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Research Article

DNA Damage Following Pulmonary Exposure by Instillation to Low Doses of Carbon Black (Printex 90) Nanoparticles in Mice

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We previously observed genotoxic effects of carbon black nanoparticles at low doses relative to the Danish Occupational Exposure Limit (3.5 mg/m³). Furthermore, DNA damage occurred in broncho-alveolar lavage (BAL) cells in the absence of inflammation, indicating that inflammation is not required for the genotoxic effects of carbon black. In this study, we investigated inflammatory and acute phase response in addition to genotoxic effects occurring following exposure to nanoparticulate carbon black (NPCB) at even lower doses. C57BL/6JBomTac mice were examined 1, 3, and 28 days after a single instillation of 0.67, 2, 6, and 162 µg Printex 90 NPCB and vehicle. Cellular composition and protein concentration was evaluated in BAL fluid as markers of inflammatory response and cell damage. DNA strand breaks in BAL

cells, lung, and liver tissue were assessed using the alkaline comet assay. The pulmonary acute phase response was analyzed by *Saa3* mRNA real-time quantitative PCR. Instillation of the low doses of NPCB induced a slight neutrophil influx one day after exposure. Pulmonary exposure to small doses of NPCB caused an increase in DNA strand breaks in BAL cells and lung tissue measured using the comet assay. We interpret the increased DNA strand breaks occurring following these low exposure doses of NPCB as DNA damage caused by primary genotoxicity in the absence of substantial inflammation, cell damage, and acute phase response. *Environ. Mol. Mutagen.* 56:41–49, 2015. © 2014 The Authors. Environmental and Molecular Mutagenesis published by Wiley Periodicals, Inc. on behalf of Environmental Mutagen Society

Key words: nanoparticles; lung inflammation; genotoxicity; comet assay

INTRODUCTION

Carbon black is a high volume industrial chemical used in numerous industrial applications including the production of rubber, tires, paints, toners, and printing inks, with an annual production of 10 million tons [International Agency for Research on Cancer, 2012]. There is a risk of occupational exposure through inhalation during handling of dry powders.

The carbon black Printex 90 has a primary particle size of 14 nm and a correspondingly large surface area per mass unit [Saber et al., 2005]. It is composed almost purely of carbon with very little impurities like PAH and endotoxins [Jacobsen et al., 2007; Jackson et al., 2011b]. Carbon black particles have been classified as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer [Baan et al., 2006; International Agency for Research on Cancer, 2012]. The current Danish

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occupational exposures limit is 3.5 mg/m³ carbon black for an 8 hour working day [The Danish Working Environment Authority, 2007] for all particle sizes. Inhalation (1 hour inhalation of 42 mg/m³ aerosolized Printex 90 for 11 consecutive days; sampled 5 days postexposure [Jackson et al., 2012a]) and pulmonary deposition (from 18 µg to 162 µg/mouse; 1, 3, and 28 days post-exposure [Jacobsen et al., 2009; Bourdon et al., 2012]) of Printex 90 causes pulmonary inflammation in terms of increased neutrophil influx [Jacobsen et al., 2009; Bourdon et al., 2012; Jackson et al., 2012a]. In addition, both inhalation and pulmonary deposition of Printex 90 particles induce DNA strand breaks in lung [Saber et al., 2006; Jacobsen et al., 2009; Bourdon et al., 2012; Jackson et al., 2012a] and liver tissue of exposed mice and rats [Totsuka et al., 2009; Bourdon et al., 2012; Jackson et al., 2012a; Saber et al., 2012]. In general, pulmonary deposition by intratracheal instillation generates a stronger inflammatory response than pulmonary deposition by inhalation of the same dose [Jacobsen et al., 2009; Jackson et al., 2012a], as was also recently reported for TiO₂ [Baisch et al., 2014]. However, induction of DNA strand breaks seems to be independent of the inflammatory response, since DNA strand breaks are observed in lung tissue in the absence of neutrophil influx following inhalation exposure to Printex 90 [Saber et al., 2005].

Printex 90 is an efficient generator of reactive oxygen species (ROS) in both cellular and acellular assays [Jacobsen et al., 2008]. Furthermore, Printex 90 is mutagenic in vitro [Jacobsen et al., 2007] and the mutation spectrum is consistent with the genetic fingerprint of ROS [Jacobsen et al., 2010]. Thus, there is ample evidence that Printex 90 induces DNA damage with ROS generation as the likely mediator. However, the dose-response relationship and no observable effect level have not been established in lung. Indeed, a dose corresponding to pulmonary deposition over the course of one 8-hr working day at the Danish Occupational Exposure limit induced DNA strand breaks in lungs of mice [Bourdon et al., 2012]. The aim of the present study was to investigate whether exposure to even lower doses of Printex 90 induces DNA damage in lung tissue in mice. The test doses ranged from 0.67 to 6 µg, corresponding to 20 min to ~3 hr exposure of a mouse to NPCB at the Danish Occupational Exposure limit.

MATERIAL AND METHODS

Animals

Female mice C57BL/6J BomTac aged 6–7 weeks were obtained from Taconic Europe (Ejby, Denmark). The mice were allocated randomly to the experimental groups and were acclimatized for 1–2 weeks before the start of experiment. All mice were housed in polypropylene cages with bedding (sawdust) and enrichment at controlled environmental conditions; temperature (21 ± 1°C), humidity (50 ± 10%), and 12 hr light/dark period. The mice had access to food (Altromin 1324) and tap water ad libitum. The animals were assigned to intratracheal (i.t.) instillation at 8 weeks of age. The average weight on the day of instillation was 19.1 ± 1.1 g.

All procedures complied with the EC Directive 86/609/EEC and Danish law regulating experiments with animals (The Danish Ministry of Justice, Animal Experiments Inspectorate, permission 2006/561–1123).

