國科會專題研究計劃成果報告

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計劃名稱: An Investigation on the Presence of Nitric Oxide Synthase and the Role of Nitric oxide in Crustacean Eyestalk Ganglia.

甲殼類眼柄神經節一氧化氮合成酶與一氧化氮功能之探討

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摘要:

本研究目的是探討螯蝦 (Procambarus clarkii) 眼柄神經節氧化氮合成酶之生化特性與分布, 以及氧化氮之生理功能. 利用以亞硝酸根生成爲氧化氮合成酶活性之分析發現眼柄神經節之氧化氮合成酶活性需要 L-arginine, ß-NADPH, Ca^{2+}, 並且受 L-NNA (氧化氮合成酶之抑制物) 與 calmodulin inhibitor 之抑制. 免疫組織化學法發現氧化氮合成酶之免疫反應位於兩個視神經叢絡之間的 first chiasma. 生理的研究顯示注射氧化氮之釋放物顯著增加血糖的含量. 這些結果第一次證實氧化氮合成酶存在於甲殼類 眼柄神經節. 根據免疫組織化學的結果, 氧化氮可能涉及視覺訊息的處理. 此外, 氧化氮也可能間接透過對眼柄內的神經內分泌系統的升血糖激素分泌之影響而引發血糖之增加.

Abstract:

The objective of the present study was to determine the presence and localization of nitric oxide synthase (NOS) in crustacean eyestalk. Biochemical assay using nitrite formation as an indicator for NOS activity was employed to characterize the NOS-like activity in the eyestalk ganglia of the crayfish, *Procambarus clarkii*. The data indicated that the NOS-like activity was inhibited by L-NNA, a NOS inhibitor, as well as by omitting L-arginine, ß-NADPH, Ca^{2+} from, or by adding calmodulin inhibitor into the incubations. Immunohistochemical studies using α-NOS localized the immunoreactivity to the first chiasma situated between two visual neuropiles, the lamina and the medulla externa. Physiological studies implicated NO as a hyperglycemic regulator as injection of SNP, a NO releaser, significantly increased glucose levels. In summary, these results demonstrated for the first time the existence of NOS-like enzymatic activity in crustacean eyestalk ganglia. The locality of the NOS-immunoreactivity suggests NO might be acting as a signal molecule in the processing of visual information in crustaceans. Further, the possibility that NO might be involved in regulating the release of crustacean hyperglycemic hormone from an eyestalk neuroendocrine system (the X-organ/sinus gland) is a question worth investigating.

Background

Since it was identified in the late '80 as the endothelium-derived relaxing factor[1], nitric oxide (NO), a short-lived gaseous molecule, has been found acting as an intercellular messenger in regulating a wide variety of physiological functions ranging from muscle relaxation, macrophage cytotoxicity, inhibition of platelet aggregation, to neurotransmission[2].

Production of NO is catalyzed by a family of isozymes, the nitric oxide synthase (NOS), of which three forms are recognized, i. e., brain (bNOS, isoform I), inducible (iNOS, isoform II), and endothelial (eNOS, isoform III) isozymes. These isozymes have differential tissue distributions[3]. bNOS is predominantly found in neuronal tissues, iNOS in macrophage and other cell types (hepatocyte, neutrophil, etc.), and eNOS in endothelia[3]. Studies of mammalian isozymes have revealed their biochemical characteristics. Both bNOS and eNOS are constitutively expressed and Ca2+/calmodulin-regulated, whereas iNOS is inducible (in response to cytokines) and Ca2+-independent. All isozymes catalyze the formation of NO and citrulline using L-arginine and O2 as substrates; several cofactors, such as NADPH, FAD, FMN, biopterin, are required for catalytic functions[4].

Emerging evidences, predominantly derived from mollusks and insects, indicated that NOS

is also present in invertebrates[5]. The majority of the evidences came from immunocytochemical studies. NOS-like immuno-reactivities, detected by antibodies raised against mammalian NOS, have been reported in nervous tissues and several other tissues of two snails (*Lymnaea stagnail*, and *Helix aspersa*)[7,8], and a starfish (*Marthasterias glacialis*)[9].

