Mutation Research 696 (2010) 114–121

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Oxidative damage induced by carcinogenic polycyclic aromatic hydrocarbons and organic extracts from urban air particulate matter

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ARTICLE INFO

Article history:
Received 28 April 2009
Received in revised form 3 November 2009
Accepted 8 December 2009
Available online 14 January 2010

Keywords:
Carcinogenic polycyclic aromatic hydrocarbons
Extractable organic matter
Oxidative damage *in vitro*
HepG2
HEL
Cell lines

ABSTRACT

We investigated the role of oxidative damage in the mechanism of action of selected individual carcinogenic PAHs (c-PAHs: benzo[*a*]pyrene, B[*a*]P; dibenzo[*a*,*l*]pyrene, DB[*a*,*l*]P), an artificial mixture of c-PAHs (c-PAHs mix) and extractable organic matter (EOM) from urban air particulate matter (PM). Two cell lines (human hepatoma cells, HepG2; human diploid lung fibroblasts, HEL) were treated for 24 and 48 h with various concentrations of compounds and mixtures. A panel of oxidative stress markers included 8-oxodeoxyguanosine (8-oxodG), 15-F_{2t}-isoprostane (15-F_{2t}-IsoP) and protein carbonyl groups. The response of the cell lines to the test compounds was substantially different. In HepG2 cells, oxidative damage to DNA was generally not induced by individual c-PAHs and the c-PAHs mix, but EOM increased 8-oxodG levels in these cells. In HEL cells, none of the compounds induced oxidative DNA damage. Lipid peroxidation, measured as the level of 15-F_{2t}-IsoP, was induced by c-PAHs in HepG2 cells only after 48 h of incubation, while the effect of EOM was detected already after 24 h. In HEL cells, individual c-PAHs and the c-PAH mix generally decreased 15-F_{2t}-IsoP levels. This effect was even stronger for EOM treatment. Protein oxidation, assessed as carbonyl levels in cell lysates, was not induced after 24 h of treatment with any compound in either cell line. Individual c-PAHs and the c-PAH mix generally induced protein oxidation in both cell lines after 48 h treatment, with the exception of DB[*a*,*l*]P in HepG2 cells. Oxidative damage to proteins caused by EOM was generally increased in HepG2 cells after 48 h of incubation, while in HEL cells the effect was observed for only one dose of EOM. In summary, our results demonstrate the ability of EOM to induce oxidative damage to DNA and lipids after 24 h of treatment, and to proteins after 48 h, in HepG2 cells, while the effect of c-PAHs was substantially less. The induction of oxidative stress by c-PAHs and EOM in HEL cells was weak.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous group of chemical compounds polluting the environment. They are mostly produced by the incomplete combustion of organic material and are present in the air, soil, as well as water. Some PAHs have been identified as carcinogenic to humans [1]. In the ambient air, PAHs are mostly adsorbed on the surface of dust particles. Regarding the effects on human health, respirable particulate matter with an aerodynamic diameter < 10 μm (PM10) is of most concern, since long-term exposure to PM has been associated with an increased incidence of pulmonary and cardiovascular diseases and cancer [2,3].

Apart from their carcinogenicity, PAHs may exert their negative effects on the human organism by the induction of oxidative stress. Upon entering the organism, PAHs are first metabolized to *trans*-dihydrodiols by the activity of CYP enzymes and epoxide hydrolase [4] and then oxidized to reactive electrophiles via two pathways [5]. The first pathway includes the formation of diol-epoxides that bind to DNA to form stable DNA adducts [6]. Radical-cation formation was suggested to be a major mechanism of activation of some potent carcinogens, including dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P) [7]. When unrepaired, the presence of these adducts may lead to errors in replication and the formation of mutations. The second pathway, catalyzed by aldo–keto reductases, results in the oxidation of PAH *trans*-dihydrodiols to *o*-quinones [8]. These compounds have the ability to enter redox cycles, increase the formation of reactive oxygen species (ROS) and thus cause oxidative stress.

PM consists of a complex mixture of chemicals bound to solid, liquid and aerosol particles. The effect of PM on organisms depends on its chemical composition: a higher content of c-PAHs increases

Abbreviations: 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 15-F_{2t}-IsoP, 15-F_{2t}-isoprostane; B[*a*]P, benzo[*a*]pyrene; c-PAHs, carcinogenic polycyclic aromatic hydrocarbons; DB[*a*,*l*]P, dibenzo[*a*,*l*]pyrene; DMSO, dimethyl sulfoxide; DNPH, dinitrophenylhydrazine; EOM, extractable organic matter; FBS, fetal bovine serum; HEL, human diploid lung fibroblasts; HepG2, human hepatoma cells; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; PM, particulate matter.