Study Design

Mice were intratracheally instilled with a single dose of 0.67, 2, or 6 µg/animal, one reference high dose of NPCB 162 µg/animal or vehicle only. Mice were killed after 1, 3, or 28 days. The chosen doses correspond to pulmonary deposition doses in mice at the current Danish occupational exposure limit (3.5 mg/m³) for 20 min, 1hr and 3 hr, respectively, and the reference dose corresponds to 9 working days at the Danish occupational exposure limit [Bourdon et al., 2012]. The reference dose was chosen based on previous studies that showed strong inflammatory and genotoxic responses following instillation of this dose of NPCB and because we routinely include NPCB as a benchmark exposure [Saber et al., 2011a, b, 2012]. NPCB groups consisted of 8 mice for each dose and time point; vehicle control groups included 12 mice for each time point. The organs and broncho-alveolar lavage (BAL) were collected 1, 3, and 28 days post-exposure. A total of 132 mice were used in this study.

Particle Characterization and Preparation of Exposure Stock

The NPCB (Printex 90) was a gift from Evonik (Frankfurt, Germany). Particle preparation, characterization, and instillation procedures were described in detail previously [Jacobsen et al., 2007; Saber et al., 2009; Bourdon et al., 2012; Boisen et al., 2013]. NPCB has a primary particle size of 14nm and the particles were composed of 99% C, 0.8% N, and 0.01% H₂ [Jacobsen et al., 2007]. The total content of polycyclic aromatic hydrocarbons in Printex 90 was 0.0742 mg/g, that is, about 3000-fold less than diesel exhaust particles [Jacobsen et al., 2007]. The endotoxin level was 0.142 EU/mg Printex 90 [Jackson et al., 2011b]. The specific surface area was 295–338 m²/g, corresponding to a theoretical average spherical particle size 8.1–9.5 nm [Saber et al., 2005]. NPCB particles were suspended in 0.2 µm filtered, γ-irradiated Nanopure Diamond UV water (Pyrogens: < 0.001 EU/ml, total organic carbon: < 3.0 ppb [Jackson et al., 2012a, b]), and sonicated on ice, for 16 min without pause using a Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT) equipped with a disruptor horn (model number 101–147-037). Two suspensions were prepared at concentrations 0.12 mg/ml (6 µg/instillation) and 3.24 mg/ml (162 µg/instillation). A suspension with a final concentration of 0.12 mg/ml was subsequently diluted 1:3 to obtain 0.04 mg/ml (2 µg/instillation) and diluted further 1:3 for the lowest dose of 0.00134 mg/ml (0.67 µg/instillation). After each dilution, suspensions were sonicated for 2 minutes. Particle suspensions were instilled in mice directly after sonication up to 1 hr to assure their homogeneity. Vehicle solution was prepared by sonication of Nanopure Diamond water in the same conditions as described above.

Dynamic Light Scattering (DLS)

The hydrodynamic size distribution of NPCB suspensions was determined by DLS using Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Data were analyzed on Dispersion Technology Software v5.0 (Malvern Instruments). The size distribution was determined directly on the instillation dispersions at 25°C in 1 ml disposable polystyrene cuvettes. All data were obtained based on six consecutively repeated analyses of the same sample with no pause. For calculation of hydrodynamic size, we used the refractive (R_1) and absorption indices (R_2) of 2.020 and 2.000, respectively, for Printex 90 and standard optical and viscosity properties for H₂O.

Particle Exposure–Intratracheal Instillation

Eight 8 weeks old female mice were instilled intratracheally once as described in detail previously [Jackson et al., 2011a]. Briefly, mice were

anesthetized with 4% isofluran and instilled through the trachea with vehicle or vehicle containing NPCB (50 μ l solution followed by 200 μ l air). The total instilled doses were 0.67, 2, 6, and 162 μ g/animal. Animals instilled with 162 μ g NPCB were considered to be references of inflammatory response and pulmonary genotoxicity. After instillation, the mice were weighed and transferred to the animal unit until termination.

BAL Preparation and Isolation of Organs

Upon termination, the mice were anesthetized by subcutaneous injection of Hypnorm/Dormicum mixture diluted in sterile water 1:2 (Hypnorm: fentanyl citrate 0.75 mg/kg and fluanisone 23.8 mg/kg, VetaPharma Leeds, UK; Dormicum: midazolam 11.9 mg/kg, Roche a/s, Hvidovre, Denmark). Heart blood was withdrawn. Lungs were flushed twice with sterile 0.9% NaCl through the trachea to obtain BAL fluid, the used volume was calculated as 1 ml 0.9% NaCl/25g mouse weight and varied from 0.7 to 0.9 ml. BAL fluid was stored on ice until centrifugation at 400g for 10 min at 4°C. The BAL cells were re-suspended in 100 μ l medium (HAM F-12 with 1% penicillin/streptomycin and 10% fetal bovine serum). Acellular BAL fluid was recovered and stored at -80°C. The total number of living and dead cells in BAL was determined by NucleoCounter NC-200TM (Chemometec, Denmark) from diluted cell suspensions following manufacturer's protocol. The total cell counts were calculated for each mouse.

Samples for COMET assay were prepared from 40 μ l re-suspended BAL cells and 60 μ l freezing media (HAM F-12, 1% penicillin/streptomycin, 15% fetal bovine serum and 10% DMSO). Samples were divided into two aliquots and immediately frozen at -80°C. The rest of the cell re-suspension (40 μ l) was used to estimate the number of granulocytes (neutrophil and eosinophil), macrophages, lymphocytes, and epithelial cells in BAL fluid. The cell suspension was centrifuged at 55g for 4 min in Cytofuge 2 (StatSpin, TRIOLAB, Brøndby, Denmark) and fixed for 5 min in 96% ethanol. All slides were stained with May-Günwald-Giemsa stain, randomized, and blinded before counting 200 cells/sample under light microscope with 100 \times magnification (using immersion oil). The numbers of counted cells were expressed as % observations based on cell distribution of the 200 counted cells multiplied with the total number of cells for each mouse. Lung and liver tissue were divided into 4 parts, snap frozen in liquid nitrogen, and stored at -80°C.

