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Evidence for phosphatidylinositol-3-OH-kinase (PI3-kinase) involvement in Cd-mediated oxidative effects on hemocytes of mussels

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ABSTRACT

This study investigated phosphatidylinositol-3-OH-kinase (PI3-kinase) involvement in the induction of cadmium-mediated oxidative effects on hemocytes of mussel *Mytilus galloprovincialis*. PI3-kinase was investigated with the use of wortmannin, a specific covalent inhibitor of PI3-kinase. Moreover, phorbol-myristate acetate (PMA), a well-known protein kinase C (PKC)-mediated NADPH oxidase and nitric oxide (NO) synthase stimulator, was also used for elucidating PI3-kinase involvement during the respiratory burst process in challenge hemocytes. According to the results, cells pre-treated with non-toxic concentrations of wortmannin (1 and/or 50 nM, as revealed by neutral red retention assay) for 15 min, showed a significant attenuation of cadmium ability (at concentration of 50 μM) to promote cell death, superoxide anion ($\bullet O_2^-$) production, NO generation and lipid peroxidation (in terms of malondialdehyde equivalents). On the other hand, wortmannin-treated cells showed a significant attenuation of PMA ability to induce NO generation but not $\bullet O_2^-$ production. These findings reveal that PI3-kinase could lead to a PKC-independent induction of NO synthase activity in cells faced with pro-oxidants, such as cadmium, while its activation could be fundamental for the regulation of NAPDH oxidase activity, probably through a PKC-dependent signaling pathway.

1. Introduction

Immune response is highly complex and includes a variety of different cellular and molecular processes. In particular, the immune system of invertebrates, and especially bivalve mollusks, such as the mussel *Mytilus galloprovincialis*, is based on their hemocytes, whose main features are the phagocytic activity and the production of oxidizing elements during the respiratory burst process (Garcia-Garcia et al., 2008).

Hemocytes of mussels are known to have a complex network of cell signaling processes that allow them to modulate the immune response. Although there is evidence that these signaling pathways show high homology with those of vertebrates (Gonzalez-Riopedre et al., 2009; Plows et al., 2005) the molecular basis for the action of signaling molecules that are involved in the signaling cascades induced in hemocytes of mussels has still to be demonstrated. In fact, the involvement of a huge number of signaling molecules, including protein kinase C (PKC) and phosphatidylinositol-3-OH-kinase (PI3-kinase) have been reported during immune system stimulation by various stimuli, such as bacteria, cytokines, hormones and environmental chemicals in hemocytes of mussels (Dailianis, 2009; Barcia and Ramos-Martinez, 2008; Garcia-Garcia et al., 2008; Malagoli et al., 2007; Canesi et al., 2006; Ottaviani et al., 2000) but little is known concerning the transduction of these signaling molecules in hemocytes of mussels faced with inorganic substances, like heavy metals.

Heavy metals, such as cadmium, are considered as potent catalysts in the oxidative deterioration of biological molecules and their toxicity depend merely on the production of reactive oxygen species (ROS) and perturbation of anti-oxidant efficiency (Micic et al., 2001; Pourahmad and O'Brien, 2000). In particular, micromolar concentrations of cadmium could enhance the respiratory burst process in hemocytes of mussels via a PKC-mediated signaling pathway (Banakou and Dailianis, 2010; Dailianis, 2009). Since respiratory burst products, such as superoxides ($\bullet O_2^-$) and nitric oxides (NO) could regulate the activation of the PI3-kinase/Akt signaling pathway (Barthel et al., 2007), it was of great interest to investigate the possible involvement of PI3-kinase during cadmium-mediated oxidative effects in hemocytes of mussels.

