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Nitric-oxide generation induced by metals plays a role in their accumulation by *Phallusia nigra* hemocytes

Cintia Monteiro de Barros[a,d,*], Danielly da Fonte Carvalho Martins[a,d], Andressa de Abreu Mello[a,d], Leonardo Tavares Salgado[c], Silvana Allodi[b]

[a] *Laboratório Integrado de Morfologia, Núcleo em Ecologia e Desenvolvimento Sócio Ambiental de Macaé – NUPEM, Universidade Federal do Rio de Janeiro, Campus UFRJ - Macaé, Macaé, RJ, Brazil*

[b] *Laboratório de Neurobiologia Comparativa e do Desenvolvimento, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, UFRJ, Rio de Janeiro, RJ, Brazil*

[c] *Instituto de Pesquisas, Jardim Botânico do Rio de Janeiro, Rio de Janeiro, RJ, Brazil*

[d] *Pós-Graduação em Produtos Bioativos e Biociências, Universidade Federal do Rio de Janeiro, Campus UFRJ - Macaé, Macaé, RJ, Brazil*

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ABSTRACT

Ascidians are good monitors for assessing water quality, since they filter large volumes of water; however, little is known about how xenobiotics, including metals, can affect ascidian hemocytes. Metals can be either toxic or beneficial to health, inducing many different responses. The response mechanism depends on the class of metals to which organisms are exposed: essential, nonessential, and borderline. To analyze the influence of metals from different classes on the protective mechanisms of an ascidian, we investigated the production of nitric oxide (NO) after exposure to various concentrations of Mg, Mn and Pb over different time periods. We also determined the amounts of each metal in the hemocytes. Our results indicated that especially Pb could stimulate NO production. Although Pb induced the highest NO production, cell viability was not severely altered in all Pb concentrations and time periods. Ascidians might serve as biomonitor for Pb, since their vanadocytes accumulate Pb.

1. Introduction

More than 100,000 chemicals are released annually into the environment as a consequence of their production, disposal and use. Among these chemicals, around 30 metals and metalloids are potentially toxic to humans, and affect cells and living organisms in many ways (Chiarelli and Roccheri, 2014). In contrast to vertebrates, invertebrates have relatively simple immune systems that are highly sensitive to pollutants, and are therefore excellent organisms to monitor their effects on the environment (Galloway and Depledge, 2001). Ascidians are especially good bioindicators, since their distribution may reflect some of the predominant factors that affect the benthic community. As filter-feeders, ascidians sieve large volumes of water and consequently can accumulate toxic and non-toxic metals, such as V and Hg, in their tissues (Papadopoulou and Kanias, 1977; Parrinello et al., 2017). Ascidian vanadocytes, a type of hemocyte also named signet-ring cell (Sawada et al., 1993; De Barros et al., 2009, 2014), concentrate the elements V, Fe and Mn and consequently accumulate higher concentrations of these elements than the concentrations found in seawater (Michibata et al., 1986, 2002). The ascidian *Phallusia nigra* has the capacity to accumulate larger amounts of Pb and other metals in its tissues than other species, even in areas where these metals are present at very low concentrations (Abdul Jaffar Ali et al., 2015).

Metals and metalloids can be separated into three classes, essential, nonessential, and a borderline class, according to their activity or function in living organisms. In general, essential metals participate in enzymatic reactions and in RNA stability, and are toxic only in overdoses, whereas nonessential metals are xenobiotics and highly toxic (Fraústo da Silva and Williams, 1993; Tamás and Martinoia, 2005).

The effects of metals on ascidians and other invertebrates are little known. In the ascidian *Halocynthia roretzi*, Mg induces hemocyte aggregation (Takahashi et al., 1994), and in the colonial ascidian *Botryllus schlosseri*, Mg is essential for phagocytosis (Ballarin et al., 1994). Mn exposure induces abnormalities in embryos of the sea urchin *Paracentrotus lividus*, and impairs fertilization processes, causing modifications in the reproductive state, mediated by nitric oxide (NO) (Migliaccio et al., 2014; Migliaccio et al., 2015). Mn affects the pollination activity of honeybees and can accumulate in their tissues (Søvik et al., 2015). In addition, Mn reduces the bactericidal capacity of circulating hemocytes in the Norway lobster *Nephrops norvegicus* (Oweson

* Corresponding author at: Núcleo em Ecologia e Desenvolvimento Sócio Ambiental de Macaé - NUPEM, Universidade Federal do Rio de Janeiro, Av. São José do Barreto 762, São José do Barreto, 27910-970 Macaé, RJ, Brazil.

