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Nitric oxide synthase activity in tissues of the blowfly *Chrysomya megacephala* (Fabricius, 1794)

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Key words: NO synthase, nitric oxide, NADPH-diaphorase, *Chrysomya megacephala*, blowfly, tissues

ABSTRACT: Although insects lack the adaptive immune response of the mammalians, they manifest effective innate immune responses, which include both cellular and humoral components. Cellular responses are mediated by hemocytes, and humoral responses include the activation of proteolytic cascades that initiate many events, including NO production. In mammals, nitric oxide synthases (NOSs) are also present in the endothelium, the brain, the adrenal glands, and the platelets. Studies on the distribution of NO-producing systems in invertebrates have revealed functional similarities between NOS in this group and vertebrates. We attempted to localize NOS activity in tissues of naïve (UIL), yeast-injected (YIL), and saline-injected (SIL) larvae of the blowfly *Chrysomya megacephala*, using the NADPH diaphorase technique. Our findings revealed similar levels of NOS activity in muscle, fat body, Malpighian tubule, gut, and brain, suggesting that NO synthesis may not be involved in the immune response of these larval systems. These results were compared to many studies that recorded the involvement of NO in various physiological functions of insects.

Introduction

Calliphorid blowflies have substantial medical and veterinary importance because they produce myiasis in humans and other animals and may be mechanical vectors of enteric pathogens and parasites (Furlanetto *et al*., 1984; Wells, 1991). Because both immature and adult stages are the primary invertebrate consumers of decomposing animal organic matter (such as carrion), blowflies can play a valuable role in forensic medicine, helping to determine time, manner, and place of death (Catts and Goff, 1992; Wells and Kurahashi, 1994). *Chrysomya* is an abundant blowfly genus that is native of Africa. This genus was established in South America in 1975 (Guimarães *et al*., 1978) and its distribution reaches also North America (Greenberg, 1988). These insects are holometabolous, and the larvae go through three instars when they live in or feed on recently dead animals or carrion. During this period, they feed on a substrate with a high concentration of micro-organisms. When they disperse outside their original food source to search for a suitable site for pupariation, they are susceptible to parasitism and physical stress. Consequently, these larvae must possess an extremely efficient immune system. Therefore, they may be an excellent model for studying insect defense mechanisms.

Although lacking the components that characterize the acquired immunity systems of vertebrates, in-

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sects are known to possess efficient mechanisms for combating pathogens by building up defense responses, which exhibit striking parallels with those of the innate immunity of vertebrates. These innate immune systems include both cellular and humoral elements. The cellular components of insect immunity, called hemocytes, are able to phagocytose, nodulate, and encapsulate. Phagocytosis is the first barrier against foreign bodies, and it has been described in the hemolymph of many insect species to fight against biological (Ratcliffe and Rowley, 1979; Ratcliffe *et al.*, 1985; Götz and Boman, 1985; Ratcliffe, 1986) and non-biological agents (Wiesner, 1991, 1992; Slovák *et al.*, 1991; Faraldo and Lello, 2003). If a considerable number of elements invade the hemocoel, the elements are isolated by hemocyte aggregation and form nodules that may or may not be melanized (Ratcliffe and Rowley, 1979; Lackie, 1980). The encapsulation by hemocytes occurs when the foreign body is too large to be phagocytosed. Many studies have revealed that both humoral and cellular factors contribute to this encapsulation reaction (Götz and Vey, 1987; Götz *et al.*, 1987; Rizki and Rizki, 1987; Faraldo *et al.*, 2005). Humoral immunity involves (a) the induction of proteolytic cascades that cause hemolymph coagulation (Muta and Iwanaga, 1996) and localized melanization (Nappi and Vass, 1993; Carton and Nappi, 1997); (b) the oxygen-dependent mechanisms that include the synthesis of lysozyme, proteolytic, and hydrolytic enzymes, and antimicrobial peptides (Meister *et al.*, 1997); and finally (c) the generation of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) (Nappi *et al.*, 1995; Nappi and Vass, 1998; Luckhart *et al.*, 1998; Nappi *et al*., 2000; Nappi and Ottaviani, 2000; Whitten *et al*., 2001). In mammalian immunity, among the nitrogen radicals, nitric oxide (NO) is best known for its anti-microbial activity by killing microorganisms by nitrosylation, nitration, and oxidation of essential microbial components (DNA, lipids, and proteins) (Fang, 1977).

