

Oxidative DNA damage in vitamin C-supplemented guinea pigs after intratracheal instillation of diesel exhaust particles

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Abstract

The health effects of diesel exhaust particles (DEP) are thought to involve oxidative damage. We have investigated the effect of intratracheal DEP instillation to guinea pigs in three groups of 12 animals each given 0, 0.7, or 2.1 mg. Five days later guinea pigs exposed to DEP had increased levels of oxidized amino acids (γ -glutamyl semialdehyde), DNA strand breaks, and 7-hydro-8-oxo-2'-deoxyguanosine (8-oxodG) in the lung. Bulky DNA adducts were not significantly elevated in the lung. The antioxidant enzyme activity of glutathione reductase was increased in the lung of DEP-exposed guinea pigs, whereas glutathione peroxidase and superoxide dismutase enzyme activities were unaltered. There was no difference in DNA strand breaks in lymphocytes or urinary excretion of 8-oxodG at the two doses tested. Protein oxidations in plasma and in erythrocytes were not altered by DEP exposure. The concentrations of ascorbate in liver, lung, and plasma were unaltered by the DEP exposure. The results indicate that in guinea pigs DEP causes oxidative DNA damage rather than bulky DNA adducts in the lung. Guinea pigs, which are similar to humans with respect to vitamin C metabolism, may serve as a new model for the study of oxidative damage induced by particulate matter.

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There is growing concern over health effects of air pollution, especially those related to diesel engine exhaust particles (DEP). Epidemiological studies have demonstrated an association between levels of particulate matter in ambient air and increased incidence of cardiovascular and respiratory mortality and morbidity (U.S. Environmental Protection Agency, 2002). Inhalation of DEP is clearly carcinogenic in rats, whereas studies in Syrian hamsters and mice have been negative or inconclusive, and there is limited evidence of carcinogenicity in humans, with the strongest association with lung cancer (IARC, 1989).

The mechanism by which DEP induces lung cancer is a matter of controversy. DEP consists of elemental carbon particles, which have large surface areas that absorb organic compounds such as polyaromatic hydrocarbons (PAH), heterocyclic compounds, phenols, and nitroarenes (IARC, 1989). Some of the organic components are clearly carcinogenic, either alone or as mixtures, yet the concentrations in DEP may not be high enough to explain the carcinogenicity. Studies of bulky DNA adducts following inhalation of DEP in rats have shown mixed results, with increased DNA adduct levels in some studies (Mauderly et al., 1994; Sato et al., 2000; Bond et al., 1990; Wong et al., 1986) and no effect in other studies (Gallagher et al., 1994; Iwai et al., 2000). Taken together, the induction of DNA adducts appears to be without a clear doseresponse relationship; e.g., low DEP doses were not tumorigenic by inhalation yet

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produced a level of DNA adducts similar to that of a 30-fold higher dose in rats (Bond et al., 1990). Furthermore, particles such as TiO_2 and carbon black contain little or no low-molecular-weight organic compounds, yet are able to induce lung cancer by inhalation in rats (Mauderly et al., 1994; Nikula et al., 1995). DEP, from which the organic fraction had been removed by repeated extractions in organic solvents, had lung tumorigenicity similar to that of DEP in rats exposed by intratracheal instillation (Iwai et al., 1997). Collectively, it appears that the extractable organic fraction of DEP does not account for the carcinogenicity, and the induction of aromatic DNA adducts is only one type of DNA damage involved in the development of lung tumors following DEP inhalation in rats.

An alternative mechanism by which DEP could be carcinogenic is by the generation of oxidative DNA damage. An *in vitro* study has shown that reactive oxygen species (ROS) are generated by DEP without any biological activation system (Sagai et al., 1993). DEP contain oxides of sulfur and nitrogen and transition metals such as iron, copper, and chromium that are capable of catalyzing the generation of ROS (McClellan, 1987). Moreover, inflammation is an important source of ROS and reactive nitrogen species and it has been suggested that the carcinogenic mechanism of particles is related to induction of inflammatory responses in rats (Mauderly et al., 1994).

