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Note

Nitrite released in haemocytes from *Mytilus galloprovincialis*, *Crassostrea gigas* and *Ruditapes decussatus* upon stimulation with phorbol myristate acetate

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Abstract − In order to demonstrate the involvement of nitric oxide in the defence systems of marine bivalves, we investigated the production of superoxide and nitrite, following in vitro phorbol myristate acetate stimulation of *Mytilus galloprovincialis*, *Crassostrea gigas* and *Ruditapes decussatus* haemocytes. Whereas *M. galloprovincialis* and *C. gigas* haemocytes were found to produce superoxide and nitrite, *R. decussatus* haemocytes were found to be unable to generate either of these mediators. Nitrite is a stable end product of nitric oxide and peroxynitrite as well; it appeared therefore that some marine bivalves, to kill microbial pathogens, use NADPH-oxidase and nitric oxide-synthase pathways. This was confirmed at an experimental level where inhibitors of both enzymatic pathways blocked the production of nitrite. Moreover, this notion was strengthened by the inability of the haemocytes from *R. decussatus*, which cannot produce superoxide, to release nitrite when stimulated.

haemocytes / immune defence / bivalve / nitrite / reactive oxygen intermediates

1. INTRODUCTION

Molluscs are protected against foreign invaders by an internal defence system consisting of both cellular and humoral limbs. The haemocytes are the most important defence cells because they are involved in phagocytosis and produced most of the cytotoxic factors, which degrade and kill foreign organism (Adema et al., 1991).

Haemocytes move freely in the open circulatory system of molluscs and, upon contact with non-self antigens, generate superoxide anions (O_2^-), the initial species of reactive oxygen intermediates (ROI) (Torreilles and Guérin, 1996).

Generation of ROI has been observed in several bivalves such as *Crassostrea virginica* (Nakamura et al., 1998), *C. gigas* (Bachère et al., 1991), *Patinopecten yessoensis* (Nakayama and Maruyama, 1998), *Ostrea edulis* (Bachère et al., 1991), *Mytilus edulis* (Pipe, 1992; Noël et al., 1993) and *M. galloprovincialis* (Torreilles and Guérin, 1999). However, some bivalves, such as *Ruditapes decussatus* (López et al., 1994), *Corbicula japonica* and *Mercenaria mercenaria* (Cheng et al., 1975), kill microbial pathogens without using ROI.

Nitric oxide (NO) is another oxygen intermediate produced from L-arginine by NO-synthase (Ichiropoulos et al., 1992). Studies of mammalian phagocytes have shown that NO is involved in killing foreign micro-organisms by reacting with O_2^- to form peroxynitrite, a strong bactericidal oxidant agent.

NO-synthase activity has been detected in many molluscs (Moroz and Gillette, 1995; Martinez, 1995; Conte and Ottaviani, 1995). The most frequently reported site for the production of NO is the nervous systems (Elofsson et al., 1993; Winlow et al.,1993; Moroz et al., 1994) and, for a smaller part, the muscle cells (Elofsson et al., 1993; Moroz et al., 1993a, b).

Radomski et al. (1991) detected NO-synthase in the haemocytes from the horseshoe crab and Ottaviani et al. (1993) reported that the haemolymph bacterial clumping activity stimulated by lipopolysaccharide in *M. edulis* was selectively and significantly reduced by NO-synthase inhibitors indirectly suggesting an in-

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volvement of NO production in the antibacterial protection of this species.

Here we show that haemocytes from some marine bivalves are able to produce NO as measured by its final end product nitrite (NO_2^-) which also possesses bactericidal properties (Klebanoff, 1993; Nakayama and Maruyama, 1998). We compared the nitrite release of two species already known to generate O_2^-: *M. galloprovincialis* and *C. gigas*, with that of one species that is unable to produce this intermediate: *R. decussatus* as previously shown by Lopez et al. (1994). Interestingly, we found that only the bivalves demonstrating the ability to generate O_2^- were also efficient in producing NO_2^- upon stimulation with phorbol myristate acetate.

This suggests that O_2^-, peroxynitrite and NO are involved in the internal defence system of *M. galloprovincialis* and *C. gigas* but not in the internal defence system of bivalve species of the order Veneroida.

