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Accumulation of lipids and oxidatively damaged DNA in hepatocytes exposed to particles☆

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ABSTRACT

Exposure to particles has been suggested to generate hepatosteatosis by oxidative stress mechanisms. We investigated lipid accumulation in cultured human hepatocytes (HepG2) and rat liver after exposure to four different carbon-based particles. HepG2 cells were exposed to particles for 3 h and subsequently incubated for another 18 h to manifest lipid accumulation. In an animal model of metabolic syndrome we investigated the association between intake of carbon black (CB, 14 nm) particles and hepatic lipid accumulation, inflammation and gene expression of *Srebp-1*, *Fasn* and *Scd-1* involved in lipid synthesis. There was a concentration-dependent increase in intracellular lipid content after exposure to CB in HepG2 cells, which was only observed after co-exposure to oleic/palmitic acid. Similar results were observed in HepG2 cells after exposure to diesel exhaust particles, fullerenes C_{60} or pristine single-walled carbon nanotubes. All four types of particles also generated oxidatively damaged DNA, assessed as formamidopyrimidine DNA glycosylase (FPG) sensitive sites, in HepG2 cells after 3 h exposure. The animal model of metabolic syndrome showed increased lipid load in the liver after one oral exposure to 6.4 mg/kg of CB in lean Zucker rats. This was not associated with increased iNOS staining in the liver, indicating that the oral CB exposure was associated with hepatic steatosis rather than steatohepatitis. The lipid accumulation did not seem to be related to increased lipogenesis because there were unaltered expression levels in both the HepG2 cells and rat livers. Collectively, exposure to particles is associated with oxidative stress and steatosis in hepatocytes.

Introduction

Non-alcoholic fatty liver disease (NAFLD), characterized by accumulation of lipids in the liver, is now the most common cause of chronic liver disease in the Western countries. It is a relatively benign condition in humans when it presents as simple steatosis without inflammation (Preiss and Sattar, 2008). Steatosis may progress to steatohepatitis, which is a more severe stage of NAFLD where deposition of lipids occurs concurrently with inflammation. Nevertheless, NAFLD is a risk factor of cardiovascular disease and hepatocellular carcinoma (Bhatia et al., 2012). The accumulation of lipids in hepatocytes is considered to be a stress response, which is associated with oxidative stress or inflammation (Neuschwander-Tetri, 2010; Tilg and Moschen, 2010). Interestingly, it has been shown that pulmonary exposure to diesel exhaust particles (DEP) was associated with oxidative stress and accumulation of lipid in the liver of obese diabetic mice (Tomaru et al., 2007). Another study has shown that wild type mice on a high fat diet had exacerbated progression of NAFLD after inhalation exposure to concentrated air particulate matter (Tan et al., 2009). The findings are in keeping with observations that inhalation of concentrated air particulate matter was associated with elevated levels of hepatic lipid peroxidation in dyslipidemic ApoE knockout mice (Araujo et al., 2008). It is also well established that pulmonary exposure to particles is associated with systemic health effects such as accelerated plaque progression, which is also a condition of unfavorable accumulation of lipids in foam cells and endothelial dysfunction (Møller et al., 2011).

Oxidative stress and inflammation are key mechanisms of action of particle-generated health effects (Møller et al., 2010). Particle-mediated oxidative stress can occur because of increased generation of reactive oxygen species (ROS) or decreased activity of the antioxidant defense system. It is relatively easy to measure the ROS production in cultured cells, whereas it is more common to measure oxidation products of lipids, proteins or DNA in animals. The most commonly

Abbreviations: ABCG5, ATP-binding cassette, sub-family G (WHITE), member 5; ABCG8, ATP-binding cassette, sub-family G (WHITE), member 8; CB, carbon black; DEP, diesel exhaust particles; FASN, fatty acid synthase; FFA, free fatty acids; FPG, formamidopyrimidine DNA glycosylase; iNOS, inducible nitric oxidase synthase; NAFLD, non-alcoholic fatty liver disease (NAFLD); ROS, reactive oxygen species; SCD-1, steroyl-coenzyme A desaturase 1; SRM2975, standard reference material 2975; SWCNT, single-walled carbon nanotube; SREBP-1, sterol regulating element-binding protein 1; SREBP-2, sterol regulating element-binding protein 2; 8-oxodG, 8-oxo-7,8-dihydro-2′deoxyguanine.

