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Short communication

Insight into the messenger role of reactive oxygen intermediates in immunostimulated hemocytes from the scallop *Argopecten purpuratus*

Daniel Oyanedel [a], Roxana Gonzalez [b], Katherina Brokordt [b], Paulina Schmitt [a, *], Luis Mercado [a, **]

[a] Laboratorio de Genética e Inmunología Molecular, Instituto de Biología, Pontificia Universidad Católica de Valparaíso, 2373223 Valparaíso, Chile
[b] Laboratorio de Fisiología y Genética Marina (FIGEMA), Centro de Estudios Avanzados en Zonas Áridas (CEAZA), Universidad Católica Del Norte, 1781421 Coquimbo, Chile

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ABSTRACT

Reactive oxygen intermediates (ROI) are metabolites produced by aerobic cells which have been linked to oxidative stress. Evidence reported in vertebrates indicates that ROI can also act as messengers in a variety of cellular signaling pathways, including those involved in innate immunity. In a recent study, an inhibitor of NF-kB transcription factors was identified in the scallop *Argopecten purpuratus*, and its functional characterization suggested that it may regulate the expression of the big defensin antimicrobial peptide *ApBD1*. In order to give new insights into the messenger role of ROI in the immune response of bivalve mollusks, the effect of ROI production on gene transcription of *ApBD1* was assessed in *A. purpuratus*. The results showed that 48 h-cultured hemocytes were able to display phagocytic activity and ROI production in response to the β-glucan zymosan. The immune stimulation also induced the transcription of *ApBD1*, which was upregulated in cultured hemocytes. After neutralizing the ROI produced by the stimulated hemocytes with the antioxidant trolox, the transcription of *ApBD1* was reduced near to base levels. The results suggest a potential messenger role of intracellular ROI on the regulation of *ApBD1* transcription during the immune response of scallops.

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1. Introduction

The immune response of marine mollusks has been widely studied with a focus on the identification and characterization of genes homologous with vertebrates (Little et al., 2005). However, many processes remain unknown, and a systemic notion of the interaction between molecular components is required for a functional understanding of mollusk immune response. Among aquacultured mollusk species, the scallop *Argopecten purpuratus* represents one of the most important species in northern Chile (López et al., 2000). During the last decade, hatchery-reared larvae of scallops have suffered mass mortality events associated among others factors to the Gram-negative bacterium *Vibrio splendidus* (Rojas et al., 2015). Therefore, a deeper understanding of the immune response mechanisms in this species is essential for the improvement of management strategies in aquaculture.

Like all invertebrates, the immune response of mollusks depends exclusively on innate immune mechanisms, mediated by cellular and humoral components. Marine mollusk immunity has been reviewed in detail (Song et al., 2015; Bachère et al., 2015), and can be summarized in three main stages carried out through circulating and infiltrating immunocompetent cells, the hemocytes. These cells mediate (i) the recognition of non-self molecules by soluble and membrane receptors, (ii) the consecutive activation of intracellular signaling cascades and (iii) the triggering of cellular and humoral responses, such as phagocytosis and the expression of antimicrobial peptides, which are key effectors of antimicrobial immune response (Canesi et al., 2002). Antimicrobial peptides (AMPs) are present in virtually all organisms, displaying diverse roles in immunity (reviewed in (Bulet et al., 2004)). To date, two AMPs have been characterized from scallops; a fragment of the histone H2A and a big defensin. The big defensin displays antimicrobial properties and it is upregulated during immune response (Song et al., 2015), and as with many other AMPs, it appears to be regulated by a putative NF-kB pathway (Oyanedel et al., 2016).

Reactive oxygen intermediates (ROI) are metabolites

* Corresponding author.
** Corresponding author.
E-mail address: paulina.schmitt@pucv.cl (P. Schmitt).

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constitutively produced mainly in the mitochondria as a side reaction of the respiratory chain. They can be induced in response to damage associated molecular patterns (DAMPs) such as pollutants and ionizing radiation (Bartosz, 2009; Lodovici and Bigagli, 2011), and in response to microorganism associated molecular patterns (MAMPs) during the phagocytic process (Flannagan et al., 2012; Donaghy et al., 2014). Evidence reported in higher vertebrates suggests that ROI can also act as messengers regulating a variety of signaling molecules, including transcription factors, G protein-coupled receptors, phosphatases and proteases that affect several cellular processes (Forman et al., 2010). Despite the fact that hemocytes produce ROI as part of their base metabolism as well as in response to immune stimuli, the multiple intracellular sources of ROI are poorly described in marine mollusks (Donaghy et al., 2014). Moreover, to date there is no information on the potential role of ROI as an immune messenger in invertebrates.

Considering that the main strategies of the innate immune response in mollusks involves both, ROI production and antimicrobial peptide expression derived from signaling events, the aim of this study is to investigate the effect of intracellular ROI production on the expression of the big defensin antimicrobial peptide in the scallop *Argopecten purpuratus*. Results obtained in this study constitute, to the best of our knowledge, the first evidence of a positive association between the ROI produced during phagocytosis and the expression of an antimicrobial peptide in mollusks.

2. Material and methods

2.1. Scallop procurement and maintenance

US National Research Council guidelines for the care and use of laboratory animals were strictly followed during this research (National Research Council, 2011). Adult scallops were collected from the Central Culture Center at the Universidad Católica del Norte (UCN), in Coquimbo, transported to the Experimental Laboratory of Aquaculture Curauma, (PUCV) and placed in a 60 L sea water tank connected to a water pump with a UV system (SERA), a biological filter (SERA) and a chiller field control circuit (HAQOS). The water was maintained at 17 °C and seawater was replaced 1:10 every day. The scallops were fed daily with the microalgae *Isochrysis galbana* and *Tetraselmis suecica* until use.