Comet Assay—Preparation and Analyses

DNA strand breaks were determined on frozen BAL cells suspension, lung (3 \times 3 mm of left lobe) and liver tissue (2 \times 2 mm piece of median lobe). Organ samples were snap frozen directly after dissection and kept at -80°C until analysis. Sample preparation and analysis was previously described in detail [Jackson et al., 2013]. Briefly, BAL cells, lung, or liver cell suspensions were embedded in agarose (0.7% final concentration) on TREVIGEN 20-Well CometSlides™. Slides were quickly immersed into lysing solution at 4°C and stored overnight. The next day, samples were alkaline treated and subjected to alkaline electrophoresis (pH >13) in ice cold circulating electrophoresis solution. Samples were neutralized, fixed, and later stained by SYBRGreen®. Comets were scored by the fully automated PathFinder™ system (IMSTAR, France). DNA strand breaks were quantified as % DNA in the comet tail (%TDNA) and the comet tail length (TL). The day-to-day variation and electrophoresis efficiency was validated by including PBS exposed and 60 μ M H₂O₂-exposed A549 cells as negative and positive controls, respectively, on each slide. Control cells were exposed for 30 min at 4°C as described [Jackson et al., 2013]. The day to day variation including all slides ($n = 21$) from this experiment was 28%.

Total Protein Concentration in BALF/Mice Lung Epithelial Permeability

Total protein content in BAL fluid was measured by Pierce® BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's

protocol. Briefly, protein concentrations of all unknown acellular BAL fluid samples were compared to a standard curve of known albumin concentrations. Samples were prepared in duplicate and were incubated for 30 min in 37°C. Absorbance was measured at 550 nm on 1420 Multilabel counter VICTOR WALLAC2006™ (Turku, Finland).

Saa3 mRNA Expression Analysis

RNA from the right lung (16–20 mg) of each mice was isolated on Maxwell® 16 (Promega) using Maxwell® 16 LEV simply RNA Tissue Kit (AS1280, Promega) according to the manufacturer's protocol. RNA was eluted by 50 μ l nuclease free (DEPC) water. cDNA was prepared from DNase treated RNA using Taq-Man® reverse transcription reagents (Applied Biosystems) as described by manufacturer's protocol. Total RNA and cDNA concentration was measured on NanoDrop 2000c (Thermo-Fisher). The *Saa3* gene expression was determined using real-time RT-PCR with 18S RNA as reference gene as previously described [Saber et al., 2009]. In brief, each sample was run in triplicate on the ViiA7 sequence detector (Applied Biosystems). The sequence of the *Saa3* primers and probe were *Saa3*forward: 5' GCC TGG GCT GCT AAA GTC AT 3', *Saa3*reverse: 5' TGC TCC ATG TCC CGT GAA C 3', and *Saa3*probe: 5' FAM-TCT GAA CAG CCT CTC TGG CAT CGC T-TAMRA 3'. In all assays, TaqMan predeveloped mastermix (Applied Biosystems) was used. Target and 18S RNA levels were quantified in triplicate in separate wells. The relative expression of the target gene was calculated by the comparative method 2^{- Δ Ct} [Livak and Schmittgen, 2001]. mRNA measurements were excluded if the 18S content fell outside the range in which the PCR was found to be quantitative as defined by the validation experiments. Negative controls, where RNA had not been converted to cDNA, were included in each run. The day-to-day variation was 1.6% for the plate control.

Statistical Analyses

The data were analyzed by nonparametric statistics. The data were ranked and assessed by two-way ANOVA with post-hoc Tukey-type multiple comparison test for effects showing statistical significance in the overall ANOVA test (dose of NPCB and post-exposure time were used as categorical variables). For the analyses, where the interaction was significant, one-way ANOVA was performed for each of the post-exposure time-points with post-hoc Tukey-type multiple comparison test for effects showing statistical significance in the ANOVA test. Statistical significance was tested at the $P < 0.05$ level. The statistical analyses were performed in SAS version 9.2 (SAS Institute, Cary, NC).

RESULTS

Particle Characterization

NPCB were dispersed in Nanopure water as previously described [Jackson et al., 2012a, b]. Size distribution was measured by DLS (Fig. 1). The Z-average particle size distribution was similar for 13.4 μ g/ml (corresponds to 0.67 μ g/instillation) and 40 μ g/ml (corresponds to 2 μ g/instillation) particle suspensions; 674.2 and 740.6 nm with a polydispersity index of 0.22 and 0.18, respectively. The hydrodynamic number size-distribution had a mode at 531 nm for 13.4 μ g/ml and 615 nm for 40 μ g/ml. The Z-average particle size of the 120 μ g/ml suspension (corresponding to 6 μ g/instillation) NPCB was 131.7 nm (polydispersity index 0.14) and the hydrodynamic number size-distribution had a peak size at ~106 nm. The most concentrated suspension (3240 μ g/ml, corresponding to 162 μ g/instillation) had a Z-average size of

127.5 nm, and a polydispersity index of 0.55. The size distribution varied from nanometer to micron-sized, free, and agglomerated particles with spherical to subspherical agglomerates and occasional free single primary particles [Saber et al., 2011a; Jackson et al., 2012a].

