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DNA adduct formation and oxidative stress in colon and liver of Big Blue® rats after dietary exposure to diesel particles

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Abbreviations: DEP, diesel exhaust particles; PAH, polycyclic aromatic hydrocarbons; ROS, reactive oxygen species; EndoIII, endonuclease III; AAS, 2-aminoadipic semialdehyde; GGS, γ-glutamyl semialdehyde; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline.

Abstract
Exposure to diesel exhaust particles (DEP) via the gastrointestinal route may impose risk of cancer in the colon and liver. We investigated the effects of DEP given in the diet to Big Blue® rats by quantifying a panel of markers of DNA damage and repair, mutation, oxidative damage to proteins and lipids, and antioxidative defence mechanisms in colon mucosa cells, liver tissue, and the blood compartment. Seven groups of rats were fed a diet with 0, 0.2, 0.8, 2, 8, 20 or 80 mg DEP/kg feed for 21 days. DEP induced a significant increase in DNA strand breaks in colon and liver. There was no effect on oxidative DNA damage (8-oxodG) in colon or liver DNA or in the urine. However, the mRNA expression of *OGG1*, encoding an enzyme involved in repair of 8-oxodG, was increased by DEP in both liver and colon. DNA adduct levels measured by ^{32}P-postlabelling were elevated in colon and liver, and the expression of *ERCC1* gene was affected in liver, but not in colon. In addition to these effects, DEP exposure induced apoptosis in liver. There was no significant change in mutation frequency in colon or liver. The levels of oxidative protein modifications (oxidised arginine and proline residues) were increased in liver accompanied by enhanced vitamin C levels. In plasma, we found no significant effects on oxidative damage to proteins and lipids, antioxidant enzymes, or vitamin C levels. Our data indicate that gastrointestinal exposure to DEP induces DNA adducts and oxidative stress resulting in DNA strand breaks, enhanced repair capacity of oxidative base damage, apoptosis and protein oxidation in colon mucosa cells and liver.

Introduction

Diesel exhaust particles (DEP) are important contributors to air pollution in some workplace atmospheres and in urban air, and there is concern that they are lung carcinogens in exposed humans (1). It has been reported that rats develop lung tumours when they are exposed to high concentrations of whole diesel exhaust by inhalation exposure in long-term studies (2,3). Research on DEP has mainly focused on the pulmonary effects after inhalation, the primary route of exposure. However, a large proportion of the inhaled diesel particles is rapidly transported via the mucociliary clearance system (depending on particle size and dose) to the oral cavity, swallowed and eliminated via the gastrointestinal tract (1). Moreover, foodstuff, e.g. plant material, may be polluted with DEP during growth or transport. A few epidemiological studies have demonstrated associations between occupational exposure to diesel exhaust and colon or liver cancer (4-6). Accordingly, effects of exposure via the gastrointestinal route are important for risk assessment. So far, no data are available on DEP or whole diesel exhaust in this respect.

At present, the mechanisms underlying the carcinogenic response of DEP are uncertain. Diesel particulate extracts contain polycyclic aromatic hydrocarbons (PAH) and nitro-arenes, and the results of several studies suggest a potential role for the organic chemicals associated with DEP as causative factors in the lung carcinogenic response of rodents (7,8). The mechanism of action of many PAH carcinogens has been attributed to the formation of bulky DNA adducts (9-11). However, the carcinogenic mechanism of DEP is controversial, since carbon black, which is almost devoid of genotoxic organic compounds such as PAHs, has been found to be similarly potent in causing lung tumours in rats (12).

Experimental studies have shown that oxidative damage to DNA, in addition to bulky DNA adduct formation of DEP-associated organic compounds, is important in the initiation of DEP-induced lung carcinogenesis (11,13). Reactive oxygen species (ROS) are compounds, e.g. superoxide and hydroxyl radicals, possessing high reactivity with DNA, proteins and lipids. The most abundant and mutagenic ROS-induced DNA lesion, 8-oxodG, causes G:C to T:A transversions and, thus, could be responsible for mutations that lead to carcinogenesis. It has previously been reported that DEP can produce superoxide and hydroxyl radicals *in vitro* without any biological activating systems and that DEP washed with methanol no longer could produce these radicals, indicating that the active components were extractable with organic solvents (14). The involvement of ROS in the induction of toxic and potentially carcinogenic effects of DEP in the lungs was suggested by the finding that extraction of the organic fraction prior to intratracheal instillation in mice or intravenous pre-treatment with the superoxide dismutase, an antioxidant enzyme, markedly reduced lung injury due to dosing with DEP (14). Whether oral administration of DEP leads to similar local effects in the colon has not previously been investigated.

The mammalian organism possesses a spectrum of defence mechanisms to counteract oxidative stress. Plasma antioxidants, e.g. ascorbic acid, which scavenge the ROS prior to their interactions with cellular components, are the first line of defence against oxidative stress. Superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase are some of the enzymes constituting the secondary defensive system against oxidative stress. The system detoxifies ROS by reductive and oxidative metabolism. A repair system exists in case ROS escape the first two defence lines and react with the cellular DNA. An example of this system is the *OGG1* gene product, which repairs 8-oxodG. It was recently observed that inactivation of the *OGG1* gene in yeast and mice leads to elevated spontaneous mutation frequency (15).

