REVIEW

Effects of interleukin-2 on nitric oxide production in molluscan innate immunity

R Barcia, JI Ramos-Martinez

Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Santiago de Compostela, Lugo, Spain

Summary

The hemocytes are the cells responsible for the immunity in molluscs. Cytokines, growth factors, etc, present in the soluble fraction of the hemolymph modulate the immune response. The inflammatory cytokine interleukin-2 (IL-2) induces the synthesis of nitric oxide (NO), responsible for oxidizing processes. In hemocytes of mollusks, the presence of IL-2 induces the synthesis of the three subunits of the receptor of high affinity, and the subsequent activation of the signaling pathway where the cAMP-dependent protein kinase (PKA) plays the main role, with a secondary role of PKC. In the presence of IL-2, PKA activates NO synthesis through the constitutive enzyme *mc*-NOS. In winter, the action of PKC on a novel inducible form *mw*-NOS leads to an increase of the IL-2-induced NO synthesis. According to our studies, this set of metabolic reactions explains the seasonal variations in NO production by hemocytes of *Mytilus galloprovincialis*. However, after two years of studies, and coinciding with the *Prestige* oil spill in November 2002, this effect was cancelled for at least the two years following the catastrophe.

Key words: hemocyte; immune response; interleukin-2; nitric oxide; mollusc; *Mytilus galloprovincialis*

Introduction

Molluscs constitute the widest phylum existing on Earth after arthropods, with over 100,000 species present in all the ecosystems. Many mollusc species, mainly marine, are very important from the economic point of view because of their use as human food resource. Molluscs are also interesting because of their susceptibility to infection by parasites, bacteria, and viruses, which makes them transmitters of many diseases affecting different vertebrate species. Finally, their ability to accumulate diverse toxins allows considering several mollusc species as excellent biosensors of the biologic quality of the ecosystems.

Unlike vertebrates, molluscs only have an ancestral defensive line, comparable to that known as immune innate system in vertebrates (Medzhitov and Janeway, 2000; Plows *et al.*, 2005), and constituted by a cellular component and the products that it generates (i.e. humoral component).

The circulating cells, called hemocytes, are responsible for the phagocytosis, cytotoxic reactions, and the synthesis of the humoral factors, comprising antimicrobial peptides, agglutinins, lectins, cytokines, nitric oxide, etc. (Ottaviani, 2006). Due to their immune activity, hemocytes can be also referred to as immunocytes. Although molluscan immunocytes show a wide heterogeneity, the cells involved in the defensive processes are similar to the macrophages from vertebrates. Their morphological features include an irregular shape, a small nucleus in relation to cell size and presence of numerous granules (Krupa *et al.*, 1977; Joky *et al.*, 1983; Ottaviani and Franchini, 1988; Ottaviani *et al.*, 1998a; Cao *et al.*, 2003). Moreover, molluscan hemolymph contains other hemocytes, roundish and with a bigger nucleus. In the particular case of *Mytilus galloprovincialis*, both cell types seem to correspond to two maturation stages of the same cell (Ottaviani *et al.*, 1998a). As far as the humoral component is concerned, it promotes several responses involving cell shape changes, chemotaxis, phagocytosis, cytotoxicity, encapsulation, and neuroendocrine responses, thus suggesting the involvement of stress mediators in the immune response (for review see, Ottaviani, 2006; Ottaviani *et al.*, 2007).

Corresponding author:
Juan Ignacio Ramos-Martinez
Departamento de Bioquímica y Biología Molecular,
Facultad de Veterinaria,
Universidad de Santiago de Compostela,
E-27002 Lugo, Spain
E-mail: bniramos@lugo.usc.es

In invertebrates, physiologic responses suggesting the presence of molecules with cytokine-like functions have been observed in molluscs, insects, annelids, echinoderms, and tunicates (Ottaviani *et al.*, 1995a, 2004; Ottaviani, 2006). As for molluscs, cytokine-like molecules with immune and neuroendocrine functions have been detected in both marine and freshwater species. In this sense, heterologous cytokines provoking cell shape changes, chemotaxis or phagocytosis are also capable to induce the synthesis of biogenic amines (BA), nitric oxide (NO) or oxygen radicals. These results suggest the existence of an ancestral immune-mobile brain that imitates the hypothalamic-pituitary-adrenal axis (HPA) from vertebrates (Ottaviani *et al.*, 1993a; Ottaviani, 2004, 2006). Heterologous cytokines, growth factors, and cell differentiating factors increase epinephrine, norepinephrine and dopamine synthesis in molluscan hemocytes, both in those freshly extracted and those cultured for several days (Franchini *et al.*, 2000; Cao *et al.*, 2003, 2004b, 2007b).

