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The regulation of cardiac activity by nitric oxide (NO) in the Vietnamese stick insect, *Baculum extradentatum*

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ABSTRACT

This study examines the role of the unconventional gaseous signaling molecule nitric oxide (NO) on the regulation of heart rate in the Vietnamese stick insect, *Baculum extradentatum*. Using nicotinamide dinucleotide hydrogen phosphate (NADPH)-diaphorase histochemistry, as well as immunohistochemistry and Western blotting with an antibody against NO synthetase (NOS), we identified the presence of NOS in hemocytes present throughout the lumen of the dorsal vessel. We propose that NO is delivered to heart muscle tissue via hemocytes circulating within the hemolymph. In the present study, stimulation of NO levels by the application of the NO donor MAHMA-NONOate and L-arginine led to a dose-dependent decrease in heart rate. Treatment of tissues with the NOS inhibitor, L-NAME, in equimolar concentrations with L-arginine, led to a recovery of heart rate, without modifying heart rate on its own. Finally guanosine 3′,5′-cyclic monophosphate (cGMP) analog, 8-bromo-cGMP, elicited similar inhibitory effects on stick insect heart rate as did the guanylate cyclase activator, YC-1, and the phosphodiesterase inhibitor, dipyridamole, indicating that cGMP is most likely the second messenger in the stick insect NO signaling pathway. Contrary to the cardioexcitatory effect of NO on other insect hearts, we have found that NO inhibits stick insect heart rate independently from any nervous system input, in a similar inhibitory fashion as that of vertebrate hearts.

1. Introduction

The insect dorsal vessel is a cylindrical organ that comprised a helical arrangement of striated muscle fibers that stretches the entire length of the insect [1]. In the Vietnamese stick insect, *Baculum extradentatum*, the dorsal vessel is divided into a thoracic aorta and an abdominal heart [2], which is subdivided into nine chambers separated by incurrent ostia (or valves). Pairs of striated alary muscles are located just posterior to each pair of incurrent ostia [3], and are postulated to aid in dorsal vessel contraction and hemolymph uptake into the vessel lumen [4], as seen in other Orthopteroid insects [5]. The heart of *B. extradentatum* is also attached to a dorsal diaphragm [2], which anchors the dorsal vessel to the dorsal body wall, and may play a role in heart contractions [3]. Excurrent ostia also line the dorsal vessel of Orthopteroid hearts [5], and are small openings in the vessel wall that allow for flow of hemolymph from the vessel lumen to the pericardial cavity [5]. In stick insects, three pairs of excurrent ostia are associated with the dorsal vessel, and are found in the metathoracic, 1st abdominal and the 2nd abdominal segments [2].

Dorsal vessel contractions originate in the posterior end of the heart, and travel in peristaltic waves anteriorly toward the head [2,3]. The insect dorsal vessel is under both myogenic and neurogenic controls, with innervation arising from the central nervous system (CNS), as seen in *Carausius morosus* [1,3]. Two classes of nerves project to the heart of *B. extradentatum*: the segmental nerves, which originate from the tergal nerves of all thoracic and abdominal ganglia and branch onto the heart anterior to the incurrent ostia, and a pair of lateral cardiac nerves (LCN), which originate from ganglia in the head and run along both sides of the entire length of the dorsal vessel [2,3,6]. Nerve processes from the segmental nerves have also been shown to innervate the incurrent and excurrent ostia in both *B. extradentatum* and *C. morosus* [2,3].

The heart and associated structures can be regulated by neurotransmitters, neuromodulators, and neurohormones, which can induce excitatory or inhibitory responses in cardiac activity [1]. Previously, it has been shown that excitatory neurochemicals, such as the neuropeptides

Abbreviations: 8-bromo-cGMP, 8-bromoguanosine 3′,5′-cyclic monophosphate; ATPases, adenosine-5′-triphosphate hydrolases; CAP2b, cardioacceleratory peptide 2b; CCAP, crustacean cardioactive peptide; cAMP, adenosine-3′,5′-cyclic monophosphate; cGMP, guanosine-3′,5′-cyclic monophosphate; CNS, central nervous system; GTP, guanosine-5′-triphosphate; GTPase, GTP hydrolase; eNOS, endothelial nitric oxide synthetase; iNOS, inducible NOS; nNOS, neuronal NOS; uNOS, universal NOS; FaRP, FMRF-amide related peptides; LCN, lateral cardiac nerves; L-NAME, Nω-Nitro-L-arginine methyl ester hydrochloride; MAHMA-NONOate, 6-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine; NADPH, nicotinamide dinucleotide hydrogen phosphate; NBT, nitroblue tetrazolium; NO, nitric oxide; NOS, nitric oxide synthetase; PKG, cGMP-dependent protein kinase; sGC, soluble guanylate cyclase; SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside.