For elimination of bulky DNA adducts, cells have evolved the multi-enzyme nucleotide excision repair system. The ERCC1 enzyme is part of the 5' nuclease in the nucleotide excision pathway. We have previously demonstrated that mRNA levels of *ERCC1* are correlated with DNA repair capacity in human primary lymphocytes (16) and others have observed that an increased mRNA expression of *ERCC1* leads to resistance against DNA adduct formation (17,18).

In the present study, we investigated the effects of DEP in colon, liver and plasma after oral administration in Big Blue® rats, on various pathways involved in DNA damage and repair, oxidative damage to proteins and lipids, and antioxidative defence mechanisms, in order to see whether the local effects observed in the lung after inhalation are also observed in colon epithelium after oral administration and whether systemic effects may occur.

Materials and methods

Diesel particles

Diesel particles were Standard Reference Material 1650 from the National Institute of Standards & Technology (Gaithersburg, MD, USA). They had been collected from heat exchangers of a dilution type facility after 200 engine hours, representing combustion particles from a heavy-duty diesel engine.

Animals, exposure protocol and preparation of subcellular fractions

Forty-two male Big Blue® (Fischer) rats, approximately 8 weeks of age from Stratagene (La Jolla, USA), were acclimatized for 7 days while maintained on Altromin 1320 pellets (Altromin, Lage, Germany). The chemical composition of the diet is based on defined standards and is available on www.altromin.de. Animals were assigned to 7 groups (six animals/group), which were fed with 0, 0.2, 0.8, 2, 8, 20 or 80 mg DEP/kg Altromin diet prepared by Altromin in Germany. An extra group of animals was given a diet with 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) in Altromin, 70 mg/kg, and used as positive controls for the mutation assay. Animals were kept in a 12 hours light and 12 hours dark cycle at an average temperature of 22°C and at 55% humidity. The diets were administered for a total of 21 days. Throughout the study the animals had free access to the diet and water. A 24-hour urine was collected on day 20 and stored at -80°C. At day 21, all rats were healthy, and they were anesthetized with carbon dioxide/oxygen, decapitated and blood was collected. Liver and colon were isolated, weighed and frozen in liquid nitrogen until storage at -80ºC. Before freezing, the isolated colon was washed with 0.15 M NaCl and cut longitudinally into two pieces. The heparinized blood samples were separated into plasma, white and red blood cells by centrifugation on Ficoll Paque®, and stored at -80ºC. Care and treatment of the animals was conducted in accordance with guidelines established by the Danish government.

Detection of DNA strand breaks and endonuclease III sensitive sites by the alkaline comet assay

The isolation of liver cells was essentially carried out as described previously (19). Cells from one piece of the divided colon were scraped off the mucosa with a glass microscope slide and placed in ice-cold Merchant-EDTA solution (0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 10 mM NaEDTA, pH 7.4) (20). The detection of DNA strand breaks was carried out as described previously (21). The level of endonuclease III sensitive sites was obtained as the difference in scores of parallel slides incubated with and without endonuclease III enzymes at 37°C for 45 min (endonuclease III enzyme was a kind gift from Serge Boiteaux, UMR217 Centre National de la Recherche Scientifique et Commissariat a l'Energie

Atomique, France). A total of 50 nuclei were scored for each sample, using the Kinetics Imaging Limited version 4, Liverpool, UK software system to determine the amount of DNA migrated from the comet head to the tail.

Detection of 8-oxo-deoxyguanosine by HPLC
Levels of 8-oxodG relative to dG were measured in colon mucosa cells and liver by HPLC with electrochemical detection after isolation and digestion of nuclear DNA as described elsewhere (22). Urinary concentrations of 8-oxodG were measured by HPLC with tandem mass spectrometry detection as described elsewhere (23).

^{32}P-postlabelling
DNA was extracted by standard phenol/chloroform extraction procedure, and the ^{32}P-postlabelling assay with butanol enrichment was carried out in at least duplicates as described previously (24).

Quantification of the rERCC1 and rOGG1 mRNA levels in colon and liver
Total RNA was purified from 10 mg liver or from 5 x 10^6 colon cells using a Qiagen total RNA purification kit as recommended by the manufacturer. The RNA was DNase treated as recommended by Qiagen. Subsequent quality control showed that all genomic contaminations were removed by the DNAse treatment. The integrity of the RNA was checked by gel electrophoresis. An aliquot of 200 ng RNA was used for cDNA synthesis in a reaction volume of 10 µl using the Taqman Gold RT-PCR kit as recommended by Applied Biosystems. For quantification of the mRNA levels, Taqman probes were used. For *rERCC1* the following oligonucleotides were used: forward primer 53F: 5'-CCT GGG AAG GAC GAG GAA A-3', reverse primer 121R: 5'-TGG GAT AAC AAA CTT CTT CCT GGT-3', Taqman probe 74T: 5'-FAM-CGG CCA CAG CCC TCA GGA CC-Tamra-3' (TAG Copenhagen, Denmark). For *rOGG1* the following oligonucleotides were used: forward primer: 5'-ACT TAT CAT GGC TTC CCA AAC C-'3, reverse primer: 5'-CAA CTT CCT GAG GTG GGT CTC T-'3, Taqman probe: 5'-FAM-TCA TGC CCT GGC TGG TCC AGA AG-TAMRA-'3. The PCR reactions were performed in duplicate or triplicate in an ABI 7700 Sequence Detection System in 15 µl reactions containing 200 nM primers, 300 nM *rERCC1* / 150 nM *rOGG1* Taqman probe, 0.1-0.5 µl cDNA in 1 x Mastermix (Applied Biosystems). For normalization, 18S was quantified in a separate PCR reaction using an endogenous control pre-developed assay reagent for 18S quantification (Applied Biosystems) in duplicate or triplicate. For each animal the average value of *rERCC1* or *rOGG1* was divided by the average value of the 18S. The signals from *rERCC1, rOGG1,* and 18S were linear over a 100-fold dilution. Likewise, normalization of the mRNA signals to 18S yielded the same signal over a 100-fold dilution. Repeated measurements of the same sample yielded a standard deviation of 20%. Standard deviation on triplicates was on the average 15%.