Interleukin-2 (IL-2) receptor in molluscan hemocytes

Early studies on the involvement of diverse cytokines in the activation of the immune response in molluscan hemocytes suggest the existence of a receptor with low specificity (Ottaviani *et al.*, 1994, 1995a, b). IL-2 was one of the cytokines assayed, as it induces phagocytosis and provokes the strongest response in the synthesis of BA, NO, etc (Ottaviani *et al.*, 1995a, b). Studies on the interference of the inflammatory cytokine and corticotrophin-releasing hormone (CRH) on BA production provided the initial clues about the possible presence of an IL-2 receptor (Ottaviani *et al.*, 1994). Subsequent studies on hemocytes of *M. galloprovincialis* allowed the detection of the three subunits constituting the IL-2 receptor (Barcia *et al.*, 1999). Flow cytometry confirmed the presence of a subunit of high affinity, termed IL-2Rα, and two of low affinity, termed IL-2Rβ and IL-2Rγ, which only one group of hemocytes expresses (Barcia *et al.*, 1999). Also lipopolysaccharide (LPS) induces the expression of the IL-2 receptor, which can be explained as a collateral effect, similar to that detected in vertebrates, as LPS might stimulate the hemocytes to synthesize molecules comparable to TNF-α, and the necrosis factor might in its turn induce IL-2 the synthesis of molecules structurally/functionally related to IL-2 (Barcia *et al.*, 1999).

Nitric oxide production in molluscs

Nitric oxide (NO) is a gas that performs multiple biologic functions. Among them outstands its character of intra and extracellular signal (Moncada *et al.*, 1991). In recent years, several studies have proved the presence of nitric oxide synthase (NOS) and the involvement of NO in cellular signaling in organisms dispersed through the whole animal kingdom (Palumbo, 2006). The involvement of NO in
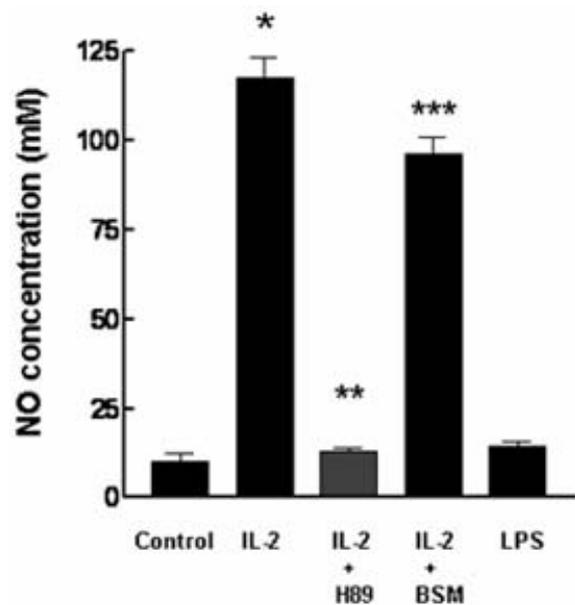
Fig. 1 Effect of different representative protein kinase inhibitors on the stimulation of NO production by IL-2. Control: non-activated hemocytes. *Compared with control $p < 0.001$. ** Compared with IL-2 $p < 0.001$. *** Compared with IL-2. (Modified from Novas *et al.*, 2004).

such processes as memory (Korneev *et al.*, 2005), bioluminescence (Trimmer *et al.*, 2001), sight (Elphick *et al.*, 1996), or cell proliferation (Kuzin *et al.*, 1996) was studied in invertebrates. Particularly, in molluscs, NO and the different forms of NOS seem to be involved in the modulation of neuron activity (Gelperin, 1994; Moroz *et al.*, 1996; Hurst *et al.*, 1999; Stefano and Ottaviani, 2002), metamorphosis (Leise *et al.*, 2004) and preferably in processes related to the immune defense (Ottaviani, 2006).

NO may act directly, but it also reacts with free oxygen radicals, thus generating peroxinitrite ($ONOO^-$), a potent oxidizing agent whose action affects the peroxidation of the membrane lipids, the inhibition of tricarboxylic acid (TCA) cycle, and the mitochondrial respiration (Nappi and Ottaviani, 2000).

