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Nitric oxide production inhibited by xenobiotic compounds in the protozoan *Paramecium primaurelia*

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ABSTRACT

The notable increase in agricultural and industrial activities over the last decades has caused a considerable increase in anthropogenic waste and, consequently, the presence of pollutants in both water and sediments. For this reason, there is great interest in identifying alternative models and bioassays complying with the 3Rs strategy (aimed at Reducing, Refining and Replacing tests on vertebrate organisms in toxicological studies). Protozoa seem to be well suited to this strategy and it is widely accepted that assays with protozoa are relevant to the study of environmental modifications due to the presence of xenobiotic compounds.

Recently, we detected the presence of nitric oxide synthase (NOS)-related NADPH-diaphorase activity and neuronal NOS-related molecules, immunologically recognized by the anti-rat brain NOS antibody, in a single-cell freshwater eukaryote, *Paramecium primaurelia*. In this work we have looked for the basal NO production in living cells of *P. primaurelia* using the specific fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2 DA) and measuring the intracellular NO levels with image analysis. The NO production was sensitive to compounds modulating NOS activity such as: S-methyl-tiocitrulline, an NOS activity inhibitor, L-NAME, an analogue of arginine that inhibits NO production, arginine, an NOS substrate, or sodium nitroprusside, an NO donor. The NO production in *P. primaurelia* was also shown to be sensitive to μM concentrations of heavy metals ($HgCl_2$ and $CdCl_2$), or μM concentrations of pesticides (diazinon and AFD 25), thus representing a potential biomarker for environmental biomonitoring. The possible involvement of cellular Ca^{2+} concentration, assayed by the fluorescent probe chlortetracycline hydrochloride, in NO production was examined after xenobiotic exposure.

1. Introduction

There is increasing evidence that nitric oxide (NO) is a molecular mediator involved in signal-transduction pathways and stress–response mechanisms (Colasanti and Suzuki, 2000) and its production has been found to be sensitive to a variety of environmental contaminants, including pesticides and heavy metals such as mercury and copper (Morrall et al., 1998; Smith et al., 2000; Kim et al., 2002; Ji et al., 2005; Casares and Mantione, 2006). In a recent study, we detected the presence of NO-synthase (NOS)-related NADPH-diaphorase activity and molecules immunologically recognized by the anti-rat brain (b)NOS antibody in a single-cell eukaryote, *Paramecium primaurelia* (Amaroli et al., 2006). Our previous results on the presence of transmitters in protozoa revealed that *P. primaurelia* also synthesizes molecules functionally related to the GABAergic and cholinergic signalling systems, playing a role in modulating cell–cell communications (Trielli et al., 1997; Delmonte Corrado et al., 1999, 2001, 2002) as well as a cholinesterase activity detected in both *Dictyostelium discoideum* (Falugi et al., 2002; Amaroli et al., 2003) and *Euplotes crassus* (Trielli et al., 2007). A remarkable finding of our investigations was that the acetylcholinesterase (AChE) enzyme activity detected in *P. primaurelia* was inhibited by low concentrations of neurotoxic drugs, such as organophosphate and carbamate, compounds used world-wide for agricultural pest control and potentially harmful for non-target wildlife. Thus, as in higher organisms (Hassal, 1990), the inhibition of AChE activity in *P. primaurelia* represents a potential specific biomarker of exposure to neurotoxic drugs (Delmonte Corrado et al., 2005, 2006). Due to its features as a single-cell eukaryotic organism, widely occurring in freshwater ecosystems where it plays a key role in the food chain as a component of the microfauna, *P. primaurelia* has been proposed as a bioethical and excellent test organism for standardized laboratory procedures to evaluate environmental quality and the effects of xenobiotic compounds on a simple alternative biosystem (Delmonte Corrado et al., 2005, 2006). *P. primaurelia*, and the protozoa in general, is well suited to being included in the increasing panel of organismic systems that could

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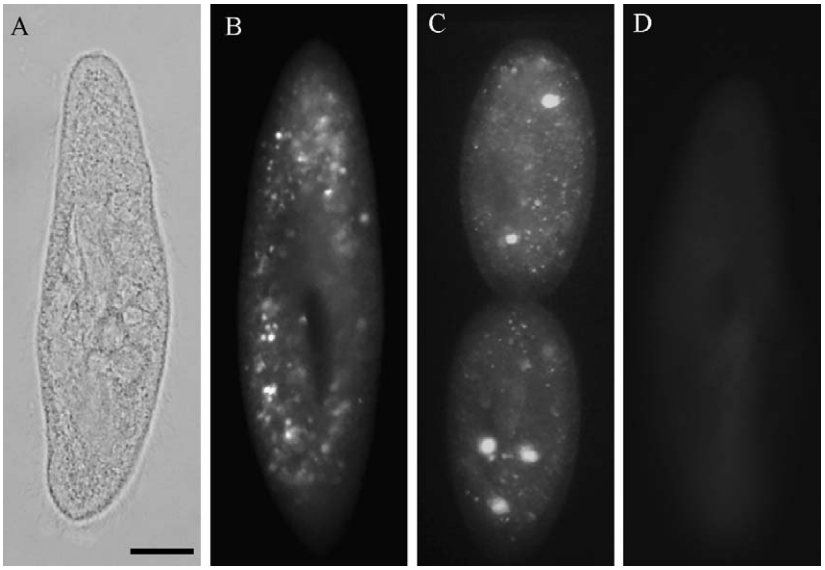