The production of NO results from ligand-receptor interactions and is generated among citrulline during the NOS-catalyzed hydrolysis of L-arginine. These enzymes require NADPH as co-factor and are inhibited by some analogues of L-arginine. Three isoforms of NOS have been described in vertebrates: two are constitutive (cNOS) and one is inducible (iNOS). In mammals, cNOSs are present in the tissues of the endothelium, the brain, the adrenal glands, and in platelets (Moncada *et al.*, 1991). The iNOS, synthesized in response to cytokines and inflammatory mediators, has been identified in macrophages, hepatocytes, vascular smooth muscle, endothelial cells and neutrophils (Moncada *et al*., 1991; Rimele *et al*., 1991). NOSs were originally identified in mammalian tissues. However, NOS genes were cloned from some insect species (*Drosophila melanogaster*, *Anopheles stephensi*, *Anopheles gambiae*, *Rhodnius prolixus*) and the amino-acid sequences deduced exhibited similarities with those of Ca^{2+}/calmodulin-dependent NOSs from mammals (Regulski and Tully, 1995; Luckhart *et al*., 1998; Luckhart and Rosenberg, 1999; Dimopoulos *et al*., 1998; Yuda *et al*., 1996). Martínez (1995) has showed evidence for NOS activity in invertebrate tissues. However, there are few studies that relate NOS activity to immune responses in insect tissues (Ribeiro and Nussenzveig, 1993; Luckhart *et al.*, 1998; Dimopoulos *et al*., 1998; Whitten *et al*., 2001; Foley and O'Farrel, 2003; Faraldo *et al*., 2003).

To elucidate the relation between NO generation in insect tissues and immune mechanisms, this study aims to identify tecidual NOS activity among naïve larvae, yeasts inoculated-larvae, and saline injected-larvae of *Chrysomya megacephala* and to outline comparisons with other insect orders.

Materials and methods

Insects

Newly hatched larvae of *Chrysomya megacephala* were obtained from adults kept at constant temperature (25°C), and raised in vials containing minced meat. The postfeeding larvae were removed from the vials, washed in 10% clorhexidine solution, immersed in 10% sodium hypochloride (NaClO) solution for 10 minutes, and washed in distilled water.

Microorganisms

Saccharomyces cerevisae yeasts were used in all experiments. *C. megacephala* reactions against these microorganisms were the model used to study the immune responses in this insect.

S. cerevisae yeasts (*Fleischmann*®) were suspended in saline solution for insects (SSI: 154mM NaCl, 126mM KCl, 7.2mM $CaCl_2$, 0.24mM $NaHCO_3$, pH 7.0) and stored at 4°C. Before each experiment, the yeasts were resuspended in the solution mentioned above, and the suspension was adjusted to 10^5 yeast/ml. The volume of yeast suspension injected in each larva was approximately 20 μL.

