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Effect of fluoranthene on plant cell model: Tobacco BY-2 suspension culture

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) belong to the group of the most important pollutants of the living environment, which are present in air, soils, freshwater, seawater and sediments. They have very substantial effects on all living organisms including plants and animals. Plants represent important point in PAHs food chain entry. Despite the fact that PAHs influence on animals is intensively studied, effect on plants is almost unknown. In our study, action of polycyclic aromatic hydrocarbon fluoranthene on a plant cell experimental model – tobacco BY-2 cell suspension culture – was studied. BY-2 cells were exposed to the fluoranthene in concentration range from 0 to 1000 μM, duration of treatment was 120 h, respectively 5 days. Samples were collected in the strictly defined time intervals of 24 h. Exposure of the BY-2 cells led to significant changes in viability, changes in autofluorescence due to accumulation of fluoranthene in lipophilic cell compartments, especially biomembranes, and production of reactive oxygen species, which resulted in damage of biomembranes and disruption of their semipermeability and initiation of process of programmed cell death. Obtained results bring new knowledge about phytotoxicity of fluoranthene and mechanism of its action.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are lipophilic chemical compounds consisting of fused aromatic rings without heteroatoms. They are widely distributed organic pollutants, which are able to enter a soil as well as aquatic systems with subsequent deposition in the living organisms, especially thanks to their lipophilicity. They are formed anthropogenically during incomplete combustion of organic materials including fossil fuels; steel and iron industry, where PAHs are released from iron and steel making in foundry industry; coke manufacturing and mold poring and cooling belong to the group of the most important sources (Bhargava et al., 2004; Ciaparra et al., 2009; Dellantonio et al., 2010; Fang et al., 2004; Nizzetto et al., 2008; Tsai et al., 2001b,c, 2002). Petrochemical industry belongs to the most important producers of PAHs. Carbon black as important commodity and source for manufacturing of paints and lacquers represents important source of PAHs (Achten and Hofmann, 2009; Tsai et al., 2001a). Natural sources, such as forest fires and volcanic eruptions contribute also to their releasing into the living environment (Bourotte et al., 2009; Gabos et al., 2001; Yang et al., 2002). PAHs are worldwide distributed not only in air, soils, waters, but also in river and ocean sediments, and sludge (Fuoco et al., 2005; Gambaro et al., 2004; Ravindra et al., 2006; Thompson et al., 2002; Wilcke, 2000). They are able to entry food chain. Composting, which is widely employed for treatment of organic wastes, can be one of the most important entries of PAHs to food chain, especially in connection with the agriculture (Carlstrom and Tuovinen, 2003; Eom et al., 2007; Lashermes et al., 2010; Yang et al., 2010). Due their lipophicity and low water solubility, PAHs in waters are bound to suspended particles and accumulate in bottom sediments (Tam et al., 2001); subsequently, they are accumulated in tissues and organs of wide range of organisms, including freshwater and marine organisms, especially of those, which are associated with sediments (Fabbri et al., 2006). On the other hand, organisms capable of PAHs biodegradations are known. The most important are bacteria (*Pasteurella*), fungi (especially not only *Zygomycota*, but also *Basidiomycota*, such as *Pleurotus ostreatus*), and algae (*Cyclotella, Nitzschia*, *Skeletonema*) (Hong et al.,

Abbreviations: DHE, dihydroethidium; FDA, fluorescein diacetate; FLT, fluoranthene; HPLC, high performance liquid chromatography; PAHs, polycyclic aromatic hydrocarbons; PCD, programmed cell death; PI, propidium iodide; MDA, malondialdehyde; PCD, programmed cell death; ROS, reactive oxygen species.

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2008; Liu et al., 2006; Pozdnyakova et al., 2006; Salicis et al., 1999; Sepic et al., 2003; Schutzendubel et al., 1999; Thongkred et al., 2011; Valentin et al., 2007). Recently, new technological procedures for removing of PAHs are intensely studied (Hwang et al., 2007; Sayara et al., 2011; Sponza and Gok, 2010).

PAHs are at the focus of the interest due to their toxicity. Some PAHs appear to be non-toxic; some of them are activated biologically through cytochrome P450-mediated monooxygenation or photochemically, especially by the UV radiation, which leads to formation of more reactive PAHs structures (Ren et al., 1994). Benzo[*a*]anthracene and chrysene, benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, benzo[*ghi*]perylene, coronene, dibenz[*ah*]anthracene, indeno[1,2,3-*cd*]pyrene and ovalene are the most toxic PAHs, which have carcinogenic and mutagenic effects. This fact connected with the DNA damage and formation of PAHs-DNA adducts was demonstrated on *in vitro* (animal as well as human cell lines) and *in vivo* models in tissues and organs of organisms exposed to PAHs (Cavallo et al., 2006; Knuckles et al., 2004; Qu and Stacey, 1996; Taioli et al., 2007; Toyooka and Ibuki, 2007; Walker et al., 2007). Connection between different types of malignant tumors and PAHs has been demonstrated (Armstrong and Gibbs, 2009). One of the recent studies demonstrates fact than activation of some PAHs, which were considered non-toxic without UV radiation activation (e.g. benzo[*a*]anthracene, fluoranthene or pyrene), is not necessary to DNA damage. PAHs exhibit ability to induce oxidative stress (Sun et al., 2011). Oxidation stress is connected with generation of the reactive oxygen species (ROS), which may serve not only as signals of initiation of the processes leading to apoptosis, but also with damage of crucial cell structures and functions. Human exposure may occur via environmental pollution, processed foods, contaminated water, and green vegetable (Camargo et al., 2008; Camargo and Toledo, 2003; Schoket, 1999).

