Contribution of cGMP but not peroxynitrite to negative feedback regulation of penile erection elicited by nitric oxide in the hippocampal formation of the rat


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Running Title: Hippocampal NO/cGMP and penile erection

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Abstract

We established previously that nitric oxide (NO) in the hippocampal formation (HF) participates actively in negative feedback regulation of penile erection. This study further evaluated whether this process engaged soluble guanylyl cyclase (sGC)/cGMP cascade or peroxynitrite in the HF. Intracavernous pressure (ICP) recorded from the penis in adult, male Sprague-Dawley rats anesthetized with chloral hydrate was employed as our experimental index for penile erection. Microinjection bilaterally of a NO-independent sGC activator, YC-1 (0.1 or 1 nmol) or a cGMP analog, 8-Bromo-cGMP (0.1 or 1 nmol), into the HF elicited a significant reduction in baseline ICP. Bilateral application into the HF of equimolar doses (0.5 or 1 nmol) of a sGC inhibitor, LY83583 or a NO-sensitive sGC inhibitor, ODQ significantly antagonized the decrease in baseline ICP induced by co-administration of the NO precursor, L-arginine (5 nmol); along with significant enhancement of the magnitude of papaverine-induced elevation in ICP. In contrast, a peroxynitrite scavenger, L-cysteine (50 or 100 pmol) or an active peroxynitrite decomposition catalyst, 5,10,15,20-tetrakis-(N-methyl-4'-pyridyl)-porphyrinato iron (III) (10 or 50 pmol) was ineffective in both events. These results suggest that NO may participate in negative feedback regulation of penile erection by activating selectively the sGC/cGMP cascade in the HF.

Key Words: Nitric oxide; Soluble guanylyl cyclase/cGMP cascade; Peroxynitrite; Hippocampal formation; Intracavernous pressure; Negative feedback regulation of penile erection
1. Introduction

A well-documented signaling pathway for the actions of the gaseous molecule nitric oxide (NO) engages activation of the soluble form of guanylyl cyclase (sGC), leading to synthesis of cGMP (Knowles et al., 1989). In addition to its actions as an endothelial-derived relaxing factor that promotes relaxation of the blood vessels (Rees et al., 1989), NO is critically involved in erectile functions (Andersson and Wagner, 1995; Meston and Frohlich, 2000). At the end-organ level, NO is derived mainly from neuronal nitric oxide synthase (nNOS) in the nitrergic nerves and to a lesser extent from the endothelial NOS (eNOS) in the sinusoidal endothelia of the penis (Seyam et al., 1999). In addition, the notion that penile erection results from relaxation of cavernous smooth muscle induced by NO via activation of cGMP synthesis is widely accepted (Martinez-Pineiro et al., 1993; Trigo-Rocha et al., 1993; Miller et al., 1994).

There are also indications that the NO/sGC/cGMP cascade participates in central regulation of penile erection. Neural substrates that have been mentioned include paraventricular nucleus (Melis and Argiolas, 1995) and medial preoptic area (Sato et al., 2001). Another central site for NO in the regulation of penile erection is the hippocampal formation (HF). Our laboratory demonstrated previously (Chang et al., 1998) that a negative feedback regulatory mechanism on penile erection exists in the HF, and is triggered by sending sensory inputs initiated by tumescence of the penis during normal erectile processes. Recent results (Chang et al., 2002) further showed that NO generated by both neuronal and inducible nitric oxide synthase (nNOS and iNOS), but not endothelial NOS (eNOS), in the HF may play an active role in this feedback inhibition on erectile functions. Whether this regulatory action of NO engages the sGC/cGMP cascade requires further elucidation. We are also aware that, instead of the sGC/cGMP pathway, some actions attributed to NO may engage peroxynitrite (Beckman et al., 1990; Szabo, 1995), a potent reactive oxidant formed by reaction between superoxide anion and NO (Blough and Zafiriou, 1985). Whether NO at the HF also involves peroxynitrite in the negative feedback regulation of penile erection also requires investigation.
The present study addresses two hypotheses. First, the NO/sGC/cGMP cascade at the HF participates in the negative feedback regulatory machinery on penile erection. Second, the actions of NO in this negative feedback regulatory process do not engage peroxynitrite at the HF. Our results support both hypotheses.

