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Short Communication

Norepinephrine depresses the nitric oxide production in the ascidian hemocytes

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ABSTRACT

Norepinephrine (NE) is a neuro-hormone released by vertebrates and invertebrates during acute stress, and can influence their immune function. We found that NE depressed the production of nitric oxide (NO) by the hemocytes of ascidians. Our results with a fluorescent indicator for NO in assays using both NE and either α or β-antagonist revealed that NE down-regulated NO production by the ascidian hemocytes. Our data suggest that NE may be acting via specific hemocyte receptors to induce a decrease in immune function.

1. Introduction

Ascidians are marine invertebrates, whose phylogenetic position makes them good subjects for the study of evolutionarily ancient mechanisms of biological systems, such as the immune system. The immune system is constituted by hemocytes (blood cells), which circulate in the hemolymph and perform a wide variety of functions, such as phagocytosis, production of microbicide peptides, such as nitric oxide (NO) (de Barros et al., 2009; Hartenstein and Mandal, 2006). Because of these characteristics, hemocytes have been used in many studies as indicators of environmental stress.

Some species from diverse phyla coordinate their acute stress response using chemical derivates of the amino acid tyrosine. For example, vertebrates, crustaceans and mollusks release norepinephrine (NE) during acute stress and their responses prepare the animal to physiological changes that can influence their immune function (Adamo, 2008; Charmandari et al., 2005; Madden, 2003). In hemocytes of the white shrimp *Litopenaeus vannamei* and the Sydney rock oyster *Saccostrea glomerata*, NE can reduce phagocytosis and burst capacity (Aladaileh et al., 2008; Cheng et al., 2006). However, there are still unclear issues regarding the physiological role of adrenergic stress responses in invertebrates (Adamo, 2008).

NO is important in modulation of the immune system by the nervous system in normal and pathological situations, since its role as a signaling molecule has been demonstrated throughout the animal kingdom. However, few data concerning the regulation of NO production by NE exist for vertebrates and invertebrates. Therefore, we investigated the in vitro immunomodulation of NE on the production of NO by hemocytes of the ascidian *Phallusia nigra*. This study will help us to understand, from a comparative point of view, the consistent connection between neurohormones and immune function during acute stress as an important adaptive function.

2. Materials and methods

2.1. Hemolymph collection

Adult specimens 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, pH, and salinity for five days before the experimental procedures. The animals were fed every other day with microalgae and Artemia. The ascidians were bled from incisions in their incurrent siphons, and the hemolynph was collected in a marine anticoagulant solution (0.45 M NaCl, 100 mM glucose, 1.5 mM trisodium citrate, 13 mM citric acid, 10 mM EDTA in 1 L distilled water, pH 7.0) (Smith and Peddie, 1993). Hemocytes from individual tunicates, after centrifugation at 800 Xg for 10 min at room temperature, were washed and finally suspended in Ca^{2+}-, Mg^{2+}-free Herbst's artificial sea water prior to being incubated in F-HASW. Cells were counted using a Neubauer hemocytometer. Suspensions of hemocytes containing 3×10^6 cells/mL were prepared before the experiments.

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2.2. Hemocyte treatment and cell viability

The hemocytes from five different animals were incubated in 5 μM of the fluorescent indicator for NO, 4-amino-5-methylamino-2′,7′-difluororescein diacetate (DAF-FM DA – Molecular Probes) diluted in Ca^{2+}-, Mg^{2+}-free Herbst's artificial sea water for 30 min in the dark (Nakatsubo et al., 1998). The cells were then washed in F-HASW prior to being incubated in F-HASW for 15 min to de-esterify the intracellular DAF-FM DA. The cell suspensions were divided into 3- mL aliquots and the hemocytes were incubated either with Zimosan A (50 μg) (Gordon et al., 2001), to mimic an infection, or with NE (0.1, 1.0 or 10.0 μM). Additionally, they were incubated either with Zimosan A (50 μg) plus 1.0 μM NE and propranolol (0.1, 1.0 or 10.0 μM), a β-adrenoceptor antagonist, or Zimosan A (50 μg) plus 1.0 μM of NE and phentolamine (0.1 or 1.0 or 10.0 μM), a α-adrenoceptor antagonist, all from Sigma France. Control hemocytes were suspended in F-HASW. Following challenge, the fluorescent signal was measured after 30 and 60 min using a Varian Cary Eclipse Fluorescence, calibrated for excitation at 485 nm and emission at 520 nm. Hemocyte mortality was evaluated by the trypan blue dye (1% in F-HASW) exclusion test (Ford and Ashton-Alcox, 1998).