Molluscan hemocytes remove bacteria by means of phagocytosis and NO production. Both processes are interdependent, although phagocytosis seems to happen before NO synthesis. This means that the bacterial clumping takes place prior to NO elimination. Hemocyte incubation with sodium nitroprusside provokes bacterial clumping, and LPS also display a similar action (Ottaviani *et al.*, 1993b; Franchini *et al.*, 1995; Tafalla *et al.*, 2002). In addition, incubation of hemocytes from the mussel *M. galloprovincialis* or the oyster *Crassostrea gigas* with phorbol myristate acetate (PMA) or laminarin leads to the synthesis of NO and superoxide ions. These induce the synthesis of peroxinitrite, which has the cytotoxic properties commented above (Arumugan *et al.*, 2000,
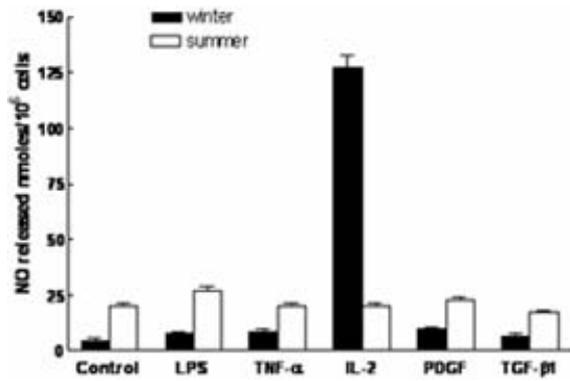
Fig. 2 NO synthesis by haemocytes of *Mytilus galloprovincialis* collected in winter and summer. A) The cells were cultured for 3 days in L-15 medium and then incubated for 24 h with the referred cytokines as expressed in Novas *et al.*, 2007c. The bars represent the standard deviation of six individual assays. Results of a representative assay of each season. Tumor necrosis factor-α (TNF-α); Platelet-derived growth factor (PDGF); Transforming growth factor-β1 (TGF-β1).

2001; Gourdon *et al.*, 2001; Torreilles and Romestand, 2001). These authors, however, discard LPS as inducer of NO synthesis. The existence of NOS activity in molluscan hemocytes was proved in *Viviparus ater*. The activity was stimulated by LPS and was located in the soluble fraction of hemolymph (Conte and Ottaviani, 1995). The use of NOS inhibitors promoted the canceling of the bacterial clumping induced by LPS in the molluscan hemocytes of *M. edulis* and *V. ater* (Ottaviani *et al.*, 1993b).

Studies performed in hemocytes from freshwater molluscs show that the incubation with different cytokines induces a significant increase of NO production (Ottaviani *et al.*, 1995a). Similar works performed with hemocytes from marine molluscs offer similar results, with IL-2 as the cytokine inducing the maximal response (Ottaviani *et al.*, 1995a). The results are similar, either if the hemocytes were freshly extracted or if they were subjected to culture for several days, and this was related to the capability of molluscan hemocytes to express the complete structure of the IL-2 receptor (Barcia *et al.*, 1999; Novas *et al.*, 2004). During culturing, the hemocytes from *M. galloprovincialis* keep their ability to synthesize NO induced by IL-2. The results about the effect of LPS on NO production are different, maybe because of the use of LPS of diverse origins LPS does not induce the NO synthesis in hemocytes of *Mytilus* (Arumugan *et al.*, 2000, 2001; Gourdon *et al.*, 2001; Torreilles and Romestand, 2001), a different result to the obtained with cells of other models (Conte and Ottaviani, 1995).-

Mytilus hemocytes respond to the presence of mammalian IL-2 synthesizing BA and NO, although the difference between the kinetics of NO and BA synthesis (Cao *et al.*, 2004a, b; Novas *et al.*, 2004, 2007b) suggest different pathways of signal

internalization. Apparently, BA synthesis involves preferably the enzyme protein kinase C (p105) (Cao *et al.*, 2004a, 2007a), whereas the cAMP-dependent protein kinase (PKA) plays a secondary role (Cao *et al.*, 2004a). Both enzymes were purified from different tissues of *M. galloprovincialis* (Mercado *et al.*, 2002a, b, 2003; Diaz-Enrich *et al.*, 2003; Bardales *et al.*, 2004), which led to the obtaining of specific antibodies that allowed learning more about the function of these enzymes in the action of IL-2 on NO production.

