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Research paper

Identification and molecular characterization of nitric oxide synthase (*NOS*) gene in the intertidal copepod *Tigriopus japonicus*

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ABSTRACT

In copepods, no information has been reported on the structure or molecular characterization of the nitric oxide synthase (*NOS*) gene. In the intertidal copepod *Tigriopus japonicus*, we identified a *NOS* gene that is involved in immune responses of vertebrates and invertebrates. *In silico* analyses revealed that nitric oxide (NO) synthase domains, such as the oxygenase and reductase domains, are highly conserved in the *T. japonicus NOS* gene. The *T. japonicus NOS* gene was highly transcribed in the nauplii stages, implying that it plays a role in protecting the host during the early developmental stages. To examine the involvement of the *T. japonicus NOS* gene in the innate immune response, the copepods were exposed to lipopolysaccharide (LPS) and two *Vibrio* sp. After exposure to different concentrations of LPS and *Vibrio* sp., *T. japonicus NOS* transcription was significantly increased over time in a dose-dependent manner, and the NO/nitrite concentration increased as well. Taken together, our findings suggest that *T. japonicus NOS* transcription is induced in response to an immune challenge as part of the conserved innate immunity.

1. Introduction

Nitric oxide (NO) is a gaseous intra- and intercellular signaling molecule and a key biological messenger (Kröncke et al., 1997). NO has been found to play pivotal roles in metabolism in all types of organisms (Nathan and Xie, 1994). NO is biosynthesized by several NO synthases (NOS) through a reaction of L-arginine with molecular oxygen to form L-citrulline with the aid of cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH_4), calcium (Ca^{2+}), and calmodulin (CaM) (Hope et al., 1991; Griffith and Stuehr, 1995). Vertebrates have three NOS isoforms, neuronal NOS (nNOS or NOSI), inducible NOS (iNOS or NOSII), and endothelial NOS (eNOS or NOSIII), that share a similar structure and function. nNOS and eNOS are constitutively expressed to synthesize NO in response to the intracellular Ca^{2+} concentration. Most invertebrates have a single *NOS* gene that is likely to encode a constitutive neuronal-like isoform from an ancestral *NOS* gene (Andreakis et al., 2011), while the diversity of invertebrate *NOS* isoforms is occasionally driven by gene/genome duplication. Thus, the annotation of invertebrate *NOS* as "inducible" may not be justified based solely on physiology (Andreakis et al., 2011).

The endogenous NO and NOS functions have been extensively studied in mammals, and information on NO biosynthesis, biological activity, and signaling associated with antibacterial, antiviral, and antiparasitic activity is available for invertebrates (Knowles and Moncada, 1994; Müller, 1997). The insect NOS system and its rapid inducibility in response to many different pathogens have been extensively studied (Nappi et al., 2000; Rivero, 2006; Davies, 2000; Marmaras and Lampropoulou, 2009; Park et al., 2013). NOS is also a pivotal component of the innate immune system and a cellular signal of environmental stress in aquatic invertebrates (Giovine et al., 2001; Palumbo, 2005). Particularly in aquatic crustaceans, LPS-triggered inducibility of NOS activity and *NOS* transcript level have been highlighted in the haemocyte tissues that play a crucial role in the innate immunity of invertebrates (Rodríguez-Ramos et al., 2010, 2011; Yao et al., 2010; Li et al., 2012; Jiang et al., 2013). However, inconsistent correlation between *NOS* transcription and activity was also observed in response to immune challenge. For example, in the tiger shrimp (*Penaeus monodon*), *NOS* mRNA expression was not significantly stimulated in response to *in vivo* LPS exposure. Instead, the NOS was quickly induced at the early stage of wound inflammation (Wu et al., 2013). In copepods, the structure of the *NOS* gene and its molecular response remain unclear,

Abbreviations: *iNOS*, inducible nitric oxide synthase; NO, nitric oxide; LPS, lipopolysaccharide; NADPH, nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide; BH_4, tetrahydrobiopterin; CaM, calmodulin; CDD, Conserved Domain Database; GTR, general time-reversible; EtBr, ethidium bromide; ORF, open reading frame; pI, isoelectric point.

