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Microarray analysis of gene expression in rat alveolar epithelial cells exposed to fractionated organic extracts of diesel exhaust particles

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ABSTRACT

Diesel exhaust particles (DEP) affect health adversely. Our previous studies revealed that DEP extracts up-regulated expression of genes related to drug metabolism, antioxidant enzymes, cell cycle/apoptosis and coagulation, and in addition, *n*-hexane soluble fraction (n-HSF) of DEP extracts contains aliphatic and polycyclic aromatic hydrocarbons, and *n*-hexane insoluble fraction (n-HISF) contains oxygenated compounds and has strong oxidative property. However, the relationship between the properties of chemicals in DEP extracts and the gene expression has not been fully elucidated. Here, we used a microarray analysis to identify and characterize genes whose expression is regulated by exposure to fractions of DEP extracts. A dichloromethane-soluble fraction (DMSF) of DEP was further fractionated into n-HSF and n-HISF. We exposed rat alveolar epithelial (SV40T2) cells to these fractions (30 μg/ml) for 6 h and identified regulated genes. DMSF predominantly up-regulated genes associated with drug metabolism (Cyp1a1, Gsta3), oxidative stress response (HO-1, Srxn1) and cell cycle/apoptosis (Okl38). Genes up-regulated by n-HSF were mainly associated with drug metabolism (Cyp1a1, Gsta3). The genes up-regulated by n-HISF included antioxidant enzymes (HO-1, Srxn1); genes response to cell damage, such as those functioning in cell cycle regulation or apoptosis (Okl38); and genes in coagulation pathways. Our present results suggested that n-HSF and n-HISF regulated characteristic genes which respond to chemical properties of each fraction.

1. Introduction

Previous studies showed that particulate matters (PM) including diesel exhaust particles (DEP) have adverse effects on respiratory system and cardiovascular system. In the lung, DEP can induce carcinogenesis (Wong et al., 1986), oxidative stress (Yang et al., 1999), inflammation (Shima et al., 2006), and thrombosis (Mills et al., 2006), and can exacerbate allergic diseases (Nemmar et al., 2003). DEP also cause changes in the expression of various genes (Koike et al., 2004).

DEP are composed of carbonaceous cores and many organic compounds, including aliphatic and aromatic hydrocarbons, heterocyclics, quinones, aldehydes, and unknown compounds (Li et al., 2000; Schuetzle et al., 1981; Schuetzle, 1983). Several reports have suggested that adsorbed organic compounds affect cells by producing reactive oxygen species, inducing tumors, and promoting inflammation (Risom et al., 2005; Ohyama et al., 1999; Devouassoux et al., 2002). Therefore, we focused here on genes regulated by organic extracts of DEP.

DEP are deposited mainly in the alveolar region of the lungs and may affect alveolar epithelial cells adversely (Snipes, 1989). Type I alveolar epithelial cells occupy approximately 97% of the entire alveolar surface, and therefore, we used alveolar epithelial cells which phenotype are type I cells in the present study. Type II cells are known to differentiate into type I cells during incubation. Rat type II alveolar epithelial (SV40T2) cells which used in the present study also differentiate into type I cell-like phenotype during incubation (Furuyama et al., 1997). Our previous study using a microarray and SV40T2 cells showed that organic extracts of DEP up-regulated the expression of genes encoding proteins involved in drug metabolism, such as cytochrome P450s; antioxidation, such as heme oxygenase-1; and cell cycle/proliferation/apoptosis, such as pregnancy-induced growth inhibitor; and coagulation/fibrinolysis, such as plasminogen activator inhibitor 2 (Koike et al., 2004). In order to analyze the chemical properties responsible for the induction of these genes, we fractionated DEP extracts into *n*-hexane insoluble fraction (n-HISF) and *n*-hexane soluble fraction (n-HSF) (Shima et al., 2006). n-HSF contains mainly hydrocarbons, including aliphatic and polyaromatic hydrocarbons, and n-HISF contains compounds with functional groups related to oxygenation and

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oxidative ability. It also showed that this oxidative ability of n-HISF may contribute to this fraction's toxic effects on cell viability, oxidative stress, and the inflammatory response (Shima et al., 2006), but it remained to be elucidated that what genes were regulated by chemical properties of each fraction, respectively.

To compare the effects on gene expression by whole or fractionated extracts of DEP, it is necessary to perform the experiments under various conditions of concentration and duration of exposure, such as same concentration, concentration based on composition ratio of fractions and concentration based on state of cells exposed to fractions.