2. MATERIALS AND METHODS

2.1. Chemicals

All reagents used were of analytical grade. NO-synthase inhibitors (N^5-(1-iminoethyl)-L-ornithine monohydrochloride (L-NIO), diphenylene iodonium chloride (DPI) and N^G-monomethyl-L-arginine (NMMA) were obtained from Alexis Corporation (San Diego, USA). Phorbol myristate acetate (PMA) and nitroblue tetrazolium (NBT) were purchased from Sigma Chemical Co. (St. Louis, USA). Phosphate buffered saline was prepared using Dulbecco's PBS salt and HPLC grade distilled water. The pH and osmolarity were adjusted to 8.3 and 1 100 mOsm, respectively. Nitrate/Nitrite fluorometric assay kit was from Cayman Chemical (Ann Arbor, USA). Stock solutions of superoxide dismutase from bovine erythrocytes were prepared in phosphate buffered saline at 15 000 U·mL^{-1} and stored at 4 °C.

2.2. Marine bivalves and haemolymph collection

Two-year-old marine bivalves: *M. galloprovincialis*, *C. gigas* and *R. decussatus* were collected from Bouzigues (France) and maintained for at least 8 days in recycled sea water. Immediately before each experiment, 1.5 mL haemolymph were withdrawn into a syringe from the posterior adductor muscle using an 18-gauge needle. The haemolymph was held in an ice bath to inhibit clumping. For each experiment, cell pools were obtained from an average of three mussels. Cell number was determined using a Malassez haemocytometer.

2.3. Assay of superoxide

Suspensions were measured according to the procedure of Anderson et al. (1994). Briefly, an aliquot of 250 µL haemocyte suspension in phosphate buffered saline (containing 1.0×10^6 cells·mL^{-1}) was incubated with an equal volume of 0.1% nitroblue tetrazolium with or without phorbol myristate acetate (10 µg·mL^{-1}final concentration) at room temperature. In controls, phorbol myristate acetate was substituted by phosphate buffered saline. After 60 min, the haemocyte suspension was centrifuged (180 *g*, 5 min, 4 °C) and finally was resuspended in 550 µL of 70 % methanol, centrifuged again (180 *g*, 5 min, 4 °C) and the pellet was resuspended in 550 µL of extraction fluid (6 mL of 2 M KOH + 7 mL DMSO) to dissolve the insoluble formazan formed. The samples were then mixed by vortexing and centrifuged (3 500 *g*, 20 min, and 10 °C). The optical density of supernatants obtained from controls and stimulated samples was measured at 630 nm with a Dynatech MR 5 000 microplate reader. The results are expressed as O.D. 630 nm·h^{-1} according to Anderson et al. (1994).

2.4. Assay of nitrite

For NO_2^- analysis, haemocyte suspensions (250 µL) were maintained with or without phorbol myristate acetate for 30 min. At the end of the incubation period, the samples were frozen immediately and stored at –75 °C until use. Prior to analysis, the samples were thawed and subjected to ultrasonication (20 kHz, 50 W, 3×20 s) on ice bath using a Vibracell apparatus from Bioblock scientific. The resulting homogenates were centrifuged (14 000 *g*, 10 min, 4 °C). The supernatants were analysed for NO_2^- content using the Nitrate/Nitrite fluorometric assay kit. The method is based on formation of 1(H)-naphthotriazole from 2,3-diaminonaphthalene and NO_2^- (Misko et al., 1993). In biological fluids, NO undergeos reactions with oxygen as well as superoxide anions leading to nitrate (NO_3^-) and nitrite (NO_2^-). Then, the relative proportion of NO_3^- and NO_2^- in biological samples is variable. To use NO_3^- and NO_2^- as an index of NO production from bivalve haemocytes, we measured the sum of both NO_3^- and NO_2^-. According to the manufacturer's instructions, samples were added with nitrate reductase to convert NO_3^- to NO_2^- and then, in a second step, added with 2,3-diaminonaphthalene and NaPOH to generate the fluorescent 1(H)-naphthotriazole. The fluorescence intensity of 1(H)-naphthotriazole formed in each sample was measured using multilabel fluorescence plate reader (Wallac 1420, Victor 1, λem. = 365 nm, λex = 450 nm). To test the effect of inhibitors on O_2^- and NO_2^- generation, 250 µL of haemocyte suspension in buffer were incubated for 30 min at RT with phorbol myristate acetate (10 µg·mL^{-1}) and either DPI (50 µM) or L-NIO (50 µM) or NMMA (50 µM) or SOD (750 U) in the absence (for NO_2^- assay) or in the presence of 250 µL of 0.1 % nitroblue tetrazolium (for O_2^- assay) (inhibitor concentrations were previously tested for cytolethality). At the end of the incubation period, the haemocyte suspensions were assayed for O_2^- or NO_2^- as described above. In positive controls the inhibitor solutions were substituted with buffer. In

