Mutat Res Gen Tox En 827 (2018) 50–58

Contents lists available at ScienceDirect

Mutat Res Gen Tox En

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

Kinetics of ROS generation induced by polycyclic aromatic hydrocarbons and organic extracts from ambient air particulate matter in model human lung cell lines

Helena Libalova, Alena Milcova, Tereza Cervena, Kristyna Vrbova, Andrea Rossnerova, Zuzana Novakova, Jan Topinka, Pavel Rossner Jr*

Department of Genetic Toxicology and Nanotoxicology, Institute of Experimental Medicine CAS, Prague, 14220, Czech Republic

ARTICLE INFO

Keywords:
Polycyclic aromatic hydrocarbons
Extractable organic matter
Reactive oxygen species
Antioxidant mechanisms
Human lung cell lines

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) associated with particulate matter (PM) may induce oxidative damage via reactive oxygen species (ROS) generation. However, the kinetics of ROS production and the link with antioxidant response induction has not been well studied. To elucidate the differences in oxidative potential of individual PAH compounds and extractable organic matter (EOM) from PM containing various PAH mixtures, we studied ROS formation and antioxidant response [total antioxidant capacity (TAC) and expression of *HMOX1* and *TXNRD1*] in human alveolar basal epithelial cells (A549 cells) and human embryonic lung fibroblasts (HEL12469 cells). We treated the cells with three concentrations of model PAHs (benzo[a]pyrene, B[a]P; 3-nitrobenzanthrone, 3-NBA) and EOM from PM < 2.5 μm (PM2.5). ROS levels were evaluated at 8 time intervals (30 min–24 h). In both cell lines, B[a]P treatment was associated with a time-dependent decrease of ROS levels. This trend was more pronounced in HEL12469 cells and was accompanied by increased TAC. A similar response was observed upon 3-NBA treatment in HEL12469 cells. In A549 cells, however, this compound significantly increased superoxide levels. This response was accompanied by the decrease of TAC as well as *HMOX1* and *TXNRD1* expression. In both cell lines, a short-time exposure to EOMs tended to increase ROS levels, while a marked decrease was observed after longer treatment periods. This was accompanied by the induction of *HMOX1* and *TXNRD1* expression in HEL12469 cells and increased TAC in A549 cells. In summary, our data indicate that in the studied cell lines B[a]P and EOMs caused a time-dependent decrease of intracellular ROS levels, probably due to the activation of the antioxidant response. This response was not detected in A549 cells following 3-NBA treatment, which acted as a strong superoxide inducer. Pro-oxidant properties of EOMs are limited to short-time exposure periods.

1. Introduction

Ambient air pollution represents a serious health problem affecting the human population worldwide. Exposure to air pollutants has been associated with increased incidence of pulmonary and cardiovascular diseases and adverse birth outcomes [1–3]. Recently, both outdoor air pollution and particulate matter (PM) in air pollution have been classified by the International Agency for Research on Cancer as carcinogenic to humans (Group 1) [4]. Reactive oxygen species (ROS) generation and subsequent oxidative stress and damage to macromolecules belong among the mechanisms underlying negative biological effects of PM. PM-induced ROS production is a complex process that depends on physico-chemical properties of PM, the presence of transition metals and organic compounds. These properties may directly affect ROS production, or alter the function of mitochondria, NADPH-oxidases or activate inflammatory cells [5] resulting in oxidative damage to macromolecules. While the presence of organic compounds, particularly polycyclic aromatic hydrocarbons (PAHs) and their derivatives, is primarily associated with genotoxicity manifested by bulky DNA adduct formation [6], organic extracts from PM may also affect ROS levels and induce oxidative stress.