Biochemical assays measuring NOS activity also produced consistent results. Thus, production of citrulline (a co-product in the formation of NO) in the presence of exogenous L-arginine has been demonstrated in *Lymnaea stagnail*, and was inhibited in the presence of N^w-nitro-L-arginine, a NOS inhibitor[10]. Similarly, insect (*Schistocerca gregaria*) brain was found capable of converting L-[^{14}C]-arginine to L-[^{14}C]-citrulline, and the catalytic activity was suppressed by omitting Ca^{2+} or NADPH in the incubation, or by adding calmodulin inhibitors (W-13 and W-5), NOS inhibitors (N^w-nitro-L-arginine and N^w-monomethyl-L-arginine)[11], suggesting that invertebrate NOS shares with its mammalian counterparts similar catalytic properties.

Although increasingly comparative physiologists and biochemists are interested in studying NO signalling pathways in invertebrates[5], crustaceans are rarely exploited as a favored system in this regard. To our best knowledge, a L-arginine- and NADPH-dependent NO production by hemocytes was demonstrated to inhibit hemocyte aggregation in horseshoe crab (*Limulus polyphemus*)[12]. In the present study, we demonstrated, using NOS immunohistochemistry and biochemical assay, for the first time, that the eyestalk ganglia in crayfish (*Procambarus clarkii*) also contain nitrergic (NO-synthesizing) neurons. The possible roles NO may play in crustacean eyestalk ganglia will be discussed in light of these findings.

Materials and Methods

Animals (Procambarus clarkii) were purchased from local fisherman, and kept in freshwater tanks as described previously[13].

Ganglia were dissected out from ablated eyestalks in cold crustacean saline, homogenized in extraction buffer (20 mM Tris-HCl, pH 7.2) containing 0.5 mM EDTA and 100 µg/ml PMSF, and centrifuged (10,000Xg, 4 °C, 15 min). The supernatants were collected, and concentrated using Microcon-30 (Amicon). To initiate the control NOS reactions, aliquots (20 µl) of the concentrate equivalent to 30 eyestalk ganglia were added to 40 µl of extraction buffer containing (in final concentration) 2 mM ß -NADPH (RBI), 0.45 mM $CaCl_2$, 25 U/ml calmodulin (Sigma), 0.2 mM L-arginine, and 1 µM tetrahydro-L-biopterin dihydrochloride (BH4, CalBiochem). In order to characterize the NOS activity, experimental reactions were conducted as mentioned for the control reactions with the exception that ß-NADPH or $CaCl_2$ was omitted from the incubations, or pharmacological inhibitor (fluphenazine-N-2-chloroethane, W-13, or N^G-nitro-L-arginine, CalBiochem) was added to the incubations (see Results). The enzymatic reactions were incubated at 20 °C for 6 h, and terminated by heating the incubations at 100 °C for 10 min, and centrifuged (10,000Xg, 4 °C, 15 min). The supernatants were collected for determination of nitrite levels using a commercially available kit (Nitric Oxide Synthase Assay Kit, # 482702, CalBiochem) based on the method of Griess reaction. In each experiment in which the same batch of tissue preparation was used, zero-time reactions prepared as described for the control reactions but heat-inactivated without incubation were also included. Nitrite levels in the zero-time samples were similarly determined by the assay kit and subtracted from those of the control and experimental samples. Thus, nitrite levels reported were the amounts of nitrite accumulated during the 6 h incubation.

Aliquots of the tissue concentrates were taken for protein determination according to the method of Bradford[14]. Amounts of nitrite accumulated during incubation was normalized to tissue protein.