Oxidative DNA damage is generated by reaction of DNA with ROS. These species are capable of reacting with virtually any cellular macromolecule, including lipids and proteins. Oxidations of cellular macromolecules by ROS have been implicated in a diverse array of conditions such as aging, cancer, and autoimmune diseases (Møller et al., 1996). 7-Hydro-8-oxo-2'-deoxyguanosine (8-oxodG) is a type of oxidative DNA damage that has been studied under several conditions of oxidative stress. Increased level of 8-oxodG is associated with the cancer process induced by several different exposures and chemicals (Loft and Poulsen, 1996). In mice and rats, increased levels of 8-oxodG were observed in lung tissue following exposure to diesel exhaust by inhalation and DEP by intratracheal instillation (Tsurudome et al., 1999; Sato et al., 2000; Ichinose et al., 2000; Iwai et al., 2000). Increased levels of 8-oxodG in the lung of ICR mice after 10 weekly instillations of DEP, washed DEP, or TiO_2 particles (0.1 mg per instillation) correlated well with the lung tumor incidence 1 year after exposure (Ichinose et al., 1997). These observations suggest that oxidative DNA damage plays an important role in the carcinogenesis of DEP.

In vitro studies have documented the antioxidant effect of low concentrations of ascorbate, and it appears to play an important role in the antioxidant defense system *in vivo* (Halliwell and Gutteridge, 1999). Although some studies have shown prooxidant effects of ascorbate and transition metals *in vitro*, ascorbate appears to possess only an antioxidant property *in vivo* in guinea pigs, even at high vitamin C doses and iron overload (Chen et al., 2000). If oxidative

stress is an essential pathway in the carcinogenesis of DEP, an important aspect in the extrapolation of animal data to human risk is that, unlike humans, mice and rats do synthesize ascorbate *de novo*. Because humans cannot produce ascorbate in response to a perturbation of the oxidative stress defense it may be misleading to study rats and mice. Moreover, the animal models used so far have yielded conflicting results with respect to carcinogenesis (IARC, 1989). Accordingly, the guinea pig, similar to humans with respect to vitamin C metabolism, may offer a more appropriate experimental model for DEP effects.

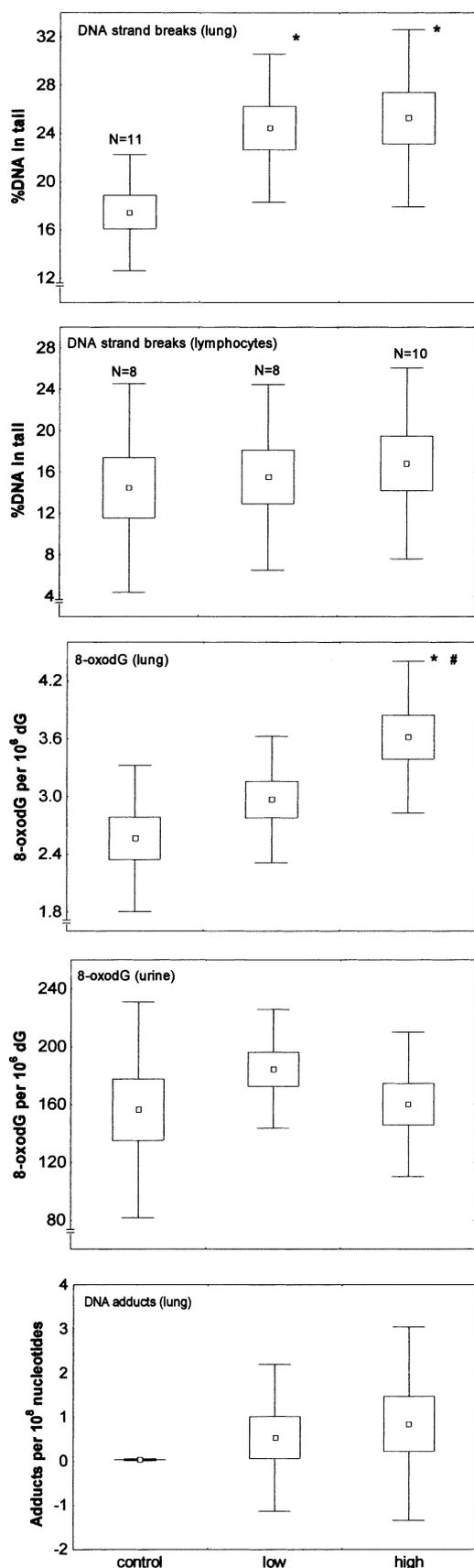
The aim of this study was to investigate genotoxic and oxidative stress effects of an intratracheal instillation of DEP in guinea pigs, while monitoring the ascorbate status. The doses were chosen within the range for which oxidative damage has been shown in rats and mice after intratracheal instillation of DEP (Tsurudome et al., 1999; Ichinose et al., 2000). Biomarkers of oxidative stress included protein oxidation, antioxidant enzyme activity, oxidative DNA damage, and DNA strand breaks. Aromatic DNA adducts were detected in the lungs to determine the effect of the PAH component of DEP.

