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Effects of the marine toxins okadaic acid and palytoxin on mussel phagocytosis

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Abstract The present study analyzes the effects of the marine toxins okadaic acid (OA) and palytoxin (PTX) on the phagocytic activity of immunocytes from the mussel *Mytilus galloprovincialis*. In particular, we describe how the effects of the two biotoxins are influenced by the temperature and experimental stress applied before hemolymph withdrawal. The collected data indicate that OA increases phagocytic activity only when hemolymph incubation is performed at 25 °C, but not at 20 °C, suggesting a certain degree of dependence of OA effects from the status of mussel immunocytes. Conversely, PTX plays an active role in immunocyte signalling transduction pathways, increases the phagocytic activity and markedly promotes the involvement of p38 mitogen-activated protein (MAP) kinase in phagocytosis. Overall, we conclude that both OA and PTX influence mussel phagocytic activity, and the toxic effects may depend on both the mollusc conditions and the activation of specific signalling pathways.

Introduction

Studies on the immunity of bivalve molluscs are becoming increasingly numerous as a result of the importance of these models from ecological, economic and public health points of view [1]. As phagocytosis is central to the cell-mediated immune response in invertebrates [2], numerous experiments aimed at evaluating bivalve status are focused on the phagocytic activity of circulating hemocytes [3]. Given their feeding behaviour, bivalves can accumulate molecules that are potentially dangerous for human health, including marine biotoxins [4] and information about bivalve ability to eliminate accumulated toxins is relatively scarce [5,6]. Even if the harmful effects of some toxins on humans are well-known and elicit concern [4], little information is available on the effects that accumulated toxins may exert on bivalve molluscs, especially in the long-term. Such data could be of fundamental importance in evaluating the real ecological impact of marine toxins and could contribute to clarifying how diarrhoeic toxins such as okadaic acid (OA) [7], or highly toxic molecules such as palytoxin (PTX) [8] can be accumulated in some bivalves without any apparent toxic effect.

The marine toxin okadaic acid (OA) is a polyether produced by some phytoplanctonic dinoflagellate species and can be accumulated in the digestive gland of shellfish as a result of their filter feeding behaviour. The consumption

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of these animals provokes diarrhoeic shellfish poisoning (DSP) in humans [9,10]. OA is a potent tumor promoter in inhibiting the activity of protein phosphatases type 1 and 2A [11]. Experiments on mammalian models demonstrated that OA activates caspases [12] and provokes a severe alteration in the structural architecture of the thymus [13]. Palytoxin (PTX) is a non-protein toxin first isolated from the soft coral of the genus *Palythoa* [14]. PTX is considered one of the most toxic molecules occurring in nature [15], and it has also recently been studied as a skin tumor promoter [8]. As for other phycotoxins, PTX can accumulate in the food chain therefore provoking severe, or even lethal, intoxications to humans [16].

In previous experiments, we have demonstrated that the algal toxin yessotoxin (YTX) increases the phagocytic activity of circulating immunocytes in the mussel *Mytilus galloprovincialis*, indicating that the innocuity of YTX for mussels might be only apparent [17]. Moreover, we have also observed that experimental stresses annulled YTX effects, suggesting that stress-mediated perturbation of the mussel immunocyte signal transduction pathways [18] could interfere with the action of the toxin [17]. Since phagocytosis is a pillar of invertebrate innate immunity, many comparative immunology studies have analyzed the response in ordinary and stressful conditions, and a link between phagocytic activity, stress and mitogen-activated protein (MAP) kinase pathways has been firmly established in several invertebrate models, including the bivalve mollusc *M. galloprovincialis* [17,19–21].

The present article therefore analyzes mussel phagocytosis in two successive steps. First, the influence of experimental procedures (i.e. incubation temperature of the hemolymph) and of a prior stressful condition, such as air exposure, thermal and salinity stress on the effects of the two biotoxins has been considered. On the basis of the results obtained, we have further considered how the diarrhoeic toxin OA and the tumor promoter PTX may influence mussel phagocytosis. Finally, the combined influence of stressful situations and biotoxins on phagocytic activity has also been analyzed by means of specific inhibitors of MAPK pathway.

