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Increased Urinary Excretion of 2-Thiobarbituric Acid Reactants in Rats Exposed to Diesel Engine Exhaust

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The inhalation of diesel engine exhaust may induce health risks (McClellan 1986). Expansion of diesel engine usage may bring to bear serious health damages on roadside residents. Therefore, sensitive biological indicators affected by inhalation of diesel exhaust are needed for estimation of the effects of air pollution on human health. Diesel engine exhaust includes oxidizing pollutants such as nitrogen dioxide (NO_2). The toxicity of NO_2 is assumed to be related to lipid peroxidation of bio-membranes, because NO_2 readily attacks unsaturated lipid in lung tissue (Menzel 1976). Recently, Draper et al.(1984) showed that urinary 2-thiobarbituric acid reactant(s) (TBAR) is closely related to lipid peroxidation in vivo. TBAR is considered as a class of metabolites of malonaldehyde excreted in urine. The major metabolites, N-ε-(2-propenal)lysine (Draper et al. 1988) and N-(2-propenal)serine (Hadley and Draper 1988), have been identified. Monitoring of malonaldehyde excretion in urine gave useful information about the toxicological effects induced by chloroform or hydroquinone (Ekstroem et al. 1988). The present paper describes the changes of urinary TBAR as an indicator of lipid peroxidation and the activities of antioxidative protective enzymes in the lungs of rats exposed to diesel engine exhaust.

MATERIALS AND METHODS

Ten-week-old, specific-pathogen-free, pregnant Fisher 344 DuCrj rats (Nippon Charles River Co., Atsugi, Japan) were exposed to diluted diesel exhaust for 2 mo. Newborn female rats were also exposed for 3 mo from the fetal period. The exposure conditions are listed in Table 1. Most of the diesel soot particles in the whole exhaust (H group) were removed by filtration (ATM 3QA, Nippon Muki Co., Tokyo, Japan) for the non dust group (F). At 16 hr after the end of exposure, each

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Table 1. Experimental conditions of diesel engine exhaust.

Group	Particles (mg/m^3)	NO_2 (ppm)
C	0.01± 0.01	0.02± 0.01
L	0.61± 0.04	0.05± 0.02
H	8.32± 0.89	1.70± 0.40
F	0.03± 0.02	1.60± 0.40

Rats were exposed to diesel engine exhaust for 5 days at 7 hr per day. C:Control group given clean air. L:Low concentration group given whole exhaust. H:High concentration group given whole exhaust. F:High gas concentration group given filtered exhaust; comparable to the H group.

animal was put into a glass metabolism cage (Sugiyama-Gen Environmental Science Co., Tokyo, Japan). Then the urine was collected for 24 hr and stored in the frozen state. The animals were given water ad libitum but without feeding during the urine collection period. Diesel exhaust was generated by running a 269 mL diesel engine (Model NS40CGE, Yanmar Diesel Co., Ohsaka, Japan) at 2,400 rpm, diluted with air in a dilution tunnel and then drawn into an inhalation chamber (1.6 m^3). Chamber ventilation was effected with 15 volume changes per hr. The temperature was held between 23 and 25℃ and the relative humidity was varied between 45 and 65% in the chambers, with a 12 hr dark/light cycle. Control rats were exposed to clean air in the same type of chamber under the same conditions. Gravimetric measurements of the particulate matter were conducted daily using an automatic β -ray dust mass monitor (Model BAM-102, Shibata Scientific Technology Co., Tokyo, Japan). The concentration of nitrogen dioxide was continuously monitored with a chemiluminescence analyzer (Model 8440, Monitor Labs Co., San Diego, CA). The particulate size was determined with a particle fractionating sampler (Andersen Type low pressure impactor LP-20, Tokyo Dylec Co., Tokyo, Japan). More than 90% of the particulate matter in diesel exhaust had a particle size below 0.5μ m.