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although the biological roles of NO have been suggested to be involved in feeding, defense, environmental stress, learning, metamorphosis, swimming, symbiosis, haemocyte aggregation, and regulation of blood pressure of marine invertebrates (reviewed in Palumbo, 2005).

Although several immune aspects of large crustaceans, including crabs, lobsters, and shrimp, have been extensively studied for their economic importance (Mydlarz et al., 2006), little attention has been given to small crustaceans, such as copepods. Copepods are likely to have more specific immune-relevant defense mechanisms than primitive mechanisms attributed to invertebrates (Huq et al., 1983; Kurtz, 2007). Particularly, the intertidal copepod *Tigriopus japonicus* (Copepoda, Harpactidae), inhabits splash pools and is directly exposed to daily environmental stimuli, including immune stressors and aquatic anthropogenic contaminants in freshwater run-off (Raisuddin et al., 2007). Thus, *T. japonicus* is a good model species to bridge laboratory-based immune relevant experiments of marine environments and field-based studies. In this study, we cloned and characterized the full-length cDNA of *NOS* from *T. japonicus*. Also we investigated changes in *T. japonicus NOS* (*Tj-NOS*) transcription in response to immune challenges. These results will be useful to better understand the potential involvement of the *NOS* gene in the innate immune response of copepods.

2. Materials and methods

2.1. Culture and maintenance

T. japonicus was originally collected from a single rockpool at Haeundae beach (35°9′29.57″N, 129°9′36.60″E) in Busan (South Korea) in 2003. It has since been continuously cultured in a laboratory (≈300 generations; Sungkyunkwan University, Suwon, South Korea) with filtered artificial sea water (TetraMarine Salt Pro, Tetra™, Cincinnati, OH, USA) with a salinity of 30 practical salinity units (psu) at 25 °C and a photoperiod of 12 h:12 h light/dark. The copepods were fed green algae *Chlorella vulgaris* (approximately 6×10^4 cells/ml). *T. japonicus* was identified based on morphological characteristics and the sequence identity of the universal barcode marker, the mitochondrial DNA *CO1* gene.

2.2. Cloning and annotation of NOS gene

A *T. japonicus* transcriptome DNA database was constructed as shown in our previous study (Kim et al., 2015). Briefly, Illumina NGS sequencing (Solexa paired-end library) and assembly with Trinity software (ver. 2.0.6; Grabherr et al., 2011) produced 140,130 contigs with Trinity with a size range of 201 to 30,174 bp. Sequence reads with a Q-score below 30 and a length shorter than 90 bp were removed for quality control. Finally, TransDecoder (http://transdecoder.sourceforge.net) found 54,761 contigs (accumulated nucleotide length: 82,984,914 bp) that contain candidate coding regions. The sequences of the transcript contigs were deposited to the Transcriptome Shotgun Assembly (TSA) database in GenBank (Accession no. GCHA01000000). In the assembled transcripts, we scanned for the presence of conserved oxygenase and reductase domains which are general features of the *NOS* gene using InterProScan (Zdobnov and Apweiler, 2001). The cDNA sequence coding for putative *NOS* was subjected to BLAST analysis in the GenBank non-redundant (NR; including all GenBank, EMBL, DDBJ, and PDB sequences except EST, STS, GSS, or HTGS) amino acid sequence database to confirm its identity. To confirm exon/intron boundaries and start/stop codons, the genomic structure was compared with the *T. japonicus* genomic DNA database (Lee et al., 2010). All gene information was registered in the GenBank database.

2.3. Conserved domain and phylogenetic analysis

NO synthase domains such as the N-terminal oxygenase domain (P450-like cysteine thiolate-ligate heme; tetrahydrobiopterin, BH_4), linker domain (calmodulin binding motif, CaM), and C-terminal reductase domain (flavin mononucleotide, FMN; flavin adenine dinucleotide, FAD; nicotinamide adenine dinucleotide phosphate, NADPH) were analyzed through a Pfam HMM search (http://pfam.sanger.ac.uk), a Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan), and web-based National Center for Biotechnology Information (NCBI)'s Conserved Domain Database (CDD).

