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Establishing A Crustacean Model of Stress Responses

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主持人: 李奇英 國立彰化師範大學生物學系

Tel.: 04-723-2105 ext. 3429; Fax: 04-721-1156; E-mail: bicylee@cc.ncue.edu.tw

Abstract:

The objective of the present studies was to investigate the effect of nitric oxide on the release of CHH. Eyestalk ganglia obtained from adult crayfish, *Procambarus clarkii* were *in vitro* incubated under various pharmacological treatments that alter NO levels, followed by analysis of CHH levels in the incubation media by a CHH ELISA. Sodium nitroprusside (a NO donor) or L-arginine (a NO precursor) significantly reduced the levels of CHH released into the media. Conversely, NMMA (a NOS inhibitor) or hemoglobin (a NO scavenger) enhanced the release of CHH. Furthermore, hemoglobin was able to counter the inhibitory effect of SNP on CHH release. The combined results demonstrate that NO tincially inhibits CHH release. This is the first demonstration that NO modulates neurosecretory activity in invertebrates. Future studies will be focused on the cellular source of NO and the physiological relevance of NO-inhibited CHH release.

Background:

Nitric oxide (NO) is formed by NO synthase (NOS) from L-arginine with stoichiometric formation of citrulline. A family of isoenzymes is currently recognized in mammalian tissues: the brain (bNOS) and endothelial (eNOS) isoforms are constitutively expressed and Ca^{2+}/calmodulin-regulated, whereas the cytokine/endotoxin-induced isoform (iNOS) is Ca^{2+}-independent (Griffith and Stuehr 1995). Recent comparative studies have shown that NOS is present in the central and peripheral nervous systems of various invertebrate species and suggested a wide variety of functional roles for NO (Elofsson et al. 1993, Martinez 1995, Müller 1997). In crustaceans, localizations of NOS in the cerebral ganglion of the crayfish (*Pacifastacus leniusculus* and *Cambarellus montezumae*) and in the cerebral ganglion and the ventral nerve cord of the lobster (*Homarus americanus*) have been investigated by nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry and NOS immunohistochemistry, respectively (Johansson and Carlberg 1994, Talavera et al. 1995, Scholz et al. 1998). In addition, Ca^{2+}/calmodulin-dependent NOS activity was detected in the crayfish brain (Johansson and Carlberg 1994). It was suggested that NO may participate in crayfish olfactory processes and the development of lobster neural networks (Johansson and Mellon 1998, Scholz et al. 1998). Recently, we showed that the eyestalk ganglia of the crayfish, *Procambarus clarkii*, contain Ca^{2+}/calmodulin-dependent NOS activity and localized NOS immunoreactivity to the fibers present in an optic chiasma (the 1st chiasma) and a neurohemal organ (the sinus gland) (Lee et al. 2000).

We are particularly interested in

the localization of NOS in the sinus gland, where several well-characterized regulatory neuropeptides are released. Such observation implicates that NO plays important role in neurosecretory activity in the sinus gland. Among these neuropeptides, crustacean hyperglycemic hormone (CHH) is involved in maintaining basal glucose levels and evoking stress-induced hyperglycemic. The objectives of the present project were to investigate whether NO modulates the release of CHH.

Materials and Methods

Animals (*Procambarus clarkii*) were purchased from local fisherman, and kept in freshwater tanks as described previously (Lee et al. 2001).

A sandwich ELISA was developed in order to quantify CHH levels. The unlabeled α-CHH IgG solution was diluted to 10 μg/ml with coating buffer (0.1 M sodium carbonate, pH 9.6) and 100 μl of the diluted solution was added to wells of an ELISA plate (2580, Corning). The plate was incubated at 4 ℃ overnight. All subsequent incubations were performed at room temperature. After 3 washes with washing buffer A (0.01 M PBS containing 0.1% Tween 20 and 0.02% sodium azide, pH 7.4), 100 μl of blocking buffer (0.01 M PBS containing 2% BSA [bovine serum albumin]) was added and the plate was incubated for 1 h. After 3 washes with washing buffer A, 100 μl of HPLC-purified Prc CHH I (1.5 – 50.0 fmol in blocking buffer, Lee et al. 2001) or sample diluted with 0.01 M PBS containing 2% BSA and 2% glycine ethyl ester was added and the plate was incubated for 2 h. After 3 washes with washing buffer A, 100 μl of biotinylated IgG (diluted to 2.5 μg/ml with blocking buffer) was added and the plate was incubated for 2 h. After 3 washes with washing buffer B (0.01 M PBS containing 0.1% Tween 20, pH 7.4), 100 μl of avidin-biotinylated peroxidase complex (32020, Pierce) was added and the plate was incubated for 0.5 h. After 3 washes with washing buffer B, 100 μl of a substrate solution of peroxidase, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS, A3219, Sigma), was added and the plate was incubated for 0.5 h. Optical density was read at 405 nm using an ELISA reader (Elx 808, Bio-Tek). CHH levels were inferred from the standard curve constructed from a series of simultaneously assayed Prc CHH I and corrected for the dilution factor using KC4 software (Bio-Tek), and reported as fmol of CHH I equivalents.