TBAR in urine was determined by the following procedure which is based on the HPLC method (Bird et al. 1983). Urine samples were centrifuged at 1000× g for 5 min. The supernatant (1 mL) was adjusted to below pH 3 with 1 N hydrochloric acid, and mixed with 1 mL of 0.6 % 2-thiobarbituric acid (TBA). Then the mixture in a reaction tube was heated in a boiling water bath for 30 min and cooled to room temperature. The sample was applied to a BOND ELUT® C18 cartridge (Analytichem International Inc., CA). The first fraction eluted with 5 mL of distilled water was discarded. The second fraction

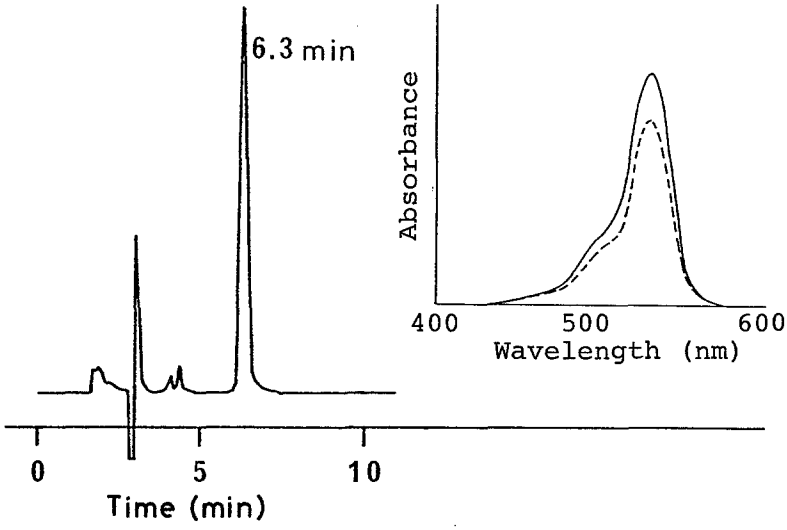

Figure 1. An HPLC chromatogram of a urine sample. Column, Nucleosil 7C18 (4.6 mm id x 250 mm); mobile phase, 20% acetonitril in 4 mM KH_2PO_4; flow rate, 1 mL/min; detector, 532 nm, 0.08 AUFS. The visible spectrum (broken line) of the major peak at 6.3 min is compared with that of authentic TBA-malonaldehyde complex (solid line).

eluted with 3 mL of 50 % V/V methanol/water was adjusted to 5 mL with distilled water. The sample was injected into an HPLC apparatus (Model 880PU, JASCO, Tokyo, Japan) equipped with a UV-VIS three-dimensional detector (Model HP 1040M, Hewlett-Packard, PE). Analytical standard, sodium salt of malonaldehyde, was prepared from tetraethoxypropane (Marnett and Tuttle 1980).

Eight daughter rats of each group were sacrificed after collection of urine. The lungs were homogenized with 9 volumes of cold 50 mM Tris buffer (pH 7.5) using an Ultra-Turrax® (Model TP18/10S2, Janke & Kunkel KG, Slaufen i. Breisgau, West Germany) and centrifuged at 16,000× g for 30 min at 4℃. Enzyme assays were carried out on the resulting supernatants. The supernatants were stored at -20℃ until assayed. The activities of glutathione (GSH) peroxidase and GSH reductase were determined by the method of Paglia and Valentine (1967) and Beutler (1969), respectively. The activities of GSH peroxidase and GSH reductase were expressed in μ moles of NADPH oxidized per min per g tissue. Glucose-6-phosphate dehydrogenase (G-6-PD) activity was determined by the method of Taketa and

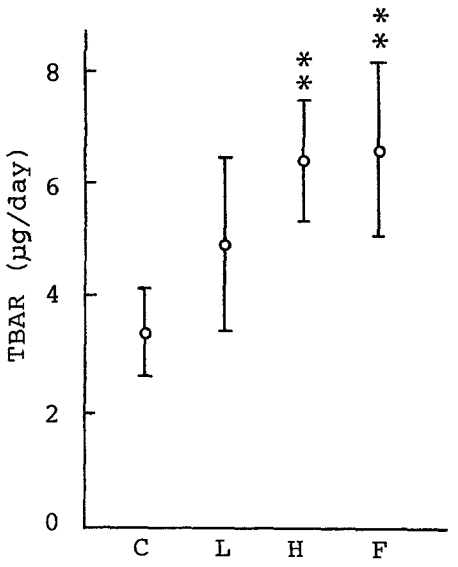

Figure 2. Effect of diesel exhaust on TBAR in urine of mother rats. Data are expressed as mean ± S.D. (n=5). Symbols indicate significant differences from the control by Student's t-test (**: P<0.01). C,L,H,F: See Table 1.

