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Accumulation of zinc protects against cadmium stress in photosynthetic *Euglena gracilis*

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ABSTRACT

To determine the interplay between zinc and cadmium on the heavy metal accumulation capacity of *Euglena gracilis*, the effects of increasing Zn^{2+} concentrations (13–65.4 ppm or 200–1,000 μM) were analyzed on growth; O_2 consumption; photosynthesis; ascorbate (APX) and glutathione peroxidase (GPX) activities; chlorophyll a and b (Chl a+b) content; essential metals, thiol-metabolites and polyphosphates (polyPs) levels; as well as on zinc and cadmium accumulation capacities. Control cells (*Eg*Zn_{20}; grown with 20 μM Zn^{2+}) showed a half-maximal inhibition of growth (IC_{50}) of 1,700 μM by external Zn^{2+}. O_2 consumption, and APX and GPX activities were unaltered by Zn^{2+} treatments. Cells cultured with 500 or 1,000 μM Zn^{2+} showed photosynthesis impairment but normal Chl a+b contents. Zn^{2+} preconditioning increased the intracellular contents of zinc (25–54 times) and calcium (2–27 times); thiol-metabolites and polyPs were only marginally altered. The growth of cells preconditioned to 400 μM Zn^{2+} (*Eg*Zn_{400} cells) was less susceptible to Cd^{2+} than that of *Eg*Zn_{20} cells, although no differences in photosynthesis and respiration were observed. In cells chronically grown with Zn^{2+}, the cadmium accumulation capacity was unchanged or slightly increased in the same culture media with high Zn^{2+}, and increased by 42–90% in media with 20 μM Zn^{2+}. The thiol-metabolites increased at similar levels in both *Eg*Zn_{20} and *Eg*Zn_{400} cells when further exposed to 200 μM Cd^{2+} and polyPs were at high levels independently of Zn^{2+} or Cd^{2+} treatments. It was concluded that chronic exposure to high Zn^{2+} (1) was innocuous for *E. gracilis* at concentrations lower than 0.5 mM and (2) promoted protection against Cd^{2+} toxicity and increased cadmium accumulation; and (3) these zinc effects involved GSH and polyPs metabolism and were associated with high intracellular zinc contents.

1. Introduction

Zinc is an essential heavy metal for humans although at high intracellular concentrations is linked to formation of β-amyloid plaques associated with Alzheimer's disease, neuronal death, and degeneration of pancreatic cells (Maret, 2013; Sekler et al., 2007). On the other hand, in water bodies polluted with metals, zinc concentrations are 2.1–134 ppm or 32–2,050 μM (Bervoets and Blust, 2003; Luís et al., 2011; Sarmiento et al., 2011), while maximal concentration ranges of some toxic metals are 0.2–1.76 ppm of cadmium [1.8–16 μM]; 0.01–2.1 ppm of chromium [0.2–40 μM]; 0.2–2.2 ppm of nickel [3–37 μM]; and 0.7–4 ppm of lead [3–19 μM] (Bervoets and Blust, 2003; Luís et al., 2011; Rehman et al., 2007; Sarmiento et al., 2011; Vázquez-Suaceda et al., 2012). In comparison, maximal reported metal concentrations in polluted soils are 13,231 ppm for zinc, 1,896 ppm for cadmium, 1,028 ppm for chromium, 13,267 ppm for lead, and 4,710 ppm for nickel (Broadhurst and Chaney, 2016; Özkul, 2016; Yanqun et al., 2005).

The simultaneous exposure to Zn^{2+} (>13 ppm or 200 μM) and Cd^{2+} (0.0079–1.1 ppm or 0.007–10 μM) induces in the green alga *Chlamydomonas reinhardtii* and free-floating aquatic plant *Ceratophyllum demersum* L. lower cadmium accumulation (up to 85%), preservation of photosynthesis, and enhanced activities of antioxidant (SOD, catalase, ascorbate peroxidase) and GSH metabolism (GSH-S-transferase and GSH peroxidase) enzymes (Aravind et al., 2009; Aravind and Prasad, 2004, 2005; Lavoie et al., 2012b). Wheat (*Triticum aestivum* L.) seedlings acclimated with

Abbreviations: APX, ascorbate peroxidase; Chl a+b, chlorophyll a and chlorophyll b; DW, dry weight; *Eg*PCS, phytochelatin synthase from *E. gracilis*; GPX, glutathione peroxidase; GSH, glutathione; γ-EC, γ-glutamylcysteine; IC_{50}, half maximal inhibitory concentration for cell growth; PolyGSH, polymers of glutathione; PCs, phytochelatins; PolyPs, polyphosphates; ppm, parts *per* million.

