Developmental and Comparative Immunology 56 (2016) 25–36

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Pathogen-associated molecular patterns activate expression of genes involved in cell proliferation, immunity and detoxification in the amebocyte-producing organ of the snail *Biomphalaria glabrata*

Si-Ming Zhang [a, *], Eric S. Loker [a, b], John T. Sullivan [c]

[a] Center for Evolutionarily and Theoretical Immunology, Department of Biology, The University of New Mexico, Albuquerque, NM 87131, USA
[b] Parasite Division, Museum of Southwestern Biology, The University of New Mexico, Albuquerque, NM 87131, USA
[c] Department of Biology, University of San Francisco, San Francisco, CA 94117, USA

ARTICLE INFO

Article history:
Received 11 September 2015
Received in revised form
16 November 2015
Accepted 17 November 2015
Available online 22 November 2015

Keywords:
Biomphalaria glabrata
Mollusk
Amebocyte-producing organ (APO)
Pathogen associated molecular pattern (PAMP)
Aminoacyl-tRNA synthetase
GiMAP

ABSTRACT

The anterior pericardial wall of the snail *Biomphalaria glabrata* has been identified as a site of hemocyte production, hence has been named the amebocyte-producing organ (APO). A number of studies have shown that exogenous abiotic and biotic substances, including pathogen associated molecular patterns (PAMPs), are able to stimulate APO mitotic activity and/or enlarge its size, implying a role for the APO in innate immunity. The molecular mechanisms underlying such responses have not yet been explored, in part due to the difficulty in obtaining sufficient APO tissue for gene expression studies. By using a modified RNA extraction technique and microarray technology, we investigated transcriptomic responses of APOs dissected from snails at 24 h post-injection with two bacterial PAMPs, lipopolysaccharide (LPS) and peptidoglycan (PGN), or with fucoidan (FCN), which may mimic fucosyl-rich glycan PAMPs on sporocysts of *Schistosoma mansoni*. Based upon the number of genes differentially expressed, LPS exhibited the strongest activity, relative to saline-injected controls. A concurrent activation of genes involved in cell proliferation, immune response and detoxification metabolism was observed. A gene encoding checkpoint 1 kinase, a key regulator of mitosis, was highly expressed after stimulation by LPS. Also, seven different aminoacyl-tRNA synthetases that play an essential role in protein synthesis were found to be highly expressed. In addition to stimulating genes involved in cell proliferation, the injected substances, especially LPS, also induced expression of a number of immune-related genes including arginase, peptidoglycan recognition protein short form, tumor necrosis factor receptor, ficolin, calmodulin, bacterial permeability increasing proteins and E3 ubiquitin-protein ligase. Importantly, significant up-regulation was observed in four GiMAP (GTPase of immunity-associated protein) genes, a result which provides the first evidence suggesting an immune role of GiMAP in protostome animals. Moreover, altered expression of genes encoding cytochrome P450, glutathione-S-transferase, multiple drug resistance protein as well as a large number of genes encoding enzymes associated with degradation and detoxification metabolism was elicited in response to the injected substances.

1. Introduction

The freshwater snail *Biomphalaria glabrata* is an intermediate host of the digenetic trematode *Schistosoma mansoni*, one of three major etiologic agents of human schistosomiasis, a disease that affects more than 230 million people worldwide (Colley et al., 2014). Recent molecular studies have focused on snail immunity, with an aim to better understand the mechanism of snail defense against schistosome parasites (Adema and Loker, 2015; Knight et al., 2014; Lockyer et al., 2012; Tennessen et al., 2015).

Snail defense cells, called amebocytes or hemocytes, are responsible for the cellular recognition of foreign bodies and for phagocytosis and cytotoxic reactions (Larson et al., 2014; Yoshino and Coustau, 2011; Zahoor et al., 2014). The circulating hemocytes are produced in *B. glabrata* primarily in the amebocyte-producing organ (APO), considered here synonymous with the anterior pericardial wall. This structure contains follicles of hemopoietic cells in a sinus located between the anterior wall of the

* Corresponding author.
E-mail address: zhangsm@unm.edu (S.-M. Zhang).

http://dx.doi.org/10.1016/j.dci.2015.11.008
0145-305X/

pericardial sac and the posterior wall of the mantle cavity (Jeong et al., 1983; Lie et al., 1975). In addition to hemopoietic cells and pericardial and mantle epithelia, the APO contains typical cells of snail connective tissue, i.e., fibroblast-like cells, muscle tissue, hemocytes, and large pore cells (Pan, 1958; Sminia, 1972). It has been demonstrated that hemopoietic cells of the APO exhibit increased mitotic activity, and the APO undergoes visible enlargement, following infection with trematode larvae (Lie et al., 1975; Sullivan et al., 1982, 1984) or injection with extracts of larval or adult parasites (Noda, 1992; Sullivan et al., 2004; Sullivan, 2007). Moreover, previous studies have shown that transplanting the APO from schistosome-resistant to schistosome-susceptible snails resulted in increased resistance in the recipients, suggesting a role of the APO in snail resistance to infection (Sullivan and Spence, 1999).

Invertebrates rely on innate immunity for their internal defense against pathogens. To defend against a variety of microbes, one of the strategies that invertebrates employ is expression of pattern recognition receptors (PRR) that recognize relatively invariant microbial ligands called pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs leads to activation of immune signaling pathways, which in turn lead to production of antimicrobial agents (Buchon et al., 2014; Hoffmann, 2003; Zipfel, 2014). Inspired by these observations, investigations have been undertaken to understand the effect of PAMPs on the APO of *B. glabrata*. It has been demonstrated that injected crude lipopolysaccharide (LPS) and crude peptidoglycan (PGN) from *Escherichia coli* (but not purified PGN) show potent mitogenic activity in the APO (Sullivan et al., 2011, 2014). A similar mitotic response occurs to the brown algal polysaccharide fucoidan (FCN), which, although not a pathogen-associated molecule, may mimic fucosylated glycans on sporocysts of *S. mansoni* (Sullivan and Belloir, 2014), and hence is referred to as a PAMP in this study.

