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Role of nitric oxide in the defenses of *Crassostrea virginica* to experimental infection with the protozoan parasite *Perkinsus marinus*

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Abstract

We investigated the role of nitric oxide (NO) in the responses of the Eastern oyster, *Crassostrea virginica*, to the protozoan parasite *Perkinsus marinus*, causative agent of Dermo disease. *P. marinus* induced a slight but significant increase in NO production by oyster hemocytes *in vitro*, comparable to the increase induced by the immune stimulants phorbol myristrate acetate (PMA) and lipopolysaccharide (LPS). *P. marinus* also activated the NO response in oysters *in vivo*, as shown by induction of a protein reacting with a universal NO synthase (NOS) antibody in hemocytes and the presence of high levels of nitrite in plasma. Treatment of experimentally infected oysters with the NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME) resulted in a transient decrease in NO levels in oyster plasma and a significant increase in the number of parasites at early time points after infection. The NO donor, *S*-nitroso-*N*-acetyl-penicillamine (SNAP) caused a significant inhibition in the proliferation of *P. marinus* cultured cells after 24 h of incubation. These results indicate that NO has a role in decreasing parasite loads at early time points after infection.
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Keywords: Oyster; Bivalvia; Mollusca; Dermo disease; *Perkinsus marinus*; Hemocytes; Nitric oxide; Host-pathogen

1. Introduction

The protozoan parasite *Perkinsus marinus* [1] is the causative agent of Dermo disease, responsible for widespread mortalities of the Eastern oyster, *Crassostrea virginica*, along the East coast of North America and the Gulf of Mexico [2]. Dermo disease is a slow progressing disease, characterized by hemocyte infiltration at early stages, followed by fibrosis and cell death, culminating in most cases in the death of the oyster by emaciation [2]. The mechanisms for the pathogenesis of *P. marinus* and the immunological and physiological responses of the oyster are still poorly understood. Phagocytosis and degradation of *P. marinus* meronts by oyster hemocytes, the key effector cells of immune response in mollusks, have been demonstrated using transmission electron microscopy. However, hemocytes are not always able to kill the parasite and

Abbreviations: ASW, artificial seawater; iNOS, inducible nitric oxide synthase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; PMA, phorbol myristate acetate; RNI, reactive nitrogen intermediates; SNAP, *S*-nitroso-acetylpenicillamine; uNOS, universal nitric oxide synthase

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may be responsible for the systemic distribution of the parasite [3]. Molecular responses involved in the host–parasite interaction between oysters and *P. marinus* include enhanced synthesis of proteases and protease inhibitors [4,5], agglutinins, and lectins [6], molecules with antimicrobial activity [7,8], and radical oxygen species (ROS) [9]. *P. marinus* is tolerant to the effects of super oxide and hydrogen peroxide [10] and appears to be able to modulate the oyster defenses, suppressing the respiratory burst response of *C. virginica* hemocytes [11,12] and degrading components of the oyster plasma [5].

Nitric oxide (NO) is a ubiquitous signaling molecule with potent immunoregulatory and antimicrobial effects [13,14]. NO is produced by a variety of cells in both vertebrates and invertebrates from the substrate L-arginine through the action of nitric oxide synthases (NOS) [13]. The inducible NOS (iNOS), the most immunologically relevant NOS in vertebrates, is activated by viral, bacterial, and parasitic stimuli, as well as pro-inflammatory cytokines [15]. Hemocytes from several marine and fresh water mollusks, including the snail *Lymnaea stagnalis* [16], the mussels *Mytilus edulis* [17,18], and *Mytilus galloprovincialis* [19–22], the Pacific oyster *Crassostrea gigas* [23,24], and the carpet shell clam *Ruditapes decussatus* [25] are able to produce NO in response to immune stimuli.

The antimicrobial effects of NO and reactive nitrogen intermediates (RNI) depend on their chemical reactive nature. NO, as a relatively nonpolar uncharged molecule with a small Stokes radius, is predicted to cross membranes readily, facilitating the damage of microbial DNA, proteins, lipids, and enzymes. NO also reacts with the superoxide anion (O_2^-) generated during the respiratory burst to produce peroxynitrite, a compound with higher toxicity [26]. NO and other RNI are of particular importance in vertebrate and invertebrate host defenses against a large variety of intracellular pathogens, including protozoan and metazoan parasites [27]. Nevertheless, NO is considered to be a “double-edged sword” molecule, since high concentrations of NO and RNI produced in response to infection have been shown in experimental animal models to contribute to pathogenic processes and immunosuppression [28,29].

In the present work, we have studied the effect of experimental infection of the Eastern oyster, *C. virginica*, with the parasite, *P. marinus*, on NO production by the host organism. We have also evaluated the role of NO production in the clearance of the parasite *in vivo* by studying the effect of an NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) on the progression of experimental infection of oysters with *P. marinus*. Furthermore, we assayed the *in vitro* inhibitory activity of the NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP) against *P. marinus* in culture. We have also characterized the effect of the immunostimulants phorbol myristrate acetate (PMA) and bacterial lipopolysaccharide (LPS), as well as *P. marinus*, in the NO production by *C. virginica* hemocytes *in vitro*.

2. Materials and methods

2.1. Oysters

C. virginica oysters free of *P. marinus* were obtained from commercial farms in the Pacific coast of USA (Taylor Shellfish Company, Samish Bay, WA) and New Brunswick, Canada (Mallet, L’Étang Ruisseau Bar Ltd., Shippagan, Canada). Upon arrival, oysters were placed in 100 l tanks filled with artificial seawater at 15 °C and a salinity of 28‰, and acclimated by a gradual increase in the temperature up to 22 °C during a week. Oysters were fed daily with an algae mixture of *Tetraselmis*, *Pavlova*, *Thallassiosa*, and *Isochrysis* (Instant Algae, Reed Mariculture Inc., San Jose, CA). To confirm the absence of the parasite *P. marinus*, a sample of 10 oysters from each oyster batch was tested using Ray’s fluid thioglycollate medium (RFTM) tissue assay [30].

2.2. Perkinsus marinus cultures

Cultures of *P. marinus* (HCTR, originally isolated from infected oysters from Charlestown Pond, RI) were kindly provided by D. Bushek (Rutgers University, NJ). Cultures were routinely maintained in sterile flasks containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with Ham’s nutrient mixture and 2% fetal bovine serum (FBS, Invitrogen, Carlsbad CA, USA) at 28 °C [31]. In order to increase infectivity of *P. marinus*, cultures were supplemented with Eastern oyster plasma (cell-free hemolymph, 0.3 mg/ml of protein in plasma) for 2 weeks prior to the experimental infections [32]. The plasma for culture supplementation was prepared as follows: 1 ml of hemolymph from the adductor muscle of 10 oysters (Wickford, RI) was withdrawn through a shell notch using a

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