Apoptosis by the TUNEL assay
The liver tissue was embedded in paraffin and cut in 2-µm sections mounted on poly-L-lysine-coated slides. The sections were deparaffinized and incubated with 20 µg/ml Proteinase K (Sigma, USA) for 5 min at room temperature to strip off nuclear proteins and then incubated in 3% H_2O_2 in PBS-buffer for 5 min at room temperature to quench endogenous peroxidase. TUNEL assay was accomplished using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Intergen, NY, USA). After preincubation in equilibrium buffer for 30 min at room temperature, the sections were incubated with a mixture of TdT-enzyme and nucleotides linked with digoxigenin in a humidified chamber at 37°C for 1 hour and hereafter immersed in a stop/wash buffer at 4°C for 10 min. The slides were incubated with an anti-

digoxigenin antibody conjugated with peroxidase. Afterwards, the sections were washed in PBS buffer and incubated in anti-digoxigenin-peroxidase solution for 30 min at room temperature. DAB (3,3'-diaminobenzidine, Sigma Fast tablet sets) was used as a chromogen and the sections were counterstained with Mayer's Hematoxylin (Sigma Diagnostics). Negative control sections were treated similarly but incubated in the absence of TdT enzyme/nucleotides. Sections were compared with positive control slides from Intergen. Morphologic criteria for apoptosis were evaluated, since the TUNEL assay may also stain necrotic cells.

Mutation analysis

One half of the frozen colon was thawed and washed thoroughly in ice-cold Merchant-EDTA solution (0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 10 mM NaEDTA, pH 7.4). The mucosa cells were scraped off with a microscope slide (20), and suspended in 2 ml Merchant-EDTA. About 20 million cells were filtered through a cell strainer (Falcon, Becton Dickinson, Franklin Lakes, New Jersey, USA) and DNA was purified by the RecoverEase™ DNA isolation kit (Stratagene, La Jolla, USA). DNA from about 60 mg frozen liver was prepared using the RecoverEase™ as described by the manufacturer. DNA preparation (8 µl) was packaged with Transpack packaging extract (Stratagene, La Jolla, USA). If the packaging mixture was viscous after the recommended standard packaging time of 180 min, the mixture was incubated for another 60 min. If the mixture was still viscous after this time, additional Transpack reagents were added and the mixture was incubated for another 60 min. This phage preparation was used to infect *E. coli* G1250 (*hfl*$^-$). Phages with mutations at the *cII* locus were identified by plaque formation under selective growth conditions at 24^{o}C and the total number of infective phages was determined by plaque formation under non-selective growth conditions at 37^{o}C as described (λ Select-cII™ Mutation Detection System for the Big Blue® Rodents, Stratagene, La Jolla, USA).

Oxidized protein and lipid

Frozen liver was thawed, homogenized, centrifuged, and the cytosolic fraction was obtained by the method of Kim and Lee (25). Cytosolic fractions from liver tissue, plasma proteins and erythrocyte proteins were assayed for oxidised lysine residues (2-aminoadipic semialdehyde, AAS) and for oxidised proline or arginine residues (gamma-glutamyl semialdehyde, GGS) in proteins as described previously (26). Protein was determined on a Cobas Mira + analyser using a commercial kit (Roche, Basel, Switzerland). Total malondialdehyde (MDA) in plasma was determined by HPLC as described elsewhere (27).

Determination of antioxidant enzymes and ascorbic acid

Automated assays for the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and glutathione reductase (GR) in blood hemolysate were performed on a Cobas Mira analyzer. SOD, GPx and hemoglobin were determined using commercially available kits from Randox, whereas the activity of GR was determined by the method of Goldberg and Spooner (28), and the activity of CAT was determined according to a method described by Wheeler et al. (29). The enzymatic activities in RBC were calculated relative to the amount of hemoglobin. Ascorbic acid and total vitamin C were determined by HPLC as described previously (30).

Statistics

All variables were tested for normality using the Anderson-Darling test. The groups were also tested for homogeneity of variance with Bartlett's test ($P > 0.05$). To fulfil the criteria for normality and homogeneity of variance some variables were logarithmically transformed

(*colon*: strand breaks, *ERCC1*, 8-oxodG, adducts, mutation frequency; *liver*: strand breaks, EndoIII sites, 8-oxodG). The groups were compared using the ANOVA function in the MINITAB program. If significant differences were found ($P < 0.05$), the DEP-exposed groups were compared with the control group using Student's t-test. For a few variables, the criteria for normality could not be achieved by logarithmic transformation and therefore the non-parametric Kruskal-Wallis test was used (*liver:* mutation frequency*; plasma*: AAS). If significant differences were found ($P < 0.05$), the DEP-exposed groups were compared with the control group using the Mann-Whitney test.

