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Significant Formation of 8-Hydroxydeoxyguanosine through Interaction of Diesel Particulate Matter with Deoxyguanosine

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Diesel exhaust emissions produce cancers in experimental animals (Iwai et al. 1986; Brightwell et al. 1989). Organic extracts of diesel particulate matter (DPM) contain a large number of highly carcinogenic and mutagenic compounds such as benzo(a)pyrene (Hayano et al. 1985). It has been considered that polycyclic aromatic hydrocarbons (PAHs) are principal causative factors in the carcinogenic response of rodents (Grimmer et al. 1987), and we suggested that PAH concentration in human lungs is correlated to prevalence of lung cancer (Seto et al. 1993).

On the other hand, recent investigations have revealed that the formation of oxy-radicals similar to hydroxyl radical (·OH) is catalyzed by DPM in the presence of cysteine and hydrogen peroxide (Vogl and Elstner 1989). DNA damage caused by reactive oxygen species may be related to cancer incidence (Fraga et al. 1990). In order to clarify the role of DPM in generating oxidative DNA damage, we investigated the effect of DPM on hydroxylation of guanine base at the C-8 position. We demonstrated significant formation of 8-hydroxydeoxyguanosine (8-OH-dG) through interaction of DPM with 2'-deoxyguanosine (dG) in aqueous solution. It is noteworthy that the most effective part of DPM is the residue obtained after organic solvent extraction, and the next most effective part is the polar material extracted with acetone. We also discuss the hydroxylating activity of organic pollutants in DPM.

MATERIALS AND METHODS

DPM (No. 1650) was obtained from the U.S. Department of Commerce National Institute of Standards and Technology (Gaithersburg, MD). 2'-Deoxyguanosine, catalase from bovine liver, superoxide dismutase (SOD) from bovine erythrocytes, and desferrioxamine mesylate (DM) were obtained from Sigma Chemical Co. (St. Louis, MO). 8-OH-dG was synthesized according to Kasai and Nishimura (1984). Other chemicals were purchased from commercial sources. Organic extracts of DPM were prepared as follows. DPM (200 mg) was extracted with 10 mL of hexane under sonication for 10 min. After centrifugation (2500rpm, 5 min) of the mixture, the supernatant

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was transferred to a flask. The precipitate was re-extracted with benzene, acetone and then methanol in the same manner. Activated charcoal (200 mg, Darco G-60) was added to each extract solution, and the mixture was shaken for 10 min. Extract-adsorbed charcoal (EAC) was obtained after evaporation of the solvent from the mixture with a rotary evaporator. Chemical-adsorbed charcoal (CAC) was prepared after drying of a mixture containing 100 mg of charcoal and 1 mg of a selected chemical in 10 mL of hexane.

A reaction mixture (2 mL) containing 2 m*M* dG, 2 mg of DPM, EAC, CAC or an inorganic salt and 0.1 *M* phosphate buffer (pH 7.0) was shaken at 37 °C in the dark. In some of the experiments, the mixture contained 1.25 m*M* hydrogen peroxide. After 16 h (or after appropriate time), the reaction mixture was filtered and subjected to HPLC analysis. 8-OH-dG was analyzed with the HPLC-electrochemical (HPLC-EC) detection technique described elsewhere (Fraga et al. 1990). The analysis was performed with a Jasco 880 PU pump from Japan Spectroscopic Co. (Tokyo, Japan) and an ESA model 5100A Coulochem detector (Bedford, MA). The potentials for electrodes 1 and 2 were adjusted to 0.20 V and 0.35 V, respectively. A preinjector guard cell set at a potential of 0.40 V was employed. 8-OH-dG in the reaction mixture was separated with a GL Science Inertsil ODS-2 column (25 cm x 4.6 mm i.d., Tokyo, Japan) in an oven (35°C) using a mobile phase containing 10 m*M* NaH_2PO_4 in 8% (V/V) methanol/water. The mobile phase was eluted at a flow rate of 1 mL/min. The data are expressed as mean values of duplicated determinations. The identification of 8-OH-dG formed in the reaction mixture was performed by HPLC in-line UV spectrometry with a Hewlett-Packard HP-1040M diode array detector (Palo Alto, CA) using the same eluting conditions. Furthermore, the fraction of the product peak in the HPLC was collected and desalted by HPLC. After drying of the fraction, 8-OH-dG in the residue was converted to a trimethylsilyl (TMS) derivative. The mass spectrum of the TMS derivative was obtained with a VG 70S mass spectrometer (VG Analytical Ltd., UK).