To place the *NOS* gene on the phylogenetic tree, multiple alignment of the *NOS* gene with those of other species was performed using Clustal X software (ver. 1.83) at the level of the deduced amino acid sequences. For phylogenetic analysis, gaps and missing data matrices were excluded from the analysis. The generated data matrix was converted to nexus format, and the data matrix was analyzed with the MrBayes program (ver. 3.1.2) and the general time-reversible (GTR) model. A total of 1,000,000 generations were conducted, and the sampling frequency was assigned as every 100 generations. After analysis, the first 10,000 generations were deleted as the burn-in process. The consensus tree was constructed and then visualized with PHYLIP Tree View software (ver. 3.5).

2.4. Developmental stage

Like all copepods, *T. japonicus* undergoes anamorphic development with distinctive post-embryonic developmental stages by molting activity, resulting in naupliar stages (N1–6), copepodid stages (C1–5), and adults (male and female). To prepare different stages of developmental samples, entire copepod cultures were separated in 4 size classes roughly with 3 sieves (90, 150, and 200 μm). Of 4 separated groups (<90, 90–150, 150–200, and >200 μm), 3 naupliar stages (N1–2, N3–4, N5–6; 120 individuals were separated into three groups as triplicate in each stage), three copepodid stages (C1–2, C3–4, C5; 100 individuals were separated into three groups as triplicates in each stage), and adults (males and females; 100 individuals were separated into three groups as triplicates in each sex) were sampled one by one with two standards (body length and phenotype) under a stereomicroscope (Olympus IX71, Olympus Corporation, Tokyo, Japan).

2.5. Lipopolysaccharide (LPS) and Vibrio exposure

LPS was purchased from Sigma (L3024; Sigma-Aldrich, Inc., St. Louis, MO, USA). Lyophilized LPS was dissolved to 10 mg/ml in deionized water. Adult copepods were exposed to different LPS concentrations (1, 5, 10, 25, 50, and 100 μg/l) for 24 h in a 50-ml culture tube (SPL Life Science, Seoul, South Korea) at a salinity of 30 psu at 25 °C and a photoperiod of 12 h:12 h light/dark. No mortality was observed for 96 h at 1 mg/l LPS exposure. Three replicates (n ≈ 200/50 ml artificial seawater for each replicate) were used for each LPS concentration. An algal diet of *C. vulgaris* (6×10^4 cells/ml) was supplied during the experiment. For a time–course experiment, copepods were exposed to 100 μg/l LPS for 0, 3, 6, 12, 24, 48, 72, and 96 h. During the experiment, 50% of the culture water was replaced after 24 h, while maintaining the desired LPS concentration, with an algal diet of *C. vulgaris* (6×10^4 cells/ml) every 24 h.

Two *Vibrio* species, *Vibrio vulnificus* and *Vibrio parahaemolyticus*, were obtained from the Korean Culture Collection of Microorganisms (KCCM, Seoul, South Korea) and were cultured in marine broth (Pronadisa, MA, USA) at 25 °C. Three replicates (n ≈ 200/50 ml artificial seawater for each replicate) were used for each *Vibrio*-exposed group, and different concentrations of both *Vibrio* species (1.0×10^1–10^6 cfu/ml) were added for 24 h in a 50 ml culture tube (SPL Life Science, Seoul, South Korea) at a salinity of 30 psu at 25 °C and a photoperiod of 12 h:12 h light/dark. No mortality was observed in either *Vibrio*-exposed group after 96 h of exposure to 10^6 cfu/ml of *Vibrio*. For a time–course experiment, copepods were exposed to 1.0×10^6 cfu/ml of each *Vibrio* species for 0, 3, 6, 12, 24, 48, 72, and 96 h in a 50 ml culture tube (SPL Life Science, Seoul, South Korea) at a salinity of 30 psu at 25 °C