2.2. Hemocyte primary cultures: immune stimulation and ROI scavenger

The hemolymph was collected from the adductor muscle of the scallops using a cold syringe coupled to an 18G needle. Hemolymph was pooled in polypropylene tubes in ice and the total hemocyte number was determined using a hemocytometer. Hemolymph was centrifuged at 800 × g for 5 min at 4 °C. Hemocyte pellets were resuspended in modified L15 medium with Glutamine (Thermofisher Scientific), supplemented with 10% FBS, 50% 0.22 μm filtered scallop plasma, 500 mOsm NaCl, penicillin (100 U/mL) and Streptomycin (50 μg/mL). Cell number counts were adjusted to 4×10^6 cell/mL, 1 mL was added to 12-well culture plates and then incubated for 3 h at 17 °C. After adhesion, the cells were washed with sterile sea water (SSW) and fresh medium was added. Cell viability was evaluated using trypan blue staining. Cultured hemocytes were incubated at 17 °C for 48 h before the experiments. For cytological analyses, hemocytes were fixed in 4% paraformaldehyde at pH 7 for 10 min and stained with May-Grunwald-Giemsa. Unfixed hemocytes were incubated with 0.4% Trypan blue and examined by phase-contrast microscopy. Samples were examined under a Leica DM5000B microscope equipped with a Leica DFC450C digital camera. Fixed hemocytes were also stained with Alexa Fluor 488 Phalloidin (Thermofisher Scientific) for actin filaments and to-pro iodide (Thermofisher Scientific) for nuclear staining. Confocal images were obtained with a Leica 40 × 1.25 Oil HCX PL APO CS lens (Leica Microsystems). For the immune stimulation, a stock of 1×10^8 particles/mL of Zymosan A from *Saccharomyces cerevisiae* (Sigma) was prepared in SSW, and 10 particles of zymosan per hemocyte were added to the culture wells in fresh medium and incubated at different time points. For the phagocytosis assay, the hemocytes were incubated for 30 min with zymosan to allow complete engulfment of the particles and phagocytic vesicle formation. For ROI neutralization, 10 μM of the scavenger trolox® (SIGMA) was added to the 4×10^6 cells 3 h before zymosan treatment, as determined by previous essays in our lab. Trolox is a cell permeable compound that works as a chemical analogue of vitamin E, acting as a potent antioxidant due to its free radical scavenging activity (Hamad et al., 2010). The cells were then incubated at 17 °C until (i) paraformaldehyde fixation, (ii) RNA extraction or (iii) ROI detection. All conditions were performed in triplicate. ROI accumulation was detected by 2′,7′−dichlorofluorescin diacetate DCFDA Cellular Reactive Oxygen Species Detection Assay (Abcam) following the manufacturer's protocol. Fluorescence was detected (EX: 495 nm/EM: 529 nm) on a microplate reader (Thermo Appliskan) and by confocal analysis with a Leica 40 × 1.25 Oil HCX PL APO CS lens (Leica Microsystems).

2.3. Total RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted from cultured hemocytes under the different conditions using TRIzol® reagent in accordance with manufacturer's instructions (Thermo Scientific). RNA was then treated with DNAse I (Thermo Scientific) for 15 min at room temperature and inactivated by heat for 10 min at 65 °C. Quantification and quality of the total RNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies) and 1% agarose gel electrophoresis, respectively. First strand synthesis was carried out from 1 μg of total RNA using 500 ng oligo-(dT)12−18 (Thermo Scientific), 1 mM dNTPs (Promega), 25 U Rnasin (Promega) and 200 U M-MLV reverse transcriptase in reverse transcriptase buffer (Promega) following the manufacturer's protocol. For RT-qPCR analyses, the 10 μl-volume reaction consisted of 1 × Brilliant II SYBR Green QPCR master mix (Stratagene), 0.3 μM of each primer and 1 μL of cDNA diluted to 1:5 in sterile ultra-pure water. Primers were for the big defensin (GenBank no. KU499992): *ApBD1f*: 5′-TGGCAACAGCGGATGGTGT-3′; *ApBD1r:* 5′-AACGCCTAAGTTCC-CACCTCG-3′ and for the β-actin (GenBank no. ES469330): *ApAct-f*: 5′-CACTGCTCTTGCTCCACAAAC-3′: *ApAct-r*: 5′-GAAGGTGGACAGA-GATGCCAA-3′. RT-qPCR assays were performed in triplicate in a Biorad C1000 Touch Thermocycler CFX96, and primer pair efficiencies (E) were calculated from the given slopes in the BioRad CFX software according to the equation: $E = 10^{[-1/slope]}$. Samples were submitted to an initial denaturation step of 10 min at 95 °C followed by an amplification of the target cDNA (40 cycles of denaturation at 95 °C for 5 s, annealing at 56 °C for 5 s and extension time at 60 °C for 15 s) and fluorescence detection. After an initial 10 s denaturation step at 95 °C, a melting curve was obtained from a start temperature of 65 °C to a final temperature of 95 °C, with an increase of 0.06 °C/s. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Calculations of means, standard deviations and statistical analysis using the Kruskal-Wallis test for expression analysis were carried out using GraphPad Prism software version 6.01 (significant value: $P < 0.05$).

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