Eyestalk ganglia used for immunohistochemistry were dissected out as described

above, fixed in 0.1 M phosphate-buffered saline (PBS, 18.9 mM NaH_2PO_4, 81.0 mM Na_2HPO_4, 136.9 mM NaCl, 2.7 mM KCl, pH 7.5) containing 4% paraformaldehyde at 4 °C for 2 h, cleaned briefly in PBS, cryoprotected in 15% sucrose in PBS at 4 °C overnight, embedded in O.C.T. (Tissue-Tek, Miles Inc., Elkhart, IN, USA), and sectioned (20 µm) at -20 °C using a cryo-microtome (Cryo-Cut II, American Optical, Buffalo, NY, USA). Tissue sections were mounted on chrome alum gelatined slides, and the NOS immunoreactivity was detected by the method of avidin-biotinylated peroxidase complex using ImmunoPure ABC Peroxidase Staining Kit (# 32032, Pierce). Briefly, sections were incubated at room temperature sequentially with 0.3% H_2O_2 in methanol for 30 min to deplete the endogenous peroxides activity, normal goat serum in 20 mM PBS (3.2 mM NaH_2PO_4, 16.8 mM Na_2HPO_4, 150 mM NaCl, pH 7.5) (as blocking solution) for 30 min, rabbit a-NOS (see below) in blocking solution for 3 h, biotinylated goat a-rabbit IgG in blocking solution for 1 h, avidin-biotinylated peroxidase complex in PBS for 30 min, and substrate solution for 5 min (Metal Enhanced DAB Substrate Kit, # 34065, Pierce). Each incubation was followed by washing (3X) with 20 mM PBS containing 0.05% Tween 20 and 0.1% BSA before proceeding to the next one. The tissues sections were then dehydrated with ethanol, cleaned with xylene, and coverslipped with Entellan (Merck). Two a-NOS antibodies were used separately as the primary antibody: a-universal NOS (# PA1-039, ABR) was used at the dilution of 1/100, and a-rat bNOS (# 482724, CalBiochem) at 1/100, 1/200, 1/400 or 1/800.
X-organ/sinus gland.

Controls was conducted simultaneously by carrying out the immunohistochemical reaction as mentioned above except that primary (rabbit a-NOS) or secondary (biotinylated goat a-rabbit IgG) antibodies were omitted from the incubations.

In order to determine if NO is involved in regulating glucose levels, NO donor, sodium nitroprusside (SNP), was injected in vivo into animals, and the effect on blood glucose levels was determined by a colorimetric assay. Briefly, 1 h after injection of NO donor, blood was withdrawn from animals, and centrifuged at 2000Xg for 10 minutes. Aliquots of the supernatant was added to Trinder assay reagents (# 315, Trinder Glucose Assay Kit, Sigma), and incubated at room temperature for 10 minutes before the optical density was read by an ELISA reader (Elx808IU, Bio-Tek, Winooski, VT, USA) at 490 nm. A standard curve was constructed for each assay using standard glucose. Glucose concentrations in the samples were inferred from the standard curve and corrected for the dilution factor using a computer software (KC4, Bio-Tek).

Results

The NOS activity in the eyestalk ganglia of the crayfish, P. clarkii was indirectly measured by the levels of nitrite, a stable NO metabolite, accumulated during incubation of tissue homogenates. In the presence of exogenous ß-NADPH (2 mM), $CaCl_2$ (0.45 mM), calmodulin (25 U/ml), L-arginine (0.2 mM), and BH_4 (1µM), the mean enzymatic activity was 4.3 ± 0.2 pmole nitrite/mg protein/min (range = 3.5 — 5.3 pmole nitrite/mg protein /min) (n = 6). As shown in Fig. 1, the activity was inhibited by 0.1 mM of N^G-nitro-L-arginine (a NOS inhibitor) to 36.4 + 4.0% of the control levels, and to 68.0 ± 12.0% by omitting L-arginine (NO precursor) from the incubations. Furthermore, the activity was inhibited to 17.2 ± 4.8 and 35.3 ± 3.5% of the control by omitting ß-NADPH and Ca^{2+}, respectively, from the incubations. Fluphenazine-N-2-chloroethane, a calmodulin inhibitor, in the concentration of 1mM decreased the activity to 53.2 ± 6.0%, whereas W-13 (another calmodulin inhibitor) only to 81.1 ± 4.5%.