Materials and methods

Male and female Ssc: AL guinea pigs (aged 40–50 days) were obtained from the Danish Serum Institute, Hvidsten, with a mean and SD weight of 450 ± 23 g. They were caged in pairs and acclimatized for a week before the experiment. They lived in an alternating dark (9 AM–9 PM) and light (9 PM–9 AM) cycle with an ambient temperature of 22–25°C. They were given standard guinea pig food (Altomin 3123, without vitamin C supplement, Lage, Germany). The drinking water contained 1 g/L vitamin C. All animals had free access to water and food.

Three groups of 12 guinea pigs each (6 females and 6 males) were given 0, 0.7, or 2.1 mg NIST 1650 DEP (National Bureau of Standards, Gaithersburg, MD) in 0.1 ml saline, respectively. The DEP suspension was homogenized using ultrasound and instilled intratracheally using a syringe equipped with a blunt stomach intubation tip for rats. The placement inside the trachea was ascertained by placing a finger on the throat of the animal and moving the tip slightly from side to side. The guinea pigs were lightly anesthetized with 0.6 ml of a 1:1 mixture of Hypnorm (Janssen-Cilag A/S, Denmark) and Dormicum (5 mg/ml, Roche, Basel, Switzerland). A 24-h urine collection was started 4 days after instillation of DEP (urine was collected in metabolic cages). The animals were killed by decapitation 5 days after DEP instillation. Samples of lung and liver tissue, plasma, red blood cells and lymphocytes were isolated and frozen at -80°C until use.

The level of 8-oxodG in nuclear DNA from lung tissue was analyzed as described elsewhere (Wellejus and Loft, 2002). The level of 8-nitroguanine in lung DNA was deter-



mined as described by Tuo et al. (2000). Urinary content of 8-oxodG was determined as described by Loft and Poulsen (1999).

DNA strand breaks were determined by the comet assay, using the amount of fluorescence in the tail of images (%DNA in tail) to quantitate the level of DNA strand breaks (Møller et al., 1998). The isolation of lung cells and lymphocytes was essentially carried out as described previously (Møller et al., 1998; Thein et al., 2000). Images were processed by the Kinetics Imaging System Version 4, and the mean for each sample was calculated from 50 randomly processed images. The level of DNA adducts was detected by ^{32}P postlabeling (Astrup et al., 1999).

Lung and liver cytosols were prepared by differential centrifugation and removal of microsomes (Kamath and Narayan, 1972). Protein oxidation was determined by the presence of γ -glutamyl semialdehyde (GGS) and 2-amino adipic semialdehyde (AAS) in lung and liver cytosols and in plasma and hemoglobin samples (Daneshvar et al., 1997). The activity of glutathione peroxidase, glutathione reductase, and superoxide dismutase was determined in lung cytosol (Young et al., 1999). Total vitamin C was determined as described previously (Lykkefeldt et al., 1997).

In the statistical analysis, the data were tested by a post hoc ANOVA, using least-significant difference, and 5% as significance level. The data were tested for homogeneity of variance within dose groups by Levene's test and for normality by Shapiro-Wilk's W test. Log transformations, using the base of 10, were performed for biomarkers, which did not exhibit homogeneity of variance. Dose-response relationships were tested by linear regression. The statistical analysis was performed in Statistica for Windows, Statsoft, Inc. (1997; Tulsa, OK).

Results

The intratracheal installation was successful in all dosed animals as determined from more or less dispersed spots of DEP inside their tracheas and lungs. From the instillation of vehicle or DEP till sacrifice all groups of animals lost weight (terminal weight and SD at sacrifice was 474 ± 26 g and weight before instillation was 499 ± 24 g). There was no difference between the weight losses in the three groups. None of the determined endpoints showed differences between the sexes. Consequently, sex was not included in the statistical analysis.

Intratracheal instillation of DEP increased the level of

Fig. 1. Box and whiskers blot of oxidative DNA damage, DNA strand breaks, and bulky DNA adducts in guinea pig tissues after intratracheal instillation of DEP at low (0.7 mg), high (2.1 mg), and zero dose. Points represents the means, boxes the standard error of mean, and whiskers the standard deviation. Numbers of guinea pigs were 12 in each group except where indicated. * $P < 0.05$ compared to control; # $P < 0.05$ compared to low dose.