2.3. Statistical analyses

The differences of all groups were tested for significance by non-parametric test Kruskal–Walls, followed by Mann–Whitney test for differences between specific pairs of groups. Significance was established at $P < 0.05$ using Graphpad Prism version 5.00 from GraphPad Software, Inc.

3. Results

After 30 min of incubation, the cells with Zimosan A plus NE in two concentrations (1.0 and 10.0 μM) were capable to significantly decrease NO production ($P < 0.05$) (Fig. 1A). In the presence of 1.0 μM and 10.0 μM NE, NO production was reduced in 40.46 ± 6.88% and 46.51 ± 7.91%, respectively, compared with the stimulation with Zimosan A. After 60 min of challenge NE was still capable to inhibit NO production by the hemocytes. The data show that in 1.0 μM and 10.0 μM NE, NO production was reduced by 83.66 ± 4.68% and 94.61 ± 5.29%, respectively, compared with hemocytes stimulated with Zimosan A only (Fig. 1B). At concentrations of 1.0 and 10.0 μM, NE caused a significant decrease in the response curve of NO production of ascidian hemocytes after both 30 ($P < 0.0079$) and 60 min ($P < 0.0317$) of incubation. After 90 min of incubation, the response to NE did not change significantly when compared with the control (Fig. 1C). Only in the presence of 1.0 and 10.0 μM propanolol the increased NO production may have prevented the inhibitory effect of NE.

In order to determine what type of biogenic amine receptor may be responsible for the decreased NO production by hemocytes, the effects of phentolamine and of propranolol, α and β-antagonists, respectively, were analyzed. When compared with the control, the samples with 1.0 and 10.0 μM propranolol showed an increased production of NO of 51.63 ± 4.93% and 62.10 ± 5.93%, respectively ($P < 0.05$) (Fig. 1D). These effects were smaller than with phentolamine. In the presence of 10.0 μM phentolamine only, the increase was of 111.80 ± 7.53% when compared with Zimosan A (Fig. lE). Hemocytes changed the response by NE: in the presence of the α and β-antagonists (0.1–10 μM), phentolamine and propranolol, respectively, the effect of NE was significantly ($P < 0.05$) attenuated.

4. Discussion

In some invertebrates, catecholamines play essential roles in several physiological processes (Syed and Winlow, 1991; Teyke et al., 1993), however, few studies have explored neuroendocrine-immune relationships in invertebrates (Adamo, 2012; Lacoste et al., 2001; Ottaviani and Franceschi, 1996). In the ascidinan *Ciona savignyi* the NE and β-adrenergic receptor in the nervous system triggers early metamorphosis of larvae (Kimura et al., 2003), and analysis of the *Ciona intestinalis* genome revealed genes that encode thyrosine hydroxylase, and aromatic acid decarboxylase enzymes of the catecholamine synthetic pathway, such as dopamine β hydroxylase (Horie et al., 2009). Therefore, besides the production of dopamine, it is most probable that NE is present in the ascidian hemolymph and that circulating concentrations of this substance increase in response to stress, as occurs in other animals (Carlock et al., 1999). Immunological activity is mediated by circulating hemocytes: they participate in many immune functions that can be modulated by physiological stress in normal and pathological responses involving synthesis and excretion of catecholamines (Lacoste et al., 2001). The present experiments were conducted in order to determine whether NE can modulate ascidian hemocyte NO production.

NO production decreased 30 and 60 min after the NE stimulation. However, the activity began to return to normal levels within 90 min, suggesting that the NE-mediated effects are transient. Similar decreases in phenoloxidase activity were observed in hemocytes of both the Sydney rock oyster and the shrimp *Liptopenaus vannamei*: 120 min after stimulation the NE-mediated effects were lost (Aladaileh et al., 2008; Cheng et al., 2006).