2. Methods

The experimental procedures used in this study were similar to those reported previously (Chang et al., 1998, 2001; 2002), and conformed to the guidelines of our institutional committee on experimental animals. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

2.1. Animals and general preparations

Adult, male Sprague-Dawley rats (230–275 g) were purchased from the Experimental Animal Center, National Science Council, Taiwan, Republic of China. They were anesthetized initially with chloral hydrate (400 mg/kg, i.p.) for preparatory surgery, and maintenance of anesthetic level was provided by intravenous infusion of chloral hydrate (40 mg/kg/h) via the left femoral vein. Previous studies (Chang et al., 1998, 2001, 2002) indicated that this management scheme provided satisfactory anesthetic level while preserving the capacity of central cardiovascular regulation. The trachea was intubated to maintain patency of the airway. Systemic arterial pressure was recorded from the left femoral artery through a pressure transducer (Gould P23XL, Valley View, OH, USA) and a universal amplifier (Gould 20-4615-58), and heart rate was derived from the systemic arterial pressure signals (Yang et al., 1996). Animals were thereafter fixed to a stereotaxic headholder (Kopf 1404, Tujunga, CA, USA), and the rest of the body was placed on a heating pad to maintain body temperature at 37°C throughout the experiment.
2.2. Recording of intracavernous pressure

The increase in intracavernous pressure (ICP) was used as our experimental index for penile erection (Chang et al., 1998, 2001, 2002). In brief, a 26-gauge needle filled with saline and connected to a pressure transducer (Gould 23ID) was inserted into the corpus cavernosum on one side. Intracavernous (i.c.) administration of saline (250 µL) was routinely given at the beginning of the experiment to ensure the lack of leakage. During the experiment, ICP, systemic arterial pressure and heart rate signals were digitized (Adaptec AHA-1520A, Milpitas, CA, USA), stored on magneto-optical disk (Kyocera FRE-3651W-5P, Kyoto, Japan), and displayed continuously on a computer monitor. In some experiments, i.c. injection of papaverine was used to activate the negative feedback machinery by eliciting an increase in ICP (Chang et al., 1998, 2001, 2002). This vascular smooth muscle relaxant induces penile erection by promoting increase in inflow of arterial blood, distension of sinusoids and possible restriction of venous outflow (Lue and Tanagho, 1987).

2.3. Microinjection of test agents into the hippocampal formation

Microinjection bilaterally of test agents into the HF was carried out sequentially with a stereotaxically positioned 27-gauge stainless steel needle connected to a 0.5-µL Hamilton microsyringe (Reno, NV, USA). The stereotaxic coordinates used were 2.3–3.2 mm posterior to the bregma, 3.6–4.4 mm from the cortical surface, and 1.5–2.4 mm lateral to the midline (Chang et al., 1998, 2001, 2002). A total of 50 nL was delivered over 1–2 min to allow for full diffusion of the injected solution. In all cases, microinjection of the vehicle served as the volume and solvent control. To avoid the confounding effects of drug interaction, only one treatment schedule was delivered to each animal.