Table 1

Levels of protein oxidation products following intratracheal DEP instillation (pmol/mg protein)^a

Protein oxidation product	Control	Low	High
AAS (lung)	71.7 ± 4.4 (12)	75.2 ± 3.1 (12)	76.7 ± 5.5 (10)
AAS (liver)	77.8 ± 13.8 (12)	73.6 ± 13.0 (12)	71.0 ± 7.8 (11)
AAS (hemoglobin)	153.8 ± 30.5 (9)	145.0 ± 13.8 (8)	159.0 ± 14.1 (10)
ASS (plasma)	96.3 ± 17.3 (9)	92.3 ± 15.3 (10)	96.3 ± 15.4 (12)
GGs (lung)	180.8 ± 19.4 (12)	155.7 ± 15.6 (12)*	144.7 ± 14.7 (10)*
GGs (liver)	96.3 ± 19.4 (12)	92.1 ± 16.7 (12)	91.0 ± 14.0 (11)
GGs (hemoglobin)	413.3 ± 35.0 (9)	375.1 ± 78.5 (8)	398.8 ± 49.0 (10)

^a Data represent the means and standard deviations, and the number of samples is shown in parentheses.* $P < 0.0001$ compared to control.

8-oxodG and DNA strand breaks in lung cells (Fig. 1). The level of bulky adducts was not significantly changed by DEP ($P = 0.08$ in ANOVA), although linear regression suggested an increase with dose ($r = 0.38$, $P < 0.05$, Fig. 1). In general, the PAH-DNA adduct levels were low compared to our previous findings of DNA adducts in the range of 40 per 10^8 nucleotides in skin cells following a single coal tar painting (Thein et al., 2000). 8-Nitroguanine could not be detected in lung cells in any of the guinea pigs (data not shown). The detection limit was approximately five 8-nitroguanine adducts per 10^6 guanine (Tuo et al., 2000). The level of DNA strand breaks in lymphocytes and the excretion of 8-oxodG in the urine was unchanged by the DEP exposure (Fig. 1).

Determination of protein oxidation after DEP exposure showed decreased levels of GGS and no significant change of AAS in the lung, although linear regression suggested an increase with dose ($r = 0.43$, $P < 0.05$, Table 1). Protein oxidation remained unaltered in plasma and liver.

The total ascorbate status was unaffected by the DEP instillation (Table 2). In the lung cytosol there was a significant increase in glutathione reductase activity in the low dosage group compared to control (Table 3).

Discussion

The present study indicated that in guinea pigs, a species similar to humans with respect to vitamin C metabolism, pulmonary exposure to DEP induced oxidative stress with accompanying DNA damage in terms of base damage and strand breaks in the lung, whereas the level of bulky DNA

adducts was unchanged. Induction of DNA strand breaks has not previously been investigated in relation to DEP. The level of ascorbate was not affected by DEP exposure. The significant increase in lung glutathione reductase activity following DEP exposure may be explained by de novo synthesis of antioxidant enzyme as a response to oxidative stress. In contrast to the clear oxidative damage observed in the lungs, there were unaltered levels of 8-oxodG excreted in urine, DNA strand breaks in lymphocytes, and absence of protein oxidation in plasma and hemoglobin. This indicates that the circulatory compartment may not be affected by acute pulmonary DEP exposure in guinea pigs. This is supported by longer-term studies showing that exposure of diesel exhaust for 3 months did not increase the frequency of micronuclei or sister chromatid exchanges in circulating lymphocytes of rats (Mauderly et al., 1994).

Probably the pulmonary oxidative stress arises both from ROS generated by metal-catalyzed Fenton reactions of the trace metals in DEP and from inflammatory reactions when DEP is deposited in the lungs. The NIST1650 DEP contains small amounts of transition metals, particularly trivalent iron (690 ppm), but also traces of trivalent chromium, divalent copper, nickel, divalent lead, and divalent manganese (62, 50, 50, 23, and 15 ppm, respectively) (Huggins et al., 2000). A significant portion of the metal content seems to be extractable in neutrally buffered water (Ball et al., 2000). It is therefore plausible that the transition element content is sufficient to produce oxidative damage in the lung of guinea pigs.

A carcinogenic mechanism involving inflammatory reactions caused by DEP or other poorly soluble particles due

Table 2

The level of ascorbate in liver, lung, and plasma following DEP exposure^a

Tissue	Control	Low	High
Liver (mM)	4096 ± 2688 (12)	3420 ± 1495 (12)	2934 ± 719 (12)
Lung (mM)	305 ± 390 (10)	260 ± 339 (12)	429 ± 473 (12)
Plasma (μM)	211 ± 96 (9)	225 ± 76 (11)	216 ± 45 (11)

^a Data represent the means and standard deviations, and the number of samples is shown in parentheses.