When the α and β-antagonist adrenoceptors were used, the effect of NE was attenuated indicating the presence of biogenic amine receptors on the surface of the ascidian hemocytes. However, we have to consider that the antagonists alone affected NO production (data not shown). Comparing the effects of the α and β-antagonists indicated that α-antagonists can produce more NO than β-antagonists after 30 min. Yet, we still do not know which type of hemocyte(s) show this receptor on its surface, and whether there is a specific hemocyte type showing both α and β adrenoreceptors, or whether one type shows α adrenoreceptor and other shows β adrenoreceptor. Nevertheless, we cannot rule out the possibility that octopamine (OA) receptors are involved in this response, since they have similar pharmacological profiles to vertebrate adrenergic receptors (e.g. Adamo, 2008; Evans and Maqueira, 2005). Moreover, Evans and Maqueira (2005) demonstrated that OA receptors show some affinity for NE. Therefore, this question must be addressed in further studies.

Adrenergic receptors in mammals are divided into α and β subtypes. Lymphocytes T $CD34_+$ and B have almost exclusively β2 adrenoreceptors. In contrast, the expression of α receptors in immune cells appears to be limited to specific innate immune-cells subsets (Kin and Sanders, 2006). Negative regulation of immunocyte phagocytosis by NE occurs in vertebrates and mollusks (Abrass et al., 1985; Lacoste et al., 2001). In both, mollusks and mammals, the NE-induced inhibition of phagocytosis involves β-adrenergic receptors (Lacoste et al., 2001).

In mammals, the pathway of the neuro-immune regulation is bidirectional and both the nervous and immune systems can communicate between them (Dantzer, 2004). Generally, the activated immunological cells produce cytokines that regulate the Central Nervous System (CNS) through the binding of these molecules to their receptors. After, the CNS communicates back to the immune system releasing the neurotransmitter NE, and this event, together with other routes, including the HPA axis, maintains the immune homeostasis (Wrona, 2006). Because the ascidian hemocytes can

