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Nitric oxide synthase content of hypothalamic explants: Increased by norepinephrine and inactivated by NO and cGMP

(medial basal hypothalami/luteinizing hormone-releasing hormone/cAMP/arginine/nitroarginine methyl ester)

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ABSTRACT **Release of luteinizing hormone (LH)-releasing hormone (LHRH), the hypothalamic peptide that controls release of LH from the adenohypophysis, is controlled by NO. There is a rich plexus of nitric oxide synthase (NOS)-containing neurons and fibers in the lateral median eminence, intermingled with terminals of the LHRH neurons. To study relations between NOS and LHRH in this brain region, we measured NOS activity in incubated medial basal hypothalamus (MBH). NOS converts [^{14}C]arginine to equimolar quantities of [^{14}C]citrulline plus NO, which rapidly decomposes. The [^{14}C]citrulline serves as an index of the NO produced. NOS basal activity was suppressed by incubation of the tissue with an inhibitor of NOS, nitroarginine methyl ester (NAME) (10^{-5} M). Furthermore, incubation of MBH explants for 30 min with norepinephrine (NE) increased NOS activity and the increase was prevented by prazosine (10^{-5} M), an α_1-adrenergic receptor blocker; however, direct addition of NE to the tissue homogenate or to a preparation of MBH synaptosomes did not alter enzyme activity, which suggested that NE increased the content of NOS during incubation with the tissue. After purification of NOS, the increase in enzyme content induced by NE was still measurable. This indicates that within 30 min NE increased the synthesis of NOS *in vitro*. Incubation of MBH or the MBH homogenate with various concentrations of sodium nitroprusside (NP), a releaser of NO, reduced NOS activity at high concentrations (≥0.9 mM), which were associated with either a reduction of stimulation or a plateau of LHRH release. Finally, incubation of either MBH or the homogenate with cGMP, a major mediatior of NO action, at concentrations that increased LHRH release also reduced NOS activity. These results indicate that NO at high concentrations can inactivate NOS and that cGMP can also inhibit the enzyme directly. Therefore, the increased NOS activity induced by activation of α_1 receptors by NE is inhibited by NO itself and a principal product of its activity, cGMP, providing negative feedback on NOS. In central nervous system (CNS) infections with high concentrations of inducible NOS produced by glial elements, the high concentrations of NO and cGMP produced may suppress LHRH release, resulting in decreased gonadotropin and gonadal steroid release.**

Nitric oxide synthase (NOS)-containing neurons, termed NOergic neurons, have been localized in various parts of the central nervous system (CNS) of the rat. In particular, NOergic neurons occur in the hypothalamus where the perikarya are located in paraventricular and supraoptic nuclei; their axons project to the neural lobe of the pituitary gland, the structure that contains the largest quantity of NOS of any organ in the body (1). NO has recently been found to control release of luteinizing hormone (LH)-releasing hormone (LHRH) from the hypothalamus (2). LHRH is released into hypophyseal portal capillaries through which it is carried to the anterior pituitary gland. There it releases LH, which induces ovulation and ovarian steroid secretion in females and testosterone secretion in males (3). Since NO controls LHRH release in the arcuate nucleus-median eminence region, where axons of LHRH neurons terminate on the portal capillaries (2), we expected to find NOergic neurons in this region. Indeed, in the present study we found a large number of NOergic cell bodies and fibers in the arcuate-median eminence region.

This study was initiated to determine if we could measure NOS activity in incubated medial basal hypothalami (MBHs) by the citrulline method (4) and study the effect of various transmitters controlling LHRH release on the activity of the enzyme. We report details of the evidence that demonstrates that norepinephrine (NE) increased the content of NOS in the MBH. We also show that high concentrations of NO released by sodium nitroprusside (NP) can directly inactivate NOS, providing an ultra-short-loop negative feedback on the enzyme. This feedback can inhibit NO-induced activation of LHRH release. Furthermore, cGMP, released from the tissue by NO, can directly lower NOS activity. In CNS infections, with high concentrations of NO produced by inducible NOS in glial cells, NO and cGMP may inhibit LHRH release induced by NO leading to decreased gonadotropin release.

MATERIALS AND METHODS

Male rats of the Wister strain (200–250 g) from our colony were used. All rats were kept in group cages in a light (0500–1900 hr) and temperature (23–25°C)-controlled room with free access to laboratory chow and water.