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doi:10.1016/j.mrgentox.2009.12.018

the genotoxicity of PM, resulting in the preferential formation of PAH-DNA adducts [9]. The presence of other compounds, including *o*-quinones, or transition metals may lead to ROS formation and the subsequent induction of oxidative stress. Solid particles in PM are another source of ROS, produced mostly as a result of inflammatory processes [10,11]. Inflammation in the lungs following exposure to PM may result in the influx of alveolar macrophages, leading to the generation of free radicals and in turn to increased oxidative stress [10]. However, chemical composition may not necessarily be informative about the resulting effect of PM on the organism, because it does not take into account the interactions between various components that may cause synergistic, antagonistic, or additive effects [11].

Oxidative stress resulting from an imbalance between pro-oxidants, including ROS, and antioxidants in the organism may affect DNA, lipids, as well as proteins [12]. The attack of ROS on DNA molecules yields a number of oxidized bases [13], among which 8-oxodeoxyguanosine (8-oxodG) is the most often studied. 8-oxodG is a highly mutagenic base; its presence in DNA results in GC-TA transversions. Lipid peroxidation has two major impacts on a cell: it changes the properties of the cellular membranes, which affects their structure and the activity of membrane-bound proteins, and it causes the formation of other reactive intermediates that propagate oxidative stress [14]. Among the markers of lipid peroxidation, isoprostanes are considered the most reliable. They are formed by a free-radical attack on arachidonic acid in cellular membranes. Currently, 15-F_{2t}-isoprostane (15-F_{2t}-IsoP) is the best-characterized and most often studied isoprostane [15,16]. Protein oxidation occurs mostly on the side chains of amino acids, particularly lysine, arginine, proline and threonine, on glutamyl residues and on the protein backbone. It results in protein inactivation, a failure to fold correctly, or proteolysis [17]. Currently, carbonyl groups, a major product of protein oxidation, are widely used as a marker of oxidized proteins.

Due to the complexity of the response to oxidative stress and the numerous factors that may affect its induction, the mechanisms of action of chemical compounds are best studied in *in vitro* systems under controlled culture conditions. In the present study, we used two cell lines with distinctively different characteristics: the human hepatoma cell line (HepG2) and human diploid lung fibroblasts (HEL). While HepG2 cells have many characteristics of human hepatocytes, particularly enzymatic pathways [18], and are able to activate PAHs including benzo[*a*]pyrene (B[*a*]P) and dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P) [19], they also express a number of other genes not found in normal hepatocytes, probably reflecting their transformed phenotype. On the other hand, HEL cells, although having a lower capacity to metabolize PAHs [19], have a normal phenotype and thus are more likely to mimic *in vivo* conditions. Moreover, HEL cells are suitable as an *in vitro* model of the lung as a target tissue for inhalation exposure. Both cell lines have been used in our laboratory to test the genotoxicity of c-PAHs, as well as organic extracts from PM (extractable organic matter, EOM) [19].

The aim of the present study was to compare the ability of B[*a*]P, DB[*a*,*l*]P, an artificial mixture of c-PAHs (c-PAH mix), and EOM collected in Prague, Czech Republic in three different seasons, to induce oxidative damage to DNA, lipids and proteins in HepG2 and HEL cells after their treatment with selected compounds for 24 and 48 h. We tested the hypothesis that B[*a*]P, DB[*a*,*l*]P and the c-PAH mix would cause oxidative damage to macromolecules, but with a lower potency than EOM. We also hypothesized that due to their higher metabolic capacity, HepG2 cells would show higher levels of oxidative damage than HEL cells. The results will be used to determine which cell line is more appropriate for future testing of the oxidative damage potential of EOM.

2. Materials and methods

2.1. c-PAHs, c-PAH mix and EOM

c-PAHs used for the treatment of cells and/or the preparation of the c-PAH mix, including benz[*a*]anthracene (B[*a*]A), benzo[*a*]pyrene, benzo[*b*]fluoranthene (B[*b*]F), benzo[*k*]fluoranthene (B[*k*]F), benzo[*g*,*h*,*i*]perylene (B[*g*,*h*,*i*]P), chrysene (CHRY), dibenz[*a*,*h*]anthracene (DB[*a*,*h*]A), dibenzo[*a*,*l*]pyrene and indeno[*1*,*2*,*3-c*,*d*]pyrene (I[*1*,*2*,*3-c*,*d*]P), were obtained from Sigma–Aldrich (St. Louis, MO, USA). The c-PAH mix was prepared by mixing eight of the above-mentioned c-PAHs (excluding DB[*a*,*l*]P) in the same relative proportion as in the EOM sample collected in the winter of 2001 in Prague (Table 1).

