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An inducible nitric oxide synthase (NOS) is expressed in hemocytes of the spiny lobster *Panulirus argus*: Cloning, characterization and expression analysis

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ABSTRACT

Nitric oxide (NO) is a free radical gas involved in a variety of physiological processes in invertebrates, such as neuromodulation, muscle contraction and host defense. Surprisingly, little is known about the involvement of NO synthase (NOS) in the immune system of crustaceans. This work is focused on the study of the NOS gene of the spiny lobster *Panulirus argus*, a crustacean with commercial interest, and its relationship with the immune response to a microbial elicitor. A NOS full-length DNA was isolated from hemocytes by reverse transcription-polymerase chain reaction (RT-PCR) using degenerated primers. The open reading frame (ORF) encodes a protein of 1200 amino acids, with an estimated molecular mass of 135.9 kDa, which contains the conserved domains and binding motifs of NOS found in a variety of organisms. NOS gene expression in lobster gills, heart, stomach, digestive gland, abdominal muscle, gut and hemocytes was studied by Real Time quantitative PCR (Real Time qPCR). The expression was higher in hemocytes, heart and gills. In addition, when lobster hemocytes were exposed *in vitro* to *Escherichia coli* O55:B5 lipopolysaccharide (LPS), an increase in the NOS activity and also in the NOS gene expression evaluated by Real Time qPCR was observed, thus demonstrating the presence of an inducible crustacean NOS by a microbial elicitor of the immune response. The information is relevant in providing basic knowledge for further studies of crustacean defense mechanisms.

1. Introduction

Nitric oxide (NO) is a short-lived radical generated by nitric oxide synthases (NOS). NOS catalyze the production of NO from L-arginine and molecular oxygen, with L-citrulline as the by-product. Oxidation is carried out by the heme group, while reduction is achieved using electrons donated by NADPH due to the action of tetrahydrobiopterin (BH4), which is thought to couple the reduction of NADPH to the synthesis of NO. Electrons are then transferred via FAD and FMN to the heme group, which then oxidizes the terminal guanidine nitrogen atoms of L-arginine to NO and L-citrulline. For review see Eddy [1].

Three NOS isoenzymes have been recognized in vertebrate tissues: the brain (bNOS or NOS1) and endothelial (eNOS or NOS3) isoforms that are constitutively expressed and calcium/calmodulin-regulated, and the inducible isoform (iNOS or NOS2) that is calcium-independent and whose activity increases following exposure to certain cytokines and microbial lipopolysaccharide (LPS) [2].

NO performs a variety of functions in different tissues, in the nervous, cardiocirculatory and immune systems [3]. In vertebrates it is well established that iNOS is a very important component of the innate immune system participating in the elimination of pathogens in which its oxidation-derived compounds have antibacterial and antiviral activity [4,5]. It is expressed in macrophages, Kupffer cells, neutrophils, fibroblasts vascular smooth muscle and endothelial cells in response to pathological stimuli [1].

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In invertebrates, NO has also been involved as a modulator of nerve and muscle activity [6–12]; however, in this group of organisms, there is now an increasing number of references on NO that show, in addition, multiple biological roles of this molecule, related to feeding, defense, environmental stress, learning, metamorphosis, swimming, symbiosis, hemocyte aggregation and regulation of blood pressure. For review see Palumbo [13].

The role of NOS in the immune response of invertebrates has been reported for many taxa. NOS has been related to the immune response to LPS [14–18], yeast [19,20], laminarin [19], silica beads [17], bacteria [18,21], parasites [22–24] and viruses [15]. For instance, an inducible NOS has been demonstrated in *Anopheles stephensi* that limited the development of *Plasmodium berghei*, the malaria parasite [22]; in the mollusc, *Viviparus ater*, the LPS-stimulated snail hemocytes showed an increase in their NOS activity as compared to the control [14]; and the hemocytes of the shrimp *Marsupenaeus japonicus* were able to produce NO after stimulation with LPS or with White Spot Syndrome Virus (WSSV) [15].

Surprisingly, most of the crustacean studies on NOS and its relation to the immune system have been made at the enzymatic level, and there are not many data that relate crustacean NOS expression with the immune response. NOS cDNA has been cloned and sequenced in only three crustaceans, the decapod *Gecarcinus lateralis* [25] and the cladocerans *Daphnia pulex* [26] and *Daphnia magna* [21]. In *G. lateralis*, a study was performed on the expression of the NOS gene in some neuronal and non-neuronal tissues. The authors discussed the NOS expression in gills as a possible role of this enzyme in host defense [25], although further experimental evidence is needed to demonstrate if NOS from gills is involved in crustacean immune responses. In *D. magna* a challenge with its natural pathogen, *Pasteuria ramosa*, did not vary NOS expression, but an increase in host resistance was found when L-arginine was added to the water [21]. Until the present work, there have been not been any studies that demonstrate changes in NOS gene expression after *in vitro* or *in vivo* stimulation of the crustacean immune system.