2.4. Test agents

Test agents used were freshly prepared during the experiment. These included a NO-independent activator of sGC (Wu et al., 1995), YC-1 (RBI, Natick, MA, USA); a cGMP analog (Bawin, 1994), 8-bromoguanosine-3’,5’-cyclic monophosphate
(8-Bromo-cGMP, RBI); the NO precursor (Szabo, 1996), L-arginine (RBI); a sGC inhibitor (Di Fulvio et al., 2001), LY83583 (RBI); a NO-sensitive sGC inhibitor (Garthwaite et al., 1995), ODQ (RBI); a peroxynitrite scavenger (Salvemini et al., 1998), L-cysteine (Sigma, St. Louis, MO, USA); an active peroxynitrite decomposition catalyst (Trabace and Kendrick, 2000), 5,10,15,20-tetrakis-(N-methyl-4'-pyridyl)-porphyrinato iron (III) (FeTMPyP; Calbiochem, Temecula, CA, USA) and a vasodilator (Lue and Tanagho, 1987), papaverine (U-Liang Pharmaceuticals, Taiwan, Republic of China). The doses used were the same as in previous studies (Chan et al., 2001a,b,c, 2002; Chang et al., 2002) or modified from other studies (Bawin, 1994; Garthwaite et al., 1995; Wu et al., 1995; Di Fulvio et al., 2001) in which these test agents were used for the same purpose as in the present study. LY83583 was dissolved in 3% methanol (MeOH), ODQ and YC-1 in 0.2% DMSO, and papaverine in saline. The other test agents were dissolved in artificial cerebrospinal fluid (aCSF). The composition of aCSF was (mM): NaCl 117, NaHCO₃ 25, Glucose 11, KCl 4.7, CaCl₂ 2.5, MgCl₂ 1.2 and NaH₂PO₄ 1.2.

2.5. Histology

At the conclusion of the experiment, the brain of the animal was removed and fixed in 30% sucrose in 10% formaldehyde-saline for at least 72 h. Frozen 25-μm sections stained with Neutral red were used for histological verifications of the microinjection site in the HF, aided by the addition of 1% Evans blue to the injection medium. This vital dye was ineffective in eliciting discernible actions on ICP, systemic arterial pressure and heart rate.

2.6. Statistical analysis

All values are expressed as mean ± S.E.M. The maximal changes in baseline or evoked ICP measured during 10 min of baseline recording and within 30 min after administration of test agents were used for statistical analysis. Differences between treatment groups were statistically assessed using one-way analysis of variance, followed by the Dunnett or Scheffé multiple-range test for a posteriori comparison of
means. \( P < 0.05 \) was considered to be statistically significant.

3. Results

3.1. Activation of endogenous sGC/cGMP cascade in hippocampal formation elicited descending inhibition on penile erection

To qualify for a role in the negative feedback machinery on erectile functions, activation of sGC/cGMP cascade at the HF must be able to elicit a descending inhibition on penile erection. Our first series of experiments explored this possibility. Microinjection bilaterally of a NO-independent activator of sGC, YC-1 (0.1 or 1 nmol) elicited a dose-related reduction in baseline ICP (Fig. 1). Similar results were obtained when a cGMP analog, 8-Bromo-cGMP (0.1 or 1 nmol) was applied to the HF (Fig. 1). On the other hand, 0.2% DMSO or aCSF was ineffective. Of note was that at the doses used, the effects of both YC-1 and 8-Bromo-cGMP, similar to their vehicle controls, were not accompanied by discernible changes in systemic arterial pressure or heart rate.

3.2. Activation of NO/sGC/cGMP cascade in hippocampal formation elicited descending inhibition on penile erection

Our second series of experiments ascertained that NO at the HF elicited descending inhibition on penile erection via activation of the sGC/cGMP cascade. As a baseline (Chang et al., 2002), augmenting the endogenous production of NO by microinjection bilaterally into the HF of the NO precursor, L-arginine (5 nmol) together with 3% MeOH or 0.2% DMSO evoked a reduction in baseline ICP (Fig. 2), without significant changes in systemic arterial pressure or heart rate. On the other hand, aCSF did not induce appreciable changes in these three experimental indices (Fig. 2).

The inhibitory action of L-arginine (5 nmol) on baseline ICP was dose-dependently antagonized (Fig. 2) on co-administration with either a sGC
inhibitor, LY83583 (0.5 or 1 nmol) or a NO-sensitive sGC inhibitor, ODQ (0.5 or 1 nmol). On the other hand, co-microinjection bilaterally into the HF of a peroxynitrite scavenger, L-cysteine (50 or 100 pmol) or an active peroxynitrite decomposition catalyst, FeTMPyP (10 or 50 pmol) did not discernibly affect the suppression of ICP elicited by L-arginine (5 nmol) (Fig. 3). None of these treatments resulted in appreciable changes in systemic arterial pressure or heart rate.