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proctolin [7] and crustacean cardioactive peptide (CCAP) [8], increased heart rate dose-dependently in *B. extradentatum* in dorsal vessel preparations that were semi-isolated and fully detached from the CNS [2]. Upon further investigation, proctolin- and CCAP-like immunoreactivity was located in axons in nerves projecting to the heart [2], suggesting the regulation of heart activity by these neuropeptides in vivo. The insect dorsal vessel can also be regulated by inhibitory neuropeptides, such as the FMRF-amide related peptides (FaRP) leucomyosuppressin in *Tenebrio molitor* [9], dromyosuppressin in *Drosophila melanogaster* [10,11] and SchistoFLRFamide in *B. extradentatum* [12] with the inhibition of cardiac activity occurring at the cardiomyocyte level, mediated by the CNS.

The free radical neurotransmitter nitric oxide (NO) has been found to elicit inhibitory responses in vertebrate cardiovascular systems, and excitatory responses in insect cardiac systems [13]. NO is produced as a byproduct in the conversion of L-arginine to citrulline, aided by the cofactor NADPH and molecular oxygen, by a family of cytosolic nitric oxide synthetases (NOS), which can be located in epithelial tissue (eNOS), neural tissue (nNOS), or inducibly expressed (iNOS) in macrophages [14]. In most cases, NO production is increased through the stimulation of NOS by elevated cytosolic Ca^{2+} levels, triggered by hormones or neurotransmitters [14]. Once produced, NO travels through the plasma membrane to proximal target tissues or cells, where it binds to the heme groups of soluble guanylate cyclase (sGC), and activates the sGC GTPase domain. This leads to the synthesis of cyclic-guanosine-3′,5′-monophosphate (cGMP) from guanosine-5′-triphosphate (GTP), which then mediates a variety of downstream signaling events [13]. In vertebrates, increased NO and cGMP production by the exogenous application of NO through donors such as S-nitroso-N-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP) has been found to decrease human platelet adhesion to bovine epithelial cell cultures [15,16]. Human platelets also contain an inducible form of NOS, where the NO generated reduces platelet aggregation in human blood samples [17]. Furthermore, overexpression of nNOS in the atria of rats during acute myocardial infarction led to a decrease in heart rate, akin to parasympathetic regulation of heart rate in normal conditions [18]. Conversely, in insects such as the African migratory locust, *Locusta migratoria*, where NOS-like immunoreactivity was identified in the LCN and in segmental neurons projecting to the heart, it was found that increasing levels of exogenous NO by the application of another donor, 6-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine (MAHMA-NONOate), led to dose-dependent increases in heart rate [19].

The purpose of the present study was to investigate the prevalence of NO in *B. extradentatum* using the nitroblue tetrazolium (NBT) NADPH-diaphorase assay and immunohistochemistry for NOS, along with Western blot analysis, and to investigate the effects of NO on cardiac activity. It was postulated that, due to the similarities in cardiac morphologies and physiologies between the Orthopterans *L. migratoria* and *B. extradentatum* [5], NO would have a stimulatory effect on the heart rate of *B. extradentatum*. It was also hypothesized that the second messenger pathway elicited by NO would be cGMP-dependent, and therefore the effects of a cGMP analog, 8-bromo-cGMP, as well as the GC activator, YC-1, and the phosphodiesterase V (PDE 5) inhibitor, dipyridamole, on heart activity were also investigated.

2. Materials and methods

2.1. Animals

Stick insects were reared at moderate humidity and at 25 °C in a 12 h light:dark cycle at the University of Toronto Mississauga. Diet for *B. extradentatum* consisted primarily of ficus leaves, supplemented with red oak leaves. Immature stick insects ranging from 3 to 5 cm were used for experiments.