PI3-kinase is a key signaling molecule responsible for phosphorylating phosphoinositides at the 3· position of the inositol ring that has been implicated in a number of signaling pathways (Arcaro and Wymann, 1993). Indeed, PI3-kinase activation is seemed to support various cell functions, such as cell growth, migration and survival, via the activation of Akt/protein kinase B, which in turn triggers cytoprotective events (Shimamura et al., 2003), as well as cell interaction with the extracellular matrix both in invertebrates and vertebrates (Konstantinidis et al., 2009; Howe et al., 1998; Wei et al., 1997; Guan and Chen, 1996; Parson, 1996). When activated PI3-kinase

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could bind to tyrosine kinase receptors and associated proteins by protein—protein interactions, and lie upstream in the cascade leading to activation of the immune response (Canesi et al., 2002a). Indeed, Garcia-Garcia et al. (2008) showed the involvement of PI3-kinase in the regulation of phagocytosis and the concomitant activation of NADPH oxidase and NO synthase during the respiratory burst process, but little is known about its role in hemocytes faced with heavy metals, such as cadmium.

Susceptibility of different kinases to specifically designed inhibitors and stimulators is commonly investigated in order to define the role of different signaling pathways in phagocyte activation and bacterial killing (Perskvist et al., 2000; Hii et al., 1999; Schnyder et al., 1998). Although not entirely specific, some inhibitors and stimulators have been shown to be effective also in identifying the signaling pathways involved in the response of mussel cells to different extracellular stimuli, in the same range of concentrations utilized in mammalian systems (Dailianis, 2009; Dailianis et al., 2005; 2009; Kaloyianni et al., 2006; Dailianis and Kaloyianni, 2004; Canesi et al., 2002a; 2002b; 2002c). For example, wortmannin, a specific covalent inhibitor of PI3-kinase (Arcaro and Wymann, 1993), has been reported to inhibit cell adhesion, migration, phagocytosis and reorganization of cytoskeleton in the colonial ascidian *Botryllus schlosseri* (Ballarin et al., 2002). Moreover, PI3-kinase inhibition in hemocytes of mussels treated with wortmannin, is partly related with cells inability to promote bacterial killing (Canesi et al., 2002a; Hii et al., 1999; Schnyder et al., 1998), cell adhesion and migration (Koutsogiannaki and Kaloyianni, 2011; Canesi et al., 2002b). In addition, stimulators, such as the phorbol myristate (PMA), are commonly used for PKC activation in different cell types of mussels (Banakou and Dailianis, 2010; Dailianis, 2009; Dailianis et al., 2009; Cao et al., 2003).

Regarding the close relationship between PI3-kinase and immune-related response of hemocytes, it was of great interest to investigate its possible involvement in the signaling pathway that leads to the enhancement of cadmium-mediated effects on hemocytes of mussel *M. galloprovincialis*. PI3-kinase activity was estimated indirectly, with the use of wortmannin. In this light, treatment of hemocytes with different concentrations of this covalent inhibitor of PI3-kinase was primary performed in order to determine the non toxic concentration range of wortmannin in hemocytes of mussels, with the use of neutral red assay. Thereafter, we investigate the possible involvement of PI3-kinase in cadmium-mediated enhancement of respiratory burst, via determination of $\bullet O_2^-$ and NO generation in hemocytes of mussels, as well as its role in the induction of oxidative stress related products, such as lipid peroxides (in terms of malondialdehyde content). Furthermore, the possible interaction between PI3-kinase and PKC in the signaling pathway that leads to respiratory burst induction was investigated, after treatment of hemocytes with PMA in the presence or the absence of PI3-kinase inhibitor.

2. Materials and methods

2.1. Chemicals and reagents

Sulfanylic acid and wortmannin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Cadmium chloride ($CdCl_2$) was purchased from MERCK (Darmstadt, Germany). Nitroblue-tetrazolium (NBT), neutral red, *N*-(1-Naphthyl)ethylenediamine, sodium nitrite, phorbol-myristate acetate (PMA), hydrogen peroxide, phosphoric acid, fetal calf serum (FCS), penicillin G, streptomycin, gentamycin and amphotericin B were purchased from Applichem. Leibovitz L-15 medium was purchased from Biochrom A.G.