E-mail address: cintiabarros@macae.ufrj.br (C.M. de Barros).

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and Hernroth, 2009). In the hemolymph of the housefly *Musca domestica*, Pb affects the proportion of hemocyte types and also alters the cell morphology (Borowska and Pyza, 2011); and in the rockpool prawn *Palaemon elegans*, Pb induces a reduction in the number of hemocytes (Lorenzon et al., 2001).

The functions and effects of NO are better studied. In mammals, NO is a widespread inter- and intracellular signaling molecule, with a variety of functions in the nervous, cardiovascular and immune systems (e.g., Moncada et al., 1991). In ascidians, NO production is related not only to the immune response but also to events such as egg fertilization and notochord regression, which involve signaling that culminates in apoptosis (Grumetto et al., 1997; Comes et al., 2007; Castellano et al., 2014). In the colonial ascidian *Botryllus schlosseri*, Cima et al. (2004) identified NO production by hemocytes and suggested roles for NO in cell death and in cytotoxicity reactions. In the ascidian *Styela plicata*, nitrite (NO_2^-) generation did not change after lipopolysaccharide (LPS) stimulation, and in addition, all types of hemocytes produced NO, although lymphocyte-like cells produced the most (De Barros et al., 2009). In the hemocytes of the ascidian *P. nigra*, NO production can be regulated by norepinephrine, which reduces its production (De Barros et al., 2012). NO is regulated by a complex signaling pathway that involves protein kinase A (PKA) and protein kinase C (PKC) together with nuclear factor kappa B (NFκB) (De Barros et al., 2014).

Although some researchers have investigated essential and nonessential metals that affect NO production, few have evaluated the role and accumulation of metals in the cells and tissues of invertebrates. In this study, we analyzed the accumulation of two essential metals (Mg and Mn) and one nonessential metal (Pb) in the hemocytes of *P. nigra*, and also the effects of these metals on NO production in this ascidian.

2. Materials and methods

2.1. Animal collection

A total of 21 adults of *P. nigra* were collected from Porto do Forno, Arraial do Cabo, Rio de Janeiro, Brazil, and maintained at 20 °C in an aerated aquarium with controlled photoperiod (12 h light/12 h dark), pH (approximately 8.0), and salinity (34), for five days before the experimental procedures. The animals were fed with nauplii of *Artemia* (Sardet et al., 2011).

2.2. Isolation of hemocytes

The ascidians were bled from incisions in their incurrent siphons, and the hemolymph was collected in a marine anticoagulant solution (MAC; 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 10 mM EDTA, 0.45 M NaCl, pH 7.0) (Peddie and Smith, 1993). Hemocytes from individual tunicates were isolated by centrifuging the hemolymph at 800 × *g* for 10 min, then washing and finally suspension in artificial sea water (ASW; 420 mM NaCl, 9 mM KCl, 10 mM $CaCl_2$, 24.5 mM $MgCl_2$, 25.5 mM Mg SO_4, 2.15 mM $NaHCO_3$ and 10 mM Hepes buffer, pH 8.0, sterilized with a 0.2 µm filter). The amount of heavy metals present in the salts used was not significant (> 0.05 ppm). Cells were counted using a Neubauer hemocytometer, and suspensions of hemocytes containing 3×10^6 cells/mL were prepared before the experiments (De Barros et al., 2009, 2014).

