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Oxidative DNA damage and defence gene expression in the mouse lung after short-term exposure to diesel exhaust particles by inhalation

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Exposure to diesel exhaust particles (DEP) is suspected to contribute to lung cancer and cardiopulmonary diseases. In recent years generation of reactive oxygen species capable of inducing cellular oxidative stress has been in focus as one of the underlying mechanisms behind the genotoxic effects of particles. However, the role of the antioxidative defence system still needs to be clarified, especially in relation to low-dose DEP exposures. The aim of this study was to characterize the effects of short-term exposure to DEP in terms of DNA damage and expression of key response genes towards oxidative stress in lungs of mice. Mice were exposed by inhalation to 20 or 80 mg/m^3 DEP inhaled as either a single dose, or four lower doses (5 and 20 mg/m^3) inhaled on four consecutive days. Our results indicate that HO-1 mRNA expression in lung tissue was up-regulated after both types of DEP exposures, whereas OGG1 expression was only up-regulated after repeated exposures. The level of oxidative DNA damage in terms of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) was increased in the lung tissue after a single exposure, whereas increased levels of DNA strand breaks was observed in bronchoalveolar lavage cells after repeated DEP exposures. The levels of 8-oxodG and OGG1 mRNA in lung tissue were mirror images. This suggests that after repeated exposures, up-regulation of DNA repair counteracts an increased rate of 8-oxodG formation leaving the steady state level of 8-oxodG in DNA unchanged. In conclusion, this study indicates that a single high dose of DEP generates 8-oxodG in lung tissue, whereas the same dose inhaled as four low-exposures may up-regulate the antioxidative defence system and protect against generation of 8-oxodG.

Introduction

Diesel exhaust particles (DEP) are major pollutants in ambient air. Epidemiological studies have demonstrated associations between fine particles in ambient air and deaths from lung cancer and cardiopulmonary diseases (1). Moreover, there is compelling evidence that occupational exposure to DEP may cause lung cancer (2). In animal studies DEP have been demonstrated to be carcinogenic in the lung, although the mechanism involved is not well understood (3,4).

Diesel particles consist of carbonaceous cores with a large surface area to which hydrocarbons, including polycyclic aromatic hydrocarbons (PAH), aldehydes, quinones, metals and acids are absorbed (5,6). DEP induce genotoxic effects not only in terms of PAH–DNA adducts but also by oxidative DNA damage. The level of oxidative DNA damage has been correlated with mutagenicity and tumour formation in animal experiments (7,8). DEP can induce oxidative stress mediated by inflammation causing macrophages to release reactive oxygen species (ROS), by transition metals on the particle surface capable of generating ROS through the Fenton reaction or by quinones in the particles that produce ROS through redox cycling (9–11). Several studies have demonstrated that DEP induce production of ROS with (11,12) and without (13,14) the presence of biological activating systems. Moreover, cell culture experiments have shown increased generation of oxidative DNA damage in terms of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) after exposure to particulate matter (15,16). Because it is potently mutagenic and relatively easy to determine, 8-oxodG is probably the most studied oxidation product among several types of oxidative DNA damage (17). Studies have reported increased 8-oxodG levels in the lung tissue in animals exposed to DEP both by intratracheal installation and long-term inhalation (4,7,8,18–20). However, effects of short-term exposure by inhalation on DNA damage and potential adaptation to oxidative stress in terms of expression of key response genes have not been characterized.

Mammalian cells have evolved specific repair systems to remove the different types of damage generated after oxidative stress. Oxidized bases are generally repaired by base excision repair (BER) (21,22). The *OGG1* gene encodes the 8-oxoguanine-DNA glycosylase, which removes 8-oxodG and other oxidatively damaged bases from DNA as part of the BER pathway (23). *OGG1* knockout animals have been shown to accumulate 8-oxodG, in particular if subjected to oxidative stress (24–26). Thus, induction of OGG1 may be an important response of defence in relation to oxidative stress.

