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Full-length Article

# Catecholamines are produced by ascidian immune cells: The involvement of PKA and PKC in the adrenergic signaling pathway

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ABSTRACT

The stress response is a complex mechanism, which includes changes in the immune system to enable organisms to maintain homeostasis. The neurohormones dopamine, noradrenaline (NA) and adrenalin are responsible for the physiological modulations that occur during acute stress. In the present study, we analyzed the effects of NA on the immune system specific to nitric-oxide (NO) production by subpopulations of immune cells (hemocytes) of the ascidian *Phallusia nigra*. We also investigated the capability of immune cells to produce catecholamine (CA). Finally, we tested the involvement of protein kinase A (PKA) and C (PKC) in the NA downstream signaling pathway. The results revealed that NA can reduce NO production by *P. nigra* hemocytes threefold, and that signet-ring cells, univacuolar refractile granulocytes and morula cells are the cell types most involved in this event. A challenge effected with Zymosan A induced CA production, and co-incubation with both inhibitors of the second messengers PKA and PKC revealed the involvement of these molecules in the adrenergic pathway of *P. nigra* hemocytes. Taken together, these results suggest that NO production can be down-regulated by NA through α- and β-adrenoceptors via the second messengers PKA and PKC.



## 1. Introduction

The response to stress is a process stimulated by the exposure of organisms to intrinsic or extrinsic perturbations, and this process includes complex behavioral and physiological changes that enable homeostasis (Chrousos and Gold, 1992; Ottaviani and Franceschi, 1996). The catecholamine (CA) system, including dopamine (DA), noradrenaline (NA) and adrenalin (A), is the main physiological mechanism involved in acute stress, and in addition is responsible for inducing alterations in many systems including the reproductive, respiratory and immune systems, in both vertebrates and invertebrates (Ottaviani and Franceschi, 1996; Sabban and Kvetňanský, 2001; Hartenstein, 2006; Chen et al., 2008; Kvetnansky et al., 2009). Additionally, CA can be synthesized *de novo* by immune cells and secreted in either an autocrine or paracrine manner (Qiu et al., 2005).

In recent years, several studies have been conducted to determine the role of CA in the invertebrate immune system. For example: in the shrimp *Litopenaeus vannamei*, NA depresses phenoloxidase and phagocytic activities (Cheng et al., 2006); and in the giant freshwater prawn *Macrobrachium rosenbergii,* NA down-regulated disease resistance (Chang et al., 2011). Also, immune cells from the scallop *Chlamys farreri* and the oyster *Mytilus galloprovincialis* produce and release NA (Cao et al., 2007; Jiang et al., 2014). We reported that in the ascidian *Phallusia nigra,* NA modulation reduces the production of nitric oxide (NO) (De Barros et al., 2012). Subsequently, we described *P. nigra* hemocytes (De Barros et al., 2014): using light microscopy and transmission electron microscopy, we were able to identify eight types of hemocytes (hemoblast, signet-ring cell, univacuolar refractile granulocyte, compartment cell, morula cell, pigment cell, nephrocyte, and amebocyte).

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NA may act via adrenergic receptors (ARs), which are present in some types of immune cells. Vertebrates express ARs in T and B lymphocytes, monocytes/macrophages, natural killer cells, and the surface of granulocytes (Marino and Cosentino, 2013). In *M. rosenbergii* and *L. vannamei*, the presence of ARs was reported in granulocytes and semi-granular cells (Ottaviani and Franceschi, 1997; Chang et al., 2012; Chang et al., 2015); and Lacoste et al. (2001) found that ARs regulate specific second messengers at the intracellular level.

In vertebrate immune cells, the ARs play a role activating in G protein through NA (Massarsky et al., 2011; Marino and Cosentino, 2013). The signaling pathway of NA by α- and β-ARs has different functions, depending on specific cells and tissues. The α-AR activates phospholipase C (PLC) (Ma et al., 2000), which eventually triggers the protein kinase C (PKC) second messenger (Harris et al., 2008). The β-ARs couple to G protein and activate protein kinase A (PKA; Rybin et al., 2000). In hemocytes of the mollusk *Chlamys farreri*, it was reported that NA acts on ARs though cAMP/$Ca^{+}$ (Zhou et al., 2013; Jiang et al., 2014).

We previously demonstrated that NA down-regulated the levels of nitric oxide (NO) in *P. nigra* (De Barros et al., 2012). In ascidians, NO is produced by hemocytes after challenge (Cima et al., 2004; De Barros et al., 2009). However, the cell type and the signaling pathway involved in adrenergic receptors activated by NA were not determined. In this study we report for the first time that ascidian immune cells are capable of producing CA, and that PKA and PKC are the second messengers involved in the signaling pathway of NA. In addition, we investigated the types of hemocytes that undergo NO modulation by NA.

## 2. Materials and methods

### 2.1. Animal collection

Fifty adults of *P. nigra* were collected from Porto do Forno, Arraial do Cabo, Rio de Janeiro, Brazil, and maintained at 20 °C in an aerated aquarium with controlled photoperiod (12 h light/12 h dark), pH (approximately 8.0), and salinity (34), for five days before the experimental procedures. The animals were fed with nauplii of *Artemia* (Sardet et al., 2011).