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0.32 ppm (5 μM) zinc for 6 days also show decreased cadmium accumulation and increased catalase and SOD activities (Li and Zhou, 2012).

In contrast, the marine green alga *Dunalliela tertiolecta* preconditioned with 6.5 ppm (100 μM) zinc for 12 h was more resistant than control cells to 22.4–45 ppm (200–400 μM) cadmium, 2–5 ppm (10–25 μM) mercury, 0.37–3.74 ppm (5–50 μM) arsenate, 3.2–6.3 ppm (50–100 μM) copper, and 207.2 ppm (1,000 μM) lead (Tsuji et al., 2002). Protection against metals toxicity by zinc was attributed to increased biosynthesis of phytochelatins (PCs), which were undetectable in non-preconditioned cells (Tsuji et al., 2002). PCs are glutathione polymers that bind and inactivate heavy metals intracellularly, and were firstly described in yeast (Kondo et al., 1983; Murasugi et al., 1981), although nowadays it is known that are biosynthesized in plants, worms, yeasts, algae and protists exposed to cadmium, zinc or other essential and non-essential heavy metals and metalloids (Bräutigam et al., 2011; Brunetti et al., 2011; Clemens, 2006; Cobbett, 2000; García-García et al., 2014; Grill et al., 1985; Heiss et al., 2002; Hirata et al., 2001; Huang et al., 2012; Li et al., 2004, 2006; Oven et al., 2002; Ramos et al., 2007; Ray and Williams, 2011; Rea, 2012; Sarry et al., 2006; Tennstedt et al., 2009; Tsuji et al., 2003).

The high cadmium accumulation capacity described for the protist *E. gracilis* (4–9 mg/g dry weight) depends on an active PCs synthesis, in which Cys and GSH are key precursors and phytochelatin synthase (*Eg*PCS) is one of the key enzymes (García-García et al., 2012; Mendoza-Cózatl et al., 2002; Mendoza-Cózatl and Moreno-Sánchez, 2006a). Exposure of *E. gracilis* to Cd^{2+} (5.6 ppm or 50 μM) and Zn^{2+} (0.32–19.6 ppm or 5–300 μM) simultaneously for 8 days shows that the cadmium accumulation capacity is not affected (83–125 nmol Cd/10^7cells) by external Zn^{2+} (Mendoza-Cózatl et al., 2006b), suggesting that Zn^{2+} does not interfere with Cd^{2+} uptake and accumulation in this freshwater unicellular microorganism. In this regard, it has recently been described that the zinc-*bis*-glutathionate (Zn-GS_2) complex is the best co-substrate of *Eg*PCS over Cd-GS_2 (García-García et al., 2014).

Thus, as Zn^{2+} and Cd^{2+} are able to activate the same molecular mechanisms in *E. gracilis*, it seems plausible that chronic exposure (by more than 10 cell generations) to high Zn^{2+} concentrations could enhance both the cadmium accumulation and resistance in a process mediated by increased biosynthesis of PCs, which is a biotechnologically relevant feature of this microorganism for bioremediation of Cd^{2+} polluted aquatic systems. This hypothesis was assessed by determining the chronic effects of 13–65.4 ppm (200–1,000 μM) Zn^{2+} on several physiological parameters and zinc and cadmium accumulation capacities of *E. gracilis*.

2. Materials and methods

2.1. Growth conditions

Axenic photo-heterotrophic cultures of *Euglena gracilis* Klebs (a Z-like strain) were grown in acidic (initial pH 3.5) Hutner medium, as previously reported (García-García et al., 2012). This medium contained 34 mM glutamic acid, 15 mM malic acid, 2 mM $CaCO_3$, 0.007 mM $CoCl_2$, 0.003 mM $CuSO_4{\cdot}5H_2O$, 2 mM $MgSO_4{\cdot}7H_2O$, 0.01 mM $MnSO_4{\cdot}4H_2O$, and 0.02 mM $ZnSO_4{\cdot}7H_2O$ among other components. Inocula of 0.2×10^6 cells/mL from cultures of 5 days old (at the end of the exponential growth phase) were used to initiate a new culture. Cells grown in this culture medium (with 20 μM Zn^{2+}) were considering control cells and labeled as *Eg*Zn_{20}. The laboratory *E. gracilis* strain used in the present study proceeded from the collection of the Parasitology Department, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional. This strain was grown in culture media containing 300 μM $ZnCl_2$ for over 20 years. These cells, further cultured with 20 μM $ZnCl_2$ for over 8 years, were used in the experiments described in this work.