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investigated oxidatively damaged DNA lesion is 8-oxo-7, 8-dihydro-2′ deoxyguanine (8-oxodG), which is a mutagenic DNA lesion (Møller et al., 2012a). In addition to being relevant in hazard identification of potential mutagenic events in humans, levels of oxidatively damaged DNA are also used as biomarkers of oxidative stress in studies of human exposure to particles (Demetriou et al., 2012; Møller and Loft, 2010). We have shown that a single gastrointestinal exposure to nanosized carbon black (CB), fullerenes C_{60}, single-walled carbon nanotubes (SWCNT) or DEP was associated with elevated levels of oxidatively damaged DNA in the liver of rats (Danielsen et al., 2008b, 2010; Folkmann et al., 2009). In addition, a recent meta-analysis showed that both single oral exposure to particles and repeated exposures by incorporation of particles in chow are associated with elevated levels of 8-oxodG in the liver (Møller et al., 2013). These observations indicate that oral exposure to particles is associated with hepatic oxidative stress.

We hypothesized that the gastrointestinal exposure to particles was associated with hepatic steatosis and that oxidative stress was implicated in this process of lipid accumulation. To this end we assessed the lipid accumulation in hepatocytes after exposure to four different carbon-based particles: CB, SWCNT, C_{60} and DEP. The level of oxidatively damaged DNA in hepatocytes was determined as a biomarker of oxidative stress in order to have a point of reference between in vitro findings and earlier observations of increased levels of DNA damage in the liver after oral exposure to these particles in animal models (Møller et al., 2012b). The levels of hepatic steatosis and steatohepatitis were assessed in the liver of lean and obese Zucker rats that had been exposed to nanosized CB by intragastric administration. We have previously shown that oral exposure to nanosized CB was associated with endothelial dysfunction in both lean and obese Zucker rats (Folkmann et al., 2012). The obese Zucker rat is an animal model of metabolic syndrome and hepatosteatosis, which is caused by hyperphagia. It has been shown that both hepatic and extrahepatic factors are involved in the development of steatosis (den Boer et al., 2004). The lipid accumulation in hepatocytes can be due to passive influx, retention or increased lipogenesis. The latter was assessed by expression levels of sterol regulating element-binding protein 1 (*Srebp-1*) that activates a number of genes including *fatty acid synthase* (*Fasn*) and *steroyl-coenzyme A desaturase 1* (*Scd-1*). FASN catalyzes the final step in the synthesis of palmitate and SCD-1 is the rate-limiting enzyme catalyzing the synthesis of monosaturated fatty lipids such as oleic acid (Amacher, 2011). *Sterol regulating element-binding protein 2* (*Srebp-2*) is involved in the metabolism of cholesterol in the liver (Zhao et al., 2011). Alterations in the transport capacity of sterols were assessed by expression of ATP-binding cassette, sub-family G (WHITE), members 5 (*Abcg5*) and 8 (*Abcg8*), which encode transporters for excretion of cholesterol into bile. It has been shown that knockout mice for these genes develop triglyceridemia and steatosis when fed a high-fat diet (Mendez-Gonzalez et al., 2011; Su et al., 2012).

Materials and methods

Gastrointestinal exposure of lean and obese Zucker rats to CB. The animal model, dosing regimen and endpoints in terms of vasomotor dysfunction have been described previously (Folkmann et al., 2012). Briefly, rats were allocated randomly to 3 treatment experiments with the same number of 14 week old lean and obese Zucker rats. *Experiment 1* consisted of rats that were exposed to a single dose of CB (0.064, 0.64 or 6.4 mg/kg body weight) or distilled water and euthanized 24 h later. *Experiment 2* consisted of rats that were exposed to 10 doses of CB (one dose/week of 0.064, 0.64 mg/kg bodyweight) or distilled water and euthanized 24 h after the last dose. *Experiment 3* consisted of rats that were exposed to 10 doses of CB (one dose/week of 0.64 mg/kg bodyweight) or distilled water and allowed to recover for 13 weeks before termination. We aimed at the longest possible recovery time, balanced with observations that obese Zucker rats develop renal disease at about 40 weeks of age. The obese Zucker rats in our experiment were beginning to show signs of deprivation such as reduced fur grooming behavior and their obesity precluded movement. The rats were fed ad libitum standard Altromin 1319 chow, containing12.5 MJ/kg, with 27% protein, 13% fat and 60% carbohydrate constituting the metabolizable energy. All animal procedures followed the guidelines for the care and handling of laboratory animals established by the Danish government, and the Animal Experiment Inspectorate, Ministry of Justice, approved the study (no. 2006/561-1161).