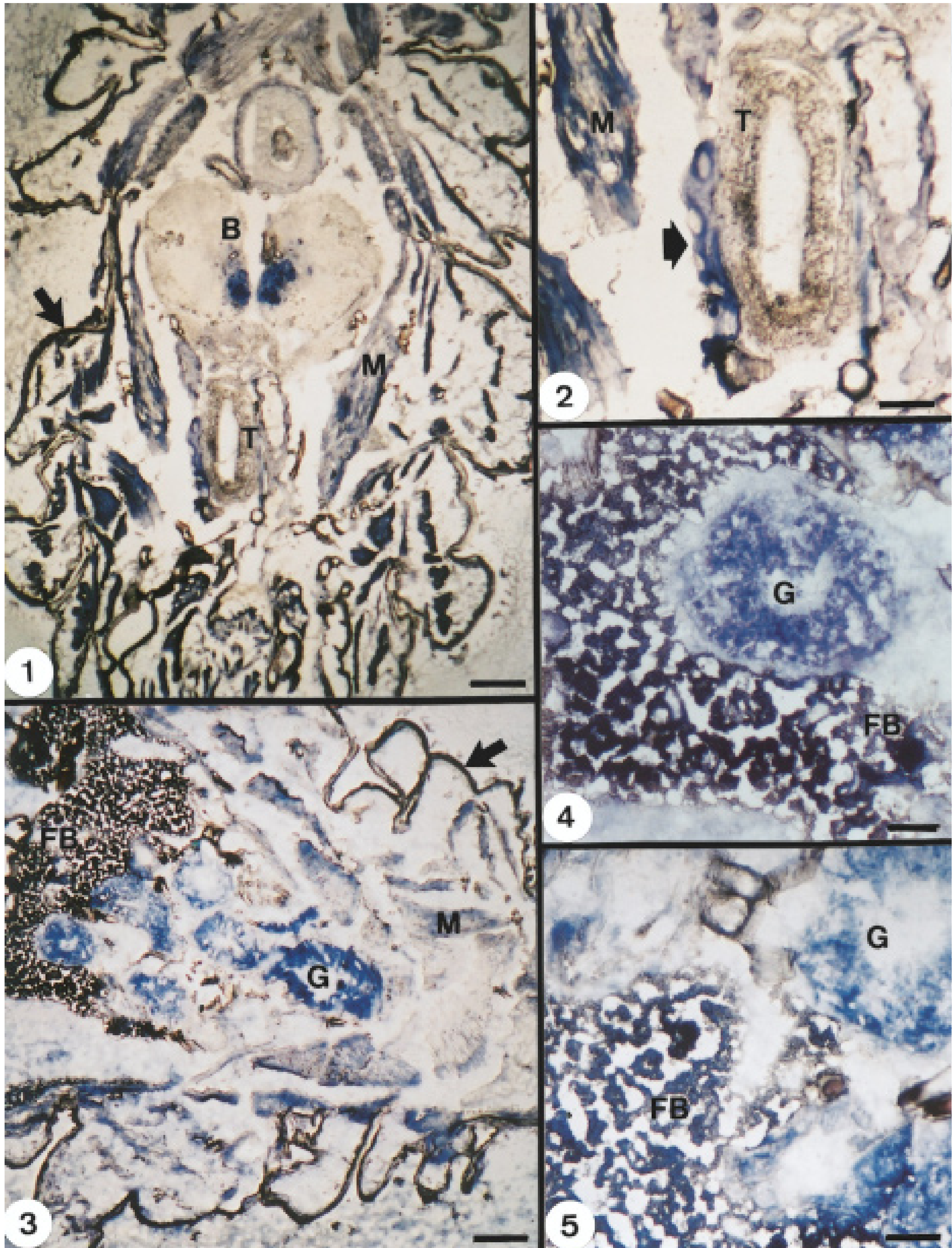

FIGURE 1-5. Cryosections of *C. megacephala* larvae. NADPH-diaphorase staining technique, which indicates NOS activity was used. 1. Brain (B) and muscle (M) of naïve larva (uninjected, UIL) showing positive reaction. Arrow: larval cuticle; T: trachea. Bar: 20 μm. **2.** Stained Malpighian tubules (arrow) of UIL at high magnification. M: muscle; T: trachea. Bar: 10 μm. **3.** Muscle (M), gut (G) and fat body (FB) of yeast-injected larvae (YIL) stained by the NADPH-diaphorase technique. Arrow: cuticle. Bar: 20 μm. **4.** Stained gut (G) and fat body (FB) of YIL at high magnification. Bar: 5 μm. **5.** Gut (G) and fat body (FB) of saline-injected larvae showing positive reaction. Bar: 5 μm.