Haeme oxygenase-1 is responsible for the degradation of haeme to biliverdin and is a well-known stress response protein inducible by oxidative stress in the lung (27). *In vitro* studies have shown that HO-1 plays an important role in the cytoprotection against redox-active compounds that are absorbed on DEP, e.g. quinones (28). An important role of *HO-1* in the defence against oxidative stress is also indicated by *HO-1*(−/−) mice showing elevated sensitivity to endotoxin-induced mortality and organ damage associated with increased oxidative stress (29). Moreover, *HO-1* is necessary for the adaptation of lymphocytes to oxidative stress after hyperbaric oxygen exposure in human subjects *in vivo* (30).

The purpose of the present study was to characterize the time course and dose-response of oxidative DNA damage and the

Abbreviations: DEP, diesel exhaust particles; GLM, general linear model; PAH, polycyclic aromatic hydrocarbons; ROS, reactive oxygen species.

oxidative stress response in lung tissue and bronchoalveolar lavage cells after single and repeated 1.5 h exposures of DEP by inhalation in mice. Oxidative DNA damage was assessed as 8-oxodG in lung tissue, whereas DNA strand breaks were investigated in BAL cells. The gene expression of *OGG1* and *HO-1* was assessed at the mRNA level in lung tissue as markers for adaptation of the DNA repair activity and enzymatic oxidative stress defence, respectively.

Materials and methods

Design

The study was conducted in two parts, in which the first part was designed to investigate the effect of single bouts of DEP inhalation at doses of 20 and 80 mg/m^3. In the second part of the study the same doses were fractionated to four single exposures of DEP on four consecutive days i.e. 5 and 20 mg/m^3, respectively. In the investigation of single exposures of DEP the time points were chosen to reflect both early and late effects of DEP (1, 3 and 22 h after exposure). As a result of experimental procedures the first sampling of tissue was practical 1 h after termination of the exposure. Based on the results from the initial single exposure experiments, animals were killed only at 1 and 22 h after exposure in the second part of the study.

Reagents

The reagents were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise stated.

Animals

Female 8-week-old (~20 g body wt) BALB/CJ mice were purchased from M & B A/S, Denmark. The animals were allowed to acclimatize for minimally 7 days in polypropylene cages with sawdust bedding (Lignolcel S8, Brogaarden, Denmark), and were given a standard diet (Altromin Standard Diet no. 1324, Brogaarden, Denmark) and tap water *ad libitum*. The mice were housed in groups of 10 animals in an environmentally controlled animal facility operating at 18–22°C, 40–60% humidity and a 12 h light/dark cycle. Institutional guidelines for animal welfare were followed and the Danish government's Danish Ethical Committee for Animal Studies approved the animal experiments.

Inhalation of DEP

Mice were exposed to a NIST standard reference material 1650 of DEP (obtained from the National Bureau of Standards, Gaithersburg, MD, USA) by head-only inhalation in a glass/stainless steel chamber with a volume of 18 l. A microfeeder aerosoled constant flow of DEP into a nozzle that atomised the DEP into fine aerosol in a flow of 25 l/min. The concentration of particles was monitored by several 8 min samplings of air samples on Teflon filters showing actual dose to be within 20% of target. The number of particles was measured using a TSI model 3022A Condensation Particle Counter. The distribution of particle size was determined using a Differential Mobility Analyser in connection with a TSI 3010 Condensation Particle Counter. Plethysmograph control of impact on respiratory function was performed as described elsewhere (31). No signs of sensory or pulmonary irritation or bronchoconstriction were found. The exposure was either by single exposure of 20 or 80 mg/m^3 DEP corresponding to 5.9×10^5 ($\pm$SD 0.9×10^5) or 54.3×10^5 ($\pm$SD 23.6×10^5) particles/cm^3, respectively; or by repeated exposures on four consecutive days to 5 or 20 mg/m^3 DEP, corresponding to 1.8×10^5 ($\pm$SD 1.09×10^5) or 12.1×10^5 ($\pm$SD 5.9×10^5) particles/cm^3, respectively. Control mice were exposed to filtered clean air. The mean number of particles for the control groups was lower than 1000 particles/cm^3. The mice were placed in the exposure chamber for 1.5 h for each bout of exposure.