Table 3

Activity of antioxidant enzymes in lung cells following DEP exposure (units/gram protein)

Antioxidant enzyme	Control	Low	High
Glutathione reductase	8 ± 2.5 (10)	11.3 ± 3.1 (10)*	9.8 ± 2.9 (10)
Glutathione peroxidase	12.9 ± 8.9 (10)	15.7 ± 7.2 (10)	8.2 ± 7.8 (10)
Superoxide dismutase	586 ± 217 (10)	613 ± 127 (10)	688 ± 190 (10)

Note. Values are means and standard deviations, and the number of samples is shown in parentheses.

* $P < 0.05$ compared to control.

to deposition in the lung is well-described for rats (Mauderly et al., 1994). The ability of particulate matter, including DEP, to induce lung tumors is not known in guinea pigs; thus, a similar phenomenon as described in rats has not been investigated in the guinea pig. However, in guinea pigs inhalation of particulate matter from residual oil fly ash increased the number of neutrophils in bronchoalveolar lavage fluid and induced cell damage in the lung (Norwood, Jr., et al., 2001). This may suggest that the inflammatory reactions are similar across species since neutrophils also are recruited in the lung of rats, mice, and humans after exposure to particulate matter (Salvi et al., 1999; Ito et al., 2000; Nightingale et al., 2000; Takano et al., 1998). Assessments of oxidative DNA damage in the lung of mice and rats indicate that DEP is associated with elevated 8-oxodG levels after both acute (8 h) and long-term exposure (12 months) (Tsurudome et al., 1999; Sato et al., 2000; Ichinose et al., 2000; Iwai et al., 2000). Moreover it seems that elevated 8-oxodG levels in the lungs coincide with lung inflammation in mice and rats and may be also for guinea pigs, although this has not been finally tested. Because the exposure in this experiment lasted for 5 days after the instillation, it is impossible to distinguish between the effect of the inflammation and metal-catalyzed oxidation.

The doses of 0.7 and 2.1 mg DEP selected for the present study were in the lower end of the range previously shown to increase 8-oxodG levels in the lungs of rats and mice (Tsurudome et al., 1999; Sato et al., 2000; Ichinose et al., 2000; Iwai et al., 2000). In rats 2–4 mg has been used, whereas in mice 10 weekly doses of 0.05 to 0.2 mg, i.e., 2 to 8 mg/kg, were instilled (Tsurudome et al., 1999; Ichinose et al., 2000). Relative to human exposure circumstances, the doses used in this experiment are rather high. The 0.7 mg dose (approximately equal to 100 mg for humans) represents approximately 2 weeks of the highest occupational exposure to DEP (based on 1280 $\mu\text{g}/\text{m}^3$ and assuming that humans inhale 8 m^3 during a work shift with hard labor and equal deposition of inhaled and instilled particles). Similarly, the 0.7-mg dose corresponded to more than 5 years of exposure in ambient urban and nonurban air, based values being reported in the range 0.2–3.6 $\mu\text{g}/\text{m}^3$ DEP (U.S. Environmental Protection Agency, 2001). However, some populations may be particularly susceptible to the effects of DEP, e.g., due to pulmonary disease or low antioxidant status. Also, bus drivers with continuous exposure to DEP and other air pollution in the urban environment have shown increased levels of protein oxidation in plasma, chromosomal damage, and PAH adducts in lymphocytes as well as urinary 8-oxodG excretion (Knudsen et al., 1999; Loft et al., 1999; Autrup et al., 1999).

In this study we supplemented guinea pigs with vitamin C at doses sufficient to maintain steady state levels in the plasma, liver, and lung. Since DEP generated oxidative DNA damage without affecting the pool of ascorbate, it indicates that oxidative DNA damage is formed independently of the vitamin C status. This is in accordance with a

previous study showing that the level of 8-oxodG in the liver of guinea pigs was unaffected by severe depletion and supplementation of vitamin C (Cadenas et al., 1997). In contrast, reduced vitamin C intake in guinea pigs increased oxidation of protein and lipid in the liver (Barja et al., 1994). Thus, it is possible that vitamin C depletion could enhance the susceptibility toward DEP in guinea pigs as shown for lipid peroxidation. Otherwise, additional depletion of other antioxidant compounds may be required for oxidative DNA damage to occur following exposure to particulate matter. In fact inhalation of residual oil fly ash, as a source of particulate matter, only induced lung injury in guinea pigs depleted of vitamin C and glutathione (Norwood, Jr., et al., 2001). It is warranted that further studies of DEP or particulate matter in guinea pigs will include animals depleted of vitamin C and other antioxidants.

In conclusion, we have shown that DEP induces substantial oxidative DNA damage and minimal amounts of bulky DNA adducts in the lungs of guinea pigs, whereas levels of ascorbate remain unchanged. Future studies should address whether depletion of ascorbate increases the susceptibility to DEP at low doses in guinea pigs.

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