RESULTS AND DISCUSSION

A new peak at 10.3 min on an HPLC chromatogram appeared after incubation of dG with DPM (Fig. 1). The peak at 10.3 min was identified as 8-OH-dG (UV λmax 250, 295 nm, MS for TMS-deriv. M^+ m/z 643). The amount of 8-OH-dG formation increased with increasing amount of DPM (Fig. 2). Prolonged reaction also gave a better yield of 8-OH-dG (Fig. 3). Hydroxylation of dG in our experiment required neither hydrogen peroxide nor cysteine, in contrast with the experiment of Vogl and Elstner (1989). Addition of hydrogen peroxide to the reaction mixture greatly enhanced the hydroxylation of dG, although hydrogen peroxide itself had very little hydroxylating activity.

The effects of some inhibitors, such as radical scavengers of reactive oxygen species, are summarized in Table 1.
Desferrioxamine mesylate (DM), a chelating agent of iron (Fe) ion, suppressed formation of 8-OH-dG in a dose-dependent manner.

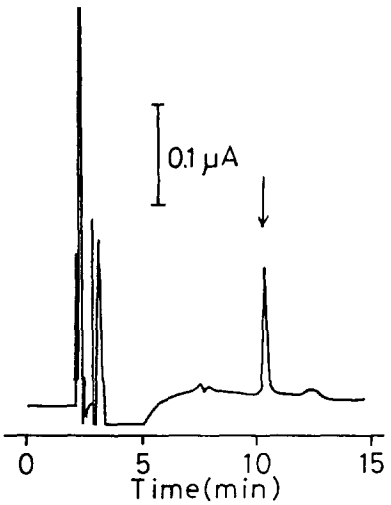

Figure 1. Chromatographic profile of dG-DPM reaction mixture. Analytical conditions are described in the text.

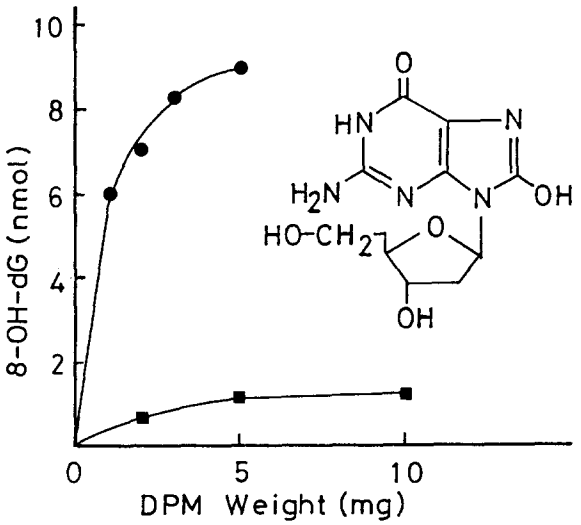

Figure 2. Effect of DPM amount on 8-OH-dG formation.
The reaction mixture (2 mL), which contained 2 m*M* dG, 0.1 *M* phosphate buffer (pH 7.0) and DPM with (-●-) or without (-■-) hydrogen peroxide, was shaken at 37°C in the dark for 16 h.

Catalase, mannitol and ethanol also suppressed formation of 8-OH-dG. These results suggest that hydroxyl radical and hydrogen peroxide are involved in the hydroxylating mechanism.

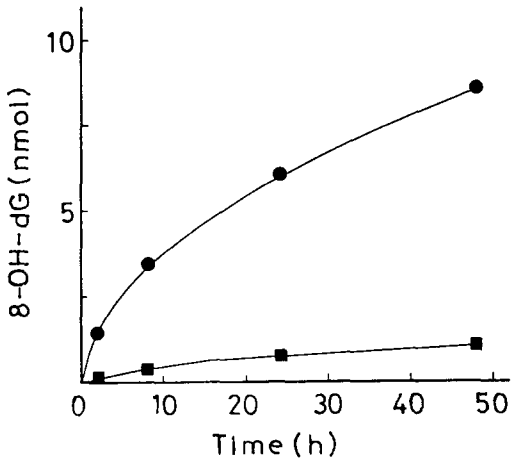

Figure 3. Time course of 8-OH-dG formation.
The reaction mixture (2 mL), which contained 2 m*M* dG, 0.1 *M* phosphate buffer (pH 7.0) and DPM (2 mg) with (-●-) or without (-■-) hydrogen peroxide, was shaken at 37°C in the dark.

Table 1. Inhibitory effects of desferrioxamine mesylate (DM) and radical scavengers on 8-OH-dG formation in dG-DPM mixture.

Inhibitor	Concentration	8-OH-dG (nmol)
DM	0 (μmol)	0.74 (0)
	0.1	0.65 (12)
	0.5	0.53 (28)
	1.0	0.29 (61)
SOD	30 (unit)	0.62 (16)
	150	0.30 (59)
	300	0.27 (64)
Catalase	10 (μg)	0.23 (69)
	50	0.17 (77)
	100	0.14 (81)
Mannitol	10 (μg)	0.74 (0)
	50	0.64 (14)
	100	0.52 (30)
Ethanol	8 (mg)	0.32 (57)
	40	0.30 (59)

Reaction mixture (2 mL) containing 2 mM dG, 2 mg of DPM, an inhibitor and 0.1 *M* phosphate buffer (pH 7.0) was shaken at 37°C in the dark for 16 h. After the reaction, 10μL of the filtered mixture was injected into an HPLC column and the yield of 8-OH-dG was determined. Values in parentheses are percent inhibition on 8-OH-dG formation.