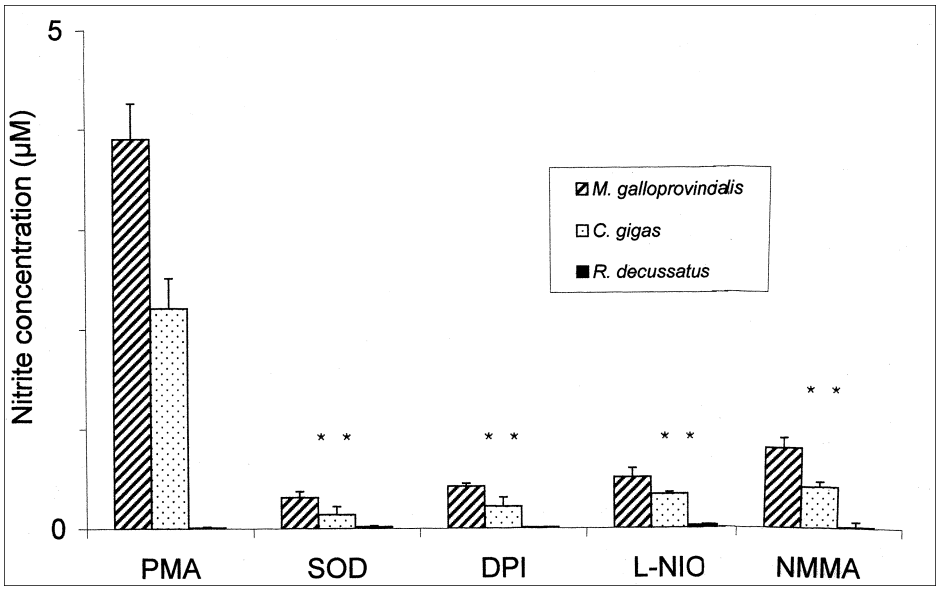

Figure 1. Effect of some chemicals on the production of nitrite by haemocytes from three bivalves: *Mytilus galloprovincialis*, *Crassostrea gigas* and *Ruditapes decussatus.* Two hundred and fifty microlitres of haemocyte suspension in phosphate buffered saline were incubated for 30 min with phorbol myristate acetate (10 µg·mL^{-1}) and either DPI (50 µM) or L-NIO (50 µM) or NMMA (50 µM) or SOD (750 U). Values are expressed as mean ± s.e.m. of six determinations with samples from different preparations.

negative controls, phorbol myristate acetate and inhibitors were substituted with buffer.

2.5. Data analysis

All assays were conducted six times. Details regarding statistical analysis are presented in figure legends.

3. RESULTS

A reduction of NBT was observed in haemocyte suspensions from *M. galloprovincialis* and *C. gigas* when stimulated in vitro with phorbol myristate acetate (*table I*). Formazan generation did not occur when haemocytes were preincubated with SOD and diphenylene iodonium before the addition of phorbol myristate acetate indicating a specific production of O_2^- by activation of haemocyte NADPH-oxidase.

In *R. decussatus*, the reduction of nitroblue tetrazolium was not increased by addition of phorbol myristate acetate indicating an absence of O_2^- production in this bivalve.

In similar experimental conditions, we next compared the generation of NO_2^- in the absence or in the presence of inhibitors of O_2^- and NO production.