Eyestalk ganglia with intact X-organ-sinus gland complex, dissected from ice-anaesthetized animals, were incubated as described previously (Lee et al. 2001) with minor modifications. Briefly, eyestalk ganglia were pre-incubated in Van Harreveld saline buffered with 20 mM HEPES (400 μl/ganglia/incubation) for 30 min at 25 ℃. After pre-incubation, each eyestalk

ganglia was transferred to a new well containing 400 µl of buffered saline without or with various concentrations of tested agents (see **Results**) and the incubation was performed for 1 additional h. The incubation media were collected and diluted (usually 10 or 15 folds) with ELISA blocking buffer. An aliquot (100 µl) of the diluted solution obtained from each incubation was assayed using the CHH ELISA as described above.

Results

Exogenous NO is able to inhibit CHH release. Sodium nitroprusside (SNP), a NO donor, decreased CHH release in a dose-dependent manner. At doses between 10^{-6} and 10^{-3} M, SNP decreased CHH release to 74.8% ~ 21.7% of control (387.5 fmol/h/ganglia). Likewise, altering endogenous NO levels by pharmacological agents changed the rate of CHH release. Thus, addition of L-arginine, a NO precursor, to the incubation also inhibited CHH release. In the range of 10^{-5} to 10^{-3} M L-arginine, CHH release was significantly decreased. The inhibitory effect was 53.2% to 21.1% of saline control (236.8 fmol/h/ganglia). Conversely, NMMA (a NOS inhibitor) in the doses between 10^{-5} and 10^{-2} M enhanced the release of CHH in a dose-dependent manner (127.7% to 287.9% of control). Removal of endogenously produced NO by hemoglobin (Hb, a NO scavenger) similarly enhanced CHH release. CHH released from Hb-treated tissues (10^{-5} and 10^{-2} M) was 124.5% to 270.6% of saline control levels (235.8 fmol/h/ganglia). Furthermore, hemoglobin significantly reversed the inhibitory effect of SNP on CHH release in a dose-dependent manner; at the dose of 10^{-6} M hemoglobin, the inhibitory effect of SNP was completely abolished so that CHH release levels (303.1 fmol/h/ganglia) is not significantly different from that of saline-treated tissues (334.2 fmol/h/ganglia).

Discussion

The combined results of the present studies provide solid evidence indicating that NO plays an important role in CHH release (that is, inhibiting CHH release). When NO levels were increased, by adding either SNP or L-arginine to the incubation, CHH release rate was decreased. Conversely, when endogenously produced NO was decreased by NMMA or HB CHH release was enhanced. These data suggest that NO exerts a tonic inhibitory effect on basal CHH levels.

Although comparative studies in the past few years indicated that NOS is widely present in invertebrate tissues and that NO is implicated in regulating many physiological processes (Elofsson et al. 1993, Martinez 1995, Müller 1997), our results represent the first demonstration that NO modulates neurosecretory activity. The source of NO is presently not known. We have previously demonstrated that the sinus

gland (SG) contains weak NOS-immunoreactivity (Lee et al. 2000). However, subsequent biochemical analysis indicated that NOS activity was undetectable in SG, suggesting that SG may not be a major site of NO production (Zou et al., 2002). It is likely that other NO-producing cells in the eyestalk ganglia are responsible for inhibiting CHH release.

Physiological significance of NO-inhibited CHH release represents a question worth of future inquiry. CHH is involved in regulating blood glucose and lipid levels, molting, and ion and water balance. Furthermore, CHH is known to mediate stress-induced hyperglycemia. Tonic inhibition of CHH release by NO as suggested by the present report is likely to play a pivotal role in the homeostasis of these physiological processes. Previous studies have shown that serotonin and dopamine, both monoamine neuroregulators, exert stimulatory effects on CHH release (Lee et al., 2001; Zou et al., 2003). Thus, NO may inhibit CHH release by modulating the levels of these monoamines. In this regard, it is relevant to mention that NO has been reported to reduce serotonin levels.

In summary, we have demonstrated that NO tonically inhibits CHH release. The source of NO is presently unknown. It is likely that nearby NO-producing cells release NO that acts as a paracrine in regulating CHH release. Physiological significance of NO-inhibited CHH release (e.g., whether NO is involved in stress-regulated CHH release) awaits future investigation.

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