BAL Fluid Cell Composition

Mice were instilled with a single dose of 0.67, 2, and 6 μg NPCB /animal and killed 1, 3, and 28 days post-

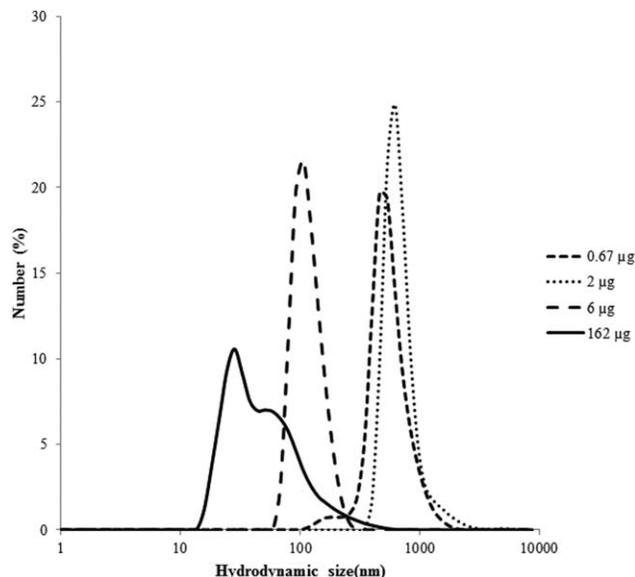


Fig. 1. DLS analysis of instillation medium.

exposure. Vehicle or 162 μg NPCB exposures were used as the negative control and reference dose, respectively. Lung inflammation was evaluated by total cell number and cell composition of BAL fluid (Table I). Mice instilled with 162 μg NPCB had increased total numbers of cells in BAL fluid as well as increased neutrophil influx in lungs at all three time-points, as previously reported [Saber et al., 2011a, 2012; Bourdon et al., 2012; Jackson et al., 2012a]. The highest number of BAL cells was observed 3 days post-exposure. The total number of BAL cells was unchanged by exposure to the three lower doses of NPCB at all time-points. The neutrophil influx observed on post-exposure day 1 was statistically significantly greater for all the three low NPCB doses, compared to the vehicle control (vehicle controls: $0.7 \pm 0.4 \times 10^3$ vs. 0.67 $\mu\text{g}/\text{animal}$: $8.8 \pm 2.0 \times 10^3$, $P < 0.001$; 2 $\mu\text{g}/\text{animal}$: $5.2 \pm 1.4 \times 10^3$, $P < 0.01$; 6 $\mu\text{g}/\text{animal}$: $5.2 \pm 1.4 \times 10^3$, $P < 0.001$). Three days after exposure, the neutrophil numbers were still elevated in animals exposed to 0.67 and 162 μg NPCB. Twenty-eight days after instillation, there was no difference in neutrophil influx between vehicle control animals and animals instilled with the three lower doses of NPCB.

The exposure also changed the distribution of macrophages, eosinophils, and lymphocytes. The change in the number of macrophages after instillation of 162 μg NPCB was similar to previous observations [Bourdon et al., 2012]. The number of lymphocytes in BAL fluid was statistically significantly increased 3 days post-exposure to 0.67 μg NPCB (vehicle controls: $0.2 \pm 0.1 \times 10^3$ vs. 0.67 $\mu\text{g}/\text{animal}$: $1.3 \pm 0.3 \times 10^3$, $P < 0.01$), and eosinophil

TABLE I. Summary of Total Cell Counts from BAL Fluid and Cells Distribution by Cells Types 1, 3, and 28 Days Postexposure to NPCB and Vehicle (Particle Exposed Groups $n = 8$, Vehicle Group $n = 12$)

| Dose | | Vehicle (H2O) | Printex 90 | | | |
|---------|------------------|-----------------|-----------------------|---------------------|--------------------|------------------------|
| | | 0 μg | 0.67 μg | 2 μg | 6 μg | 162 μg |
| 1 Day | Neutrophils | 0.7 ± 0.4 | $8.8 \pm 2.0^{***}$ | $5.4 \pm 0.8^{***}$ | $5.2 \pm 1.4^{**}$ | $128.7 \pm 14.0^{***}$ |
| | Macrophages | 67.7 ± 5.2 | 67.3 ± 6.5 | 55.5 ± 5.0 | 49.2 ± 3.0 | $35.5 \pm 4.1^{***}$ |
| | Eosinophils | 2.0 ± 1.1 | 2.9 ± 1.3 | 2.2 ± 0.8 | 0.8 ± 0.2 | 5.5 ± 3.1 |
| | Lymphocytes | 1.1 ± 0.5 | 0.3 ± 0.2 | 0.7 ± 0.2 | 0.5 ± 0.2 | $0.0 \pm 0.0^*$ |
| | Epithelial cells | 11.1 ± 1.2 | 9.3 ± 0.7 | 12.0 ± 2.3 | 14.1 ± 2.0 | 21.7 ± 5.0 |
| | Total BAL cells | 70.0 ± 9.6 | 88.8 ± 8.9 | 75.8 ± 6.2 | 69.8 ± 3.6 | $191.4 \pm 20.0^{***}$ |
| 3 days | Neutrophils | 1.9 ± 0.9 | $3.7 \pm 1.6^{**}$ | 0.9 ± 0.3 | 0.2 ± 0.1 | $132.4 \pm 4.7^{***}$ |
| | Macrophages | 56.7 ± 9.1 | 69.3 ± 6.6 | 51.2 ± 4.9 | 44.7 ± 4.3 | 58.8 ± 6.2 |
| | Eosinophils | 0.2 ± 0.1 | $20.2 \pm 10.1^{***}$ | $2.6 \pm 1.0^{**}$ | 0.7 ± 0.2 | 2.0 ± 1.5 |
| | Lymphocytes | 0.2 ± 0.1 | $1.3 \pm 0.3^{**}$ | 0.4 ± 0.2 | 0.5 ± 0.3 | 0.6 ± 0.3 |
| | Epithelial cells | 11.4 ± 2.7 | 9.7 ± 1.4 | 10.6 ± 1.3 | 9.6 ± 1.8 | 14.9 ± 2.5 |
| | Total BAL cells | 70.4 ± 11.1 | 104.0 ± 14.3 | 65.7 ± 5.3 | 55.6 ± 6.0 | $207.4 \pm 17.0^{***}$ |
| 28 days | Neutrophils | 1.5 ± 0.8 | 0.2 ± 0.1 | 0.9 ± 0.3 | 0.4 ± 0.2 | $20.8 \pm 3.6^{***}$ |
| | Macrophages | 53.2 ± 7.6 | 48.0 ± 3.7 | 37.6 ± 5.3 | 35.6 ± 4.8 | $106.1 \pm 10.4^{**}$ |
| | Eosinophils | 1.3 ± 0.9 | 0.6 ± 0.3 | 2.1 ± 1.5 | 0.0 ± 0.0 | 0.1 ± 0.1 |
| | Lymphocytes | 0.4 ± 0.2 | 0.6 ± 0.3 | 1.5 ± 0.6 | 0.8 ± 0.2 | $29.2 \pm 3.7^{***}$ |
| | Epithelial cells | 14.9 ± 2.2 | 7.0 ± 0.9 | 9.5 ± 1.9 | 8.1 ± 1.4 | 15.4 ± 3.9 |
| | Total BAL cells | 71.4 ± 8.6 | 56.4 ± 3.8 | 51.5 ± 7.9 | 45.0 ± 6.1 | $171.6 \pm 19.1^{***}$ |

