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Time-dependent changes in antioxidative enzyme expression and photosynthetic activity of *Chlamydomonas reinhardtii* cells under acute exposure to cadmium and anthracene

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ABSTRACT

Heavy metals (HM) and polycyclic aromatic hydrocarbons (PAHs) are present in the freshwater environment at concentrations that can be hazardous to the biota. Among HMs and PAHs, cadmium (Cd) and anthracene (ANT) are the most prevalent and toxic ones. The response of *Chlamydomonas* cells to Cd and ANT at concentrations that markedly reduced the growth of algal population was investigated in this study. At such concentrations, both cadmium and anthracene were recognized as oxidative stress inducers, since high concentration of H_2O_2 in treated cultures was observed. Therefore, as a part of the "molecular phase" of the cell response to this stress, we examined the time-dependent expression of genes encoding the main antioxidative enzymes: superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX), as well as the activity of these enzymes in cells, with special attention paid to chloroplastic and mitochondrial isoforms of SOD. To characterize the cell response at the "physiological level", we examined the photosynthetic activity of stressed cells via analysis of chlorophyll *a* fluorescence in vivo. In contrast to standard ecotoxicity studies in which the growth end-points are usually determined, herein we present time-dependent changes in algal cell response to Cd- and ANT-induced stress. The most significant effect(s) of the toxicants on photosynthetic activity was observed in the 6th hour, when strong depression of PI parameter value, an over 50 percent reduction of the active reaction center fraction (RC_0) and a 3-fold increase in non-photochemical energy dissipation (DI_0/RC) were noted. At the same time, the increase (up to 2.5-fold) in mRNA transcript of SOD and CAT genes, followed by the enhancement in the enzyme activity was observed. The high expression of the *Msd 3* gene in treated *Chlamydomonas* cells probably complements the partial loss of chloroplast Fe-SOD and APX activity, while catalase and Mn-SOD 5 seem to be the major enzymes responsible for mitochondrion protection. The progressive increase in SOD and CAT activities seems to be involved in the recovery of photosynthesis within 12–24 h after the application of the toxicants.

1. Introduction

The occurrence of anthropogenic stress-induced chemicals such as metals, petroleum products or herbicides in the water environment presents a serious problem today. The extent of pollution depends mainly on the use of the substance in industry/agriculture and characteristics of the area contaminated (Ahmad et al., 2010; Malik et al., 2011). Aquatic organisms are therefore often subjected to relatively high concentrations of toxicants, among which polycyclic aromatic hydrocarbons (PAHs) and heavy metals (HMs) are of great interest. These two classes of anthropogenic contaminants are widespread in all environmental

Abbreviations: ABS/RC, the amount of energy absorbed by single reaction center of photosystem II; ANT, anthracene; ANT-cells, cells treated with anthracene; APX, ascorbate peroxidase; AsA, ascorbate; CAT, catalase; Cd, cadmium; Cd-cells, cells treated with cadmium; Cu/Zn-SOD, copper-zinc superoxide dismutase; DI_0/RC, nonphotochemical energy dissipation by single reaction center of photosystem II; EDTA, ethylenediaminetetraacetic acid; ET_0/RC, the amount of energy used for electron transport by single reaction center of photosystem II; Fe-SOD, iron superoxide dismutase; HM, heavy metal; HSPs, heat shock proteins; Mn-SOD, manganese superoxide dismutase; PAH, polycyclic aromatic hydrocarbon; PI, photosynthetic "vitality"; PS II, photosystem II; PVP, polyvinylpyrrolidone; RC, reaction center of PS II; ROS, reactive oxygen species; SOD, superoxide dismutase; TR_0/RC, the amount of energy trapped in single reaction center of photosystem II

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compartments (air, soil, water, sediments). Due to their high toxicity and persistence in the environment, they are considered to be a health and environmental problem and arouse the interest not only of researchers, but also of public authorities (see e.g. EC, 2002; EU, 2002; EURAR, 2007).

Cadmium (Cd) present at high concentrations in industrial areas (Bhagure and Mirgane, 2011; D'Emilio et al., 2013) is one of the toxic metals that have been the most strictly monitored and thoroughly investigated (EFSA, 2009a, b). It enters the environment mainly from steel, pigment and pesticide production, and industrial waste disposals (Alloway, 1990). Cadmium toxicity is believed to result primarily from its interaction with sulfhydryl, carboxyl and imidazole groups of enzymes, causing inhibition of many physiological processes, including photosynthesis and respiration (Prasad et al., 1998; Aravind and Prasad, 2004; Wang and Wang, 2008). This, in turn, can be the reason for enhanced reactive oxygen species (ROS) formation leading to oxidative stress (Cuypers et al., 2012).

Oxidative stress has also been commonly observed in plant cells treated with polycyclic aromatic hydrocarbons, although the mechanism of their toxicity is not the same as that of HMs (Aksmann and Tukaj, 2008; Mujtaba et al., 2013). The risk of PAHs to the environment is connected not only with their acute toxicity but also with their mutagenic and carcinogenic effects. For this reason, the European Union Water Framework Directive (EU-WFD) (EC, 2008) and European Food Safety Authority (EFSA) (EFSA, 2008) recommended the monitoring of a set of PAHs in the environment and in food sources. Anthracene (ANT), a three-ring polycyclic aromatic hydrocarbon is commonly found in complex mixtures such as fossil fuels, automobile exhaust or crude oil (Martins et al., 2011). In the European Union Risk Assessment Report (EURAR, 2007), it has been classified as "very toxic to aquatic organisms that may cause long-term adverse effects in the aquatic environment". Anthracene, known as a highly hydrophobic compound, readily accumulates in biological membranes (Duxbury et al., 1997), thus inhibiting photosynthetic and respiratory activity of the cells (Huang et al., 1997; Aksmann and Tukaj, 2008; Aksmann et al., 2011). As a result of electron transport chain disruption, an overproduction of ROS and oxidative damages have been reported (Huang et al., 1997; Debiane et al., 2008).