2.2. NADPH-diaphorase histochemical bioassay

Stick insects were dissected in physiological saline (15 mM NaCl, 18 mM KCl, 7.5 mM $CaCl_2$, 50 mM $MgCl_2$, 185 mM glucose, 2 mM Hepes; pH 6.63), and fixed in 4% paraformaldehyde in Millonig's buffer (0.13 M $NaH_2PO_4 \cdot H_2O$, 0.1 M NaOH, 1.2% glucose, 0.3 mM $CaCl_2$; pH 7) for 2 h, at 4 °C. Tissues were then incubated at 4 °C in phosphate buffered saline (PBS; containing 0.9% NaCl, pH 7.2) with 0.5% saponin, overnight. Tissues were subsequently washed with PBS for 1 h with rinses every 5 min, and then incubated for 60–90 min at 25 °C in 50 mM Tris–HCl (pH 7.8), containing 0.1% Triton X-100, 0.1 mM nitroblue tetrazolium (NBT; Sigma-Aldrich, Oakville, ON, Canada) and 0.1 mM NADPH (Sigma-Aldrich, Oakville, ON, Canada). The staining process was stopped by washing tissues with PBS for 1 h, with rinses every 5 min. Tissues were dehydrated with an ascending ethanol series, mounted in 100% glycerol, and viewed using a Zeiss compound light microscope (Carl Zeiss, Jena, Germany).

2.3. Universal NOS immunohistochemistry

Insects were dissected in physiological saline and fixed in 4% paraformaldehyde for 2 h at room temperature, followed by washing with PBS for 1 h with rinses every 5 min. An ascending ethanol series was used to dehydrate the tissues, which were rehydrated with a descending ethanol series, in order to remove lipids surrounding tissues. Tissues were then washed in PBS at 37 °C, followed by a 20 min incubation at 37 °C in collagenase/dispase (2 mg; Sigma-Aldrich, Oakville, ON, Canada) and hyaluronidase (2 mg; Sigma-Aldrich, Oakville, ON, Canada) in 2 ml Tris–HCl (50 mM total; pH 7.8). Tissues were then washed in PBS containing 0.5% Triton X, followed by incubation in 10% normal goat serum (NGS) in PBS for 1 h at room temperature. Tissues were then left overnight at 4 °C in rabbit anti-universal NOS antibody (Affinity Bioreagents; Cedarlane Laboratories, Burlington, ON, Canada), diluted at 1:100 in PBS with 1% NGS. After washing with PBS, tissues were further incubated at 4 °C in Cy3 goat anti-rabbit secondary antibody, diluted at 1:400 in PBS, for 16–18 h. Tissues were then washed with PBS, prior to dehydration with an ascending glycerol series and mounted in 100% glycerol. Preparations were observed with a Zeiss Confocal microscope (Carl Zeiss, Jena, Germany) and Zen 2009 viewing software. No staining was observed in controls that were performed without primary antiserum.

2.4. Western blot analysis

Stick insect hemolymph was collected and spun at 2000 *g* for 5 min at 4 °C. The supernatant was subsequently removed and the pelleted hemocytes were lysed in ice cold RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate; pH 7.4) in the presence of protease and phosphatase inhibitor cocktails (Sigma-Aldrich, Oakville, ON, Canada). Samples were subsequently centrifuged at 13,000 *g* for 20 min at 4 °C. Supernatant was collected and analyzed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio Rad Laboratories, Mississauga, ON, Canada). Blots were blocked for 1 h in 5% skim milk made up in tris-buffered saline and Tween 20 buffer (TBST; 50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5), and then incubated with shaking overnight at 4 °C in a polyclonal rabbit anti-universal NOS antibody (Affinity Bioreagents; Cedarlane Laboratories, Burlington, ON, Canada), diluted at 1:1000 in 1% skim milk in TBST. Following washing with TBST, blots were incubated with donkey anti-rabbit peroxidase-coupled secondary antibody diluted at 1:1000 for 1 h and bound antibody was visualized using the Supersignal® West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Blots were imaged with a ChemiDoc XRS Molecular Imager System (Bio Rad Laboratories, Mississauga, ON, Canada).