Mean \pm SEM ($\times 10^3$), an asterisk (*, **, and ***) denotes $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, using nonparametric two-way ANOVA with post-hoc Tukey-type multiple comparison test with time after exposure and dose as categorical variables.

TABLE II. Summary of Comet Assay Results from BAL Cells, Lung, and Liver Tissue 1, 3, and 28 Days Postexposure to Vehicle and NPCB (Vehicle Group $n = 12$, Particle Exposed Groups $n = 8$)

| | Dose | Vehicle (H2O) | | Printex 90 | | |
|--------------|---------------|-----------------|--------------------|------------------|-------------------|-------------------|
| | | 0 μg | 0.67 μg | 2 μg | 6 μg | 162 μg |
| 1 Day | BAL TL | 14.3 \pm 0.6 | 18.2 \pm 1.4 | 16.9 \pm 1.3 | 15.0 \pm 0.9 | 15.3 \pm 0.7 |
| | BAL % TDNA | 4.6 \pm 0.4 | 9.1 \pm 0.9*** | 7.9 \pm 0.7* | 7.0 \pm 0.9 | 5.5 \pm 0.2 |
| | Lung TL | 9.9 \pm 0.4 | 9.3 \pm 0.5 | 9.1 \pm 0.4 | 8.9 \pm 0.4 | 11.9 \pm 0.6* |
| | Lung % TDNA | 4.3 \pm 0.3 | 4.2 \pm 0.5 | 4.3 \pm 0.5 | 3.2 \pm 0.2 | 7.9 \pm 1.4** |
| | Liver TL | 16.3 \pm 1.4 | 17.6 \pm 1.3 | 17.9 \pm 1.0 | 19.1 \pm 0.6 | 16.8 \pm 1.2 |
| 3 days | Liver % TDNA | 4.5 \pm 0.5 | 5.5 \pm 0.5 | 5.1 \pm 0.5 | 5.3 \pm 0.2 | 4.0 \pm 0.3 |
| | BAL TL | 12.7 \pm 0.8 | 17.9 \pm 1.3 | 18.6 \pm 0.5** | 19.6 \pm 0.7*** | 14.6 \pm 1.6 |
| | BAL % TDNA | 5.1 \pm 0.4 | 7.3 \pm 0.8 | 6.5 \pm 0.7 | 6.1 \pm 0.8 | 6.5 \pm 0.6 |
| | Lung TL | 10.7 \pm 0.6 | 9.5 \pm 0.2 | 10.1 \pm 0.2 | 8.8 \pm 0.7 | 11.1 \pm 0.5 |
| | Lung % TDNA | 2.1 \pm 0.2 | 2.3 \pm 0.4 | 2.5 \pm 0.2 | 2.1 \pm 0.5 | 2.5 \pm 0.2 |
| 28 days | Liver TL | 18.2 \pm 1.4 | 20.6 \pm 1.5 | 16.3 \pm 0.9 | 16.7 \pm 0.8 | 17.7 \pm 1.0 |
| | Liver % TDNA | 4.9 \pm 0.5 | 6.5 \pm 0.7 | 6.5 \pm 0.5 | 5.7 \pm 0.4 | 4.2 \pm 0.5 |
| | BAL TL | 13.9 \pm 0.7 | 19.6 \pm 0.9*** | 18.3 \pm 1.2** | 15.7 \pm 0.6 | 12.4 \pm 0.6 |
| | BAL % TDNA | 4.8 \pm 0.5 | 6.1 \pm 0.5 | 5.6 \pm 0.7 | 5.7 \pm 0.7 | 3.4 \pm 0.2 |
| | Lung TL | 9.1 \pm 0.4 | 7.8 \pm 0.2 | 12.3 \pm 0.5** | 11.0 \pm 0.3** | 9.0 \pm 0.3 |
| | Lung % TDNA | 2.0 \pm 0.4 | 1.8 \pm 0.2 | 3.3 \pm 0.4** | 2.4 \pm 0.3 | 2.0 \pm 0.2 |
| | Liver TL | 18.4 \pm 1.1 | 17.0 \pm 0.7 | 17.8 \pm 1.3 | 16.7 \pm 1.0 | 18.4 \pm 1.5 |
| Liver % TDNA | 5.7 \pm 0.6 | 4.1 \pm 0.4* | 5.3 \pm 0.4 | 4.4 \pm 0.4* | 5.7 \pm 0.7 | |