3.3. Engagement of endogenous NO/sGC/cGMP cascade in hippocampal formation in negative feedback inhibition on penile erection

Our results from the above experiments suggest that the sGC/cGMP cascade, rather than peroxynitrite formation, may be engaged in the negative feedback regulation of penile erection elicited by NO at the HF (Chang et al., 2002). This suggestion was evaluated in our third series of experiments. As in our previous studies (Chang et al., 2001, 2002), i.c. administration of papaverine (400 µg), which evoked a discernible elevation in ICP in animals that received hippocampal application of the vehicles (Figs. 4 and 5), was used to trigger the negative feedback mechanism. Pretreatment with microinjection bilaterally into the HF of either LY83583 (0.5 or 1 nmol) or ODQ (0.5 or 1 nmol) (Fig. 4) significantly and dose-dependently potentiated the papaverine-evoked elevation in ICP. Pretreatment with L-cysteine (50 or 100 pmol) or FeTMPyP (10 or 50 pmol), however, was ineffective (Fig. 5). Of note was all test agents did not by themselves affect discernibly the baseline ICP, systemic arterial pressure or heart rate.

3.4. Microinjection sites

Histological verifications confirmed that all effective microinjection sites in the HF were distributed randomly within the CA1 or CA3 subfield or dentate gyrus (Fig. 6). Microinjection of test agents into the hilus, stratum radiatum or subiculum was essentially ineffective.
4. Discussion

We demonstrated previously (Chang et al., 1998) that a negative feedback regulatory mechanism on penile erection exists in the HF. We further showed (Chang et al., 2002) that NO generated by nNOS and iNOS plays an active role in this regulatory process. The present study expounded on these findings by revealing that activation of the sGC/cGMP cascade represents the signaling process subsequent to generation of NO in the HF. We further ascertained that the action of NO in this negative feedback regulatory process does not involve the formation of peroxynitrite in the HF.

The clinical success of sildenafil (Carter et al., 1998; Chuang et al., 1998), a phosphodiesterase-5 inhibitor, has epitomized the critical involvement of NO/sGC/cGMP cascade as a peripheral modulator of erectile functions. In the HF, NO is engaged in synaptic plasticity via activation of sGC (Bon and Garthwaite, 2001) or cGMP-dependent protein kinases (Hawkins et al., 1994). It follows that NO may also act as a synaptic modulator of penile erection via activation of the sGC/cGMP system in the HF. This notion is supported by our observations. We demonstrated that activation of sGC or application of a cGMP analog in the HF elicited a descending inhibition on penile erection. On the other hand, hippocampal administration of sGC inhibitors blunted the reduction in ICP induced by increasing NO production in the HF, and potentiated the papaverine-evoked elevation in ICP. Taken together, it is likely that ascending sensory inputs initiated by tumescence of the penis may trigger the negative feedback regulatory mechanism in the HF via the NO/sGC/cGMP signaling pathway. Membrane-bound and soluble forms of GC have been identified (Chinkers and Garbers, 1991), and NO induces cGMP synthesis via activation of sGC (Ignarro, 1991). In this regard, sGC mRNA is present in the HF, mainly localized in the pyramidal layer of CA1–3 subfields and the granular layer of the dentate gyrus (Burgunder and Cheung, 1994). These are the same sites where our test agents elicited their respective pharmacological effects.