Localization of nitric oxide synthase (NOS)

NOS was localized *in situ* among naïve larvae (uninjected, UIL), yeasts-injected larvae (YIL), and larvae injected with SSI (SIL). Samples were taken from injected-groups 24 h post-injection, once our previous experiments showed there was increased NO production in the blowfly hemolymph at this time after yeast injection (Faraldo *et al*., 2005). Because NOSs have NADPH diaphorase activity, a histochemical technique for NADPH diaphorase was used to locate the enzyme.

Nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase technique

Injected or uninjected larvae (YIL, SIL, and UIL) were washed in phosphate-buffered saline (PBS, 0.01 M NaCL and 10 mM NaPO4, pH 8.4), fixed in 4% paraformaldehyde (PFA) for 24 h and incubated in PFA + 15% sucrose or PBS + 15% sucrose for 24 h.

The assays were performed in larval sections. All the samples were frozen at -70°C, embedded in Tissue Tek® (*Sakura Finetek*) at the same temperature, and 16 µm-cryo sectioned in a cryostat *Leica CM 1850*. The sections were placed in slides and incubated in 0.2 M PBS with 1 mM NADPH, 1 mg/ml nitroblue tetrazolium, 3% Triton X-100, and distilled water at 37°C. After 90 min, the sections were examined for specific staining by the diaphorase technique under light microscopy and documented in a Photomicroscope (*Zeiss II*).

Results

The NADPH diaphorase staining method revealed NOS activity in tissues of the muscles (M), the fat body (FB), the Malpighian tubules, the gut (G) and the brain (B) of naïve (UIL), yeast-injected (YIL), and saline-injected (SIL) larvae of *Chrysomya megacephala* (Figs.1-5). These larval tissues showed similar staining patterns among the three groups treatments.

Discussion

The NADPH diaphorase histochemical technique was applied to locate NOS activity in UIL, YIL and SIL tissues to attempt to link this activity to immune mechanisms. In this reaction, the oxidation of NADPH by diaphorase is coupled with the reduction of nitroblue tetrazolium (NBT), which precipitates as dark blue formazan and indicates the location of NOS, the enzyme responsible for biosynthesis of nitric oxide (NO) (Dawson *et al*., 1991). Presumably, the NBT-formazan precipitation observed here does not correspond to NO generation in response to the yeast inoculation, but to the activity of NOSs probably involved in distinct physiological functions of the blowfly tissues.

NOS activity has been demonstrated in invertebrate tissues (Martínez, 1995; Elphick *et al.*, 1993; Stefano and Ottaviani, 2002). In recent years, more evidence for insect NOSs has been reported (Davies *et al*., 1997; Müller, 1997; Luckhart *et al*., 1998; Chiang *et al*., 2000; Whitten *et al*., 2001; Ott and Elphick, 2003); however, there has not been data indicating NOS activity in insect muscles. In his review, Martínez (1995) reported the presence of diaphorase reaction in muscle cells of a mollusk species. He also inferred that NOS activation in muscles of more evolved invertebrates might be regulated by the nervous system. Wildemann and Bicker (1999) investigated an NO signaling system at the neuromuscular junction (NMJ) of the *Drosophila melanogaster* larvae. By combining immunocytochemical and exocytosis images, these authors demonstrated the involvement of NO in the regulation of synaptic vesicle release at the NMJ level in this dipteran. Our results suggest the NOS activity located in muscle tissue of *C. megacephala* might be located at the nerve ending, where NO might play a role in neurotransmission, as it does in *Drosophila* (Wildemann and Bicker, 1999) and mammals (Moncada *et al.*, 1991).