Results

Body and liver weight

DEP treatment had no effect on body or liver weight. The final average body weight in rats fed the control diet was 300 g, and the corresponding value in the groups fed the DEP diet ranged from 294 to 303 g (table I). The average weight of the liver in control rats was 10.9 g and in DEP-exposed rats 10.3 to 10.9 (table I).

Food consumption

There was no significant difference in food intake between rats fed the control diet and the DEP diets. The average weekly dietary consumption in control rats was 254 g, and the corresponding value in the DEP groups ranged from 245 to 256 g (table I).

DNA damage and DNA repair

Colon mucosa cells showed an increase in strand breaks as determined by the comet assay at all doses tested (table II, figure 1). The increase in strand breaks was statistically significant in the dose range 0.2-20 mg/kg DEP. There was no increase in the level of oxidised bases determined as EndoIII sensitive sites in the comet assay or 8-oxodG in DNA in colon cells. The level of DNA adducts (^{32}P-postlabelling) in colon was proportional with the dose and significantly elevated at all doses, except at 0.8 mg/kg (table II, figure 2). The expression of two DNA repair genes, *ERCC1* and *OGG1*, was determined in the colon cells. Colon *OGG1* but not *ERCC1* mRNA levels were increased by DEP exposure. The increase in *OGG1* was statistically significant in rats exposed to 0.8 and 2 mg/kg DEP (table II, figure 3), with 2.4 and 2.7-fold increased mRNA levels, respectively.

In liver cells, the level of strand breaks in DEP-exposed animals was significantly increased at 8 and 80 mg/kg (table II, figure 1). No increase was seen in EndoIII sensitive sites or the level of 8-oxodG. Adduct formation in liver cell DNA had a bell-shaped dose-response relationship and was significantly increased at 0.2, 2 and 8 mg/kg DEP (table II, figure 2). There was a 2-fold increase in the expression of liver *OGG1* mRNA in rats treated with 20 and 80 mg/kg DEP (table II, figure 3), and the expression of *ERCC1* mRNA was significantly increased at the highest DEP dose. The marker for apoptosis (TUNEL assay) was significantly induced in the liver in the dose interval 0.8-20 mg/kg (table II). It was not possible to measure apoptosis in colon tissue due to insufficient number of crypts for quantification.

The urinary excretion of 8-oxodG was determined as a marker of general oxidative stress to DNA and the nucleotide pool. There was no effect of DEP on this marker.

Mutation frequency

The IQ-treated rats served as positive controls in the mutation assay (table III). There were statistically significant increases in mutations in the *cII* gene in both the colon and liver after a dose of 70 mg/kg IQ (31). The DEP-treated animals were also scored for mutations in the *cII* gene in colon mucosa and liver, but no significant changes were observed (table III).

Oxidative damage to protein and lipids
Markers for damage to protein and lipids were determined in liver and plasma (table IV). In liver the level of γ-glutamyl semialdehyde (GGS) was significantly increased at all DEP doses. The level of 2-aminoadipic semialdehyde (AAS) was generally higher in liver in DEP-exposed rats than in control rats, but this was not statistically significant. No effect was seen in plasma protein and lipid oxidation.

Antioxidant activity
Vitamin C status was measured in plasma and liver (table V). In plasma, the levels of both ascorbic acid and total vitamin C were increased in the whole DEP dose range, but this was not statistically significant. In liver, the level of total vitamin C was significantly increased at the two highest DEP doses and the ascorbic acid concentration at the highest DEP dose. No correlation was seen between vitamin C measurements in plasma and liver (data not shown). In the erythrocyte compartment, a number of enzymes in oxidative defence were measured (table V). No effect was seen with any of these enzymes.

Discussion
In this study, we found that dietary exposure to DEP increased the formation of DNA adducts and induced strand breaks in both colon mucosa cells and the liver in rats. The apparent lack of effects on DNA base oxidation in terms of EndoIII sensitive sites and 8-oxodG levels could be related to the increased expression of the base excision repair gene *OGG1* in both colon and liver. However, it is also possible that base oxidation did not occur at all. Moreover, apoptosis as well as direct signs of oxidative stress in terms of protein oxidation and enhanced vitamin C levels were also seen in the liver. The data indicate that risk assessment of DEP should include consideration of oral exposure.

In the present experimental set-up, Big Blue® rats were fed with different levels of DEP for 21 days and sacrificed immediately afterwards. The set-up was chosen as a compromise between the requirements for the mutation assay and the requirements for the other biomarkers. The mutation assay requires a minimum of 1-2 weeks of incubation time for establishment of the induced mutations, whereas most of the other biomarkers require that the animals be exposed to the test agent until sacrifice. The mutation fixation time is dependent on the proliferation time, and for liver tissue the optimal fixation time may be as long as 5 weeks, whereas the fixation time is somewhat shorter in the colon (32,33). It is therefore possible that a mutagenic effect of DEP is missed in this experiment. However, it is of course possible that the relatively short feeding time with DEP used in this study is inadequate to induce mutations. On the other hand, we have shown that a 21-day diet containing 14% or 34% sucrose is adequate to induce mutations in the rat colon (34). Likewise, the IQ-treated rats, used as positive controls for the present study, had increased mutation frequencies in both colon and liver (31).