Preparation of cells and tissue

After exposure the mice were allowed to recover 1, 3 or 22 h before they were killed. The mice were anaesthetized by i.m. injection of a mixture of 1.5 mg/kg Hypnorm (Janssen, Titusville, NJ) and 1.5 mg/kg Dormico (Roche, Basel, Switzerland). Immediately after withdrawing the heart blood, a bronchoalveolar lavage (BAL) was performed three times with 1 ml 0.9% sterile saline through the trachea. The BAL was immediately put on ice until isolation of the cells (max 90 min after the lavage) at 1250 r.p.m. at 4°C for 10 min. The cells were re-suspended in 100 µl HAMF12 medium with 10% fetal bovine serum. One half of the suspension was stored at −80°C for later analysis in the comet assay. The lungs were obtained and snap frozen in liquid nitrogen and stored at −80°C.

Detection of 8-oxo-deoxyguanosine

The level of 8-oxodG relative to dG was measured in the right lung tissue by HPLC with electrochemical detection after isolation and digestion of nuclear DNA as described (32). We have participated in the European Standard Committee in Oxidative DNA Damage (ESCODD), and in inter-laboratory tests our assay has demonstrated low background levels of 8-oxodG and dose-responsiveness at low level of damage (33).

The comet assay

The isolation and detection of DNA strand breaks in lung cells were carried out as described previously (34). Briefly, freshly prepared BAL cells were mixed with 1% low melting point agarose (Invitrogen, Taastrup, Denmark) and were applied onto a Gelbond slide. The embedded cells were lysed in lysis buffer (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris, 1% Triton X-100, pH 10) overnight. After lysis the nuclei were immersed into an alkaline electrophoresis solution (0.3 M NaOH, 1 mM Na_2EDTA, pH >13) for 40 min, and electrophoresed in the solution at 25 V and 300 mA for 20 min. The nuclei were washed twice in 0.4 m Tris buffer (pH 7.5), and stained with 0.6 mM TOTO^{TM}-1 dye (Molecular Probes, Eugene, OR). The level of DNA damage was analysed in 50 randomly selected nuclei as the tail length by the Kinetics version 3.1 software.

Measurements of mRNA expression

Total RNA was purified from lung tissue using Qiagen total RNA purification kit and DNase treated as recommended by the manufacturer (Qiagen, Hilden, Germany). Subsequent quality control showed that all genomic contaminations were removed by the DNAse treatment. 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 UK, Nærum, DK.

For quantification of the mRNA levels, TaqMan probes were purchased from Applied Biosystems UK, Nærum, DK and primers were from TAG Copenhagen, Denmark. Probes were designed to span the intron region between two exons and were thus cDNA specific. For the 18S rRNA analysis, we used a commercially available probe and primer solution (TaqMan® Ribosomal RNA control reagents: VIC^{TM} Probe, Applied Biosystems, Foster City, CA).

For *OGG1* the following oligonucleotides were used: forward primer: 752F: 5′-tgg ctt ccc aaa cct cca t-3′, reverse primer: 816R: 5′-ggc cca act tcc tca ggt g-3′, TaqMan probe: 772T 5′-FAM-ccc tgg ctg gtc cag aag cag aga c-TAMRA-3′.

HO-1: forward primer: 392F: 5′-cct gga gca gga cat ggc-3′, reverse primer: 454R: 5′-ggc gtg caa ggg atg att-3′, TaqMan probe 411T: 5′-FAM-ttc tgg tat ggg cct cac tgg cag g-TAMRA-3′.

OGG1, HO-1 and *18S* RNA levels were quantified in separate tubes. The final concentrations of probes and primers were between 100 and 200 µM.