***In Vitro* Studies.** After decapitation and removal of the brain, the MBH was dissected from each brain (5). The hypothalami were preincubated in a Dubnoff metabolic shaker (50 cycles per minute; 95% O_2/5% CO_2) for 30 min in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 0.1% glucose before replacement with fresh medium or medium containing the substances to be tested. The incubation continued for 30 min, followed by removal of the medium and storage of samples at −20°C before assay for LHRH. LHRH was measured by RIA using highly specific LHRH antiserum kindly provided by A. Barnea (University of Texas Southwestern Medical Center, Dallas). The sensitivity of the assay was 0.2 pg per tube and the curve was linear up to 100 pg of LHRH.

Determination of NOS Activity. For determination of NO release from the incubated MBH explants, we first used the method of Bredt and Snyder (4) that measures the conversion of [^{14}C]arginine incubated with tissue into [^{14}C]citrulline. Since citrulline remains in the sample, whereas the equimolar

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Abbreviations: LH, luteinizing hormone (lutropin); LHRH, LH-releasing hormone; NOS, nitric oxide synthase; MBH, medial basal hypothalamus; NP, sodium nitroprusside; NE, norepinephrine; CNS, central nervous system.

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amounts of NO produced are rapidly destroyed, the method indirectly measures NO production, an index of NOS activity.

After preincubation (15 min) in Krebs–Ringer bicarbonate buffer (equilibrated with 95% O2/5% CO_2) at 37°C, MBH explants (2 per tube) were incubated in medium with the addition of [^{14}C]arginine (0.3 μCi; 1 Ci = 376Bq) with or without test substances for 30 min. After the incubation the hypothalami were homogenized in 0.4 ml of 20 mM Hepes (pH 7.4), 2 mM NADPH, 0.10 mM Ca^{2+}. After 30 min of incubation at 37°C, the homogenates were centrifuged at 10,000 × *g* for 10 min at 4°C and the supernatants were applied to 2-ml columns of Dowex AG WX-8 (Na^+ form) and eluted with 3 ml of double distilled water. [^{14}C]citrulline was quantified by liquid scintillation counting.

In the modified method we used, the only change was to add the [^{14}C]arginine to the homogenate or to a synaptosomal preparation instead of the incubated tissue.

Purification of NOS. The enzyme was purified by the method of Bredt and Snyder (6). Fractions containing NOS activity were incubated in a buffer that contained 20 mM Hepes, 10 μM [^{14}C]arginine (0.3 μCi), 1 mM NADPH, 1.25 mM $CaCl_2$, 1 mM EDTA, and 10 μg/ml calmodulin in 200 μl of incubation volume. After 10 min incubation at 37°C, the reaction was stopped by adding 1 ml of ice-cold 10 mM EGTA, 1 mM citrulline, 100 mM Pipes, pH 5.5. Then, [^{14}C]citrulline formation was measured as indicated above.

Effect of Sodium NP and cGMP on NOS Activity. To determine if NP, a releaser of NO, or cGMP, an active metabolite produced in tissue by the action of NO, would alter NOS activity, various concentrations of each of these compounds were added to either incubated MBH explants or to the homogenates as just described; the effect on NOS activity was determined by the modified [^{14}C]citrulline method.

Localization of NOS. For immunohistochemistry, serial coronal frozen sections (25 μm) were cut through the hypothalamus and median eminence and processed for immunohistochemistry (7). After blockade of endogenous peroxidase activity, consecutive sections were incubated in rabbit antisera to neuronal NOS from rat brain (8) or LHRH (a gift from A. Barnea, University of Texas Southwestern Medical Center). Bound antibodies were detected with biotinylated goat anti-rabbit serum and avidin-biotin-peroxidase complex (Vectastain Elite). Peroxidase activity was revealed using diaminobenzidine with nickel-enhancement (9).

Chemicals. The compounds used were purchased from Sigma: NE HCl, NP, nitroarginine methyl ester, and 8-monobutyryl cGMP. LHRH was purchased from Peninsula Laboratories.

Statistics. Data were analyzed by one-way ANOVA and the Student–Newman–Keuls multiple comparison test for unequal replicates. Differences were considered significant at $P < 0.05$.

RESULTS

LHRH and NOS Immunoreactivity in the MBH. A large number of NOS neurons and fibers were closely associated with LHRH immunoreactive neurons and terminals (Fig. 1). No specific synaptic associations were seen between LHRH-containing fibers and NO neurons. This evidence suggests that the lateral median eminence is a major site of interaction between LHRH-containing axons and NOergic neurons.