Mean \pm SEM ($\times 10^3$), an asterisk (*, **, and ***) denotes $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, using nonparametric two-way ANOVA with post-hoc Tukey-type multiple comparison test with time after exposure and dose as categorical variables).

influx was observed for the 0.67 and 2 μg NPCB groups (vehicle controls: $0.2 \pm 0.1 \times 10^3$, 0.67 μg : $20.2 \pm 10.1 \times 10^3$, 2 μg : $2.6 \pm 1.0 \times 10^3$, $P < 0.001$).

Successful pulmonary deposition was confirmed by presence of free particles and particles inside macrophages observed using light microscopy one day after exposure at all doses and up to 28 days post-exposure for mice given 162 μg (results not shown). Macrophages containing black particles were observed for all doses and at all postexposure time-points for all NPCB exposed mice.

DNA Strand Breaks

Levels of alkali labile sites and DNA strand breaks were evaluated using the comet assay (Table II). Overall, increased levels of DNA strand breaks assessed as %TDNA were observed in the BAL cells one day after exposure to 0.67 μg (2-fold: $P < 0.001$) and 2 μg (1.7-fold: $P < 0.05$) NPCB, respectively. TL was 1.5-fold increase in BAL cells 3 days post-exposure to 2 μg NPCB ($P < 0.01$) and 6 μg NPCB ($P < 0.001$). TL of BAL cells was increased 28 days post-exposure to 0.67 μg NPCB ($P < 0.001$) and 2 μg NPCB ($P < 0.01$). Pearson's correlation coefficient between %TDNA and TL was 0.667 (Fig. 2). There was no statistically significant increase in DNA strand breaks for the 162 μg NPCB group at any time point.

There was no difference in DNA strand breaks in lung tissue between the three low doses of NPCB and vehicle exposed mice one day after instillation, whereas there were more DNA strand breaks in the lung tissues of mice

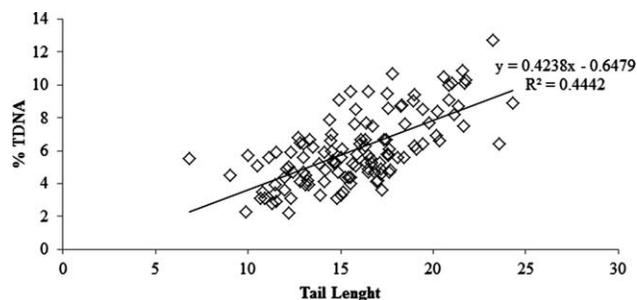


Fig. 2. Correlation between TL and %TDNA in the comet assay.

exposed to the high dose of NPCB (1.2-fold by TL: $P < 0.05$, and 1.8-fold by %TDNA: $P < 0.01$). After 28 days, 2 and 6 μg caused an increase in the levels of DNA strand breaks determined as TL (2 μg : 1.4-fold, $P < 0.01$, 6 μg : 1.2-fold, $P < 0.05$) and for 2 μg determined as %DNA (1.6-fold: $P < 0.01$). The levels of DNA strand breaks in liver cells were not affected by NPCB exposure at any of the time-points.

Total Protein in BAL Fluid

We measured the protein content in BAL fluid as a marker of increased membrane permeability due to cell damage (i.e., cytotoxicity). The reference group had significantly more protein in BAL fluid on post-exposure day 1 (3.5-fold: $P < 0.001$), day 3 (3.4-fold: $P < 0.001$), and day 28 (2-fold: $P < 0.001$) than controls. The protein concentration in BAL fluid was unaffected by exposure to the three lower doses of NPCB.

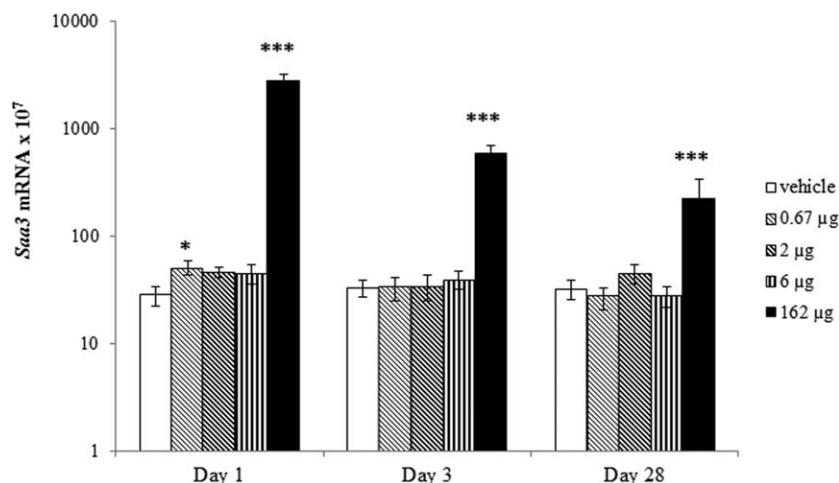


Fig. 3. Pulmonary *Saa3* mRNA expression level - dose response effect in mice 1, 3, and 28 days after intratracheal instillation of NPCB. The values represent mean of all animals in group \pm SEM ($n = 8-12$). An asterisk (*) denotes $P < 0.05$, (***) $P < 0.001$, *Saa3* mRNA expression levels in NPCB treated group versus vehicle controls.