Activated nNOS releases short "puffs" of NO, and iNOS produces long-lasting
generation of NO (Nathan, 1992). This difference in enzyme kinetics, which regulates the amount of NO generated by these two NOS isoforms, has been used to explain the opposing actions of nNOS and iNOS in central cardiovascular regulation (Chan et al., 2001b,c) and endotoxemia (Chan et al., 2001a). On the other hand, NO produced by both nNOS and iNOS in the HF triggered by ascending sensory inputs initiated by tumescence of the penis contributes synergistically to negative feedback regulation of penile erection (Chang et al., 2002). This seeming contradiction between the role of NOS isoforms in central regulation of cardiovascular and erectile functions may be rectified by our recent observations (Chan et al., 2002). Whereas both sGC/cGMP cascade and peroxynitrite are engaged in the cardiovascular suppressive actions of NO derived from iNOS in the brain stem, only the formation of peroxynitrite is pivotal to the elicitation of fatality. It is therefore intriguing that our present results suggest that the regulatory action of NO at the HF on penile erection does not involve the formation of peroxynitrite. This finding implies that the level of NO generated by both nNOS and iNOS in the hippocampal negative feedback mechanism on penile erection is in the physiological range, and engages selectively the sGC/cGMP system. The notion that iNOS is functionally active under physiologic conditions seemingly contradicts the general contention (Szabo, 1996) that iNOS is induced only by proinflammatory stimuli. We noted, however, that in addition to our recent finding (Chang et al., 2002), a physiological role for iNOS has been reported in the regulation of arterial pressure via an action on renal tubules (Mattson et al., 1998). Our laboratory (Chan et al., 2001a,b,c) also demonstrated recently that iNOS in the rostral ventrolateral medulla, the medullary origin of sympathetic vasomotor tone, is tonically active under physiological conditions at the levels of functional expression and molecular synthesis.

An important premise for the interpretation of our results is the selectivity of our test agents. In this regard, the selectivity, efficacy and doses of all test agents used in the present study have been documented (Bawin, 1994; Garthwaite et al., 1995; Wu et al., 1995; Chan et al., 2001a,b,c; Di Fulvio et al., 2001; Chang et al., 2002). In addition to being a sGC inhibitor, LY83583 also generates superoxide anion. It
follows that our observed actions of LY83583 may suggest a role for peroxynitrite in feedback regulation on penile erection. In this regard, we demonstrated recently (Chan et al., 2002) that at the doses we used, L-cysteine and FeTMPyP effectively reduce the production of nitrotyrosine, an experimental index for peroxynitrite. Thus, the lack of effects by both test agents on L-arginine-induced reduction in baseline ICP or potentiation of the papaverine-induced increase in ICP strongly supports the notion that peroxynitrite is not involved in the action of NO on hippocampal negative feedback regulation on penile erection.

Four additional observations confirmed the specificity of our experimental observations. First, by definition, a negative feedback mechanism must be triggered and should not be tonically active. This prerequisite was satisfied when microinjection bilaterally of ODQ or LY83583 into the HF did not significantly alter baseline ICP. Second, all our results were obtained under minimal alterations in systemic arterial pressure and heart rate. Thus, the observed changes in ICP may not be secondary to hemodynamic perturbations. Third, only test agents applied locally to the CA1 or CA3 subfield or dentate gyrus were effective, signifying the site-specific nature of our pharmacological treatments. Fourth, the lack of significant effects by the vehicles on both baseline and papaverine-evoked increase in ICP ascertained that the physical action of microinjection and the chemical properties of the solvents were not a confounding factor. These observations also indicated that anesthesia may not be a confounding factor in the present study.

ICP is a recognized predictor of penile rigidity and erectile functions in dog (Trigo-Rocha et al., 1993), rat (Chang et al., 1998, 2001, 2002) and human (De Meyer and Thibo, 1998). At the same time, t The HF has long been taken as an important central integration site that is associated with sexual behavior and penile erection (MacLean and Ploog, 1962; Dua and MacLean, 1964). Our previous studies further demonstrated that HF participates in the erectile process both as an inducer (Chen et al., 1992) and a feedback regulator (Chang et al., 1998). Activation of granule cells in the dentate gyrus of HF elicits multiple episodes of increase in ICP or penile erection (Chen et al., 1992), and sustained waxing and waning fluctuations in ICP are present
under physiological conditions (Chang et al., 1998). Both observations suggest that penile erection is subject to asynchronous excitatory and inhibitory influences from the HF. In physiological terms, the inhibitory influence entails the negative feedback regulation machinery at the HF to avoid harmful hyperemia of the penis under continuous penile erection. Using ICP as the experimental index for penile erection, together with pharmacological manipulations in the HF, we conclude that NO participates in this regulatory machinery on penile erection by activating selectively the sGC/cGMP cascade in the HF.