The PCR reactions were performed in triplicate in the LightCycler^{TM} system in 15 µl reactions. For the LightCycler reaction 0.5 µl of the cDNA preparation was mixed with $MgCl_2$ (5 mM final concentration) and 11 µl LightCycler master mix (FastStart DNA Hybridisation Probes, Roche 137 Molecular Biochemicals, Mannheim, Germany). Water was added to a final volume of 100 µl. Aliquots of 47 µl were mixed with primers and probe and then transferred to three LightCycler glass capillaries (15 µl in each). The other aliquot of 47 µl was mixed with 3 µl of the ribosomal RNA probe and primer solution and likewise split into three glass capillaries. For the PCR reaction the following protocol was used: activation of TAQ polymerase (95°C for 10 min), 45 cycles of 95°C for 0 s, 60°C for 30 s followed by single fluorescence measurement and cooling 40°C for 3 min. For each animal the individual level of initial target cDNA was expressed as the difference in Ct values between the average of the triplicate *OGG1*/*HO-1* and the average of the triplicate of *18S* in the parallel samples. The relative amount of target mRNA normalized to *18S* mRNA was calculated as 2^{-dCt}.

The average standard deviation on triplicates was <15%. The standard deviation of repeated measurements of the same sample (the control) in separate experiments was <20%. The probes and primers had been validated, and the PCR was shown to be quantitative over a range of 100-fold dilution.

Statistics

All data were tested for normal distribution using the Shapiro–Wilks test. The groups were also tested for homogeneity of variance with Levene's test ($P > 0.05$). To fulfil the criteria for normality and homogeneity of variance some variables were logarithmically transformed using the base of 10 (single exposure: HO-1; repeated exposures: 8-oxodG, OGG1 and HO-1). The effects of biomarkers were tested for interactions by general linear model (GLM) analysis, using 5% as significance level. If the test for interaction was not significant, the effects of single factors (time and dose) were tested at 5% significance level. For biomarkers being statistically significant, interactions or

single-factor effects were analysed by post hoc GLM analysis as the least significant difference. The statistical analysis was performed in Statistica 5.5 for Windows, StatSoft (1997), Tulsa, OK.

Results

Single exposure of DEP

For single exposures to DEP, samples were obtained after 1, 3 and 22 h after the 1.5 h DEP exposure. The time points were chosen to reflect both induction of oxidative DNA damage and the expression of genes involved in the antioxidant defence system. After a single exposure to 20 and 80 mg/m^3 DEP, the level of 8-oxodG levels increased dose-dependently in the lung tissue ($P < 0.01$) (Figure 1a). The mice that inhaled the highest dose of DEP (80 mg/m^3) had higher 8-oxodG levels than both control mice and mice exposed to 20 mg/m^3 of DEP ($P < 0.01$ and $P < 0.05$, respectively). The number of SB in BAL cells was unaffected by the single 1.5 h exposure of DEP (Figure 1b).

Inhalation of DEP increased the *HO-1* gene expression ($P < 0.001$, single factor effect of dose, GLM). The expression of *HO-1* was significantly higher than control level after both 20 ($P < 0.01$) and 80 mg/m^3 ($P < 0.001$) of DEP inhalation, whereas there was no difference between the doses (Figure 1c). The level of OGG1 was unchanged by single exposures of DEP (Figure 1d).

Repeated exposure to DEP

Based on the results generated in the first part of the study, we omitted the sampling 3 h after the last exposure, i.e. samples were collected 1 h and 22 h after exposure.

The level of 8-oxodG in lung tissue was unchanged after the exposures on four consecutive days (Figure 2a). DEP significantly increased the SB level in BAL cells by dose both 1 and 22 h after exposure ($P < 0.001$) (Figure 2b). The SB level was significantly higher than control level after both 20 ($P < 0.001$) and 80 mg/m^3 ($P < 0.001$) of DEP inhalation, whereas there was no difference between the doses.