As commented above, phorbol 12-myristate 13-acetate (TPA) induces NO synthesis, thus involving PKC in the process (Arumugan *et al.*, 2000, 2001; Gourdon *et al.*, 2001; Torreilles and Romestand, 2001). Since IL-2 has the same effect of TPA, all suggests that PKC is involved in the action induced by IL-2. Similar works on freshwater molluscs such as *Lymnaea stagnalis* link PKC and ERK (extracellular signal-regulated kinase) to the signaling mechanisms of the regulation of NOS activity (Wright *et al.*, 2006).

Despite the involvement of PKC in mediating the signal borne by IL-2, further experiments with specific PK inhibitors indicate that the signaling pathway by which IL-2 induces NO synthesis preferably involves PKA. The action of inhibitors specific for these protein kinases suggests that the signal of the IL-2-induced NO synthesis preferably involves PKA (Fig. 1). The kinase activates a form of NOS specific of *Mytilus* hemocytes (Novas *et al.*, 2004), initially identified as a form of 130 kDa and immunologically similar to iNOS from vertebrates (Novas *et al.*, 2004). The different kinetics of BA and NO production that the cell shows in the presence of IL-2 seems to have its base on the different protein kinase involved in each process.

As it happens in many aspects of the physiology of marine molluscs (Robledo *et al.*, 1995; Ramos-Martinez *et al.*, 1993), hemocyte immune response shows a seasonal variation in basal and IL-2-induced NO synthesis (Novas *et al.*, 2007c).
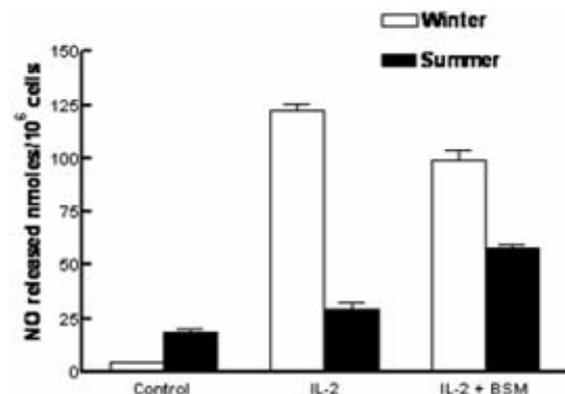
Fig. 3 IL-2-induced production of NO. Control: basal non-stimulated hemocytes. IL-2: hemocytes incubated with IL-2. The PKC inhibitor bisindolylmaleimide (BSM) was added to the cells simultaneously with IL-2. Error bars are the SD of six assays as described in Novas *et al.*, 2007b.
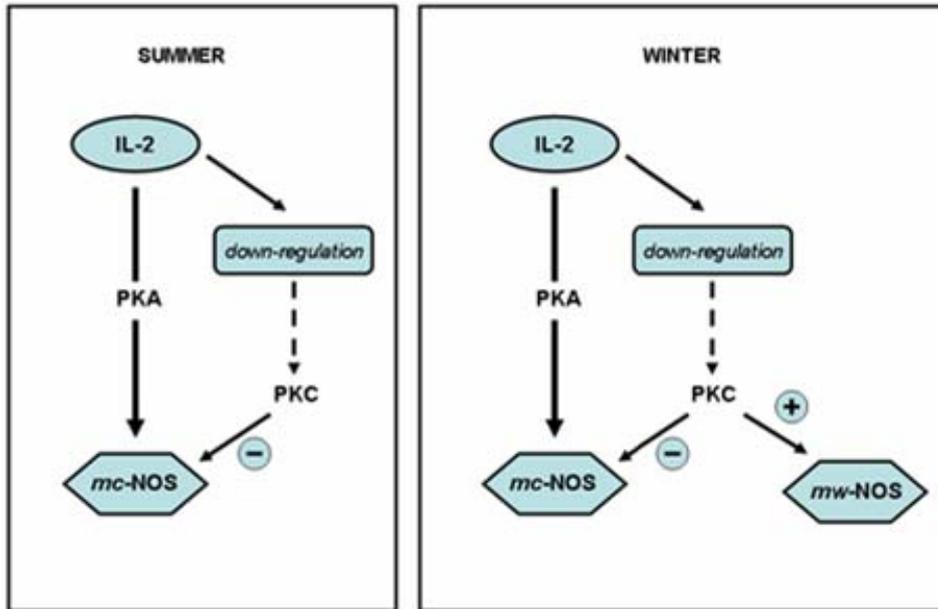
Fig. 4 Schematic diagram illustrating the hypothetical implication of PKA and PKC in the two seasonal pathways of hemocyte IL-2-induced NO synthesis. Dotted lines represent the inhibiting effect of down-regulation (Novas *et al.*, 2007b).