Watanabe (1971) and expressed in μ moles of NADPH formed per min per g tissue.

The Student's t-test was used to determine the statistical significance between the groups. All data are expressed as Mean± S.D.

RESULTS AND DISCUSSION

An HPLC chromatogram of a urine sample of the control group and a set of VIS spectra of the major peaks are shown in Figure 1. The major peak at 6.3 min was due to TBA-malonaldehyde complex. The complex in urine samples of the rats exposed to diesel exhaust (L, H and F) was determined in the same way.

The effect of diesel engine exhaust on urinary TBAR of the mother rats is illustrated in Figure 2. The TBAR value was expressed as μ g of malonaldehyde excreted in urine during 24 hr per rat. A promotive effect of diesel exhaust on lipid peroxidation is clearly demonstrated. A significant increase of TBAR levels in urine was observed in the H and F groups compared to the control. A difference in urinary TBAR between H and F groups was not discernible. It is noteworthy that the gaseous pollutants generated by diesel engines are quite oxidative to living organs. The occurrence

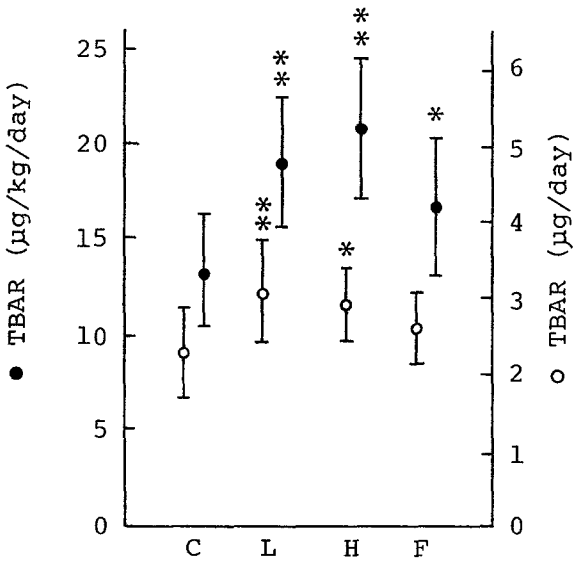

Figure 3. Effect of diesel exhaust on TBAR in urine of daughter rats. Data are expressed as mean ± S.D. (n=10). Symbols indicate the significant differences from the control by Student's t-test (*, $P<0.05$; **, $P<0.01$). C,L,H,F: See Table 1.

of in vivo lipid peroxidation caused by NO_2 exposure was reported as an increase of conjugated dienes (Thomas et al. 1968) or TBAR in lung and ethane in the breath (Sagai et al. 1981). Our results suggest that inhalation of oxidative gas such as NO_2 in diesel engine exhaust stimulates lipid peroxidation in vivo.

The effect of diesel engine exhaust on urinary TBAR of daughter rats is illustrated in Figure 3. A significant increase in urinary TBAR ($P<0.01$) was observed in the L group. TBAR excretion per day did not appear to be dose-dependent, probably owing to the different body weights among the groups (-○-). The body weight gain of H group was less than the L group. In contrast, on a body weight basis, TBAR excretion increased with the dose of exposure (-●-). It seems that the TBAR level of the rats exposed to the highest dose of whole exhaust (H) is higher than that of the rats exposed to the filtered exhaust (F) ($P<0.05$, body weight basis). Since diesel soot particles catalyze the production of oxy-radicals and promote lipid peroxidation (Vogl and Elstner 1989), the difference of TBAR levels between H and F groups is probably due to the particle matter.

