Toxicology and Applied Pharmacology 289 (2015) 573–588

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Carbon black nanoparticles induce biphasic gene expression changes associated with inflammatory responses in the lungs of C57BL/6 mice following a single intratracheal instillation

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ARTICLE INFO

Article history:
Received 15 August 2015
Revised 5 November 2015
Accepted 5 November 2015
Available online 10 November 2015

Keywords:
Carbon black nanoparticles
Biphasic response
Inflammation
DNA repair
Cell cycle regulation
Muscle contraction

ABSTRACT

Inhalation of carbon black nanoparticles (CBNPs) causes pulmonary inflammation; however, time course data to evaluate the detailed evolution of lung inflammatory responses are lacking. Here we establish a time-series of lung inflammatory response to CBNPs. Female C57BL/6 mice were intratracheally instilled with 162 μg CBNPs alongside vehicle controls. Lung tissues were examined 3 h, and 1, 2, 3, 4, 5, 14, and 42 days (d) post-exposure. Global gene expression and pulmonary inflammation were assessed. DNA damage was evaluated in bronchoalveolar lavage (BAL) cells and lung tissue using the comet assay. Increased neutrophil influx was observed at all time-points. DNA strand breaks were increased in BAL cells 3 h post-exposure, and in lung tissues 2–5 d post-exposure. Approximately 2600 genes were differentially expressed (±1.5 fold; $p \le 0.05$) across all time-points in the lungs of exposed mice. Altered transcript levels were associated with immune-inflammatory response and acute phase response pathways, consistent with the BAL profiles and expression changes found in common respiratory infectious diseases. Genes involved in DNA repair, apoptosis, cell cycle regulation, and muscle contraction were also differentially expressed. Gene expression changes associated with inflammatory response followed a biphasic pattern, with initial changes at 3 h post-exposure declining to base-levels by 3 d, increasing again at 14 d, and then persisting to 42 d post-exposure. Thus, this single CBNP exposure that was equivalent to nine 8-h working days at the current Danish occupational exposure limit induced biphasic inflammatory response in gene expression that lasted until 42 d post-exposure, raising concern over the chronic effects of CBNP exposure.

1. Introduction

Carbon black particles with diameters less than 100 nm are referred to as ultrafine powder or carbon black nanoparticles (CBNPs) and are used as reference material for diesel exhaust particles stripped of their adsorbed compounds in toxicological studies (Levy et al., 2012; Kyjovska et al., 2015b). CBNPs are one of the most widely produced and used nanomaterial. Approximately 70% of CBNPs are used by the tire industry, with the remaining 30% used for other rubber products and some non-rubber applications (e.g., as pigments and in printing ink) (IARC, 2010). Given the widespread production of CBNPs, a broad understanding of their toxicity and potential long-term health effects is required.

Pulmonary oxidative stress and inflammation are known responses to CBNPs. Substantive dose- and time-dependent neutrophil influxes have been observed in mice following intratracheal instillation and inhalation of CBNPs (Saber et al., 2009; Bourdon et al., 2012b; Jackson et al., 2012a; Saber et al., 2013; Kyjovska et al., 2015a). Generation of reactive oxygen species and induction of oxidatively damaged DNA has

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http://dx.doi.org/10.1016/j.taap.2015.11.003
0041-008X/

also been documented in cellular and acellular systems following exposure to CBNPs (Jacobsen et al., 2008). Increased oxidative DNA damage has been observed in mouse lungs following intratracheal instillation of CBNPs (Bourdon et al., 2012c); CBNPs have been shown to induce oxidative DNA damage, DNA strand breaks, and mutations both in vivo and in vitro, and in offspring following prenatal exposure (Driscoll et al., 1996; Saber et al., 2005; Jacobsen et al., 2008; Jacobsen et al., 2009; Saber et al., 2009; Jackson et al., 2012a). The mutation spectrum supports the notion that oxidative DNA damage as a result of CBNP-induced generation of reactive oxygen species may be the cause of this mutagenicity (Jacobsen et al., 2011). Furthermore, pulmonary exposure to CBNPs induces a pulmonary acute phase response in mice leading to alterations in cholesterol homeostasis (Bourdon et al., 2012a), which may increase the risk of cardiovascular effects (Saber et al., 2013; Saber et al., 2014).

In a previous study we showed that intratracheal instillation of 162 μg CBNPs (a dose corresponding to pulmonary deposition during nine 8-h working days at the current Danish occupational exposure limit for carbon black) resulted in increased neutrophil influx, changes in the expression of genes associated with inflammation, and genotoxicity in the lungs of C57BL/6 mice that lasted for 28 d following a single exposure (Bourdon et al., 2012a; Bourdon et al., 2012c). However, it remains unclear whether these inflammatory responses persist at time-points beyond 28 d, and how these inflammatory responses evolve over time. In this study, we investigate lung neutrophil influx, global gene expression changes, and genotoxicity at multiple post-exposure time-points following exposure to 162 μg CBNPs via intratracheal instillation. We used intratracheal instillation in order to have an exact time point following which to measure the onset of the pulmonary response to CBNP exposure to increase precision in measurement of temporal changes in gene expression. In addition, although inhalation is a more physiologically relevant exposure model, the generation of aerosol for the desired dose of nanomaterials is complex, expensive, time-consuming, and poses hazards to laboratory personnel (Brain et al., 1976). Moreover, inhalation methodologies require high concentrations of experimental dose and it can be difficult to deliver the desired dose to the experimental animal (Brain et al., 1976). Intratracheal instillation enables the delivery of the desired dose and provides a fairly uniform distribution of the experimental materials throughout the lungs of the exposed mice (Brain et al., 1976; Mikkelsen et al., 2011). Overall, our experiment examines the effects of CBNP exposure at early (3 h), intermediate (1, 2, 3, 4, 5, and 14 d), and late (42 d) time-points to understand the implications of CBNP exposure.