NO basal production is higher during the summer months, whereas IL-2-induced NO synthesis by cells obtained in winter is 5-fold that of the cells obtained in summer (Fig. 2). When studying the effect of PKC inhibitors on the seasonal variation of IL-2-induced NO production, PKA appeared as the major activating agent of the *mytilus constitutive-*NOS (*mc*-NOS) (Novas *et al.*, 2004). Also, the results about NO seasonal production in the presence of bisindolylmaleimide (BSM) suggest a participation of PKC that would be compatible with the hypothesis expressed in Fig. 4 (Novas *et al.*, 2007b). PKC appears as a potent *mc*-NOS inhibitor. In summer, and as a consequence of the presence of IL-2, takes place PKC inhibition by *down-regulation*, which implicates PKA in keeping an activating action of NO synthesis. The action described persists in winter, but in this season, a new NOS-inducible enzyme was detected. This was termed *mytilus winter*-NOS (*mw*-NOS) and has similarities with the constitutive form of vertebrates susceptible of activation by PKC (Fig. 4). PKC activating action on the new *mw*-NOS inducible enzyme explains the result presented in Fig. 3.

Concluding remarks

In marine molluscs, there is a remarkable biologic paradox such as the fact that the widest mortalities occur in the seasons with most abundant food and when the immunologic potential is seems at its maximum (summer). Apparently, the reason for the high mortality could be the increase of not only food but also parasites and pathogens occurring in phytoplankton blooms (Robledo *et al.*, 1995; Lacoste *et al.*, 2001; Hernroth, 2003). When considering the basal NO synthesis, the low production detected in winter is still compatible with a frame of reduced immune response in winter, hich may seem rather odd, considering the high degree of mortality registered in summer. Indeed, this supposed immunodepression during wintertime is false, because an inflammatory phenomenon, and especially the increased production of molecules related to IL-2 and to the activation of *mw*-NOS trigger the mechanism that ensures the response, despite the low basal levels on NO.
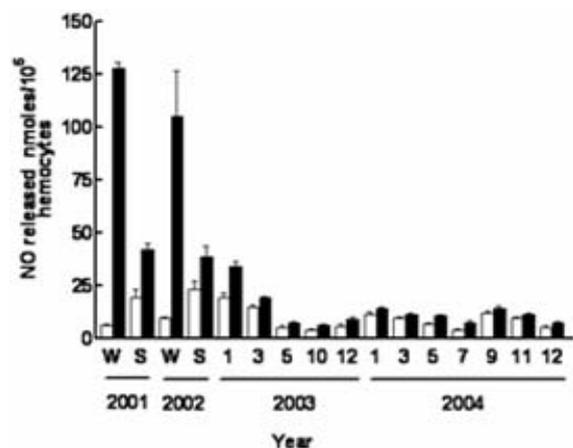
Fig. 5 Seasonal variation of nitric oxide production by hemocytes of *Mytilus galloprovincialis* Lmk. In 2001 and 2002 the assays were performed during winter (W) and summer (S) (Novas *et al.*, 2007c). Empty bars represent the basal production and solid bars represent NO production of hemocytes incubated with 10 μg/ml IL-2 for 24 h. Error bars are the SD of 6 assays.

The results of the assay on NO basal production and, mainly, hemocyte response against IL-2 accounts for the utility of *Mytilus* as biosensor, to judge by the consequences of the *Prestige* oil spill in the immune response of the mollusc (Ruiz-Villareal *et al.*, 2006; Soriano *et al.*, 2006). As shown in Fig. 5, NO synthesis ability was stable in the years before the catastrophe; then it drops and does not recover until two years later. The process is especially dramatic if assayed NO production by hemocytes treated with IL-2. Pollution inactivates NO basal production, but it also cancels the NO production mechanism induced by IL-2 during the assay. Hemocyte lack of response during assay time is parallel to other assays, such as the study of cell necrosis, apoptosis and others (Novas *et al.*, 2007a), and is compatible with a situation of immunodepression (Laffon *et al.*, 2006).

Finally, the test on IL-2-induced NO production by molluscan hemocytes may join other parameters assayed for the study of the effect of diverse stressing factors on the viability of edible molluscs, considered as biosensors (Malagoli *et al.*, 2006).

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