Otherwise, NOS activity has been described in insect fat body (FB) and Malpighian tubules (MT) (Martínez, 1995; Choi *et al*., 1995; Davies *et al*., 1997; MacPherson, 2001). These organs have similar functions to those attributed to the mammalian liver and kidney, respectively. Choi *et al*. (1995) identifies distinct types of NOSs in the FB and MT of the silkworm *Bombyx mori*. According to the authors, the major FB NOS is inducible by lipopolysaccharade (LPS), such as iNOSs from mammal hepatocytes (Moncada *et al.*, 1991). The two NOSs found in MT were constitutive. However, Choi *et al.* (1995) strongly suggest that these NOSs are related to the insect metamorphosis. By contrast, it is proposed that the activation of NOS in MT of *Drosophila* is involved in the stimulation of fluid secretion (Davies *et al.,* 1997). A similar function is described for NOS for the mammalian kidney (Springall *et al.*, 1992), which suggests that this dipteran's MT NOS is also involved in the regulation of the excretory system. NOSs activities detected in both FB and MT of *C. megacephala* larvae occurred in all the groups and may

represent constitutive NOSs. Nonetheless, experimental evidence is needed to establish whether detected NOSs are involved in the control of these systems or in the metamorphosis, as described for locusts (Choi *et al.*, 1995).

NOS activity in the insect gut has been previously described (Luckhart *et al.*, 1998; Dimopoulos *et al.*, 1998; Hao *et al.*, 2003). Using diaphorase staining, Luckhart *et al.* (1998) have already demonstrated the presence of NOS in the midgut of the malaria vector *Anopheles stephensi*. This inducible NOS activity is higher in *Plasmodium*-infected mosquitoes and is able to limit parasite development (Luckhart *et al.*, 1998; Lim *et al.*, 2005). In a similar way, there is recent evidence of increased NOS activity at the foregut/midgut junction (proventriculus) in tsetse fly, upon microbial challenge (Hao *et al.*, 2003). We examined sections of naïve, yeast-injected, and saline-injected larvae of *C. megacephala*, and in all the groups, the use of the NADPH diaphorase technique revealed NOS activity in the gut. This NOS activity could be due to the elevated concentration of microorganisms, commonly present in the insect meals, which suggests that NOS may also exert an anti-microbial effect in the *C. megacephala* gut. Further studies are required to determine whether NOS activity in the blowfly larval gut is inducible or constitutive.

Finally, in most of the invertebrates studied, NOS appear to be involved in neurotransmission in both the central and the peripheral nervous systems (Martínez, 1995). The brain in insects, as well as in vertebrates (Salter *et al.*, 1991), have been described to posses the highest activity of constitutive NOS (Müller, 1997). Using NADPH diaphorase staining, Chiang *et al.* (2000) detected cNOS activity in the cerebral ganglion of *Periplaneta americana*. Subsequently, they demonstrated that the high cNOS activity expressed in cockroach corpora allata (CA) was also expressed in other insects, such as the house cricket, a lepidopteran species, and the fruit fly. The authors assumed the occurrence of cNOS in the CA of most, if not all insects, and suggested that CA releases NO as a messenger molecule in these arthropods. Activity of cNOS was also identified in several parts of the *Schistocerca gregaria* brain (Elphick *et al.*, 1995). Based on the particular abundance of cNOS in the olfactory system, NO involvement in the locust olfaction was suggested.

To date, in addition to the olfactory signal transdution, NO generation seems to be implicated in memory formation in the honeybee *Apis mellifera*, which is a finding which provides remarkable parallels with findings in vertebrates (Menzel, 2001). In a recent review, Bicker (2005) indicated that NOS affects the wiring of insect nervous systems by regulating cell motility. Our histochemical essays revealed NOS activity in the central region of the *C. megacephala* larval brain. Interestingly, the stained portion seems to correspond to the central complex of adults, which contains the olfactory center. Olfaction is thought to be essential for larval nutrition (Hancock and Foster, 1997) and, consequently, for successfully reaching the adult stage.

The main objective of this study was to investigate NOS activity in blowfly tissue in response to yeast inoculation by the NADPH diaphorase method. Our findings revealed NOS staining levels in the tissues of the muscle, the fat body, the Malpighian tubules, the gut, and the brain, were similar before and after the challenge. This is the first study that shows the synthesis of NO in larval tissues of the blowfly *Chrysomya megacephala*. Many studies are emerging that register the implication of NO in various physiological functions of insects, and functional role of NOS in these blowfly systems certainly warrants consideration in future investigations.

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