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The role of phosphatidylinositol-3-OH-kinase (PI3-kinase) and respiratory burst enzymes in the [omim][BF_4]-mediated toxic mode of action in mussel hemocytes

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ABSTRACT

The present study investigates the role of phosphatidylinositol-3-OH-kinase (PI3-kinase) and respiratory burst enzymes, NADPH oxidase and NO synthase, in the 1-methyl-3-octylimidazolium tetrafluoroborate ([omim][BF_4])-mediated toxic mode of action in mussel hemocytes. Specifically, cell viability (using the neutral red uptake assay) was primarily tested in hemocytes treated with different concentrations of [omim][BF_4] (0.1–10 mg L^{-1}) and thereafter [omim][BF_4]-mediated oxidative (in terms of superoxide anions/$O_2^{\bullet -}$ and nitric oxide/NO generation, as well as the enhancement of lipid peroxidation by-products, in terms of malondialdehyde/MDA) and genotoxic (in terms of DNA damage) effects were determined in hemocytes treated with 1 mg L^{-1} [omim][BF_4]. Moreover, in order to investigate, even indirectly and non-entirely specific, the role of PI3-kinase, NADPH oxidase and NO synthase, the [omim][BF_4]-mediated effects were also investigated in hemocytes pre-incubated with wortmannin (50 nM), diphenyleneiodonium chloride (DPI 10 μM) and N^G-nitro-$_L$-arginine methyl ester ($_L$-NAME 10 μM), respectively. The results showed that [omim][BF_4] ability to enhance $O_2^{\bullet -}$, NO, MDA and DNA damage, via its interaction with cellular membranes, was significantly attenuated in the presence of each inhibitor in almost all cases. The current findings revealed for the first time that certain signaling molecules, such as PI3-kinase, as well as respiratory burst enzymes activation, such as NADPH oxidase and NO synthase, could merely attribute to the [omim][BF_4]-mediated mode of action, thus enriching our knowledge for the molecular mechanisms of ILs toxicity.

1. Introduction

Ionic liquids are mainly consisted of organic cations (i.e., imidazolium, pyridinium, pyrrolidinium, ammonium, phosphonium) and inorganic anions (i.e., tetrafluoroborate and bromide), and are considered as environmental friendly substitutes for conventional organic solvents [1–3]. Due to their low melting points (<100 °C), negligible vapor pressure, non-volatility, non-flammability and their excellent chemical and thermal stability, those salts are widely used in a broad range of industrial, catalytic and chemico-biological applications/processes, including electrochemistry, polymer science, and nano-chemistry [4], drug delivery and cellulose dissolution [5–7]. Nevertheless, since most of ILs are water soluble [8] and characterized by low degree of environmental degradation [9] and biodegradability [10–12], a lot of studies have reported their potential toxic behavior on numerous living organisms, including aquatic species and different cell lines [13–16]. However, little is known about the molecular mechanisms of ILs toxicity.

Among the most investigated imidazolium-based ILs, 1-methyl-3-octylimidazolium tetrafluoroborate [omim][BF_4] has been recently reported to affect a battery of species [16], including bivalve mollusks, such as the mussel *Mytilus galloprovincialis*, systematically used as biological models for assessing the adverse effects of xenobiotic compounds [14,15]. Although, there is evidence that such a type of imidazolium-based ILs, acting as surfactants, could interact, via the alkyl chain, with the polar group region of cellular membrane bilayers, thus attributing to the disturbance of membrane integrity and the concomitant cellular damage and death [17–21], our knowledge about their molecular-based mode of action remains still scarce.

Mussel hemocytes are widely used for assessing the adverse

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effects of xenobiotic compounds with great precision and reproducibility [22–31]. Those cells have a complex signaling network like those of vertebrates [32,33], and are responsible for the phagocytic activity and the production of oxidizing elements during the respiratory burst process [34], via the involvement of a huge number of signaling molecules, including phosphatidylinositol-3-OH-kinase (PI3-kinase). PI3-kinase is a key regulator of phagocytosis [35,36] and has been proposed as a survival signal in mild oxidative stress [37,38]. Namely, various stimuli, such as bacteria, cytokines, hormones and environmental chemicals, like heavy metals, have been reported to activate PI3-kinase in mussel hemocytes [34,39–42]. Moreover, during the immune response of mussel hemocytes against non-self substances, the generation of superoxide anions ($O_2^{\bullet -}$) and nitric oxides (NO) via the main enzymes of the respiratory burst process, NADPH oxidase and NO synthase, could regulate the activation of PI3-kinase/Akt signaling pathway [43]. However, although various effectors have been shown to induce NADPH oxidase and NO synthase activation, little is known concerning the involvement of those molecules in hemocytes of mussels exposed to non-self substances, such as ILs.