2. Materials and methods

2.1. Animals

Six-to-seven week old female C57BL/6 mice were purchased from Taconic (Ry, Denmark) and were allowed 1–2 weeks to acclimate. A total of 112 mice were divided into 8 experimental (N = 8 per group) and 8 control groups (N = 6 per group), and were maintained in polypropylene cages with sawdust bedding and enrichment, at 20–22 °C temperature and relative humidity of 40–60% with a 12 h light-to-dark cycle. All mice received food and water ad libitum during the period of the whole experiment. All animal experiments were approved by the Danish "Animal Experiments Inspectorate" and performed according to their guidelines for ethical conduct and care for animals in research (The Danish Ministry of Justice, Animal Experiments Inspectorate, permission 2010/561-1779).

2.2. Carbon black nanoparticle characterization

CBNPs were a kind gift from Evonik/Degussa (Frankfurt, Germany) and were extensively characterized in previous studies (Jacobsen et al., 2007; Bourdon et al., 2012c; Saber et al., 2012). Dynamic Light Scattering (DLS) was employed to determine the hydrodynamic particle size distributions in the exposure media, using a Malvern Zetasizer Nano ZS (Malvern, UK). Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used to determine the aggregation levels of CBNPs.

2.3. Mouse exposures and tissue collection

Preparation of exposure stock, exposures, and tissue collection were described previously (Bourdon et al., 2012a; Bourdon et al., 2012c; Jackson et al., 2012a; Jackson et al., 2012b; Kyjovska et al., 2015a). Briefly, CBNPs were suspended in 0.2 μm filtered, γ-irradiated Nanopure Diamond UV water (Pyrogens: <0.001 EU/ml, total organic carbon: <3.0 ppb), and subjected to sonication using a Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a disruptor horn (model number 101–147-037) as described (Jackson et al., 2012a; Jackson et al., 2012b; Kyjovska et al., 2015a). Total sonication time was 16 min without pause; samples were kept cool on ice during the sonication procedure. Vehicle control solutions contained only Nanopure Diamond UV water and were also sonicated as described above.

Prior to intratracheal instillation, mice were anesthetized with 4% Isoflurane (Jackson et al., 2011). Mice from the experimental groups received a single dose of 162 μg in a 50 μl volume (162 μg per mouse) of CBNPs suspension, followed by 200 μl air to ensure proper dispersion and deposition of the instilled suspension. Mice from matching control groups received 50 μl of Nanopure water followed by 200 μl air. At the time of the necropsy, mice were anesthetized with a subcutaneous injection of Hypnorm® (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml from Janssen Pharma) and Dormicum® (Midazolam 5 mg/mL from Roche). The lungs were flushed twice with 0.8 ml sterile 0.9% NaCl through the trachea to obtain BAL fluid. BAL fluids were stored on ice until centrifugation at 400 ×*g* for 10 min at 4 °C. The BAL cells were re-suspended in 100 μl 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 suspension following the manufacturer's protocol, the total cell counts were determined for each mouse. Lung and liver tissue were collected following BAL collection, snap frozen in liquid nitrogen, and stored at −80 °C until analyzed.

2.4. Inflammatory cell counts and protein content in BAL

Differential cell count in BAL was conducted as described previously (Bourdon et al., 2012c). Briefly, cells from 40 μl of BAL fluid were collected on microscope slides by centrifugation at 10,000 rpm for 4 min in a Cytofuge 2 (StatSpin, TRIOLAB, Rødovre, Denmark). Cells were fixed for 5 min in 96% ethanol and stained with May-Grünwald-Giemsa stain. Cellular composition of BAL fluid was determined by differentiation of cell types in 200 cells from each slide, under light microscope with 100× magnification (immersion oil). The total amount of cells of each type was recalculated using the total number of BAL cells. Protein content in BAL was determined as previously described (Kyjovska et al., 2015a).

2.5. Comet assay

The comet assay was performed to determine DNA strand breaks; frozen BAL cell suspension, lung (3 × 3 mm of right lobe), and liver (3 × 3 mm of median lobe) tissue from control and CBNP exposed mice were used as samples. The detailed experimental protocol for sample preparation and comet analysis was described previously (Jackson et al., 2013; Kyjovska et al., 2015a). Briefly, cells from BAL, lung, and liver tissue were suspended and embedded in 0.7% agarose gel on