehand L, Hammond SK, Seila RL, Zweidinger RB, Lewtas J. Characterization of environmental tobacco smoke. Environ Sci Technol 23:610–614 (1989).
4. Löfroth G. Environmental tobacco smoke: overview of chemical composition and genotoxic components. Mutat Res 222:73–80 (1989).
5. U.S. Environmental Protection Agency. Respiratory health effects of passive smoking: lung exposures to mainstream and sidestream smoke: the report of the U.S. Environmental Protection Agency. Smoking and Tobacco Control Monograph 4. Bethesda, MD:National Cancer Institute, 1993.
6. Keller LH, Garfinkel L, Correa P, Haley N, Hoffmann D, Preston-Martin S, Sandler D. Contribution of passive smoking to respiratory cancer. Environ Health Perspect 70:57–69 (1986).
7. Wells AJ. An estimate of adult mortality in the United States from passive smoking. Environ Int 14:249–265 (1988).
8. Wald NJ, Nanchalal K, Thompson SG, Cuckle HS. Does breathing other people’s tobacco smoke cause lung cancer? Br Med J 293:1217–1222 (1986).
9. Russell MAH. Estimation of smoke dosage and mortality of non-smokers from environmental tobacco smoke. Toxicol Lett 35:9–18 (1987).
10. Güten MR, Higgins CE, Jenkins RA. Measuring environmental emissions from tobacco combustion: sidestream cigarette smoke literature review. Atmos Environ 21:291–297 (1987).
11. Pryor WA, Prier DG, Church DF. Electron-spin resonance study of mainstream and sidestream cigarette smoke: nature of the free radicals in gas-phase smoke and in cigarette tar. Environ Health Perspect 47:345–355 (1983).
12. Pryor WA, Hales BJ, Premovic PI, Church DF. The radicals in cigarette tar: their nature and suggested physiological implications. Science 220:425–427 (1983).
13. Stone K, Bermúdez E, Pryor WA. Aqueous extracts of cigarette tar containing the tar free radical cause DNA nicks in mammalian cells. Environ Health Perspect Suppl (in press).
14. Borish ET, Cosgrove JP, Church DF, Deutsch WA, Pryor WA. Cigarette tar causes single-strand breaks in DNA. Biochem Biophys Res Commun 133:780–786 (1985).
15. Pryor WA, Dooley MM, Church DF. Human alpha-1-proteinase inhibitor is inactivated by exposure to sidestream cigarette smoke. Toxicol Lett 28:65–70 (1985).
16. Kohn KW, Ewing RAG, Erickson IC, Zwelling LA. Measurement of single-strand breaks and cross-links by alkaline elution. In: DNA repair: a laboratory manual of research procedures (Friedberg E, Hanawalt P, eds). New York: Marcel Dekker, 1981:379–401.
17. Birnboim HC. Fluorometric analysis of DNA unwinding to study strand breaks and repair in mammalian cells. In: Methods in enzymology, vol 186. Oxygen radicals in biological systems (Packer L, Glazer AN, eds). San Diego, CA: Academic Press, 1990:550–555.