Since the molecular mechanism of [omim][BF_4]-mediated toxicity is not yet well understood, the present study investigates the role of PI3-kinase and respiratory burst process in the [omim][BF_4]-mediated toxicity in mussel hemocytes. In this context, inhibitors such as wortmannin (Wort; a non-specific, irreversible and noncompetitive inhibitor of PI3-kinase [44,45]), diphenyleneiodonium chloride (DPI; an inhibitor of NADPH-oxidase, but also an unspecific flavoprotein inhibitor of eNOS enzymes among others [46–48]) and N^G-nitro-$_L$-arginine methyl ester (L-NAME; a non-specific NO synthase inhibitor), previously being found to inhibit PI3-kinase, NADPH oxidase and NO synthase respectively, in mussel hemocytes [23,25,49–52] were used. Thereafter, the [omim][BF_4]-mediated cytotoxic, oxidative and genotoxic effects were determined in mussel hemocytes primarily incubated or not with each inhibitor, in order to elucidate, even indirectly and non-entirely specific, the role of those molecules in the [omim][BF_4]-mediated toxic mode of action.

2. Materials and methods

2.1. Chemicals and reagents

The IL [omim][BF_4] (Cas No. 244193-52-0, analytical grade ≥97%, HPLC, currently used without further purification), as well as DPI (Diphenyleneiodonium chloride), L-NAME (N^G-nitro-$_L$-arginine methyl ester) and wortmannin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All the other reagents and solvents used were of the highest analytical grade and purity.

2.2. [omim][BF_4] stock solution preparation and concentrations tested

[omim][BF_4] stock solutions were prepared by diluting analytical grade [omim][BF_4] in 2dH_2O. Thereafter, appropriate amounts of [omim][BF_4] stock solutions were diluted in sterilized hemolymph and further used. The range of [omim][BF_4] concentrations currently tested (0.1–10 mg L^{-1}) was based on initial experimental measurements, using known [omim][BF_4] calibration curves, commonly performed with the use of UV-Vis spectrophotometric analysis as previously described [15,53].

2.3. Mussel collection and handling

Mussels (5–6 cm long) were collected from a mussel farm located at the north side of Korinthiakos Gulf (Gulf of Kontinova, Galaxidi, Greece). The selected nature reserve area is characterized by negligible, even undetectable, levels of inorganic and organic contaminants and thus widely used as a reference area in (eco) toxicological studies (for more details see Ref. [15]). Mussels were immediately transferred to the laboratory and maintained, without feeding, for 7 days in static tanks containing UV-sterilized, recirculating and filtered artificial sea water (35‰ salinity, 15 °C; acclimatization period). After the end of their acclimatization and during the experimental procedure, mussels were fed daily with *Isochrysis galbana* (almost 2×10^6 cells mL^{-1}). The health status of mussels currently used was assessed by the application of the neutral red retention time (NRRT) assay in their hemocytes (values ≥ 110 min).

2.4. Collection of mussel hemolymph

Hemolymph from 10 mussels was extracted from the posterior adductor muscle with a sterile 1 mL syringe (18G1/2′ needle), containing 0.1 mL of Alseve buffer (ALS buffer; 20.8 g L^{-1} glucose, 8 g L^{-1} sodium citrate, 3.36 g L^{-1} EDTA and 22.5 g L^{-1} NaCl, pH 7 and 1000 mOsmol). In order to eliminate impurities, the cell suspension was centrifuged at 1000×*g* for 10 min and the cell pellet was re-suspended in sterilized hemolymph (Whatman syringe filter, pore size 0.22 μm). Before each experiment, cell viability was assayed with eosin exclusion test, indicating that viable cells were about 95%, and thereafter 1 mL of mussel hemocytes suspension (almost 10^6 cells mL^{-1}) was used in all cases.

2.5. Determination of hemocytes viability (in terms of neutral red uptake) after exposure to different concentrations of [omim][BF_4]

Cell viability was primarily determined in mussel hemocytes, using the cationic dye neutral red (NR), according to the method described by Dailianis [23], with a few modifications. Briefly, hemocytes were treated for 1 h with different concentrations of [omim][BF_4] (0.1–10 mg L^{-1}). Thereafter, the cell suspension was centrifuged (1000×*g*, 10 min) and the supernatant was removed carefully. Cells were re-suspended in ALS buffer, containing 0.004% NR, and maintained in a dark place for 2 h at 4 °C, in order cells to adherent to the walls and to allow the uptake of the dye. Then, cells were centrifuged at 1200×*g* for 10 min and washed twice with ALS. Neutral red was extracted from intact cells with an acetic acid:-ethanol solution (1% v/v:50% v/v), and absorbance was determined spectrophotometrically at 550 nm. The results (expressed as the optical density obtained at 550 nm per mg of protein in each case) are mean ± SDs from 6 independent experiments in each case. In each experiment, hemocytes were pooled from hemolymph collected from 10 mussels. The protein content was determined by an ultrasensitive hydrophobic method based on Coomassie Brilliant Blue-G assay [54], with the use of known concentrations of bovine serum albumin (BSA), in all cases.

2.6. Determination of [omim][BF_4]-mediated effects on mussel hemocytes

Based on cell viability currently tested, the rest of the parameters was determined in mussel hemocytes treated with sub-lethal concentrations of [omim][BF_4] (1 mg L^{-1}), thus avoiding the interference of cell death with the obtained results. Similar approach was also followed in case of hemocytes primarily incubated (pre-incubated) with well-known concentrations of each inhibitor widely used for the investigation of the respiratory burst process and signaling cascades in mussel hemocytes [23,25,49,52]. Specifically, superoxide anions ($O_2^{\bullet -}$), nitric oxides (NO, in terms of nitrites/NO_2^-), lipid peroxidation by-products (in terms of