The localization of NOS in the eyestalk ganglia was investigated immunohistochemically using two antisera. The a-rat bNOS did not detect any immunoreactivity at the dilution tested (data not shown). On the contrary, a-universal NOS revealed NOS-immunoreactivity in the eyestalk ganglia. The immunoreactivity was mainly localized in the first chiasma situated between the lamina and the medulla externa (data not shown).

Injection of a NO releaser, SNP, into animals produced significant hyperglycemia (Fig. 2).

Discussion

The present report provids for the first time data indicating the presence of NOS-like activity in crustacean eyestalk ganglia. The conclusion was based on the observations that NOS activity of the ganglion preparations was inhibited by a NOS inhibitor. The absence of exogenous L-arginine did not significantly decreased the activity indicating that the tissue homogenates contained sufficient endogenous precursor for NO production. The NOS-like activity in crustacean eyestalk ganglia is Ca2+/calmodulin- and β-NADPH-dependent. In this respect, it is similar to the mammalian constitutive NOS[4].

The immunohistochemical studies provide further evidences that NOS is indeed present in crustacean eyestalk ganglia, and localize the NOS-immunoreactivity to the first chiasma, which contains fibers connecting two visual neuropiles, the lamina and the medulla externa. The locality of NOS-immunoreactivity strongly suggests that NO might be involved in the processing of visual information as has been found in mammals and insects[15-16]. Further electrophysiological studies of the role NO plays in crustacean eyestalk ganglia should provide interesting comparative data.

Finally, we also demonstrated that sodium nitroprusside, which spontaneously releases NO in aqueous environment, significantly increased glucose levels when injected into animals. The mechanism by which the released NO employed in eliciting hyperglycemic response is still unclear. However, it is well-known that the X-organ/sinus gland complex contained in the eyestalk secrets, among other neurohormones, the crustacean hyperglycemic hormone (CHH)[17]. It is possible that NO increases glucose levels by enhancing the release of CHH. In this respect, it is relevant to point out that NO has been shown acting as a neuromodulator in regulating mammalian neuroendocrine systems[18]. Whether NO has a similar role in crustaceans is an interesting question awaits future studies.

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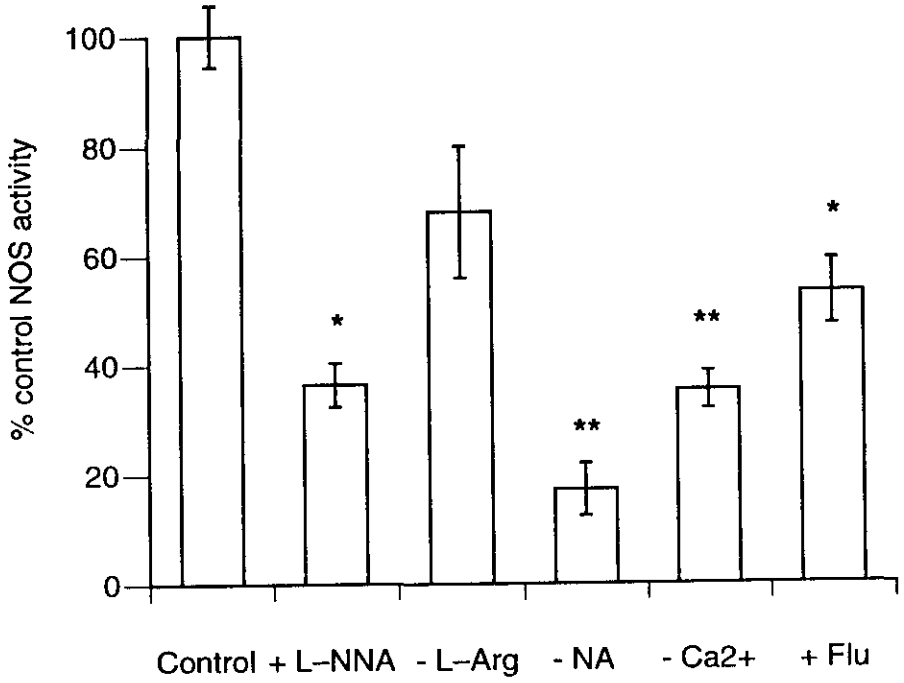
Figure Legend