### 2.2. Isolation of hemocytes

The ascidians were bled from incisions in their incurrent siphons, and the hemolymph was collected in a marine anticoagulant solution (MAC; 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 10 mM EDTA, 0.45 M NaCl, pH 7.0) (Peddie and Smith, 1993). Hemocytes from individual tunicates were isolated after centrifugation at 800*g* for 10 min, and then were washed and finally suspended in artificial sea water (ASW; 420 mM NaCl, 9 mM KCl, 10 mM $CaCl_2$, 24.5 mM $MgCl_2$, 25.5 mM Mg $SO_4$, 2.15 mM $NaHCO_3$ and 10 mM Hepes buffer, pH 8.0, sterilized with a 0.2 μm filter). Cells were counted with a Neubauer hemocytometer, and suspensions of hemocytes containing $3 \times 10^6$ cells/mL were prepared before the experiments (De Barros et al., 2009, 2014).

### 2.3. Quantification of nitrite

The method of Griess was used in order to quantify the levels of nitrite ($NO_2^-$), a stable product resulting from the degradation of NO (Leone et al., 1994; Bernardes et al., 2014). Briefly, 400 μL of the ASW solution containing $3 \times 10^6$ hemocytes/mL from 4 ascidians was exposed to different NA concentrations (0.01, 0.1, 1 or 10 μM) for 30, 60 or 90 min. Next, the samples were sonicated on ice 3 times for 15 s each, and were then centrifuged at 10,000*g* (10 min, at 4 °C). The supernatant was carefully removed, and after 10 min incubation, the samples, each containing 50 μL hemocyte lysate, were measured at an optical density of 540 nm (Molecular Devices Spectra Max 190). The molar concentration of $NO_2^-$ in the samples was determined from standard curves generated using known concentrations of $NO_2^-$, adjusted for protein concentration and expressed on the graph as mg protein/μM $NO_2^-$. The protein concentration of the hemocyte lysate was determined by the Bradford method (Bradford, 1976).

### 2.4. Quantification of NO in living cells

Hemocytes (330 μL; $3 \times 10^6$ cells/mL) from 8 animals were incubated in 5 μM of the fluorescent indicator for NO, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA, Molecular Probes; D23842) for 30 min in the dark (Nakatsubo et al., 1998). Cells were then washed in ASW prior to incubation in ASW for 15 min to de-esterify the intracellular DAF-FM DA. The hemocytes were stimulated to produce NO with 25 μg/mL of Zymosan A (ZnA) from *Saccharomyces cerevisiae* only, or incubated with ZnA and different concentrations of NA (0.1, 1.0 or 10.0 μM) for 30 min. Control hemocytes were suspended in ASW only. The fluorescent signals were measured using a Varian Cary Eclipse Fluorescence spectrophotometer, calibrated for excitation at 485 nm and emission at 520 nm.

For the assays of NO synthase (NOS) inhibition, inhibitors were added to hemocytes from 4 animals 30 min prior to incubation with DAF-FM-DA. Either 0.5 or 1 mM of the NO synthase inhibitor Nω-nitro-L-arginine (L-NAME) (Sigma, Saint Louis, USA; N5751) was used. Then, the hemocytes were incubated with NA only or with NA and ZnA as described above. NO production was measured in living hemocytes using a Varian Cary Eclipse Fluorescence Spectrophotometer, followed by the procedures described below (De Barros et al., 2014).

### 2.5. Discontinuous density gradient

The Percoll density gradient described by Camaratta et al. (1993) was used. In brief, Percoll solution (Sigma) was equilibrated with ASW to a final concentration of 90% (density, 1.122 g/mL). The 90% Percoll solution was diluted with ASW in order to obtain three more fractions, of 80%, 40% and 30%. Aliquots of 1 mL of each percentage solution were overlaid sequentially into centrifuge tubes. Then, 2 mL of hemocyte suspension in ASW was added onto the Percoll gradient and centrifuged at 800*g* for 35 min at 4 °C. The fraction of cells was removed and washed 3 times with ASW and then suspended in ASW and adjusted to $3 \times 10^6$ cells/mL. The assays to evaluate NO production were performed as described below. A sample of each fraction was collected, prepared for observation under a light microscope, and used to analyze the cell types. The cells were prepared according to De Barros et al. (2014).

### 2.6. Hemocyte viability

Hemocytes were removed from 3 animals in order to evaluate viability. The trypan blue stain (1% in ASW) exclusion test (Uliasz and Hewett, 2000) was used for each concentration treatment and time. A solution of 50 μL of sterile trypan blue dissolved in ASW (final concentration 0.05%) was added to each hemocyte suspension (50 μL; $3 \times 10^6$ cells/mL) and left to incubate for 10 min. The excess dye in the supernatant solution was discarded. The hemocytes were then lysed with 200 μL of sodium dodecyl sulfate (SDS; 1% v:v), sonicated on ice 3 times for 15 s each, and then centrifuged at 10,000*g* (10 min, at 4 °C). Finally, 200 μL of SDS/trypan blue solution was transferred to a 96-well dish and analyzed