Since SOD also suppressed hydroxylating activity of DPM, superoxide radical anion may also be involved. However, the spin-trapping technique using DMPO (5,5-dimethylpyrroline-*N*-oxide) for ESR measurement revealed only hydroxyl radical generation by DPM

(data not shown). From these findings, it is suggested that dG is hydroxylated with hydroxyl radical which is generated by the Fenton reaction caused by DPM components. We considered that certain components of DPM stimulated the Fenton reaction.

The hydroxylating activity of various EACs in the presence of hydrogen peroxide was determined. The EAC obtained with hexane or benzene showed very weak hydroxylating activity. On the other hand, the EAC obtained with acetone exhibited about 35% activity compared to the original DPM's activity. There is no hydroxylating activity in the next fraction (methanol-EAC). It is noteworthy that hydroxylating activity of the residue obtained after extraction with the organic solvents is comparable with the original DPM's activity (75%). Similar results were observed in the case of no addition of hydrogen peroxide. Thus, the stimulation of hydroxylation was most likely due to polar organic substances and inorganic substances in DPM.

Table 2. Effects of selected inorganic salts on 8-OH-dG formation.

Compound	8-OH-dG (nmol)
Na_2SO_3	19.6
Na_2SO_4	1.52
$Na_2S_2O_3$	<0.04
$NaNO_2$	<0.04
$NaNO_3$	0.80

The reaction conditions and methods for measuring the yields were the same as for Table 1, except that 2 mg of a salt was used instead of 2 mg of DPM.

Table 2 shows the effects of some inorganic salts, usually present in DPM, upon 8-OH-dG formation. Catalytic activity was in the order SO_3^{2-} >> SO_4^{2-}, NO_3^-. These anion species and iron (II) ion are regarded as major catalytic agents in the insoluble residue obtained after organic solvent extraction. Incubation of bisulfite with dG resulted in the formation of reactive OH radical, considered responsible for the hydroxylation (Kasai and Nishimura 1984).

Table 3 shows the effects of CAC on 8-OH-dG formation. Most of the chemicals examined are air pollutants. PAHs and nitrated PAHs have little catalytic ability. Some reducing agents having -OH in the molecule, such as hydroquinone, show significant formation of 8-OH-dG. It is considered that oxygenated PAH compounds are usually present in DPM (Tong et al. 1984).

Cigarette smoke, which contains similar carbonaceous particulate matter, can cause DNA single-strand breaks in cultured human lung cells (Nakayama et al. 1985). Leanderson and Tagesson (1992) suggested that the ability of cigarette smoke to cause the DNA breaks was due to mechanisms involving OH radical attack on DNA and endonuclease activation. They also suggested that hydroquinone was

an important contributor to the DNA-damaging effect of cigarette smoke in human lung cells.

Table 3. Effects of selected chemicals (CAC) on 8-OH-dG formation

Compound	8-OH-dG (nmol)
Anthrahydroquinone	0.88
Anthraquinone	0.12
Benzaldehyde	0.32
Benzoic acid	0.20
Benzo(k)fluoranthene	0.04
Benzo(g,h,i)perylene	<0.04
Benzo(a)pyrene	<0.04
1,8-Dihydroxy-anthraquinone	0.08
1,3-Dinitropyrene	0.12
1,6-Dinitropyrene	<0.04
1,8-Dinitropyrene	<0.04
Hydroquinone	1.52
8-Hydroxyquinoline	0.72
1-Nitropyrene	0.12
Vanillin	0.60

Preparation of CAC was described in the text. The reaction conditions and methods for measuring the yields were the same as for Table 1, except that the mixture contained 1.25 m*M* H_2O_2 and 2 mg of a CAC was used instead of 2 mg of DPM.

The present study showed that significant formation of 8-OH-dG occurs upon interaction of dG with DPM. We also observed transformation of thymine to thymine glycol and DNA cleavage caused by DPM (unpublished data). It is suggested that such DNA damage by DPM is attributable to generation of reactive oxygen species such as superoxide and OH radical in the aqueous phase. The catalytic effects are presumably caused by polar substances such as iron (II) ion, sulfur oxide anions (mainly SO_3^{2-}) and reducing agents having -OH in the molecule. These chemical species are regarded as able to permeate into cells. Recently, Sagai et al. (1993) reported that DPM is able to produce reactive oxygens in mice without participation of any biological activating system. They showed that active components in DPM were extractable with methanol. It is still unclear what chemical species of DPM is the key compound for reactive-oxygen production *in vivo*. The correlation of endogenous oxidative DNA damage by DPM with cancer and other diseases should be investigated.

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