2.2. Mussel collection and handling

Mussels (5–6 cm in length, approximately 1-year old) were collected from Gulf of Kontinova, located at the north side of Korinthiakos Gulf (Galaxidi, Greece), transferred to the laboratory and maintained in static tanks, containing recirculated UV-sterilized and filtered artificial sea water (35–40‰ salinity) for 7 days at 15 °C, in order to be acclimated in laboratory conditions. During the acclimation period no mortality was observed among mussels. Furthermore, in order to exclude parameters possibly related with mussels' adaptation in cadmium-polluted environment, cadmium levels were determined in soft tissues of mussels, using Flame atomic absorption spectrophotometry (Perkin Ellmer AAnalyst 800). The quality of measurements was assured by the use of Dorm-2 dogfish muscle (Certified Reference Material for trace metals, National Research Council of Canada), thus verifying minimal levels of cadmium in mussel tissues (lower than 0.5 $\mu g\ g^{-1}$ wet mass of tissue), such as digestive gland, gills and mantle/gonad complex. Concentration of cadmium used in the present study was close to that found in heavily-polluted areas (Ravera, 1984), while similar concentration was also used in other studies, investigating the effects of cadmium on cell signaling and its ability to induce cellular toxic effects (Banakou and Dailianis, 2010; Dailianis et al., 2009; 2005; Dailianis and Kaloyianni, 2004; Pruski and Dixon, 2002; Olabarrieta et al., 2001; Misra et al., 1998; Coogan et al., 1992). During the acclimation period, animals were maintained without food and then fed daily with approximately 30 mg of dry-microencapsules/mussel (Myspat, Inve Aquaculture^{NV}, Belgium).

2.3. Collection of mussel hemolymph

Hemolymph from 10 mussels was extracted from the posterior adductor muscle with a sterile 1 mL syringe (equipped with an 18 G1/2 in. needle), containing 0.1 mL of Alseve buffer (ALS buffer; 60 mM glucose, 27.2 mM sodium citrate tribasic, 9 mM EDTA and 385 mM NaCl, pH 7 and 1000 mOsmol). In order to eliminate impurities, the cell suspension was centrifuged at 150 *g* for 15 min, at room temperature and the pellet containing the hemocytes was re-suspended in modified cell culture medium (Leibovitz L-15 medium, supplemented with 350 mM NaCl, 7 mM KCl, 4 mM $CaCl_2$, 8 mM $MgSO_4$, 40 mM $MgCl_2$, 10% v/v FCS, 100 U mL^{-1} penicillin G, 100 $\mu g\ mL^{-1}$ streptomycin, 40 $\mu g\ mL^{-1}$ gentamycin, 0.1 $\mu g\ mL^{-1}$ amphotericin B, at pH 7 and 1000 mOsmol). The medium was then filtered through 0.45 μm filters and kept on 15 °C. Cells were counted in a Neubauer hemocytometer, re-suspended to obtain a concentration of 10^{-6} cells mL^{-1} and kept at 15 °C for at least 1 h before being used for the experiments (Cao et al., 2003). Throughout this period, cell viability test carried out with the use of Eosin exclusion test, showed that viable cells before the beginning of experimental procedure was about 95%.

2.4. Neutral red uptake determination in hemocytes of mussels

Estimation of the cationic dye neutral red (NR) uptake was assessed as reported by Dailianis (2009). Briefly, 500 μL of cell suspension (10^{-6} cells mL^{-1}) were exposed for 1 h to different concentrations of wortmannin (1–100 nM, from a stock solution of wortmannin dissolved in DMSO) or cadmium chloride ($CdCl_2$ at a final concentration of 50 μM). In order to compare results obtained after wortmannin treatment, viability of cells treated with DMSO (at a final concentration of 0.001% v/v) was also measured. After the exposure period, cell suspension was centrifuged at 150 *g* and supernatant was removed carefully. Hemocytes were then re-suspended in ALS buffer and maintained in a dark place, for 1 h at 4 °C, in order cells to adherent to the walls. Thereafter, the non-adherent cells were removed carefully and 500 μL of ALS, containing 0.004% w/v NR, was finally added. After 2 h of incubation to allow uptake of the dye, cells were centrifuged at 150 *g* for 10 min and washed twice with ALS. Afterwards dye was extracted from intact cells with an acetic acid–ethanol solution (1% v/v acetic acid and 50% v/v ethanol) and absorbance was determined spectrophotometrically (Perkin-Elmer 551) at 550 nm. Results are means ± SD from 6 different