GLM analysis revealed interactions of time and dose on the mRNA levels of *HO-1* ($P < 0.001$) and *OGG1* ($P < 0.01$). The interactions indicate that there was a rapid induction of mRNA 1 h after the last exposure to 20 mg/m^3 of DEP (Figure 2c and d); *HO-1* expression increased 2.6-fold compared with the control mean level and expression of *OGG1* was increased 3.2-fold. Twenty-two hours after exposure the mRNA level had returned to the control level. Exposure to 5 mg/m^3 had no effect on the mRNA expression levels of *HO-1* or *OGG1*.

Correlations

It can be inferred from Figure 1a and d and Figure 2a and d that there may be an inverse relationship between mRNA expression of *OGG1* and the level of 8-oxodG. A scatter plot of *OGG1* mRNA expression and 8-oxodG (Figure 3), also indicates that an inverse relationship across all control and exposure groups (linear regression results: $r^2 = 0.14$, $P < 0.01$). When 8-oxodG was analysed with *OGG1* mRNA levels, as a continuous predictor variable there was an interaction of time and dose after single DEP exposure ($P < 0.05$) Thus, increased OGG1 mRNA levels were associated with lower 8-oxodG levels and vice versa. There was no correlation between SB levels in BAL cells and mRNA levels of either HO-1 or OGG1.