Airborne particles of an aerodynamic diameter <10 μm (PM10) were collected in the center of Prague in three different periods: in summer 2000 (June 15th 2000–September 15th 2000), in winter 2001 (December 4th 2000–March 3rd 2001) and in winter 2005 (November 7th 2005–December 22nd 2005) using HiVol (high volume) air samplers (Anderson, USA) with 20 cm × 30 cm Pallflex filters (T60A20). Extractable organic matter (EOM) was obtained from the filters by dichloromethane extraction within several days after the sample collection; before the extraction the filters were stored at −80 °C. Extracts were divided in two equal parts. The first was used for analysis of PAHs and the second for *in vitro* tests. Quantitative chemical analysis of PAHs was performed in the certified laboratory ALS Czech Republic (ISO 17025). Right after the extraction, EOM samples were evaporated to dryness under a stream of nitrogen and re-dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50 mg/ml and stored at −80 °C until the *in vitro* tests.

2.2. Cell cultures—cultivation and treatment with the test compounds

The human HepG2 cell line, derived from a primary hepatoblastoma isolated from a 11-year-old Argentinian boy [20], was kindly provided by Dr. A. Gabelova (Cancer Research Institute, Bratislava, Slovakia). The cells were cultivated in 75 cm^2 flasks in William's medium containing 10% FBS, 2 mmol/l glutamine and 50 U/ml penicillin, at 37 °C and 5% CO_2. The cells were seeded at an initial concentration of ~25,000 cells/cm^2. Human embryonic lung diploid fibroblasts (HEL, Sevapharma, Czech Republic) were grown in minimal essential medium E-MEM supplemented with 10% FBS, 0.2% sodium bicarbonate and 100 U/ml penicillin. The cells were seeded in plastic cell culture flasks (75 cm^2) at an initial concentration of ~17,000 cells/cm^2 and incubated at 37 °C in 5% CO_2. After reaching 90% confluence, the medium was replaced with fresh medium supplemented with 1% bovine serum.

B[*a*]P, DB[*a*,*l*]P, the c-PAH mix and EOM were diluted in DMSO and added to the medium at the test concentrations. The cells were treated for either 24 or 48 h. The test concentrations were as follows—B[*a*]P: 1, 10 and 100 μM; DB[*a*,*l*]P: 0.1 and 0.5 μM; c-PAH mix: 5 and 50 μM B[*a*]P (the concentrations of the other c-PAHs in the mixture were calculated in relation to the B[*a*]P content in the proportions described in Table 1): the 'summer 2000' and 'winter 2001' EOM: 10, 25 and 50 μg/ml; the 'winter 2005' EOM: 10 and 25 μg/ml. The test concentrations and treatment periods were selected in part on the basis of published data [19,21] and partly based on unpublished results on toxicity. Each concentration was tested in triplicate in at least two independent experiments. For each experiment, at least three negative controls incubated in the medium containing only DMSO were used. As positive controls, cells treated with hydrogen peroxide were used. After treatment, cells were harvested by scraping, washed twice with PBS and split into two parts in a 1:4 ratio. The smaller part was used to make cell lysates for the analysis of lipid peroxidation and protein oxidation, while the larger part was used for DNA isolation and analysis of DNA oxidative damage. Cells were stored at −80 °C until further processing.

2.3. Analysis of cytotoxicity

To study the relationship between oxidative stress and the cytotoxicity of the test compounds, we used a commercial kit (BioVision, CA, USA) to measure the release of lactate dehydrogenase (LDH) from cells following damage to their plasma membranes. The analysis was performed according to the manufacturer's recommendations. Briefly, suspensions of HepG2 and HEL cells in fresh medium with 1% FBS were seeded in a 96-well plate in triplicates. The test compounds were added and the plates were incubated for an adequate time (24 or 48 h) in an incubator at 37 °C and 5% CO_2. Culture medium without cells was used as a blank, and a cell suspension without any compounds added was used as a low control. The high control was established by adding 10 μl of cell-lysis solution to the triplicate wells with the cells only (no test compounds), 30 min before the end of the incubation. At the end of the incubation, the plate was gently shaken and centrifuged at 250 × *g* for 10 min. The media were transferred to the corresponding wells of another 96-well plate, LDH reaction mix was added and the plate was incubated for 30 min at room temperature. Finally, the absorbance was read at 450 nm and the cytotoxicity was calculated. The results are expressed as percentage increase in LDH release when compared with the control sample.

2.4. Analyses of oxidative damage to macromolecules

To ensure maximum reliability of the data and to control for inter-experiment variability, we analyzed each test compound and mixture within a single incuba-