The comet assay is frequently used to evaluate the genotoxicity of test substances. The interpretation of the results is based on the hypothesis that the DNA damage caused by a non-crosslinking genotoxic agent produces DNA strand breaks, either directly or indirectly by excision-repair of damaged DNA or formation of alkali-labile sites. The DNA breaks allow relaxation of the DNA supercoiling during lysis and unwinding steps of the comet assay procedure and migration of the DNA form the comet tail upon electrophoresis. It cannot be excluded that the initial DNA fragmentation occurring during apoptosis may appear as highly damaged cells in the comet assay. However, the distribution of the amount of DNA in the tail in this study did not indicate particular prevalence of highly damaged cells. Thus, the

responses in terms of apoptosis determined by the TUNEL assay and strand breaks in the liver were likely to be independent effects of exposure to DEP. The finding in this study of an induction of apoptosis has also been demonstrated in a few other studies (35-37). Some of these *in vitro* results indicate that organic compounds in DEP are responsible for apoptosis through generation of reactive oxygen radicals (37).

The present study included measurement of the expression of two DNA repair genes, *OGG1* and *ERCC1*, in colon cells and in liver. The expression of *ERCC1* mRNA increased in liver, but not in colon, suggesting that a repair response to bulky DNA adducts was induced in liver. In colon, the extent of formation of DNA adducts was higher than in liver, and this may be a consequence of a low nucleotide excision repair activity. We found that *OGG1* mRNA levels were increased in both colon cells and liver in DEP-exposed rats. Moreover, the increase in DNA strand breaks followed a similar pattern, which could correspond to an increased incision activity on 8-oxodG in DNA. In a previous study, the 8-oxodG levels in lung tissue of rats increased markedly from 2 to 8 hours after a single intratracheal dose of DEP with a subsequent decrease to control level after 5-7 days concomitant with a detectable induction of *OGG1* mRNA (38). Those findings suggest that the increases of *OGG1* mRNA levels in our study reflect a compensatory induction caused by formation of 8-oxodG at an early phase of DEP exposure. This may indicate that all 8-oxodG nucleosides are repaired by accumulated OGG1 enzyme, and that the extent of 8-oxodG damage did not exceed the repair capacity of OGG1.

With respect to direct oxidative stress, DEP exposure increased the levels of oxidative protein modifications in the liver in terms of γ-glutamyl semialdehyde (GGS), but not 2-aminoadipic semialdehyde (AAS). Previous *in vitro* studies have shown that ROS formed by Fenton reactions generated more GGS than AAS, whereas AAS was more prevalent after peroxidase-catalyzed protein oxidations (26). We have previously observed that urban air pollution induced plasma protein oxidation and influenced erythrocyte antioxidant levels in bus drivers but these systemic effects were not seen in the present experimental system indicating that other factors than DEP in the urban air may be responsible (24). The exposure to DEP was also accompanied by increased levels of vitamin C in the liver, indicating an increased defence against reactive species. Increase in rat liver ascorbate synthesis has been observed previously after exposure to either genotoxic hepatocarcinogens or to peroxisome proliferators (39,40). Moreover, it was recently shown that whole diesel exhaust exposure to humans resulted in a substantial increase in nasal cavity lining fluid ascorbate concentrations that returned to basal levels 5.5 hours after exposure (41).

For many of the biomarkers of DNA damage and protein oxidation, a flat or even bell-shaped dose-response relationship was apparent in the liver and/or colon, and significant effects were seen only at the low or middle doses. The decrease in effects at higher doses is unlikely to result from direct toxicity at least as revealed by apoptosis in the liver, which also showed bell-shaped dose-response relationship. It is possible that uptake and/or transport of DEP and/or the active compounds is limited or decreased due to toxicity in the intestine, although we have no data to support such a mechanism. In retrospect, even lower doses than employed in the present study would have been interesting for a better description.

The mechanism involved in generation of oxidative stress in colon cells could be similar to that of alveolar cells, where transition metals and other soluble toxic substances on the surface of DEP as well as inflammation appear to be important (35,42,43). However, it is not known whether DEP can be taken up directly from the intestine and transported to the liver or

whether the toxic substances are released from DEP and then transported to the liver where effects are exerted. In a recent study, the translocation of ultrafine carbon particles to the liver after inhalation exposure was demonstrated (44). Since the results indicated that deposits in the respiratory tract were not fully responsible for the hepatic burden, input from particles present in the gastrointestinal tract needs to be considered as well. Thus, these mechanisms should be addressed in future studies. The lowest dose of DEP in our study (0.2 mg/kg feed, corresponding to 24 μg/kg body weight/d) induced the formation of DNA adducts and strand breaks in the colon cells as well as protein oxidation in the liver. At higher doses, e.g. 0.8 mg/kg feed (96 μg/kg body weight/d), most of the other markers of oxidative stress showed significant effects. With respect to strand breaks and *OGG1* expression, colon cells reacted at lower doses than liver, suggesting higher exposure or higher sensitivity. For human exposure, the levels of DEP in ambient urban air and non-urban air have been estimated to 1.7-3.6 $\mu g/m^3$ and 0.2 –2.6 $\mu g/m^3$, respectively (1). However, occupational exposure levels to DEP range from 10 to 1280 $\mu g/m^3$. With a normal ventilation of 16 m^3 per day, total exposure will be 3.2 to 58 and 53 to 6,800 μg per day in ambient air and occupational (8-h workday) exposure, respectively. Accordingly, given that a substantial part of the inhaled DEP is swallowed, the daily dose to the gastrointestinal tract via ambient air will approach the lowest dose used in the present study, whereas the intake via occupational exposure may even approach the higher doses.