The growth medium with 20 μM Zn^{2+} (control medium) was further supplemented with different aliquots from $ZnCl_2$ stock solutions (163–272 mM), calibrated by atomic absorption spectrophotometry (AAS) and sterilized by ultra-filtration through 0.22 μm pore diameter sterile cellulose esters membranes (Millipore, Bedford, MA, USA), to achieve 200–3,000 μM Zn^{2+}. Cell inocula were added after fixing the indicated final Zn^{2+} concentrations. For Zn^{2+} preconditioning, cells were cultured at the indicated Zn^{2+} concentration and a cell aliquot transferred to fresh medium with the same Zn^{2+} concentration every 5 days for at least 2 subsequent sub-cultures. *E. gracilis* cells preconditioned to 200, 300, 400, 500 and 1,000 μM Zn^{2+} were labeled as *Eg*Zn_{200}, *Eg*Zn_{300}, *Eg*Zn_{400}, *Eg*Zn_{500}, and *Eg*Zn_{1000}, respectively.

Cell cultures with 1–500 μM $CdCl_2$ were carried out as described above. The $CdCl_2$ stock solutions were also calibrated by AAS, sterilized by filtration, and added to the culture medium before the cell inoculum. It should be noted that the $ZnCl_2$ and $CdCl_2$ concentrations used in the present study yield fully ionized solutions with negligible binding by the culture medium components because the culture medium initial pH is 3.5, and remains in the acidic range (pH of 5–6) after 5–8 days of cell culture.

The incubation conditions for all cell cultures were cycles of 12 h light (70 μmol quanta $m^{-2}\,s^{-1}$)/12 h dark at 20–25 °C. Cell growth and viability were determined by counting HCl-immobilized cells with a hemocytometer and incubating with 0.05% (w/v) trypan blue for 2 min at 25 °C, respectively.

2.2. Determination of dry weight

Dry weight was determined for *Eg*Zn_{20}, *Eg*Zn_{200}, *Eg*Zn_{300}, *Eg*Zn_{400}, *Eg*Zn_{500} and *Eg*Zn_{1000} cells grown for 5 and 8 days under control conditions and in media supplemented with 200 μM Cd^{2+}. Cells were harvested by centrifugation for 2 min at 1,464 *g* and 4 °C and washed with SHE buffer (225 mM Sucrose, 10 mM HEPES, 1 mM EGTA pH 7.3). Thereafter, cells were counted and aliquots of $0.5–2 \times 10^8$ cells were dried at 60 °C for 24 h, although no changes in dry weight (DW) were observed after 6 h heating. The equivalences obtained (see Table 3) were similar to values previously reported (Buetow and Levedahl, 1962).

2.3. Determination of the rates of photosynthesis and respiration

Cells were harvested by centrifuging for 1 min at 1,464 *g* and 4 °C and washed with KME buffer (120 mM KCl, 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 1 mM ethylene glycol-bis(aminoethyl ether)-*N*,*N*'-tetra acetic acid (EGTA), pH 7.2. The cells were re-suspended in KME buffer and stored in ice. A cell aliquot ($1–2 \times 10^7$ cells/mL) was added to the oxymeter chamber (which was placed inside a dark box) that contained KME buffer at 25 °C. A Clark-type electrode was used to determine the oxygen consumption/production rates. Initially, it was registered the cellular O_2 consumption rate in the dark (2–5 min), followed by irradiating a white light beam (10,000 μquanta $m^{-2}\,s^{-1}$) to the cellular suspension to determine the maximal cellular O_2 production rate (*i.e.*, photosynthesis). Thereafter, light was turned off to repeat once the same dark/light cycle to determine the rates of respiration and photosynthesis.

2.4. Determination of ascorbate peroxidase (APX) activity

Cells grown under control conditions, preconditioned to Zn^{2+} and *Eg*Zn_{20} cells exposed to 200 μM Cd^{2+} were harvested after 5 days by centrifugation for 5 min at 1,464 *g* and 4 °C, and washed