In the organism, PAHs and their derivatives bind to aryl

Abbreviations: A549 cells, human alveolar basal epithelial cells; B[a]P, benzo[a]pyrene; GSH/GSSG, reduced/oxidized form of glutathione; HEL12469 cells, human embryonic lung fibroblasts; HMOX, heme oxygenase; 3-NBA, 3-nitrobenzanthrone; PAHs, polycyclic aromatic hydrocarbons; PM, particulate matter; ROS, reactive oxygen species; TAC, total antioxidant capacity; TXNRD, thioredoxin reductase

* Corresponding author at: Department of Genetic Toxicology and Nanotoxicology, Institute of Experimental Medicine CAS, Videnska 1083, Prague, 14220, Czech Republic.
E-mail address: prossner@biomed.cas.cz (P. Rossner).

https://doi.org/10.1016/j.mrgentox.2018.01.006
Received 4 September 2017; Received in revised form 30 November 2017; Accepted 12 January 2018

hydrocarbon receptor (AhR), an important transcription factor that, among other targets, activates cytochrome P450 (CYP450). Protein products of CYP450 genes are responsible for the initial steps of PAHs activation. Following the CYP450-catalyzed reaction, PAHs are predominantly metabolized by epoxide hydrolase to diol epoxides that bind to DNA resulting in bulky DNA adducts formation. However, they may also be converted by aldo-keto reductases to catechol which is unstable and undergoes oxidation first to *o*-semiquinone anion radical and finally to *o*-quinone. This process is accompanied by a generation of hydrogen peroxide and superoxide anion. As *o*-quinone may be reduced either to catechol or semiquinone radical, futile redox cycles that generate more ROS are established [7]. Mechanisms of ROS formation by PAH nitro-derivatives depend on the actual compound. Specifically, 3-nitrobenzanthrone (3-NBA), a potent mutagen often found in diesel exhaust, is first metabolized to *N*-hydroxy-3-aminobenzanthrone (N-OH-ABA) by xanthine oxidase, NADP(H):quinone oxidoreductase and NADPH:cytochrome P450 oxidoreductase. This product may undergo autooxidation to 3-nitrosobenzanthrone (3-NOBA) which is accompanied by the production of superoxide anion and hydrogen peroxide. In the presence of NADH, 3-NOBA may be reduced back to N-OH-ABA forming a redox cycle [8].

Apart from direct metabolism-associated mechanisms of ROS generation, PAHs and their derivatives may induce ROS formation via modulation of the immune response mediated by AhR. Among other activities, AhR regulates the expression of immunomodulatory genes, and the function and differentiation of inflammatory dendritic cells [9]. AhR is essential for the differentiation and activation of T helper 17 cells which play a central role in the development of inflammation [10]. It is also involved in cytokines production which further increases ROS generation [11,12]. It should also be noted that the expression of AhR is regulated by NF-κB signaling [9]. NF-κB is activated by ROS which in turn increase the expression of pro-inflammatory genes [13]. Thus, several lines of evidence, supported by a number of studies [14–26], suggest that PAHs, either alone or in mixtures in EOMs from PM increase ROS levels in the organism and may thus induce oxidative damage of macromolecules.

ROS include both radicals (e.g. superoxide, hydroxyl, peroxyl or lipid peroxyl radicals) and non-radical compounds (hydrogen peroxide, hypochlorous acid, hypobromous acid, peroxynitrite). Superoxide radical is particularly reactive and may cause inactivation of antioxidant enzymes and oxidation of glutathione [27]. Hydrogen peroxide is relatively stable and can thus travel long distances and, unlike superoxide radical, even cross cellular membranes. It is further converted to hydroxyl radical which is considered the most reactive ROS, although its lifetime is very short. The organism is protected against deleterious effects of ROS by an antioxidant system that consists of antioxidant enzymes and non-enzymatic antioxidant molecules. Glutathione is the predominant non-enzymatic antioxidant. In its reduced form (GSH) it scavenges ROS while being converted to oxidized molecule (GSSG). It is reduced back to GSH by the activity of glutathione peroxidase. Other non-enzymatic antioxidants include e.g. thioredoxins, glutaredoxins, lipoic acid, melatonin or vitamins (reviewed in [27]). The antioxidant activity of non-enzymatic molecules is commonly referred to as the total antioxidant capacity (TAC). The antioxidant enzymes consist of "primary enzymes" (glutathione peroxidase, catalase, superoxide dismutase) which prevent formation or neutralize ROS [27] and "secondary enzymes", e.g. thioredoxin reductase (TXNRD), that regulates the levels of thioredoxin which in turn affects the levels of reduced glutathione [28], or heme oxygenase (HMOX), which breaks down heme to carbon monoxide and bilirubin and increases levels of reduced glutathione [29].