In summary, the present study has shown that gastrointestinal exposure to DEP at relatively low levels can induce DNA adducts and oxidative stress with accompanying DNA strand breaks, apoptosis, protein modification and increased expression of relevant DNA repair genes locally in colon mucosa cells and systemically in the liver. The data indicate that consideration of oral exposure should be included in risk assessment of DEP.

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Table I. Food consumption and final body and liver weight

DEP mg/kg	0	0.2	0.8	2	8	20	80
Food intake (g/week)	254 ± 15	251 ± 3	245 ± 16	250 ± 8	256 ± 19	247 ± 13	246 ± 17
Body weight (g)	300 ± 28	299 ± 21	295 ± 28	294 ± 9	303 ± 30	294 ± 24	296 ± 33
Liver weight (g)	10.9 ± 1.2	10.8 ± 1.5	10.7 ± 1.2	10.3 ± 0.8	10.9 ± 1.4	10.4 ± 1.3	10.3 ± 0.9

Results are expressed as mean ± SD, n = 6.

Table II. DNA damage and DNA repair

DEP mg/kg	0	0.2	0.8	2	8	20	80
Colon							
Strand breaks[a]	6.7 ± 2.5	26.6 ± 21.0**	22.3 ± 12.7**	19.2 ± 11.6*	17.1 ± 6.4**	19.0 ± 15.2*	10.5 ± 4.1
EndoIII sites[a]	2.8 ± 2.1	4.8 ± 6.0	3.0 ± 8.4	2.2 ± 6.1	1.1 ± 5.4	0.5 ± 3.8	2.0 ± 2.4
8-oxodG / 10^5 dG	0.55 ± 0.20	0.65 ± 0.43	0.44 ± 0.22	0.43 ± 0.18	0.60 ± 0.31	0.36 ± 0.05	0.52 ± 0.24
Adducts[b]	0.30 ± 0.05	0.46 ± 0.11*	0.20 ± 0.10*	0.75 ± 0.14**	0.91 ± 0.30**	0.94 ± 0.24**	0.98 ± 0.50**
ERCC1 x 10^{-6}	11.8 ± 3.7	11.2 ± 10.6	16.9 ± 13.2	10.9 ± 3.6	15.6 ± 7.9	18.2 ± 18.1	17.6 ± 12.6
OGG1 x 10^{-6}	39.5 ± 19.3	47.0 ± 23.9	94.3 ± 52.9*	106.1 ± 35.0**	76.6 ± 51.1	52.3 ± 30.3	57.9 ± 37.3
Liver							
Strand breaks[a]	4.2 ± 2.8	6.8 ± 4.0	6.4 ± 2.6	6.8 ± 4.8	11.6 ± 7.0*	10.2 ± 7.6	16.0 ± 10.1*
EndoIII sites[a]	2.4 ± 2.7	2.0 ± 2.3	1.2 ± 1.3	2.4 ± 2.5	1.1 ± 5.4	0.3 ± 2.7	0.4 ± 1.2
8-oxodG / 10^5 dG	0.30 ± 0.14	0.41 ± 0.10	0.33 ± 0.10	0.42 ± 0.16	0.31 ± 0.06	0.36 ± 0.07	0.37 ± 0.12
Adducts[b]	0.27 ± 0.03	0.33 ± 0.07*	0.29 ± 0.05	0.45 ± 0.11**	0.59 ± 0.10**	0.28 ± 0.04	0.26 ± 0.06
ERCC1 x 10^{-6}	13.2 ± 11.5	24.1 ± 8.3	10.0 ± 3.6	18.0 ± 7.1	17.4 ± 4.9	26.3 ± 10.5	31.2 ± 9.4*
OGG1 x 10^{-6}	4.38 ± 2.28	4.43 ± 0.94	3.60 ± 0.51	4.87 ± 2.27	5.22 ± 1.05	8.84 ± 2.85*	8.18 ± 2.48*
Apoptosis[c]	1.23 ± 0.12	1.37 ± 0.11	1.53 ± 0.27*	1.58 ± 0.28*	1.83 ± 0.20**	1.60 ± 0.09**	1.36 ± 0.23
Urine							
8-oxodG (pmol/24h)	134 ± 31	121 ± 24	129 ± 35	137 ± 20	140 ± 22	140 ± 22	155 ± 30

[a] % DNA in tail, [b] pr 10^8 nucleotides, [c] % of total cells. Results are expressed as mean ± SD, n = 6.

*P < 0.05 as compared to control, **P < 0.01 as compared to control.

Table III. Mutation frequency

Diet	Rat ID	Colon			Liver		
		Total no. of plaques	MF X 10^{-6}	Mean MF ± SD	Total no. of plaques	MF X 10^{-6}	Mean MF ± SD
Control	1	840000	20.8	29.1 ± 12.5	347500	39.3	22.3 ± 11.4
	2	456960	15.3		527500	23.7	
	5	189000	46.3		114500	10.9	
	6	317100	25.2		150500	8.3	
	9	362250	24.2		484500	28.4	
	10	469500	42.6		162500	23.1	
0.2 mg/kg DEP	31	166500	52.6	35.3 ± 16.4	305000	49.1	35.9 ± 11.9
	32	234570	21.3		1225000	21.5	
	33	229110	21.8		427500	26.3	
	34	744000	20.2		546000	45.8	
	35	290500	55.9		313500	27.9	
	36	188000	39.9		281000	44.5	
0.8 mg/kg DEP	37	232680	17.2	35.6 ± 24.2	1009000	7.4	19.3 ± 8.8
	38	584000	72.8		422500	8.9	
	39	642600	28		100500	24.9	
	40	360500	10.4		633000	21.7	
	41	307500	56.9		1455000	25.8	
	42	670500	28		277000	27.1	
2 mg/kg DEP	43	387660	23.2	39.7 ± 19.7	466000	34.9	25.9 ± 13.9
	44	209000	53.8		121000	31	
	45	654360	22.9		288000	13	
	46	309000	68.8		634000	23.7	
	47	642500	46.7		1277000	7.8	
	48	493500	22.8		333500	45	
8 mg/kg DEP	49	515000	41.2	31.2 ± 7.7	558000	26.9	25.2 ± 9.8
	50	325080	18.5		419500	14.9	
	51	257000	29.2		331500	11.3	
	52	385500	35.7		314000	31.8	
	53	147750	33.8		391000	35.2	
	54	436000	28.7		162000	30.9	