Previous work on the APO has been conducted exclusively at the histological or ultrastructural level, and no gene expression studies have been carried out, in part due the difficulty in obtaining sufficient APO tissue for analysis. In the present study, we applied microarray technology to investigate gene expression in the APO of *B. glabrata* following challenge with LPS, FCN or PGN.

2. Materials and methods

2.1. Snails

Adult Salvador strain (schistosome-resistant) *B. glabrata*, measuring 10–10.5 mm in shell diameter, were used for the experiments. Snails were reared in aerated aquaria containing artificial pond water at 25–27 °C and fed with lettuce as described previously (Sullivan et al., 2011).

2.2. Reagents

Three crude (i.e., not highly purified) PAMPs from commercial sources were utilized: lipopolysaccharide (LPS) from the bacterium *Escherichia coli* O127-B8 (Sigma), peptidoglycan (PGN) from *E. coli* O111-B4 (Invitrogen), and fucoidan (FCN) from the brown alga *Fucus vesiculosus* (Sigma). All three chemicals were dissolved in 1/3 mammalian-strength phosphate buffered saline (PBS) (Kodak, Rochester, NY) at a concentration of 1 mg/ml (LPS and PGN) or 10 mg/ml (FCN).

2.3. Collection, storage and transportation of APO samples

At the University of San Francisco, individual snails were injected with 5 μl of each PAMP described above or with PBS (control) through a hole in the shell and into a hemolymph sinus anterior to the digestive gland on the left side (Sullivan, 1990). At 24 h post-injection (PI), the time of peak mitotic response (Sullivan et al., 2011), the pericardial sac was removed, and the anterior pericardial wall (or APO) was dissected free (Sullivan, 1990) (Fig. 1). As described previously, the anterior pericardial wall is fused with the lateral pericardial wall, the wall of the pulmonary cavity, saccular kidney, atrium of the heart, and albumin gland (Sullivan and Spence, 1999), and small amounts of tissue from these adjoining structures unavoidably were included. For each treatment, 80 snails were used and divided into four pooled samples of 20 APOs each. Each sample of 20 APOs was pooled in a 1.5 ml tube containing 1000 μl of Trizol (Invitrogen) at 4 °C. Because on a single day only 10 snails were injected, 10 APOs were initially added to the tube, which was then frozen at −80 °C. On a subsequent day, the tube was thawed to 4 °C, and an additional 10 APOs were added. The tube was then capped, sealed with Parafilm, and refrozen at −80 °C. After collection of all samples ($20 \times 4 \times 4 = 320$ APOs), the tubes were shipped overnight on dry ice to the University of New Mexico for RNA extraction and microarray analysis. Upon arrival, the still-frozen tubes were stored at −80 °C.

2.4. RNA extraction

A preliminary experiment was performed to determine the best method for RNA extraction, and here we briefly describe our resulting procedure, which is based on a modification of the Trizol manufacturer's instructions (Invitrogen), that yielded a good amount of high quality RNA. All steps were carried out in a sterile hood equipped with a UV light. The hood was sterilized by UV for 30 min before the experiments were conducted. Sample tubes, each containing 20 APOs in 1000 μl Trizol, were thawed at 4 °C overnight. On the following morning, 750 μl of the Trizol supernatant, containing cell lysate, was transferred into a fresh tube. The residual 250 μl Trizol, with the bulk of the APO tissues, was homogenized by hand using a small plastic homogenizer. After completion of homogenization, the supernatant (750 μl) was transferred back into the tube containing the APO homogenate, thus reconstituting the original volume. Chloroform (200 μl) was added to each tube which was then vortexed vigorously for 2 min. The samples were kept on a rotator for 1 h at room temperature, enabling complete dissociation of nucleoprotein complexes. Next, the samples were

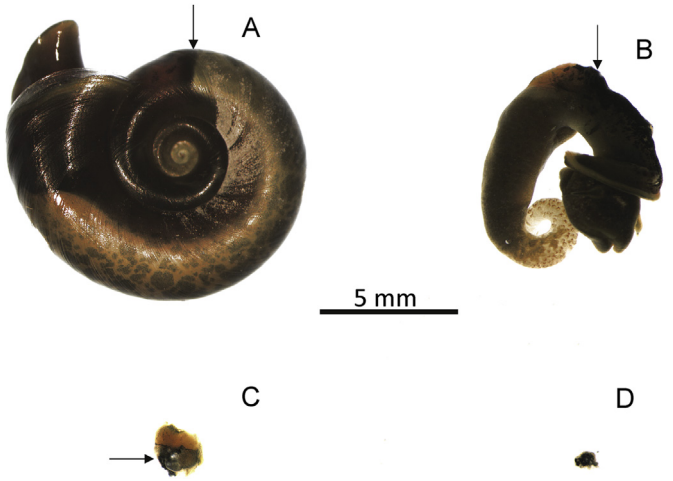

Fig. 1. Dissection of APO showing its relative size. A) 10.4 mm snail on dry surface. B) Same snail as in A, dissected from its shell and submerged in 0.03% NaCl. C) Anterior pericardial wall, inside view, still attached to portion of kidney above, mantle on left and exterior pericardial wall underneath. D) Anterior pericardial wall. Arrows show location of the anterior pericardial wall.