The role of enzyme systems that can metabolize lipid peroxides or inhibit their formation is very important for protecting cells from oxidative stress. The GSH

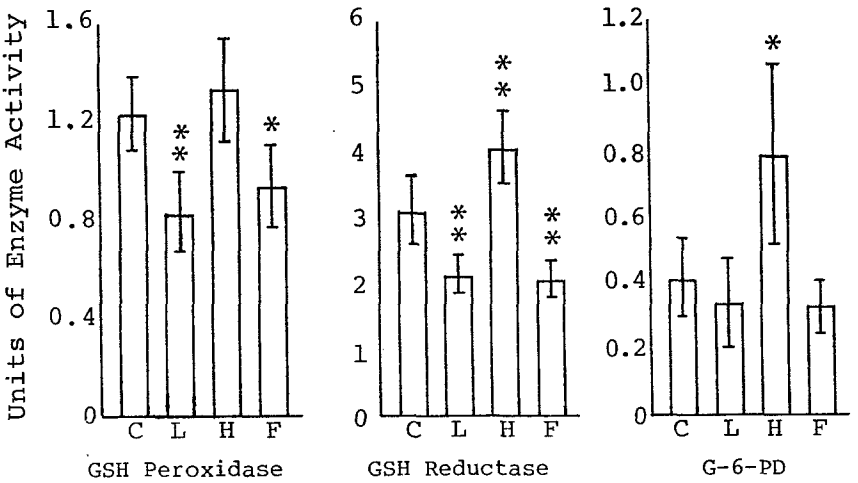

Figure 4. Changes of enzyme activity of GSH peroxidase, GSH reductase and G-6-PD on lungs of daughter rats exposed to diesel engine exhaust. GSH Peroxidase: μ moles NADPH oxidized/min/g tissue. GSH Reductase : μ moles NADPH oxidized/min/g tissue. G-6-PD: μ moles NADPH formed/min/g tissue. Symbols indicate significant differences from the control by Student's t-test (*, $P<0.05$; **, $P<0.01$). C,L,H,F: See Table 1.

peroxidase system, consisting of GSH peroxidase, GSH reductase and G-6-PD, is a typical enzyme system that protects against oxidative damage (Chow and Tappel 1972; Sagai and Ichinose 1987). As shown in Figure 4, in the L and F groups the activity levels of GSH peroxidase and GSH reductase decreased in comparison with the controls and the activity level of G-6-PD remained unchanged. The increased urinary TBAR levels of the rats in the L and F groups might be caused by the decreased activities of antioxidative protective enzymes, such as GSH peroxidase and GSH reductase. On the other hand, in the H group the activity levels of GSH reductase and G-6-PD increased and that of GSH peroxidase remained unchanged. When rats were exposed to NO_2, the antioxidative protective enzyme activities in the lungs decreased in the early stages, after that they increased correspondingly with an elevation of NO_2 level (Sagai and Ichinose 1987). The induction of these enzymes in the H group may be a compensatory reaction against lipid peroxide-induced damage.

Histochemical studies of rat lung alveolar cells confirm that the lung possesses a relative abundance of G-6-PD in Type II cells (Vijeyaratnam and Corrin 1972). White and Garg (1981) investigated the lungs of rats exposed to diluted diesel exhaust at a concentration

level of 6 mg/m^3 for periods from one day to 9 wk. They observed a highly significant scattered increase of Type II cells without any accompanying necrosis of the endothelial (Type I) cells after only 24 hr of exposure. At 6 wk, the Type II cells lining the alveoli were more numerous; this phenomenon could not be found in the control or the rats exposed to lower levels of exhaust. Therefore, in the H group the increased activities of G-6-PD may be related to an increased number of Type II cells. G-6-PD converts nicotinamide adenine dinucleotide phosphate (NADP) to reduced NADP (NADPH). GSH reductase converts oxidized glutathione (GSSG) to GSH. The increased activity levels of G-6-PD and GSH reductase in the H group should produce large amounts of NADPH and GSH, respectively which in turn will be oxidized by the anti-oxidant enzyme system of GSH reductase and GSH peroxidase, respectively. Though they protect cellular components from oxidative damage, it seems that the production of lipid peroxides in the H group exceeds the consumption of them. Although an increase of the activity level of GSH peroxidase in the H group was expected, it remained unchanged in fact. This might be attributed to delay of the growth of rats exposed to the highest dose of whole exhaust, because Pinto and Bartley (1969) observed that GSH peroxidase activity in rats increased steadily with growth.