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References


Chan, S.H.H., Wang, L.L., Ou, C.C., Chan, S.H.H., 2002. Contribution of peroxynitrite to fatal cardiovascular depression induced by overproduction of


Garthwaite, J., Southam, E., Boulton, C.L., Nielsen, E.B., Schmidt, K., Mayer, B.,


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Fig. 1. Maximal effects on intracavernous pressure (ICP) of microinjection bilaterally into the HF of YC-1, 8-Bromo-cGMP or their respective solvent (Vehicle; 0.2% DMSO or aCSF). The maximal values measured during 10 min of baseline recording and within 30 min after administration of either test agent or vehicle are presented as mean ± S.E.M., n = 5–7 animals per group. *P < 0.05 vs pretreatment control group (Basal) in the Dunnett analysis or vehicle group in the Scheffé analysis.

Fig. 2. Maximal effects on ICP of co-microinjection bilaterally into the HF of aCSF, or L-arginine (5 nmol) together with LY83583, ODQ or their respective solvent (Vehicle; 3% MeOH or 0.2% DMSO). The maximal values measured during 10 min of baseline recording and within 30 min after administration of the test agents are presented as mean ± S.E.M., n = 5–7 animals per group. *P < 0.05 vs pretreatment control group (Basal) in the Dunnett analysis or aCSF group in the Scheffé analysis; and +P < 0.05 vs L-arginine + vehicle group in the Scheffé analysis.

Fig. 3. Maximal effects on ICP of co-microinjection bilaterally into the HF of aCSF, or L-arginine (5 nmol) together with aCSF, L-Cysteine or FeTMPyP. The maximal values measured during 10 min of baseline recording and within 30 min after administration of the test agents are presented as mean ± S.E.M., n = 5–7 animals per group. *P < 0.05 vs pretreatment control group (Basal) in the Dunnett analysis or aCSF group in the Scheffé analysis.
Fig. 4. Maximal effect of intracavernous (i.c.) administration of papaverine (400 µg; Pap) on ICP, evaluated 30 min after animals were treated with microinjection bilaterally into the HF of LY83583, ODQ or their respective solvent (Vehicle; 3% MeOH or 0.2% DMSO). Values are presented as mean ± S.E.M., n = 5–7 animals per group. *P < 0.05 vs pretreatment control group (Basal) in the Dunnett analysis, and +P < 0.05 vs vehicle group in the Scheffé analysis.

Fig. 5. Maximal effect of intracavernous (i.c.) administration of papaverine (400 µg; Pap) on ICP, evaluated 30 min after animals were treated with microinjection bilaterally into the HF of aCSF, L-cysteine or FeTMPyP. Values are presented as mean ± S.E.M., n = 5–7 animals per group. *P < 0.05 vs pretreatment control group (Basal) in the Dunnett analysis.

Fig. 6. Diagrammatic representation of the HF at two rostral-caudal levels showing the location of sites in the CA1 or CA3 subfield or dentate gyrus (DG) where microinjection of test agents or vehicle was delivered. Shown are sites on which treatments elicited either reduction in baseline ICP, antagonism of L-arginine-induced decrease in ICP, or potentiation of papaverine-evoked increase in ICP, or are ineffective. For clarity, only 12% of the total microinjection sites are included. Numbers on the right side of each diagram represent the distance from the bregma.
Figure 1
L-Arginine

![Graph showing ICP (mmHg) for different conditions](Image)

- Basal
- aCSF
- Vehicle
- + LY83583 0.5 nmol
- + LY83583 1 nmol
- + ODQ 0.5 nmol
- + ODQ 1 nmol

Figure 2
Figure 3
Figure 4
Figure 5