Table III. Continued

20 mg/kg DEP	55	203280	113.1	57.1 ± 37.0	1062000	29.4	33.9 ± 14.2
	56	824000	41		960000	26	
	57	295680	47.3		211500	53.2	
	58	187750	46.6		484500	12.9	
	59	217250	86.3		315000	39.7	
	60	148950	8.4		560000	42.4	
80 mg/kg DEP	61	407500	30.7	36.9 ± 10.1	503000	24.9	32.8 ± 10.6
	62	461160	28.2		908000	33	
	63	149250	50.3		323500	38.6	
	64	305750	45		398000	15.7	
	65	438500	25.7		525500	42.8	
	66	209750	41.7		421500	41.5	
70 mg/kg IQ	19	501480	49.9	54 ± 13.9*	228000	115.1	98.5 ± 40*
	20	308000	52.8		1157000	70.2	
	21	463260	60.4		495000	35.4	
	22	243500	77		303500	131.8	
	23	153500	48.9		283500	97	
	24	106500	35.2		425000	141.2	

Results are expressed as mean ± SD, n = 6.

*P < 0.05 as compared to control.

Table IV. Oxidative damage to protein and lipids

DEP mg/kg	0	0.2	0.8	2	8	20	80
Plasma							
AAS[a]	139 ± 7	156 ± 13	141 ± 8	147 ± 7	140 ± 9	153 ± 14	138 ± 13
MDA[a]	322 ± 89	324 ± 86	354 ± 83	306 ± 131	283 ± 116	379 ± 203	396 ± 7
Liver							
AAS[a]	83 ± 5	91 ± 25	100 ± 16	89 ± 21	106 ± 15	106 ± 26	108 ± 31
GGS[a]	82 ± 6	114 ± 14**	116 ± 15**	104 ± 22*	116 ± 18**	131 ± 9**	122 ± 26**

MDA, malondialdehyde; AAS, 2-aminoadipic semialdehyde (oxidised lysine); GGS, γ-glutamyl semialdehyde (oxidised arginine/proline).

[a]Pmol/mg protein.

Results are expressed as mean ± SD, n = 6.

*P < 0.05 as compared to control.

**P < 0.01 as compared to control.

Table V. Antioxidant activity

DEP mg/kg	0	0.2	0.8	2	8	20	80
Plasma							
GR (U/g Hb)	11.4 ± 3.1	15.2 ± 6.2	19.1 ± 6.6	18.7 ± 7.5	12.2 ± 4.7	10.3 ± 2.6	13.1 ± 6.4
GPx (U/g Hb)	2328 ± 612	2936 ± 1304	3793 ± 1084	3375 ± 1206	2467 ± 852	2500 ± 381	3066 ± 1554
SOD (U/g Hb)	15428 ± 5312	18360 ± 7193	26070 ± 7674	20633 ± 5875	18040 ± 5723	18342 ± 3376	21257 ± 8192
CAT (U/g Hb)	119 ± 35	106 ± 54	125 ± 48	123 ± 52	81 ± 21	109 ± 25	111 ± 45
Ascorbate (μM)	10.3 ± 4.2	24.2 ± 20.4	25.7 ± 10.3	29.2 ± 16.4	16.5 ± 9.8	29.5 ± 10.9	23.5 ± 9.6
Total vit C (μM)	14.2 ± 3.9	27.5 ± 17.9	30.0 ± 10.3	32.2 ± 16.5	19.5 ± 10.8	31.9 ± 9.9	26.8 ± 10.4
Liver							
Ascorbate (μM)	2.05 ± 0.57	2.38 ± 0.37	2.54 ± 0.27	2.17 ± 0.49	2.36 ± 0.30	2.63 ± 0.28	3.03 ± 0.66*
Total vit C (μM)	2.10 ± 0.64	2.43 ± 0.34	2.55 ± 0.25	2.28 ± 0.57	2.38 ± 0.27	2.78 ± 0.34*	3.14 ± 0.63*

GR, glutathione reductase; GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase.

Results are expressed as mean ± SD, n = 6.

*$P < 0.05$ as compared to control.

Legends to figures

Fig 1. Formation of DNA strand breaks in colon cells and liver tissue of Big Blue® rats after oral administration of DEP for 21 days. Values are shown as mean ± SD, n = 6.
*P < 0.05 as compared to control.
**P < 0.01 as compared to control.

Fig 2. DNA adduct formation in colon cells and liver tissue of Big Blue® rats after oral administration of DEP for 21 days. Values are shown as mean ± SD, n = 6.
*P < 0.05 as compared to control.
**P < 0.01 as compared to control.