***Saa3* mRNA Expression**

Pulmonary *Saa3* mRNA levels were used as a biomarker of pulmonary acute phase response [Saber et al., 2013, 2014]. Pulmonary *Saa3* mRNA expression levels were statistically significantly increased at all three time-points following instillation of 162 μg NPCB (reference group). In the lowest dose group (0.67 μg NPCB/animal), *Saa3* levels were increased 2-fold compared with controls 1 day after exposure ($P < 0.05$). No other time or dose-related changes in pulmonary *Saa3* expression levels were seen (Fig. 3). Pulmonary expression of *Saa3* mRNA correlated closely with neutrophil influx (data not shown) as previously observed for several different nanomaterials [Saber et al., 2013, 2014].

DISCUSSION

We investigated the adverse effects of pulmonary exposure to very low doses of Printex 90 in mice. We found increased levels of DNA strand breaks in BAL cells after a single exposure to 0.67 and 2 μg NPCB/animal one day post-exposure measured as %TDNA. Statistically significant increases in DNA strand break levels were also observed for the two middle doses (2 and 6 μg /animal) three days postexposure and for the two lowest doses (0.67 and 2 μg /animal) 28 days postexposure (measured as TL). We also observed that the low doses of Printex 90 increased DNA strand break levels in lung tissue 28 days after exposure. We did not observe a dose-response relationship. Assuming that 34% of the inhaled mass ends up in the pulmonary region based on the previously observed particle size distribution during aerosolisation [Jacobsen et al., 2009] and a mouse ventilation rate of 1.8 L/h [Bourdon et al., 2012], the NPCB doses tested

correspond to pulmonary deposition of about 20 min (0.67 μg /mouse), 1 hr (2 μg /mouse), and 3 hr (6 μg /mouse) at the current Danish occupational exposure limit (3.5 mg/m^3). Mice exposed to 162 μg were used as reference group. This dose corresponds to pulmonary deposition over the course of 9 working days at the Danish occupational exposure limit. The tested doses induced small and transient inflammatory and acute phase responses, but no toxicity in terms of protein content in BAL. However, strong and long lasting inflammation and pulmonary acute phase response was observed in the reference group (given 162 μg Printex 90/mouse).

We recently validated the use of automated scoring for the comet assay [Jackson et al., 2013]. We showed that DNA strand breaks were closely correlated in BAL, lung and liver cells from methyl methanesulphonate-exposed mice using automated scoring and semi-automatic scoring of the comet assay. Furthermore, we found a close correlation between levels of DNA strand breaks in fresh and frozen tissues, demonstrating that frozen tissue can be used in the comet assay and yields very similar dose-response relationships for TL and %TDNA. In this study, we documented a strong correlation between DNA damage measured as TL and %TDNA. %TDNA is often the preferred parameter since it is much less affected by test conditions that influence electrophoresis (temperature, ionic strength, agarose concentration, and strength of the electric field), staining and scoring than TL, but TL is useful to detect low levels of DNA damage [Azqueta and Collins, 2013]. Here, we found the same close correlation between the two measures of levels of DNA strand breaks (Fig. 2). Therefore, we have chosen to use both measures of DNA damage. TL and %TDNA correlated well, but there were differences in whether or not statistical significance was reached.

In contrast to previous reports [Bourdon et al., 2012], we found no effect on DNA strand break levels in BAL and liver cells from mice exposed to 162 μg Printex 90. There are a number of differences between the two studies. In this study, C57BL/6J BomTac mice were used, whereas the parental strain C57BL/6J was used in the previous study [Bourdon et al., 2012]. Furthermore, we used pure water [Jackson et al., 2012a, b] as vehicle instead of 10% BAL fluid in 0.9% NaCl [Bourdon et al., 2012]. However, we do not believe that these experimental differences explain why low doses of NPCB induced DNA strand breaks whereas higher doses did not in the present set-up. A more likely reason is the lack of dose-response relationship for DNA strand breaks and the high interanimal variation. We have also observed no dose-response relationship in mice instilled with higher doses of NPCB [Bourdon et al., 2012] and after inhalation of diesel exhaust particles [Dybdahl et al., 2004]. Instead of increasing with dose, the level of DNA strand breaks increased by ~ 50 – 100% at all doses compared with vehicle controls. There was some interanimal variation that may result from the pulmonary instillation procedure. However, we have previously documented that pulmonary instillation leads to widespread particle deposition in the lung [Mikkelsen et al., 2011]. The 50– 100% increase in DNA strand break levels may be close to the detection level of the assay due to high interanimal variation; therefore, statistically significant differences were only obtained at some doses even though the majority of the exposed groups have numerically higher DNA strand break levels than the vehicle controls. The lack of dose-response relationship may indicate a maximal rate of particle-induced DNA strand breaks was achieved at the studied doses. This would in turn indicate that particle-induced DNA strand breaks in the lungs are formed by a mechanism that is fundamentally different from chemically induced DNA damage. Many chemicals react directly with DNA leading to the formation of DNA adducts or oxidation of DNA. Conversely, there is little evidence that inhaled particles will reach the nucleus and interact directly with DNA. Instead, nanoparticles may promote the production of ROS. It has been shown that Printex 90 (14 nm) particles generate 12- to 21-fold greater ROS in acellular medium than FineCB (95 nm) [Saber et al., 2011b] and ROS production is proportional to the surface area. We previously demonstrated that the NPCB used in this study generate free radicals, induce DNA strand breaks, and increase mutation frequency in FE1 mouse lung epithelial cells in vitro, and that the mutation spectrum is in keeping with mutations caused by ROS [Jacobsen et al., 2007, 2008, 2010]. Increased mutation frequencies were also observed in A549 human lung epithelial cells after exposure to ultrafine CB (14 nm) [Totsuka et al., 2009].