Fig. 1. Basal NO production. Image of a logarithmically-growing cell (A). Pattern of the DAF-2 fluorescent signal in a non-dividing cell (B) and dividing cell (C). Control cell (D). Scale bar = 20 μm.

meet the 3Rs strategy (aimed at Reducing, Refining and Replacing tests on vertebrate organisms in toxicological studies) and sensitive to such environmental perturbations as the occurrence of xenobiotic compounds. The sensitivity of protozoa is due to their simple eukaryotic single-cell/organism organization which exposes their receptors to the external environment, making them respond directly to environmental stimuli. Moreover, thanks to their short cell-cycles it is possible to study the effects of pollutants on a large and genetically homogeneous cell population over a short period of time as well as on subsequent cell generations (Delmonte Corrado et al., 2006). For this reason, over the last few decades, protozoa have been exploited as excellent tools for environmental biomonitoring, either as bioindicators of pollution or bioassays to evaluate the effects of toxic compounds (Apostol, 1973; Persoone and Dive, 1978; Nilsson, 1989; Lynn and Gilron, 1992; Madoni et al., 1994; Ricci, 1995; Sauvant et al., 1999; Gutierrez et al., 2003).

In this work, we looked for the basal NO production in living cells of *P. primaurelia*, using the specific fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2 DA) and measuring the intracellular NO levels with image analysis. To characterize the NO production in *P. primaurelia* we evaluated the effects of cell exposure to compounds modulating NOS activity. The aim of this study was to assay the effects of heavy metals (mercury and cadmium) and pesticides (diazinon and AFD 25) on the production of NO in *P. primaurelia*, which is a potential environmental biomarker. In addition, we looked for the possible involvement of cellular Ca^{2+} concentration in NO production after heavy metal- or pesticide-exposure.

2. Materials and methods

2.1. Reagents

The following compounds were utilized: 4,5-diaminofluorescein diacetate (DAF-2 DA) (Sigma, I); S-methyl-thiocitrulline (acetate salt) (Sigma, I); N_ω-nitro-L-arginine methyl ester (L-NAME) hydrochloride (Sigma, I); L-arginine (Sigma, I); sodium nitroprusside (Sigma, I); chlortetracycline hydrochloride (CTC) (Fluka, I); EGTA (Sigma, I); caffeine (Sigma, I) and the following compounds were tested: $HgCl_2$ (Sigma, I); $CdCl_2$ (Sigma, I); diazinon (Sigma, I), an active thionophosphate principle; AFD 25 (Cifo, I), an organochlorine (OC) hydrocarbon containing 24% dicofol, an OC active principle.

2.2. Cells and culturing methods

The experiments were carried out at 25 ± 1 °C on logarithmically-growing cells of *P. primaurelia* stock 90 (Fig. 1A), kindly supplied by Prof. Geoffrey H. Beale (Edinburgh) many years ago. The cells were cultured at 25 ± 1 °C in a lettuce medium (pH 6.8), previously inoculated with *Enterobacter aerogenes* as described by Sonneborn (1970).

2.3. Procedure for detecting NO production

NO production was detected using the DAF-2 DA fluorescent probe, which enters the cell and is hydrolyzed to DAF-2 by cytosolic esterases, binding the NO and producing triazolofluorescein, a green-fluorescent molecule. This method is very sensitive, being able to detect nanomolar amounts of NO, and highly specific, as DAF-2 does not bind any oxygen radicals other than NO and fluorescence increases in an NO concentration-dependent manner (Kojima et al., 1998). However, as shown by Planchet and Kaiser (2006), in cell suspensions derived from tobacco, DAF-2 can react with an unidentified stable compound produced and released in the medium by cells under certain conditions. Therefore, a characterization of NO production by compounds modulating NOS activity is necessary. The cells were incubated for 1 h with DAF-2 DA at the final concentration of 5 μM. After rinsing, the cells were exposed to 1% paraformaldehyde in a phosphate buffer (PB) and immediately transferred onto slides for image analysis. The controls were prepared by omitting the DAF-2 DA-incubation. To characterize the NO production, the cells were pre-incubated with 5 mM or 50 mM S-methyl-thiocitrulline, an NOS activity inhibitor, for 1 h; 100 μM L-NAME, an analogue of arginine that inhibits NO production, for 20 min; 1 mM L-arginine, an NOS substrate for NO production, for 1 h; 1 mM L-arginine in the presence of S-methyl-thiocitrulline at the concentrations of 5 mM and 50 mM, for 1 h; and 1 mM sodium nitroprusside, an NO donor, for 30 min. Each experiment was carried out in fivefold replicate and the sample size of each experiment was 30 cells.