Fig 3. *OGG1* mRNA levels in colon cells and liver tissue of Big Blue® rats after oral administration of DEP for 21 days. Values are shown as mean ± SD (n = 6), where the value 1 corresponds to control level. The mean levels of control *OGG1* were 39.5×10^{-6} and 4.38×10^{-6} in colon and liver, respectively.
*P < 0.05 as compared to control.
**P < 0.01 as compared to control.

Fig. 1: Formation of DNA strand breaks

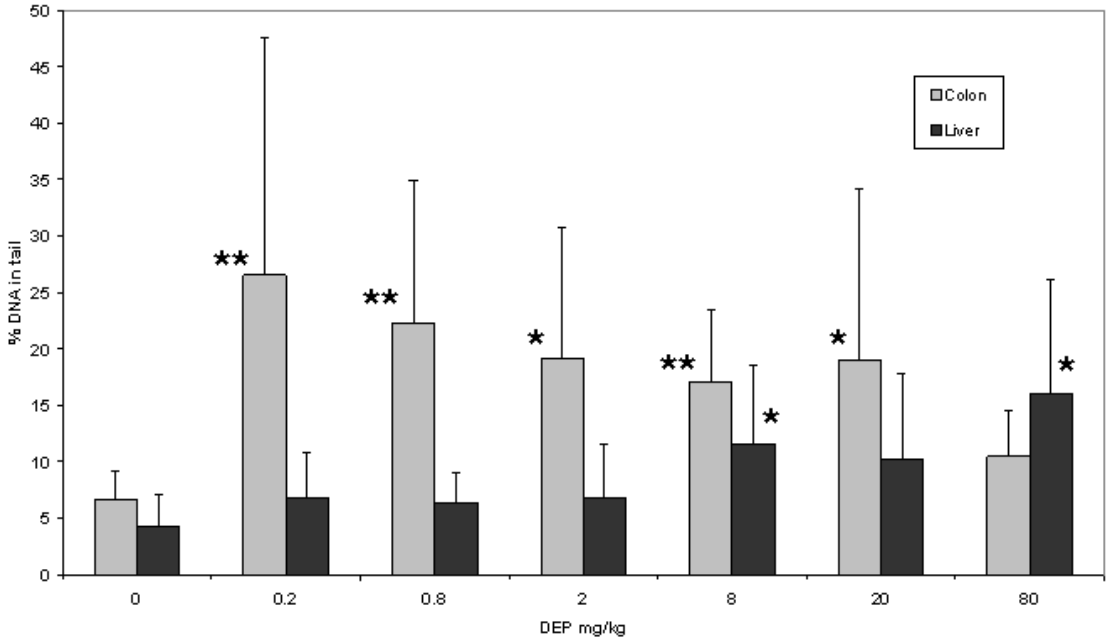

Fig. 2: Formation of DNA adducts

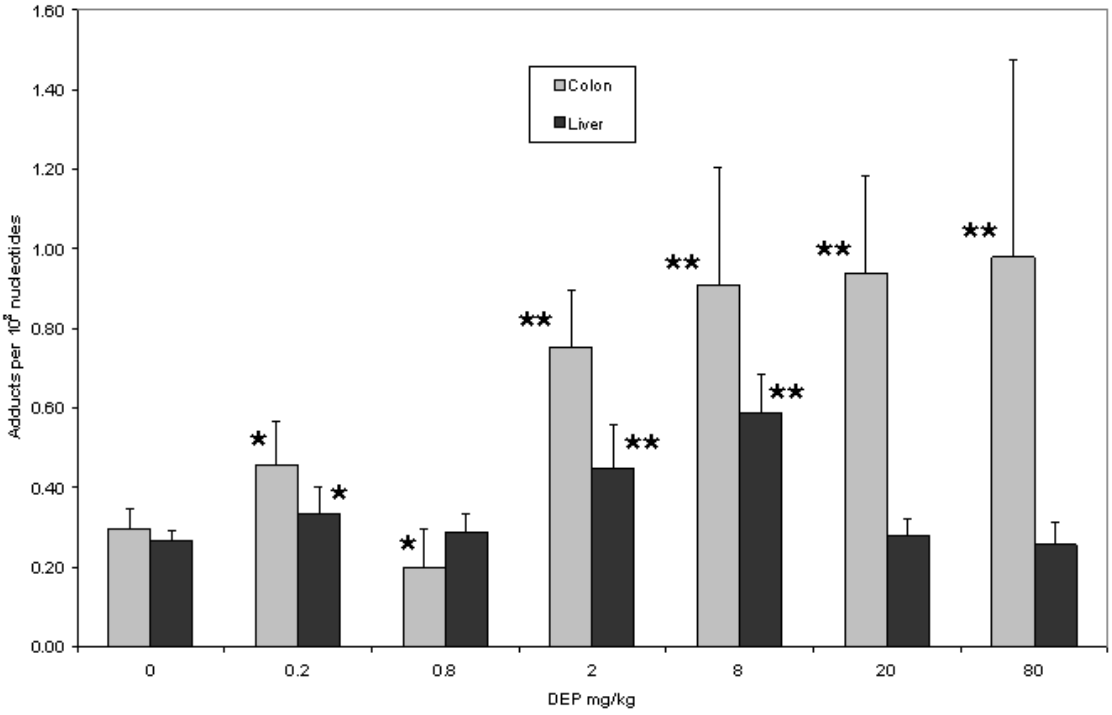

Fig. 3: *OGG1* mRNA expression