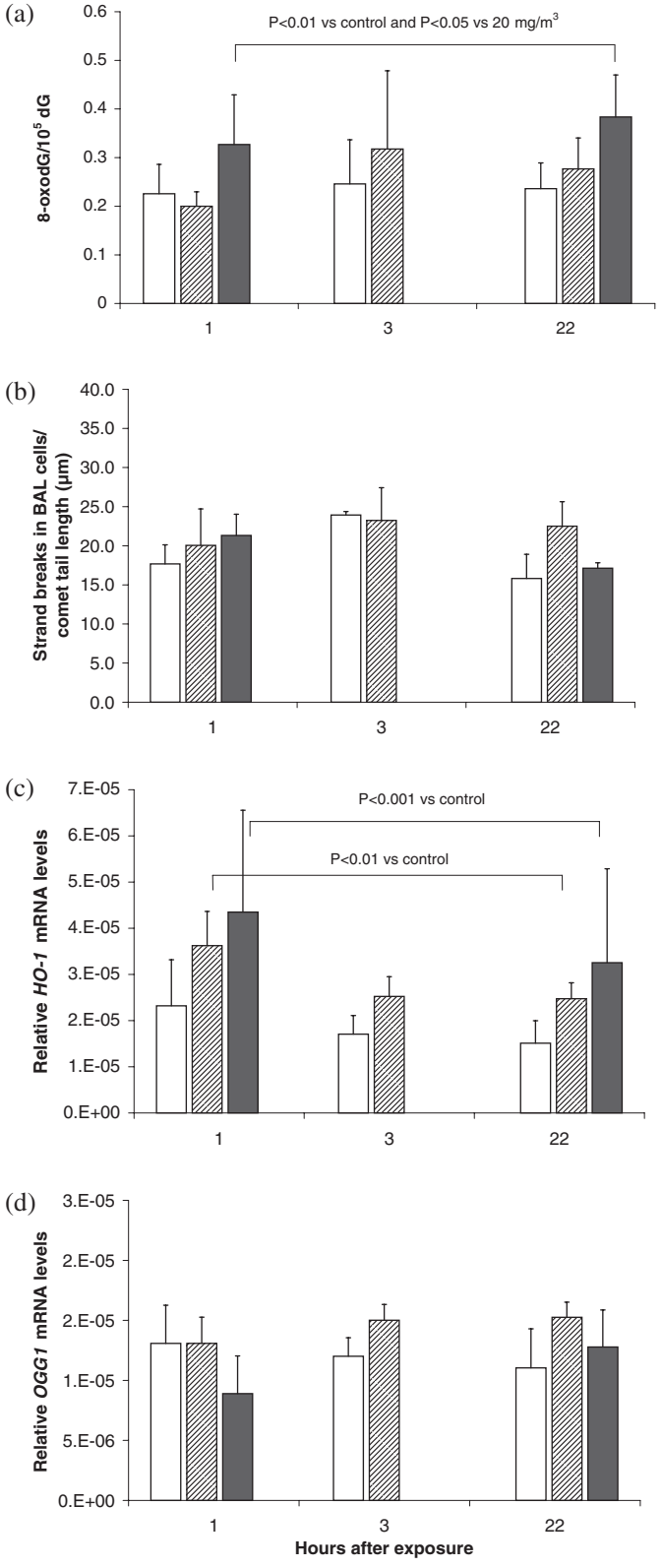

Fig. 1. DNA damage and defence gene expression of single exposure to DEP by inhalation in mice. 8-OxodG (**a**), strand breaks (**b**) and relative mRNA levels of *HO-1* (**c**) and *OGG1* (**d**). Open bars are control, shaded bars 20 mg/m^3 of DEP and black bars 80 mg/m^3 of DEP. Each bar represents means + SD ($n = 3$–7). Strand breaks are measured by comet tail length of BAL cells. mRNA levels were quantified by real-time RT–PCR normalized to the 18S mRNA level. There were single-factor effects of dose on the level of 8-oxodG ($P < 0.01$, GLM) and for *HO-1* expression ($P < 0.01$, GLM). The results of post hoc analysis of the statistically significant GLM analysis are indicated in the figure.