Spiny lobsters (Palinuridae) are interesting models for the study of the crustacean immune system. This is due to the large amounts of hemolymph that can be collected, in which important humoral and cellular effectors of the crustacean immunity can be found [27–30]. In addition, spiny lobsters have a huge economic importance as they are among the most valuable seafoods and support some of the largest commercial fisheries in the world. However, most of the spiny lobster fisheries are either over-exploited and in decline or are being managed for their maximum sustainable yield. Recently, the capture of juveniles from the wild, and on-growing them to market size, have offered a means by which spiny lobster supplies can realistically be increased until hatchery production becomes commercially viable [31,32]. On the other hand, the experience obtained with the culture of other crustaceans such as penaeid shrimps, which has been dramatically affected by the appearance and rapid spread of diseases [33], teaches us that the industry must give priority to finding ways to combat pathogens, such as immunological studies of spiny lobsters, in order to avoid future economic losses.

Surprisingly, immunity in spiny lobsters remains poorly studied as compared to other crustaceans and few spiny lobster immunity effectors and mechanisms have been examined [34–38]. In addition, there are no available studies on NOS in these species.

In this study, the full-length cDNA sequence of a nitric oxide synthase from *Panulirus argus* is reported. NOS expression was also studied by Real Time qPCR in heart, gills, gut, stomach, digestive gland, abdominal muscle and hemocytes of *P. argus*. Through the increased NOS expression in hemocytes after using a microbial elicitor, it was demonstrated for the first time the presence of an inducible crustacean NOS and its relationship to the immune response. These findings contribute to the basic knowledge which is essential for the understanding of invertebrate defense mechanisms.

2. Material and methods

2.1. Biological material and RNA isolation

Lobsters between 0.5 and 0.8 kg of weight were collected by diving in the Golfo de Batabanó, which lies in the southwestern part of the Cuban archipelago. They were kept in 100-l tanks (two lobsters per tank) connected to a flow through seawater supply system, using biological–mechanical filtration, seawater UV-sterilization, central aeration system, and controlled photoperiod of 12: 12 h light: obscurity. All experiments were performed after one week in these conditions.

To obtain hemocytes, the same amount of hemolymph from five lobsters was extracted using a modified Citrate-EDTA buffer (400 mM NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid and 20 mM EDTA, pH 4.6) as anticoagulant [39]. The hemolymph was extracted from the fourth walking leg coxa using a pyrogen-free disposable syringe containing the same volume of pre-cooled anticoagulant solution. The mixture was centrifuged at 700 g for 10 min at 4 °C. The cell pellet was washed twice with anticoagulant and suspended again in anticoagulant solution.

Samples of hemocytes, digestive gland, abdominal muscle, heart, stomach, gut and gills were collected from five lobsters, frozen immediately in liquid nitrogen and stored at −80 °C prior to RNA extraction and gene expression studies.

Hemocytes and tissues were homogenized using a tissue homogenizer (Politrón UltraTurrax, Germany). Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method [40].

Total RNA concentration and quality were determined by UV absorbance at 260 nm, the A260/A280 ratio and by denaturing gel electrophoresis. Contaminating genomic DNA was eliminated by DNase I digestion (USB, USA).

2.2. cDNA cloning of P. argus NOS (Pa-NOS)

Five micrograms of RNA from hemocytes were reverse-transcribed into cDNA using the SuperScript III RT reverse transcriptase kit (Invitrogen, USA) according to the manufacturer's instructions. Reverse transcription products were RNase H treated (Invitrogen, USA) and cDNA was purified by filtration using a Centri-sep column (Princeton Separation, Adelphia, NJ).

The primers used to obtain the cDNA sequence of *P. argus* NOS are described in Table 1 and Fig. 1.

Two pairs of degenerate primers (degFNOS1-degRNOS2 and degFNOS3-degRNOS4) designed from highly conserved regions in a wide variety of NOS genes in the GenBank database (http://www.ncbi.nlm.nih.gov) were used to obtain the first two fragments of NOS cDNA by PCR (Fig. 1). The analysis for primer design included the protein sequences from eight insect species, one crustacean and one mollusc: (i) insects, (a) Lepidopteran: *Manduca sexta* (GenBank AAC61262), *Bombyx mori* (REFSEQ NP_001036963); (b) dipteran: *Drosophila melanogaster* (GenBank AAF25682), *A. stephensi* (GenBank AAC68577); (c) hymenopteran: *Apis mellifera* (REFSEQ NP_001012980); (d) hemipteran:*Rhodnius prolixus* (GenBank AAB03810), (e) orthoptera: *Gryllus bimaculatus* (DBJ BAH14964); (f) coleoptera: *Luciola cruciata* (DBJ BAF63161), (ii) crustaceans, decapoda: *G. lateralis* (GenBank AAT46681); (iii) mollusc, *Aplysia californica*, AF288780. The ClustalW software (http://www.ebi.ac.uk/clustalw/index.html) was used for the