Our results suggest that lipid peroxidation is stimulated by cooperative effects of gaseous pollutants and soot particles in diesel engine exhaust. Urinary TBAR is a sensitive indicator, and may be suitable for evaluation of the effects of diesel exhaust exposure on human health.

REFERENCES

Beutler E (1969) Effect of flavin compounds on glutathione reductase activity : In vivo and in vitro studies. J Clin Invest 48:1957-1966

Bird RP, Hung SSO, Hadley M,Draper (1983) Determination of malonaldehyde in biological materials by high-pressure liquid chromatography. Anal Biochem 128:240-244

Chow CK, Tappel AL (1972) An enzyme protective mechanism against lipid peroxidation damage to lungs of ozone exposed rats. Lipids 7:518-524

Draper HH, Polensek L, Hadley M, Mc Girr LG (1984) Urinary malonaldehyde as an indicator of lipid peroxidation in the diet and in the tissues. Lipids 19:836-843

Draper HH, Hadley M, Lissemore L, Laing NM, Cole PD (1988) Identification of N-ε -(2-propenal)lysine as a major urinary metabolite of malonaldehyde. Lipids 23:626-626

Ekstroem T, Warholm M, Kronevi T, Hoegberg J (1988) Recovery of malonaldehyde in urine as a 2,4-dinitrophenylhydrazine derivative after exposure to chloroform or hydroquinone. Chem Biol Interact 67:25-31
Hadley M, Draper HH (1988) Identification of N-(2-propenal)serine as a urinary metabolite of malonaldehyde. FASEB J 2:138-140
Marnett LJ, Tuttle MA (1980) Comparison of the malonaldehyde and the side products formed during its chemical synthesis. Cancer Res 40:276-282
Menzel DB (1976) The role of free radicals in the toxicity of air pollutants (nitrogen oxides and ozone). In: Pryor WA (ed) Free radicals in biology, Vol 2, Academic Press. New York, p181
McClellan RO (1986) Health effects of diesel exhaust : A case study in risk assessment. Am Ind Hyg Assoc J 47:1-13
Paglia DE, Valentine WN (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 70:158-169
Pinto RE, Bartley W (1969) The effect of age and sex on glutathione reductase and glutathione peroxidase activities and on aerobic glutathione oxidation in rat liver homogenates. Biochem J 112:109-115
Sagai M, Ichinose T, Oda H, Kubota K (1981) Studies on biochemical effects of nitrogen dioxide: I . Lipid peroxidation as measured by ethane exhalation of rats exposed to nitrogen dioxide. Lipids 16:64-67
Sagai M, Ichinose T (1987) Lipid peroxidation and antioxidative protection mechanism in rat lungs upon acute and chronic exposure to nitrogen dioxide. Environ Health Perspec 73:179-189
Taketa T, Watanabe A (1971) Interconvertible microheterogeneity of glucose-6-phosphate dehydrogenase in rat liver. Biochem Biophys Acta 235:19-26
Thomas HV, Mueller PK, Lyman PL (1968) Lipoperoxidation of lung lipid in rats exposed to nitrogen dioxide. Science 159:1919-1924
Vijeyaratnam GS and Corrin B (1972) Pulmonary histiocytosis simulating desquamative interstinal pneumonia in rats receiving oral iprindole. J Pathol 108:105-113
Vogl G, Elstner EF (1989) Diesel soot particles catalyze the production of oxy-radicals. Toxicol Lett 47:17-23
White HJ, Garg BD (1981) Early pulmonary response of the rat lung to inhalation of high concentration of diesel particles. J Appl Toxicol 1:104-11

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