The population's ability to survive in the environment contaminated with HMs and PAHs depends on acclimation capacity of individuals that starts from molecular response involving stress recognition, signal transduction, gene transcription and translation (Rao et al., 2006). To explore such early stages of cells' reaction to a toxicant, ecotoxicogenomic studies use changes in the transcriptomic profile of the test organisms, giving an insight into the physiological response to the stress factor (Hutchins et al., 2010).

Superoxide dismutases (SODs), catalases (CATs) and ascorbic peroxidases (APXs) are enzymatic ROS (reactive oxygen species) scavengers that participate in cell protection under stress conditions (Pokora and Tukaj, 2013; Torres et al., 2008). SODs catalyze the reaction of disproportionation of the superoxide anion to oxygen and hydrogen peroxide that is further scavenged by catalases and peroxidases. Since biological membranes are impermeable for the superoxide anion, SOD isoforms exist in cell compartments where $O_2^{-\cdot}$ production occurs: in the chloroplast, mitochondrion, cytosol and apoplastic space (Asada, 1999). APXs are present in chloroplast, cytosol and microbody, while the CAT activity is found in peroxisomes and mitochondrium (Kato et al., 1997; Mittler, 2002). The activities of SOD, CAT and APX are mutually closely dependent due to the fact that the product of SOD-catalysed reaction becomes a substrate for APX and CAT. Moreover, the intermediates of hydrogen radical anion reduction to oxygen molecule play an important role in a number of regulatory and signaling processes (Gadjev et al., 2006).

Hydrogen peroxide (H_2O_2) plays a key role in the regulation of antioxidative enzymes expression, as well as in expression of other defense proteins, such as pathogenesis-related proteins or heat shock proteins (HSPs) (Knight and Knight, 2001). It has been proved that in higher plants hydrogen peroxide is a part of a signal transduction cascade leading to expression of CAT1 and APX1 proteins (Gadjev et al., 2006 and references therein). The importance of H_2O_2 and other ROS as signaling molecules was shown not only in higher plants, but also in cells of green alga *Chlamydomonas reinhardtii* (Fischer et al., 2007; Shao et al., 2008). *C. reinhardtii* is regarded as a model organism because of its high resemblance to a single cell of a higher plant (Harris, 2009). The organism is a useful model to investigate many physiological and biochemical processes at both cellular and molecular levels (Grossman, 2005; Harris, 2009) as well as to study plants' response to stress factors (Hema et al., 2007). The above is a result of its well-defined classical genetics, development of molecular tools that fit to *Chlamydomonas* (Rochaix, 1995), the sequencing of its nuclear genome and availability of many different mutants (Harris, 2009).

In the present work we investigate the response of *C. reinhardtii* to the acute toxicity of two different environmental pollutants, cadmium and anthracene, both known as oxidative stress inducers (Aksmann and Tukaj, 2004; Martínez-Peñalver et al., 2012). In contrast to standard ecotoxicity studies in which only the end point effects are presented (Aksmann et al., 2011; Liu et al., 2009; Perreault et al., 2011; Pokora et al., 2011; Tukaj et al., 2007; Tukaj and Aksmann, 2007), the aim of this study was to investigate the time-dependent changes in algal cell response to chemically-induced stress, analyzed during a 24-hour exposure to the toxicants.

To investigate the cell response at the "physiological level", we examined the photosynthetic efficiency of stressed *Chlamydomonas* cells by analyzing chlorophyll *a* fluorescence in vivo. In the examination of the "molecular phase" of cell response, we focused on the expression of genes encoding the main antioxidative enzymes: superoxide dismutase, catalase and ascorbate peroxidase and on the activity of the enzymes. For more complex and informative investigations, we decided to identify not only the activity but also the level of transcripts for particular enzymes' isoforms. Since in algal cells the photosynthetic and respiratory electron transport chains are believed to be affected by ANT and Cd, causing the overproduction of superoxide radical (Martínez-Peñalver et al., 2012; Perreault et al., 2011; Pokora and Tukaj, 2010), we paid special attention to the chloroplastic and mitochondrial isoforms of superoxide dismutase.

2. Materials and methods

2.1. Cultures

Chlamydomonas reinhardtii strain CC-125 was purchased from *Chlamydomonas* Resource Center, University of Minnesota, USA.

Anthracene (high purity, Aldrich Chemicals Co., USA) was dissolved in dimethylsulfoxide (DMSO) (Acros Organics, Belgium). DMSO (0.1 percent v/v) had no significant effect on the growth of *Chlamydomonas* cells (Aksmann and Tukaj, 2008). Cadmium ($CdCl_2 \cdot H_2O$, analytical grade; Merck, Germany) was dissolved in redistilled water. Final nominal concentration of chemicals in culture media was 5 μM for ANT and 95 μM for $CdCl_2$. These concentrations were chosen because they were previously found to cause inhibition of population growth but were not algicidal (Supplement, Fig. 1).

The batch cultures were set up by dilution of the pre-culture to an initial density of 0.5×10^6 cells mL^{-1}. Algae were grown in HSM medium (Harris, 2009) in 200 mL glass test tubes submerged in a thermostated water bath at a constant temperature of 30 °C, under continuous fluorescent light from TLD 58W/54 lamps (Philips), providing 80 μmol m^{-2} s^{-1} of irradiation. The cultures were aerated with a sterile gas mixture containing 2.5 percent CO_2 passed through a filter (Sartorius 2000; 0.2 μm PTFE). Chemicals were added to the cultures when set up. For each