In the present study we focused on analysis of pro-oxidant properties of model PAHs, as well as a mixture of compounds in EOMs obtained from PM2.5. We measured the time course production of ROS in two lung cell lines representing tumor and normal cells [human alveolar basal epithelial cells (A549 cells) and human embryonic lung fibroblast (HEL12469 cells)], following exposure to benzo[a]pyrene (B[a]P), 3-nitrobenzanthrone (3-NBA) and two EOMs differing in PAH content. We further evaluated the potential induction of antioxidant response by measuring TAC and expression of genes encoding antioxidant enzymes (TXNRD1 and HMOX1).

2. Materials and methods

2.1. Collection of PM2.5, preparation of EOMs and chemical analysis

PM2.5 was collected by a HiVol 3000 air sampler (model ECO-HVS3000, Ecotech, Australia) on Pallflex filters T60A20 (20 × 25 cm) in Ostrava, Czech Republic, a city characterized by high levels of air pollution, predominantly from industrial sources. The samples were collected in the winter and summer season of 2011, for 24 h each day for 4–5 weeks. Each filter was extracted with 60 mL of dichloromethane and 3 mL of cyclohexane for 3 h. Extractable organic matter from each set of filters was pooled and aliquots were used for chemical analysis and cell treatment. Quantitative chemical analysis of PAHs [including carcinogenic PAHs (c-PAHs, Group 1, 2A or 2 B according to the International Agency for Research on Cancer (IARC): benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[k]fluoranthene, chrysene, dibenzo[a,h]anthracene and indeno[1,2,3-c,d]pyrene), benzo[g,h,i]perylene, anthracene, coronene, fluoranthene, phenanthrene and pyrene] was performed by HPLC with fluorimetric detection. For the *in vitro* experiments, EOM samples were evaporated to dryness under a stream of nitrogen and the residue redissolved in dimethylsulfoxide (DMSO). The stock solution of each EOM sample contained 50 mg of EOM/ml DMSO. EOMs were kept in the freezer at −80 °C.

2.2. Cell cultures and treatment

Two cell lines derived from lungs, representing tumor and normal cells, were tested: human adenocarcinoma alveolar basal epithelial cells, type II (A549) and human embryonic lung fibroblasts (HEL12469). The cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. For HEL12469 cells, minimal essential medium EMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1% non-essential amino acids, 0.2% sodium bicarbonate, 0.5% Penicillin 10 000 U/ml and 0.5% Streptomycin 10 000 µg/ml was used. A549 cells were cultivated in Minimum Essential Medium (MEM) + GlutaMAX™-I supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 0.5% Penicillin 10 000 U/ml and 0.5% Streptomycin 10 000 µg/ml. After reaching 70%–80% confluence, the medium was replaced with complete fresh medium. Test compounds were diluted with DMSO and added to the medium at three non-cytotoxic concentrations: for B[a]P and 3-NBA, 0.1, 1 and 10 µM; for EOMs, 1, 10 and 20 µg/ml. For the analysis of ROS production, the cells were treated for 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h and 24 h; for total antioxidant capacity and gene expression analysis, the cells were treated for 4 h and 24 h. The final concentration of DMSO did not exceed 0.1% of the total incubation volume.

The cytotoxicity of B[a]P, 3-NBA and EOMs in both cell lines was analyzed after 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h and 24 h exposure using a WST-1 Cell Proliferation Assay (Roche, Basel, Switzerland) according to manufacturer recommendations. Significant cytotoxicity was not observed at any of the test concentrations (Supplementary Fig. 1 and 2).

2.3. Reactive oxygen species production

Intracellular ROS production was measured using a Cellular ROS/superoxide detection assay kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. A combination of two detection dyes allowed a separate analysis of superoxide and other ROS, including hydrogen peroxide, peroxynitrite, hydroxyl radicals, nitric oxide and peroxy radical. The cells were grown in 96 well black wall/clear bottom