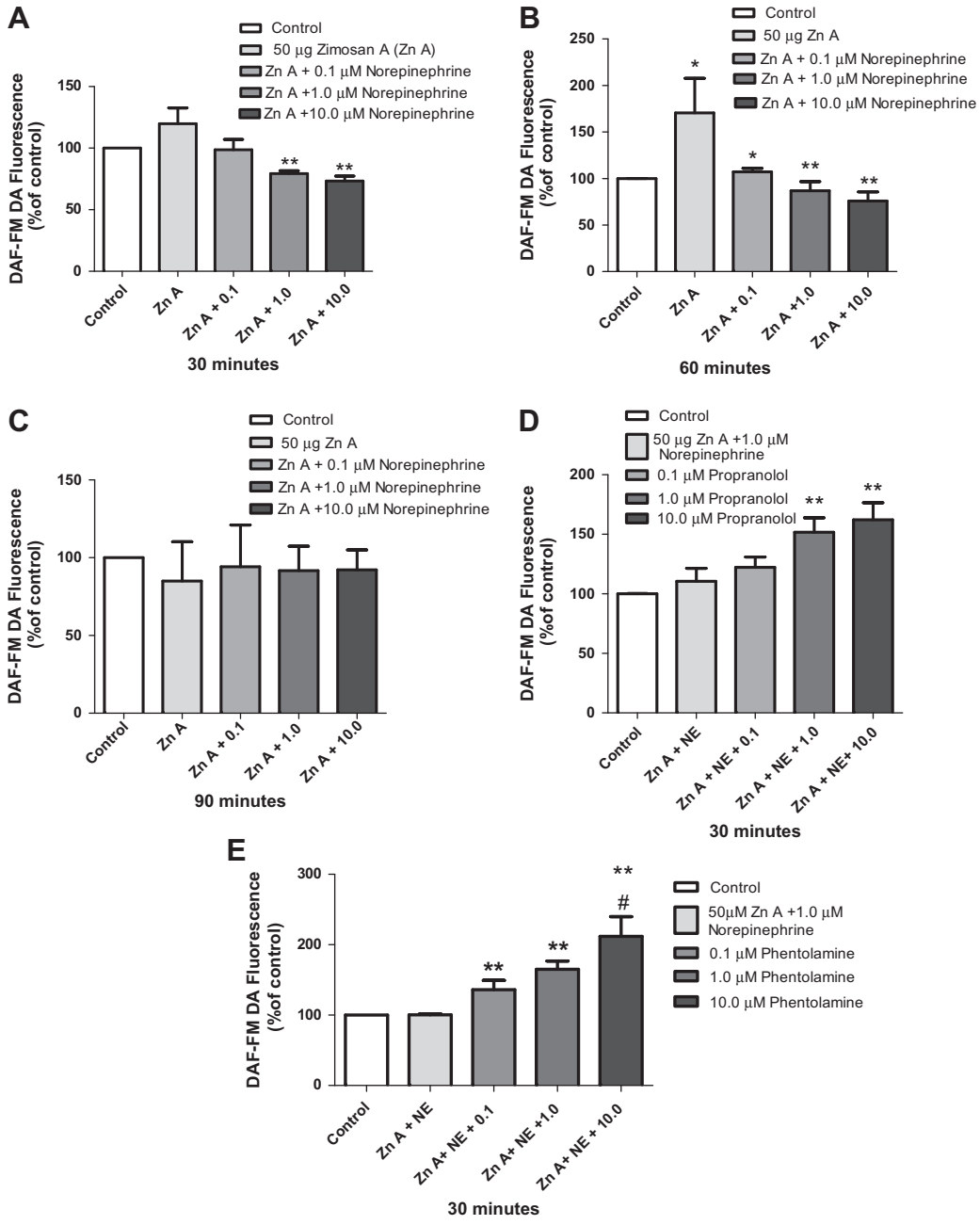

Fig. 1. Effect of noradrenaline, propranolol and phentolamine on NO production in *Phallusia nigra* hemocytes. Hemocytes incubated with 50 μg Zimosan A (ZnA) and 0.1–10.0 μM NE during the times indicated (30 and 60 min). Controls – cells incubated with DAF-FM DA alone. (A) Inhibition of DAF-FM DA fluorescence of Zimosan A NE-stimulated ascidian hemocytes by NE after 30 min incubation. (B) Inhibition of DAF-FM DA fluorescence of Zimosan A NE stimulated ascidian hemocytes after 60 min incubation. (C) Inhibition of DAF-FM DA fluorescence of Zimosan A NE-stimulated ascidian hemocytes by NE after 90 min incubation. (D) Effects of the β-adrenergic receptor antagonist propranolol on the inhibitory action of NE caused by the NO production of 50 μg Zimosan A + 1.0 μM NE-stimulated ascidian hemocytes. Cells were incubated in the presence of propranolol (0.1–10.0 μM) at the same time of the addition of NE. (E) Effects of the α-adrenergic receptor antagonist phentolamine on the inhibitory action of NE caused by the NO production of 50 μg Zimosan A + 1.0 μM of NE-stimulated ascidian hemocytes. Cells were incubated in the presence of phentolamine (0.1–10.0 μM) at the same time of the addition of NE. The fluorescence values are shown as means ± S.E.M. ($n = 5$ for all samples) and correspond to the fluorescent signal as a proportion of the control cells with vehicle alone (100%). The significance of variations between different treatments or groups was analyzed by non-parametric test Kruskal–Walls, followed by Mann–Whitney test. Asterisks denote significant differences from Zimosan A-stimulated cells. $^{*}P < 0.05$; $^{**}P < 0.01$; $\#P < 0.05$ difference from 1.0 μM phentolamine.

produce cytokines in response to Zimosan A stimulation we believe that both the CNS and the immune system communicated using NE and, possibly, the cytokine Tunicate IL-1, which is the only cytokine described so far as a product of ascidian hemocytes (Raftos et al., 1992, 1998).

For the first time in ascidians, we present evidence that NE can modulate NO production and that on the surface of their hemocytes, α and β adrenoceptors may be the sites for triggering the responses. However, further studies are needed to determine if NE has an inhibitory effect on other immune functions in ascidians. This is important because ascidians are the link between invertebrates and vertebrates, and allow us to explore evolutionary aspects of the interaction between the neuro-endocrine and the immune systems.

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