Lung cancer does not arise in BAL cells; therefore, the biological significance of the observed increases in DNA

strand break in BAL cells may be questionable. However, our results clearly indicate that even the lowest dose of CBNP was enough to induce DNA strand breaks. Thus, it is possible that similar damage also occurs in cellular subpopulations that are progenitors of lung cancer. The alkali labile sites and DNA strand breaks detected by the comet assay may be repaired by DNA repair enzymes or result in mutations if left unrepaired. Thus, DNA damage detected by the comet assay is a sensitive but nonspecific indication of genotoxic effects. We have previously detected increased DNA strand breaks in BAL and lung 28 days after exposure to higher doses of NPCB [Bourdon et al., 2012]. Here, we observed increased DNA strand breaks in both BAL at the lowest dose and in lung tissue 28 days after exposure to 2 and 6 μg Printex 90 per mouse. No increases in strand breaks were found following exposure to 0.67 μg per mouse (ca 20 g). Therefore, 0.67 μg per mouse is the no observed adverse effect level for DNA strand breaks in lung tissue in our study. NPCB is still present in the lungs after 28 days, but is restricted to macrophages as measured by light microscopy. However, the increased levels of DNA strand breaks in lung tissues 28 days after the exposure indicates that the lung epithelial cells are exposed to a genotoxic agent, which could be either NPCB or reactive oxygen species.

The reference dose was chosen based on documented inflammatory and DNA damaging effects after pulmonary exposure in rodents [Saber et al., 2005; Saber and Hougaard, 2009; Bourdon et al., 2012; Jackson et al., 2012a, b; Boisen et al., 2013]. The other biomarkers presented here are in agreement with the previous observations overall. The exposure was immediately followed by an increased pulmonary *Saa3* mRNA expression and neutrophil influx in BAL fluid. The inflammatory and acute phase responses persisted at least for 28 days post-exposure, although the strongest effect was observed 1 day post-exposure. The protein concentration in BAL fluid, which reflects alveolar-capillary permeability and/or alveolitis, was also increased all the way up to 28 days postexposure.

The exposure to very low particle doses (0.67, 2, and 6 $\mu\text{g}/\text{animal}$) led to increased neutrophil influx and *Saa3* mRNA expression one day post-exposure, but the inflammation did not persist longer than three days. We recently documented that pulmonary *Saa3* expression can be used as a biomarker of a pulmonary acute phase response and that acute phase response genes were the most differentially regulated genes 24 hr after pulmonary exposure to carbon black and TiO_2 nanoparticles [Saber et al., 2013, 2014]. The acute phase response was consistently associated with increased risk of cardiovascular disease [Taubes, 2002; Kaptoge et al., 2012] and a 4-fold increase in SAA protein levels in blood was associated with a 3-fold increase in cardiovascular risk in

prospective studies [Ridker et al., 2000]. However, pulmonary deposition by intratracheal instillation induces more inflammation than inhalation of the same dose [Jacobsen et al., 2009; Jackson et al., 2012a] and we have previously shown that inhalation exposure to a dose corresponding to an 8-hr working day for four consecutive days did not induce neutrophil influx in exposed mice [Saber et al., 2005]. Thus, the induction of DNA strand breaks in BAL cells seems independent of exposure route and of neutrophil influx as we have previously observed DNA strand breaks in BAL cells after inhalation of NPCB in the presence or absence of neutrophil influx and following instillation in presence of neutrophil influx [Saber et al., 2005; Bourdon et al., 2012; Jackson et al., 2012a].

We observed an inverse dose-response relationship for the eosinophils on day 3. We have previously observed accumulation of eosinophils 3 days after pulmonary exposure to NPCB [Bourdon et al., 2012] and we have previously observed a similar inverse dose-response relationship for eosinophils after instillation of multiwalled carbon nanotubes [Poulsen et al., 2013]. Eosinophil influx is an indication of an allergic response [John et al. 2003], but the inverse dose-response relationship is puzzling and needs further investigation.

The NPCB suspensions were agglomerated. The primary particle size of Printex 90 (powder) is 14 nm. At all concentrations the size varied from nanometer to micron-sized particles. The presence of aggregates in exposure suspension is well documented [Gilmour et al., 2004; Bourdon et al., 2012]. Suspensions used for instillation consist of nano-sized particles and larger aggregates, but DLS on unfiltered samples can only detect the largest particles/aggregates in the suspension, because they will overshadow smaller particles [Bourdon et al., 2012]. The amount of carbon agglomerates in exposure suspension increased by decreasing particle concentration and may be an artefact caused by inefficient DLS measurements at low concentrations of dispersed particles.

In summary, pulmonary exposure to low doses of NPCB induced increased levels of DNA strand breaks in BAL cells and lung tissue, and limited inflammation and acute phase response. DNA strand breaks have multiple causes, including DNA synthesis and DNA repair caused by abasic sites and nicks in one or both DNA strands of the DNA double helix. We interpret the increased DNA strand break level as an indication of increased DNA damage and repair activity, indicating induction of genotoxic effects at very low doses of NPCB.

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AUTHOR CONTRIBUTIONS

ZKY was substantially involved in sampling during necropsies; data acquisition, analysis, and interpretation of all data; statistical analyses; and drafted the manuscript. NRJ participated in designing the study and critically reviewed the manuscript. ATS participated in statistical analyses; and drafted the manuscript. SBE participated in acquisition of protein measurement data; and drafted the manuscript. PJA participated in acquisition of DNA damage data on Imstar PathFinderTM, their interpretation; and revised the manuscript. UBV drafted the manuscript and UBV and HWA were substantially involved in designing the study and acquiring the funds; interpretation of data; and revised the manuscript critically. All authors have read and approved the final manuscript.

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