Fig. 1 NOS-like activity in the eyestalk ganglia of the crayfish, *Procambarus clarkii*. In control reactions, eyestalk ganglia prepared as described in Materials and Methods were incubated in reaction buffer containing 2 mM ß-NADPH, 0.45 mM $CaCl_2$, 25 U/ml calmodulin, 0.2 mM L-arginine, and 1 µM BH_4 (control). In experimental reactions, ganglion preparations were incubated in the absence of L-arginine (- L–Arg), ß-NADPH (- NA), $CaCl_2$ (- Ca^{2+}), or in the presence of inhibitors: fluphenazine-N-2-chloroethane (+ Flu, 1 mM) or L-NAA (+ L-NAA, 0.1 mM). After incubation, nitrite levels were quantified using a NOS assay kit. Data are mean ± SEM and expressed as percentage of control enzymatic activity. N = 3 for each treatment. *, ** significantly different from control at the level of 0.05 and 0.001, respectively.

Fig. 2 Effect of SNP on glucose levels in the crayfish, *Procambarus clarkii*. Blood was withdrawn from animals 1 h after receiving *in vivo* injection of saline (A) or saline containing SNP (B: 375 µg/animals; C: 750 µg/animals), and analyzed for glucose levels. Data are mean ± SEM. N = 8 for each treatment. ** significantly different from control at the level of 0.001.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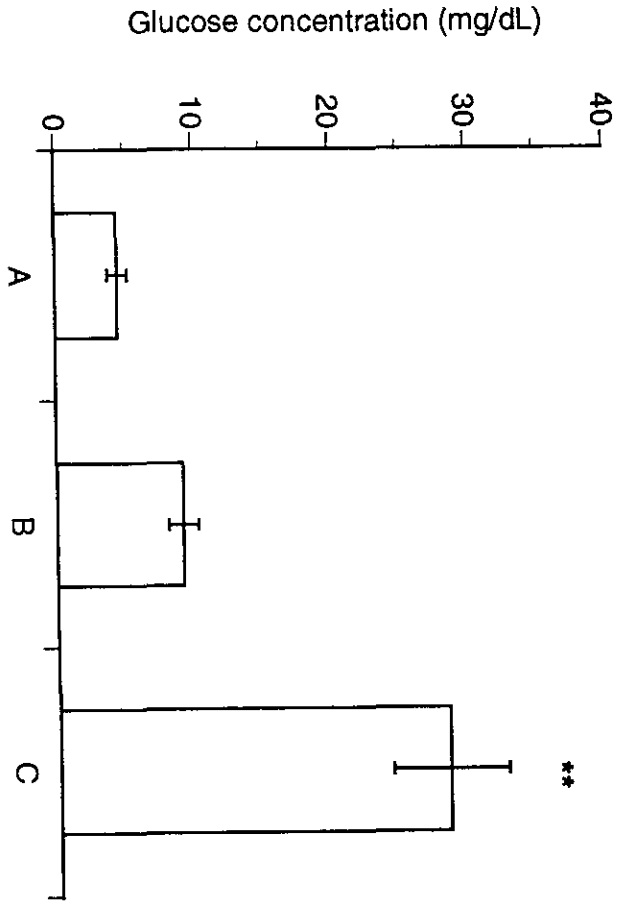
Glucose concentration (mg/dL)
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