Materials and methods

Animals

Adult specimens of the mussel *Mytilus galloprovincialis* (length 50–80 mm) were obtained from the ''Centro di Ricerche Marine'' (Cesenatico, FC, Italy) and maintained in laboratory aquaria (minimum 5 l/mussel) with artificial seawater (Tropic Marin® Sea Salt, Germany) (temperature 17 ± 1 °C, pH 8.0 ± 0.2, salinity 35 ± 1 psu) for at least 10 days before experiments. During this period, water was changed regularly (10% of aquarium capacity each 2 days) and animals were fed once a day with food for filter feeders (Liquifry Marine, Interpet, UK).

Types of stress

The following experimental stress conditions were applied to mussels before hemolymph withdrawal: (i) 120 min air exposure by maintaining the animals at 20 ± 1 °C in a 5-l tank without any water; (ii) thermal stress by maintaining the mussels in experimental aquaria at 25 ± 2 °C for 24 h; (iii) low-salinity stress by maintaining the specimens in experimental aquaria with a salinity of 25 ± 1 psu for 24 h and (iv) high-salinity stress by maintaining the mussels in experimental aquaria with a salinity of 40 ± 1 psu for 24 h. The experimental parameters for the stresses were set on the basis of previous studies regarding the effects of realistic environmental conditions on mussel immunity [17]. Control mussels were kept in laboratory aquaria (see Animals) until hemolymph withdrawal. For each type of stress, the experiments were repeated on 10 animals and the hemolymph of each mussel was utilized for all the assays described below.

Hemolymph withdrawal and cell counting

Immediately after the conclusion of the experiments, the hemolymph was collected by gently aspirating with a sterile syringe inserted between the mussel valves, paying attention not to touch the digestive gland or gonads. Phase contrast microscopy was used to assess immediately that circulating hemocytes (immunocytes) were intact and adhering, and that the hemolymph was free of any other accidentally collected cell type. The number of immunocytes was determined using a hemocytometer. After the count, the amount of hemolymph necessary for experimental procedure was retained, while the remainder was filtered (0.2 μM) and used to adjust cell concentration to a final value of 10^5 cells/ml. The hemolymph collected from each mussel was kept separately and never pooled.

Hemolymph preparation prior to the phagocytosis assay

After withdrawal, for each mussel treatment (control, 120 min air exposure, thermal stress, low-salinity, high-salinity) individual hemolymph was divided into two lots of nine subsamples each. The subsamples (100 μl, about 10^4 cells) were used for two sets of analogous treatments at 20 ± 1 °C and 25 ± 1 °C, respectively. The two incubation temperatures were, respectively, the room temperature (similar to that of control aquaria) during the air exposure stress and the maximum temperature applied in thermal stress experiments. For each temperature, nine treatments were set up: control, PD 098059-treated, SB 203580-treated, OA-treated, PTX-treated, OA + PD 098059-treated, OA + SB 203580-treated, PTX + PD 098059-treated and PTX + SB 203580-treated.

More in detail, immediately after aliquoting the hemolymph in subsamples, either 20 μM PD 098059 (BIOMOL, Plymouth Meeting, PA, USA), a mitogen-activated and extracellular regulated kinase kinase (MEK)-1 inhibitor, or 1 μM SB 203580 (BIOMOL), a p38 MAP kinase inhibitor [22,23], were added to the hemolymph and incubated for 20 min. After incubation with the inhibitors, either 100 nM (82.7 ng/ml) OA or 2 ng/ml PTX was added to the hemolymph for a further 15 min before the addition of fluorochrome-conjugated beads (fluorescent microspheres) (FluoSpheres®, Molecular Probes, OR, USA) utilized to investigate phagocytosis (see Phagocytosis assay). In control