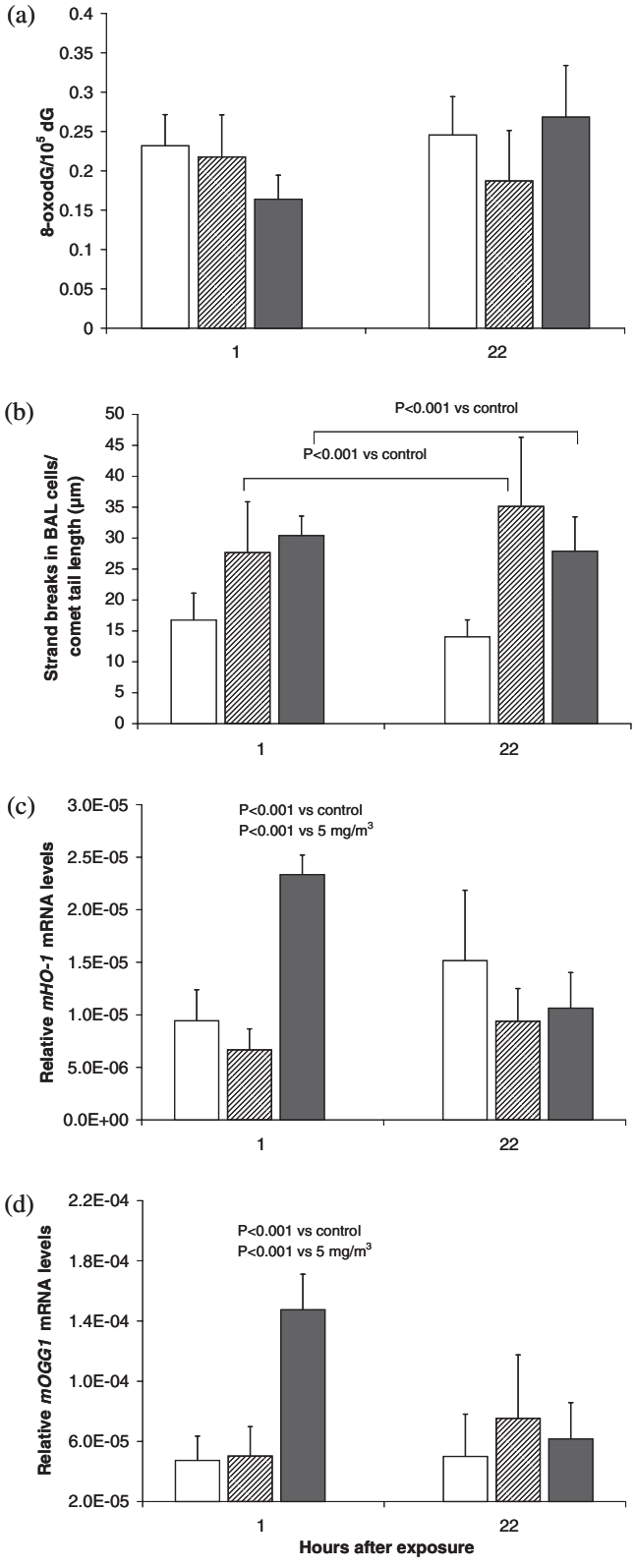

Fig. 2. DNA damage and defence gene expression of fractionated exposure to DEP by inhalation in mice. 8-OxodG (**a**), strand breaks (**b**) and relative mRNA levels of *HO-1* (**c**) and *OGG1* (**d**). Open bars are control, shaded bars 5 mg/m^3 of DEP, and black bars 20 mg/m^3 of DEP. Each bar represents means + SD ($n = 5$). Strand breaks are measured by comet tail length of BAL cells. mRNA levels were quantified by real-time RT–PCR normalized to the 18S mRNA level. There was a single factor effect of dose on the level of strand breaks in BAL cells ($P < 0.001$, GLM), and interactions between time and dose for both *HO-1* ($P < 0.001$, GLM) and *OGG1* ($P < 0.01$, GLM) expression. The results of post hoc analysis of the statistically significant GLM analysis are indicated in the figure.

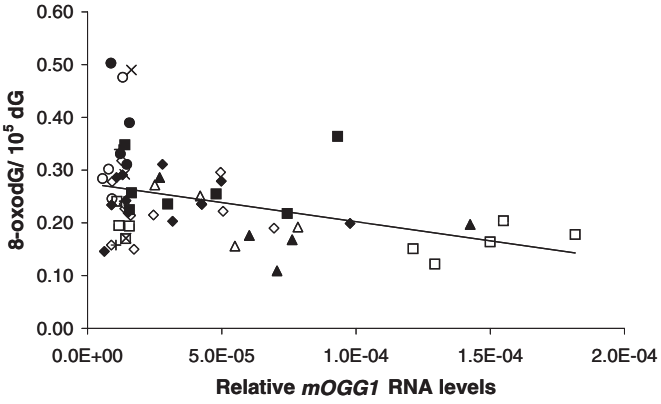

Fig. 3. Correlation between 8-oxodG and *OGG1* mRNA levels after single and repeated exposure to DEP by inhalation in mice. 8-oxodG per 10^5 dG as a function of *OGG1* mRNA levels normalized to 18S. The linear regression result: $r^2 = 0.14$, $P < 0.01$ ($n = 62$) indicates an inverse relationship. Time points 1 h after exposure: (◇) control; (△) 5; (□) 20 and (○) 80 mg/m^3 of DEP; time points 3 h after exposure: (+) control and (×) 20 mg/m^3 of DEP; and time points 22 h after exposure: (◆) control; (▲) 5; (■) 20 and (●) 80 mg/m^3 of DEP.

Discussion

In the present study we found that short-term exposure to DEP by inhalation induced the expression of HO-1 mRNA, indicating an adaptive oxidative stress response. DEP also induced oxidative DNA damage in terms of 8-oxodG in the lung tissue after a single exposure, whereas oxidative DNA damage was not observed after repeated exposures. The unchanged levels of oxidative DNA damage after repeated exposures of DEP might be due to an increased rate of repair of 8-oxodG mediated by elevated gene expression of the *OGG1* repair gene. Nevertheless, in the mainly macrophage population of BAL cells, we observed higher level of SB after repeated DEP exposures.