The Printex 90 CB is generated by combustion of fossil fuel that might generate different effects in biological systems than vegetable-based CB, which is a registered food coloring agent in Europe (E153) and with no upper limit of acceptable daily intake. It has been estimated that the mean dietary exposure to vegetable carbon is 3.7 mg/kg body weight, whereas high level consumers have a daily intake of 28.1 mg/kg body weight (EFSA, 2013). The largest dose (6.4 mg/kg body weight) in our study was originally selected to correspond to a daily dose of 7.1 mg/kg body weight for a human (70 kg) who ingests one tablet of CB (500 mg/tablet) (Folkmann et al., 2012).

The activity of alanine aminotransferase levels were determined in serum samples, collected at the time of termination by standard clinical biochemistry (Copenhagen University Hospital, Copenhagen, Denmark).

Assessment of lipid load and inducible nitric oxidase synthase (iNOS) staining in the liver of Zucker rats. We assessed the level of lipids by staining with Oil Red. Liver samples were collected, embedded in Tissue-Tek and frozen on a copper plate on dry ice and kept at −80 °C. The samples were thawed at −20 °C for 30 min before slicing thin sections (5 μm) on the Cryostat. The sections were mounted on Super Frost + slides and dried for 10 min before fixation in 100% acetone for 10 min. After drying, the slides were wrapped in aluminum foil and frozen at −80 °C until histological processing. The lipid load was assessed in four tissue sections by the intensity of red color in the ImageJ software system 1.45 (http://imagej.nih.govij). We have used the average value of the four tissue sections and subtracted the background staining intensity (corresponding to the staining in the tissue section with the least intensity).

The staining for iNOS in the liver was carried out by the same procedure as the lipid stain. Slides were thawed at room temperature for 1 h and washed with PBS (3 × 5 min). Endogenous peroxidase activity Q2 was blocked with Dako REAL Peroxidase-Blocking Solution (Dako, Glostrup, Denmark) (8 min) then washed with PBS 0.1% bovine serum albumin (BSA) and 0.3% Triton-X 100 (3 × 5 min). Unspecific binding was minimized by blocking with 10% normal serum (goat or rabbit) in 1% BSA (20 min). The slides were incubated at room temperature for 1 h with primary rabbit polyclonal antibody to iNOS (ab15323, Abcam Cambridge, United Kingdom) in a dilution of 1:400. The slides were then washed with PBS 0.1% BSA and 0.3% Triton-X 100 (3 × 5 min) and Q3 subsequently incubated with the secondary antibody (Goat polyclonal to Rabbit IgG(HRP) (ab6721), Abcam Cambridge, UK) in a dilution of 1:1000 for 30 min at room temperature. Binding was visualized with 3,3′-diaminobenzidine (DAB) according to manufacturer's instructions then washed in running tap water (10 min). The sections were counterstained with Mayer's Hematoxylin (1 min) and washed in running tap water (10 min) before dehydration in ascending alcohol series and xylene (70%, 96%, 99%, 2 × xylene 10 min each). After drying, cover slips were mounted with Pertex and the staining was evaluated in microscope. We prepared parallel slides for hematoxylin–eosin staining to assess the quality of the sections. The samples were embedded in 70% ethanol for 0.5 min then washed in running tap water and staining in Mayer's hematoxylin (2 min). They were washed again in running tap water and embedded in Eosin Y and dehydrated (95%–99% ethanol–xylene). Any section showing abnormal morphology caused by the freezing or staining procedure was discarded. Digital images were taken with at 20× magnification of areas with intact tissue and with

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