In our present study, following Koike's report (2004), we aimed to reveal the comparative effects of n-HSF and n-HISF, whose chemical properties are characterized, on gene expression at same concentrations and duration of exposure, and association of the comparative effects with chemical properties of each fraction.

2. Materials and methods

2.1. Reagents

Dichloromethane, *n*-hexane, and dimethylsulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dulbecco's Modified Eagle Medium (DMEM) and Trypan Blue Stain were obtained from Invitrogen Corp. (Carlsbad, DA). Fetal bovine serum (FBS) was purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). RNeasy Mini Kit and RNase-Free DNase Set were obtained from QIAGEN, Inc. (Valencia, CA). 2-Mercaptoethanol was purchased from Sigma–Aldrich, Inc. (St. Louis, USA). One-Cycle Target Labeling and Control Reagents kit was purchased from Affymetrix, Inc. (Santa Clara, CA), and this kit consists of Poly-A RNA Control Kit, One-Cycle cDNA Synthesis Kit, Sample Cleanup Module, IVT Labeling Kit and Hybridization Control Kit. Engine oil (CASTLE NEW SPECIAL II) was obtained from Toyota Motor Corp. (Aichi, Japan); and fuel (ENEOS light oil, sulfur content; 20 ppm), from Nippon Oil Corp. (Tokyo, Japan).

2.2. Collection of diesel exhaust particles and preparation of the DEP extract and fractions

DEP were generated by a 4-cylinder, light-duty (2740 cm^3 exhaust volume), 4JB1-type diesel engine (Isuzu Automobile Company, Tokyo, Japan) operated at a speed of 1500 rpm under a load of 10 kg m of torque. At a flow rate of 300 l/min, approximately 1 g (1.11 ± 0.03 g) of DEP was collected on a glass-fiber filter (203 mm × 254 mm, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) per 12 h in a constant-volume sampler system fitted to the end of a stainless steel dilution tunnel. The DEP were then extracted with 100 ml of dichloromethane in a Soxhlet apparatus for 6 h. The dichloromethane solution was evaporated to dryness in a rotary evaporator, and then the solvent in the oily residue was evaporated by using a vacuum pump. The residue was designated as the dichloromethane-soluble fraction (DMSF). A 0.5 ml volume of *n*-hexane was added to the DMSF and the mixture agitated gently; the *n*-hexane soluble component was then recovered. This process was repeated 3 times. The soluble component and the remaining residue were designated as n-HSF and n-HISF, respectively. These fractions were evaporated to dryness in a rotary evaporator. Then, the solvent in the oily residue was evaporated by using a vacuum pump. These fractions were dissolved in DMSO and stored in a glass vial at −80 °C until tested.

2.3. Cell culture

Immortalized rat alveolar type II epithelial cell line SV40T2 was generously provided by Prof. A. Clement (Hospital Armand Trousseau, Paris, France) (Clement et al., 1991). SV40T2 cells are progenitors of type I cells and shift to type I cell-like phenotype during incubation (Furuyama et al., 1997).

SV40T2 cells (1×10^5 cells) were placed in 60-mm Petri dishes (Becton Dickinson, Co., NJ) in a humidified incubator at 37 °C with an atmosphere of 5% CO_2. They were incubated for 3 days (until they were confluent) in 5 ml DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS.

2.4. Spectral analysis and oxidative ability of DEP extracts and fractions

Chemical properties of DEP extracts and the fractions have already shown in our previous study (Shima et al., 2006). We described briefly since the properties are almost same as we have already shown.

IR spectra were obtained by using a Fourier Transform Infrared Spectrometer (JASCO Corp., Tokyo, Japan). ^{1}H NMR spectra were obtained with a NMR spectrometer (JNM-A500, JEOL, Tokyo, Japan); and GC–MS data, with a gas chromatograph (Hewlett-Packard, HP-5890 series II)/high resolution mass spectrometer (JMS-700, Mstation, JEOL) equipped with a fused silica gel column (0.25 mm × 30 m; DB-1MS, J&W Scientific, CA). Oxidative ability was determined by the dithiothreitol (DTT) assay.

IR spectra show that the most intensive absorption was observed over the range from 2800 to 3000 cm^{-1} for all samples, and it was mainly attributed to aliphatic C–H vibration. The IR spectrum of n-HISF showed strong absorption attributed to O–H groups over the range from 2500 to 3500 cm^{-1}, whereas that of n-HSF did not show any in this range. The spectrum of n-HISF also showed intensive broad absorption from 1600 to 1800 cm^{-1} whereas that of n-HSF showed far weaker adsorption over this range. The absorption between 1600 and 1800 cm^{-1} was mainly due to oxygenated functional groups such as carbonyl and NO_2 groups. DMSF had almost the additive spectrum of n-HISF and n-HSF. These results suggest that n-HISF has many functional groups related to oxygenation.