2.3. Experimental and exposure procedure

Cell suspensions (400 µL; 3×10^6 cells/mL) from 18 ascidians were separated into 3 groups and then incubated with $MgCl_2$, $MnCl_2$,or $Pb_3(C_6H_5O_7)_2$ (lead citrate) at concentrations of 10^{-8} M, 10^{-5} M, 10^{-2} M or 10^{-1} M in a dark humidified box, for 30, 60 or 90 min. These concentrations were chosen based on the approximate reported concentrations of the metals in the marine environment (Catharino et al., 2008; Abdul Jaffar Ali et al., 2015) and also concentrations that could occur in an environmental accident. Next, NO production was quantified in the pool of cells by two different methods: cells from 9 ascidians were analyzed by the Griess method (Leone et al., 1995), and cells from the other 9 ascidians by the 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA, Molecular Probes) method (Nakatsubo et al., 1998). The experiments were conducted in duplicate for all concentrations and times.

2.4. Quantification of nitrite

The method of Griess was used in order to quantify the combined levels of nitrite (NO_2^-) and nitrate (NO_3^-), which are stable products resulting from the degradation of NO (Leone et al., 1995). Briefly, 60 µL of a suspension of hemocytes from 9 ascidians was exposed to the different metals for 30 min and in the above concentrations as described in *Experimental and exposure procedure*. Thirty minutes is the time reported for hemocytes to produce NO after Zymosan A stimulation (De Barros et al., 2014). Next, the samples were sonicated on ice 3 times for 15 s each, and were then centrifuged at 10,000 × *g* (20 min, at 4 °C). The supernatant was carefully removed and analyzed using the Nitrate/Nitrite Colorimetric Assay Kit/LDH Method (No. 760871, Cayman Chemical Company), which is based on the conversion of nitrate to nitrite by nitrate reductase (De Barros et al., 2009). After 10 min incubation, the optical density at 540 nm was measured (Molecular Devices Spectra Max 190). The molar concentration of NO_2^- in the samples was determined from standard curves generated using known concentrations of NO_2^-.

2.5. Quantification of NO in living cells

Hemocytes (3×10^6 cells/mL) were incubated in 5 µM of the fluorescent indicator for NO, DAF-FM DA for 30 min in the dark (Nakatsubo et al., 1998). Cells were then washed in ASW prior to incubation in ASW for 15 min, to de-esterify the intracellular DAF-FM DA. Control hemocytes were suspended in ASW only and experimental cells were separated into 3 groups and then incubated with Mg, Mn, or Pb at different concentrations for 30, 60 or 90 min, as specified in the Section 2.3. The fluorescence signals were measured using a Varian Cary Eclipse Fluorescence spectrophotometer, calibrated for excitation at 485 nm and emission at 520 nm (De Barros et al., 2014).

For the assays of NO synthase (NOS) inhibition, inhibitors were added to hemocytes 30 min prior to incubation with DAF-FM-DA (Molecular Probes; D23842). One or 10 mM of the NO synthase inhibitor N-ω-Nitro-L-arginine (L-NAME) (Sigma, St. Louis, USA; N5751) was used. The concentrations used are those reported to inhibit NO production by ascidian hemocytes (De Barros et al., 2014). In order to adduce NO production, the diethylenetriamine NO adduct (DETA/NO) (Sigma, St. Louis, USA; D185; 200 mM) was added to the hemocytes for 30 min prior to the incubation with DAF-FM-DA. The concentrations used are those reported to adduct NO in invertebrate hemocytes (De Barros et al., 2014). Then, the hemocytes were incubated with Mn, Mg or Pb (10^{-1} M), and after 30, 60 and 90 min, the NO production was measured using a Varian Cary Eclipse fluorescence spectrophotometer (De Barros et al., 2014).

2.6. Hemocyte viability

Hemocyte viability was evaluated by the trypan-blue stain (1% in ASW) exclusion test (Wang et al., 2006). One hundred cells of each experiment (n = 3) were analyzed as follows: 10 µL of a suspension containing 3×10^6 hemocytes/mL was incubated either with only Mg or Mn or Pb (10^{-1} M) or with metals (10^{-1} M of Mg, Mn or Pb) and either 10 mM L-NAME or 400 µM DETA-NO. After, 10 µL of trypan blue was added to 10 µL of the hemocyte suspension. After 30, 60 and 90 min the percentages of stained cells, considered unviable, were counted.