Plants are the important PAHs accumulators (Kacalkova and Tlustos, 2011). They enter plants either directly via stomata, or indirectly via root system (Kuhn et al., 2004). Lipophilic structures covering plant surface, especially wax layer and cuticle, not only represent places of PAHs accumulation and possible further transport, but also stabilization against UV radiation activation (Desalme et al., 2011; Oguntimehin et al., 2008; Oguntimehin and Sakugawa, 2008; Ratola et al., 2011). It is hypothesized that PAHs are accumulated in liphophilic compartments of cells, especially in biomembranes. Here they can be activated after sunlight activation with subsequent damage of cell structures including DNA (Ren et al., 1994). Interactions with processes of growth and especially photosynthesis with symptoms of chlorosis and necrosis (Oguntimehin et al., 2010), reduction of the photosynthetic rate and photosynthetic pigments (Kummerova et al., 2001, 2006). Fluoranthene-induced changes in root and stem anatomy – formation of lysigenous intercellular spaces – were demonstrated in the work of Vanova et al. (2011). In addition, negative effects of PAHs on seeds germination and subsequent seedlings development, especially in connection with the photoinduction expressed as a decrease of fresh weight, were demonstrated (Kummerova and Kmentova, 2004; Kummerova et al., 1997; Maliszewska-Kordybach and Smreczak, 2000; Sverdrup et al., 2003). Nevertheless, effect of PAHs on the plants on cell level is still predominantly unknown, except of studies of some PAHs on model plant – *Arabidopsis thaliana*, where induction of oxidative stress was demonstrated (Kolb and Harms, 2000). Fluoranthene (FLT), member of PAHs group, is used as model for investigation of PAHs toxicity, especially because of its reduced toxicity in comparison with other PAHs.

To better understanding the biochemical and cytological responses to FLT on cell level, tobacco BY-2 cells, which are usually compared to HeLa cells, as the most suitable plant cell model, were exposed to fluoranthene (Nagata et al., 1992). We worked with hypothesis, whether FLT is able to induce formation of ROS that are closely connected with damage of cell structures including lipid peroxidation and with processes of PCD. Changes in cell structure and viability as well as nuclear architecture including DNA fragmentation and processes of PCD were monitored. In addition, ability of FLT to generate reactive oxygen species with subsequent lipid peroxidation was investigated. Due to difficultness of PAHs analytical determination in biological samples because of their complexity, method for their rapid and accurate determination in biological samples represented by cultivation media and BY-2 cells was developed and used.

2. Materials and methods

2.1. Chemicals

Standard of the fluoranthene (purity 98.5%) was purchased from Sigma–Aldrich (USA). Murashige and Skoog cultivation medium including macro- and microelements and vitamins was purchased from Duchefa Biochemie B.V. (Denmark). All other chemicals, including fluorescence probes, chemicals for assays and solvents – ACS water and dimethylsulfoxide (DMSO) were obtained from Sigma–Aldrich (USA) unless otherwise indicated.

2.2. BY-2 cell suspension culture

Nicotina tabacum L. cv. Bright Yellow-2 suspension-cultured cells (BY-2) were grown in liquid medium (Murashige and Skoog, 1962) modified by Nagata (Nagata et al., 1992) under constant shaking (130 rpm) at 27 °C in the dark in 250 ml Erlenmeyer flasks. Cells in exponential growth phase were exposed to the fluoranthene added into cultivation medium in the form of stock solution (1 mg ml^{-1} in DMSO) in concentration range 0, 50, 100, 250, 500 and 1000 μM. As a positive control, BY-2 cells treated by DMSO (1%, v/v) were used. Samples were collected at 24 h intervals for 120 h.

2.3. Cell observation

Cell viability. The viability of the cells was measured by the addition of the fluorescein diacetate (FDA, Sigma–Aldrich, USA) and the propidium iodide (PI, Sigma–Aldrich, USA). The sample of the cell suspension culture (20 μl) was supplemented to volume of 50 μl by MS cultivation medium and incubated for 5 min at room temperature with FDA (2.4 μmol l^{-1}) and PI (30 μmol l^{-1}). PI as nucleic acid stain penetrates through damage cell membranes and intercalates DNA, so PI positive cells are dying or death. Living cells metabolize FDA to fluorogenic substrate fluorescein, so they emit green light after a excitation. The percentage of viable and death cells was evaluated by counting using the fluorescent microscope (Axioscop 40, Zeiss, Germany) equipped with the broad spectrum UV excitation, from each series was evaluated 10 random fields (minimally 1000 cells) in the microscope and the viability was determined in triplicates.

Nuclear architecture and mitotic index. For nuclei observation and mitotic index determination, cells (4 ml) were treated by adding 4 ml PEM-buffer (100 mM PIPES, 10 mM EGTA, 10 mM $MgCl_2$, pH 6.9) containing formaldehyde (4%, w/w; all Sigma–Aldrich, USA). Fluorescent probe Hoechst 33258 (Sigma–Aldrich, USA) was used. One thousand nuclei in each preparation were observed using the fluorescent microscope (Olympus AX 70, Germany) equipped with the broad-spectrum UV excitation. Each morphological changes as well as mitotic cells in individual mitotic phases were expressed as a percentage of the total cells, from each series, 10 random fields (minimally 1000 cells) were evaluated in triplicates.