The ^{1}H NMR data were divided into five chemical shift ranges (0–1.0, 1.0–2.0, 2.0–3.5, 3.5–6.0, and 6.0–10.0 ppm). The ^{1}H NMR spectra of all samples showed major peaks at 0–2 ppm, indicating the presence of aliphatic protons. The relative integrated intensity (%) of n-HISF at 2.0–3.5 ppm and 3.5–6.0 ppm was higher than that of DMSF or n-HSF. In contrast, this value at both 2.0–3.5 ppm and 3.5–6.0 ppm was similar to those values for DMSF and engine oil. This finding suggests that n-HISF has many functional groups related to oxygenation and pyrolysis during the combustion process, such as hydroxyl and carbonyl groups, as well as double bonds, as compared with n-HSF.

The GC–MS chromatograms of all samples show that the total ion chromatogram (TIC) of n-HSF was similar to that of DMSF. n-HSF had similar peaks as engine oil at retention times from 30 to 40 min and fuel at retention times from 14 to 25 min. Also for n-HSF, aliphatic peaks of low-molecular mass (C9–C16), as observed for fuel at 3–14 min, were almost disappeared. These compounds may be burned during the combustion process or not be condensed on DEP. n-HISF showed a different pattern of peaks, which could not be observed in TICs of unburned engine oil and fuel at shorter retention times from 10 to 25 min, suggesting that this pattern might be a result of pyrolysis and recombination of reactive intermediates during the combustion process.

The DTT assay shows that n-HISF had the strongest oxidative ability as compared with DMSF and n-HSF (Shima et al., 2006).

2.5. Fractionated DEP extract exposure

To further analysis of our previous experiment (Koike et al., 2004), after the cells had been grown to confluence, the cells were exposed for 6 h to 30 μg/ml DMSF, n-HSF, or n-HISF in FBS-free DMEM containing 0.1% DMSO. This concentration and duration of exposure were designed to replicate the conditions of Koike's report (2004). Cells were exposed to each fraction at the same concentration to analyze the comparative gene expression and the chemical properties of each fraction in depth. Cells for control were exposed only to FBS-free DMEM containing 0.1% DMSO.

2.6. Measurement of cell viability

Cell viability was measured as an indicator of cytotoxicity. After exposure, the cell monolayer was washed with PBS(−), treated with 500 μl trypsin–EDTA (0.05%) for 1 min, and detached by pipetting. The cells were then suspended with DMEM containing 10% FBS. Viability was calculated by using trypan blue dye exclusion to identify viable cells and a cytometer to count them in suspension.

2.7. RNA preparation

After exposure, cells were homogenized with a QIAshredder (QIAGEN). Total RNA was extracted with an RNeasy Mini Kit according to the manufacturer's instructions. DNase treatment was performed during RNA isolation with an RNase-Free DNase Set. All samples were purified to an absorbance ratio (A_{260}/A_{280}) between 1.9 and 2.1 (measured in Tris–EDTA buffer pH 7.4).

2.8. cRNA synthesis and hybridization

Sample RNA was reverse transcribed to first-strand cDNA by using a Poly-A RNA Control Kit and One-Cycle cDNA Synthesis Kit. The single-stranded cDNA was then converted to double-stranded cDNA by using One-Cycle cDNA Synthesis Kit. Complementary DNA corresponding to 10 μg of total RNA was used in a cRNA amplification step with an IVT Labeling Kit. The resulting biotinylated cRNA was cut into fragments of approximately 50 bp. Approximately 15 μg of cRNA was hybridized to a Rat Expression Array 230A (Affymetrix). The bound cRNA was visualized by binding of streptavidin/phycoerythrin conjugates to the hybridized GeneChip, followed by laser scanning of the bound phycoerythrin.

2.9. Expression data analysis

The microarray analysis was performed twice, using mRNA obtained from separate experiments conducted under the same conditions. Microarray Analysis Suite 5.0 algorithm was used to quantify microarray signals and the intensities were normalized for each chip by setting the mean intensity to 200. Though it is difficult to perform the statistical analyses as sample size is small ($n = 2$), we assumed the expression ratio was roughly Gaussian distribution because these were repeated experiments on a like-for-like basis. Though large scale replicate experiments remain to be carried out to confirm it, with common experiments,