The most novel finding in this study is the time and dose dependency of the adaptive response of the antioxidant defence system to exposure to DEP by inhalation. The single dose inhalation experiment is in agreement with previous studies showing that DEP exposure either as instillation or inhalation induced oxidative DNA damage in both the lung tissue and in the cells lining the airways (7,19,20). However, it is interesting that at the same cumulative dose, delivered as repeated lower dose, inhalations markedly increased the antioxidant response system, and this probably is the main reason that 8-oxodG levels in the lung were unaltered by repeated inhalations. In fact the mRNA expression and 8-oxodG levels in lung tissue were almost perfectly mirror-imaged. These observations are compatible with the results from single intratracheal instillations of DEP that showed increased 8-oxodG levels in the lungs concomitantly with decreased OGG1 repair activity in the first 2 days after instillation, whereas OGG1 mRNA levels were increased days 5 and 7 after the instillation (19). These results do not unravel if the increased expression of *OGG1* after DEP exposure is in response to a sudden toxic insult or because of a sustained oxidative stress or both. We also found a decreased mRNA level shortly after the single high dose of DEP and a negative correlation of 8-oxodG and the OGG1 mRNA levels. Such a relationship has been suggested previously by the finding that oxidative stress-induced reduction in 8-oxodG levels, was linked to an increase in 8-oxodG repair activity and by increased OGG1 expression in A549 cells *in vitro* (35). Recently, cloning and analysis of the

promoter region of hOGG1 showed presence of an antioxidant response element (36), which indicates that OGG1 may be regulated as a consequence of oxidative stress. Furthermore, we have found previously the mRNA expression of OGG1 to be induced in a time dependent manner with maximum 6 h after X-ray irradiation in mice (37).

There was clear induction of HO-1 determined as expression of mRNA after both single and repeated exposures by inhalation. HO-1 has been shown to be inducible in the response to oxidative stress in cell culture systems (38) and induction of the protein is likely to be mediated by the antioxidant response element pathway as shown in macrophages treated with DEP extract (28). This suggests that oxidative stress is involved in the transcription activation of this gene and that *HO-1* participates in the cellular protection against DEP exposure in the lungs. Recently, cDNA array analysis was used to investigate gene expression in alveolar macrophages after DEP extract exposure *in vitro* (39). That study revealed increased antioxidant enzyme expression with a 6-fold increase in *HO-1* mRNA levels. Early induction of *HO-1* gene translation after oxidative stress was reported with 4-fold increase in HO-1 mRNA levels 4 h after X-ray irradiation in rat liver (40). It was also shown that whole body X-ray irradiation of rats increased *HO-1* mRNA levels after oxidative stress and this was reflected in the translation to protein (40). We have found previously increased HO-1 mRNA levels in lung tissue (as early as 3 h) after partial X-ray irradiation of mice (37).

The time and dose responses of *HO-1* and *OGG1* expression suggest slight differences in the regulation of which *OGG1* may react slower and may even be suppressed shortly after a single exposure to DEP. However, after exposure for four consecutive days the patterns of mRNA expression was similar for *OGG1* and *HO-1* with early high expression followed by a return to control levels after 22 h.

Here we present a system for relatively high dose and short-term exposure to particles by nose-only inhalation in mice as an alternative to instillation. The DEP dose of 20 mg/m^3 (assuming 20 g mice inhale 40 l/day) approximately equals 3 weeks of exposure at the highest human range of occupational situations, 1280 μg/m^3 (2) (assuming humans inhale 8 m^3 during work day and equal particle deposition between man and mouse). For ambient air this would correspond to >11 years of exposure to urban air with average concentrations of 2.7 μg/m^3 (2).

In summary, this study shows that DEP induce oxidative stress, and suggest that repeated exposures of DEP increase the antioxidant defence mechanisms that may result in lower levels of 8-oxodG. This underscores the importance of assessment of repair activity in the interpretation of 8-oxodG levels after long-term DEP exposure seen in, e.g. occupational or environmental settings. The data indicate that the lungs of mice adapt to DEP induced oxidative stress. Besides the two genes analysed here it is plausible that the lungs respond with up-regulating several other genes in response to oxidative stress. The concept of adaptation is well established in radiation responses and it is possible that the mechanisms are similar in exposure to other inducers of oxidative stress.

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