As shown in *figure 1*, the stimulation of haemocytes with phorbol myristate acetate promoted NO_2^- generation in *M. galloprovincialis* and *C. gigas*, but not in *R. decussatus*. Moreover, the incubation of *M. galloprovincialis* and *C. gigas* haemocytes with SOD, DPI, L-NIO or NMNA before stimulation fully abolished the NO_2^- generation.

Table I. Superoxide production by haemocytes from *Mytilus galloprovincialis*, *Crassostrea gigas* and *Ruditapes decussatus* upon stimulation with phorbol myristate acetate (PMA) in vitro, inhibitory effect of SOD (750 U) and DPI (50 µM). Nitric oxide synthase inhibitors NMMA and L-NIO at the same concentration as DPI did not modify the superoxide production*.

Marine bivalve	PMA	+ SOD	+ DPI
M. galloprovincialis	0.134 ± 0.008	0.036 ± 0.006	0.050 ± 0.003
C. gigas	0.097 ± 0.007	0.025 ± 0.003	0.039 ± 0.005
R. decussatus	0	–	–

* Values are expressed as the nitroblue tetrazolium reduction read at 630 nm after 60 min incubation (after subtraction of the background value) and shown as mean ± s.e.m. of six determinations using samples from different preparations.

4. DISCUSSION

Bivalves do not display antigen-specific humoral mediation; their self-defence systems rely on non-specific agents such as lytic enzymes and agglutinins and on phagocytic processes mediated by haemocytes. Microbicidal agents active in phagosomes are lytic enzymes and reactive oxygen intermediates (ROI). As shown in *table I*, haemocytes from *M. galloprovincialis* and *C. gigas* produced O_2^- after in vitro phorbol myristate acetate stimulation, whereas, in the same experimental conditions, *R. decussatus* did not produce O_2^-, confirming the results reported previously by Lopez et al. (1994).

O_2^- production originates from the activation of NADPH-oxidase (Morel et al., 1991) and leads to a cascade of ROI including hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical (°OH).

The reaction of NO, a degradation product of L-arginine by NO-synthases, with O_2^- generates peroxynitrite ($ONOO^-$), which is a strong bactericidal agent (Denicola et al., 1996; Vazquez-Torres et al., 1996; Hurst and Lymar, 1997).

Production of $ONOO^-$ by mammal phagocytes has been abundantly studied (Beckman and Crow, 1993; Beckman et al.,1994; Beckman and Koppenol, 1996) but, in marine bivalves, $ONOO^-$ production has never been directly reported. However, Ottaviani et al. (1993) observed the inhibitory effect of NO-synthase inhibitors on the bacteria clumping promoted by haemocytes of a mussel, *M. edulis*, stimulated with lipopolysaccharide, and Nakayama and Murayama (1998) showed that the haemocytes from *C. gigas*, release NO_2^- when stimulated with phorbol myristate acetate.

Because NO_2^- is a stable end product of both NO and $ONOO^-$ and is itself a bactericidal agent (Klebanoff, 1993), we demonstrated the involvement of NO and $ONOO^-$ in the self-defence systems of marine bivalves by comparing the effect of SOD, a O_2^- scavenger, diphenylene iodonium, a NADPH-oxidase inhibitor with that of two NO-synthase inhibitors, L-NIO and NMNA on NO_2^- produced by haemocytes of three marine bivalve species stimulated with phorbol myristate acetate (*figure 1*).

We observed that *M. galloprovincialis* and *C. gigas*, the only species that generated O_2^-, produced NO_2^- when stimulated with phorbol myristate acetate in vitro. Moreover, NO-synthase inhibitors as well as SOD or diphenylene iodonium inhibited the NO_2^- production indicating that both NO synthase and NADPH-oxidase were involved.

Our results confirmed the absence of O_2^- generation previously reported by Lopez et al. (1994) with bivalve species of the order Veneroida and demonstrate that various defence systems are used by marine bivalves to kill microbial pathogens. The reduction in NO_2^- production observed in the presence of inhibitors of NO-synthase as well as NADPH-oxidase suggests that these enzymes are associated with *M. galloprovincialis* and *C. gigas* defence systems.

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