Determination of NOS Activity. In the method of Bredt and Snyder (4), the MBH were incubated for 30 min in the presence or absence of the compounds to be tested and [^{14}C]arginine. In our initial experiments, significant counts of [^{14}C]citrulline were obtained from control tissue but stimulation of the activity with NE was not reproducible.

Modifying the method by incubating the MBH homogenate with [^{14}C]arginine in the presence of the various cofactors resulted in a 3-fold increase in counts and a highly significant increase in labeled citrulline production after incubation of

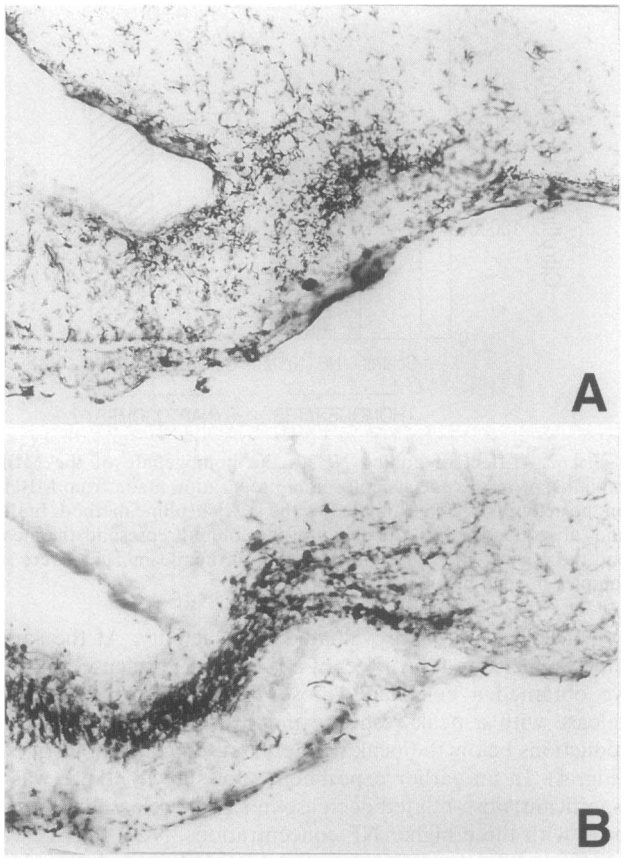

FIG. 1. Consecutive sections (30 μm) from the basal hypothalamus immunostained for LHRH and neuronal NOS. (*A*) Numerous fine fibers showing LHRH immunoreactivity were observed in the lateral region of the median eminence. Fibers were also present around the ventricular lumen in the region of the arcuate nucleus. (*B*) The lateral portion of the median eminence contained a large number of NOS-ir fibers. Similar fibers were also observed close to the midline. Isolated fibers were found around meningeal vessels. Comparison of *A* and *B* shows that the lateral area occupied by NOS-ir fibers includes the smaller area containing the lateral cluster of LHRH-ir fibers.

MBH with 10^{-5} M NE, which suggested that NE increased the NOS content in the tissue. This increase was completely blocked by the α_1 receptor prazosine (5). As expected, incubation of MBH with the inhibitor of NOS, nitroarginine methyl ester (10^{-5} M), caused a dramatic reduction in citrulline formation indicative of suppression of NOS activity (5).

Effect of Adding NE to Homogenized MBH or to Synaptosomes. Since the method estimates the content of NOS in the tissue, NE appeared to increase the tissue NOS content. Consequently, we attempted to see if we could increase the enzyme content by incubation of the MBH homogenate or a synaptosomal preparation with NE (10^{-5} M). It had no effect on production of labeled citrulline, indicating that the NOS content could not be increased in the MBH homogenate or synaptosomes (Fig. 2).

Effect of Purification of NOS on the NE-Induced Activation. At this point, we tentatively concluded that NE had increased the NOS content in the tissue. When purification of NOS from control or NE-incubated MBHs was carried out by the method of Bredt and Snyder (6), we found that the enzyme not only was active on addition of the appropriate cofactors, but also that the increased content induced by NE was still measurable (Fig. 3).

Ability of NP to Inhibit NOS. It was possible that there might be an ultra-short-loop negative feedback of NO to suppress the activity of the enzyme. Consequently, we added different concentrations of NP, which spontaneously releases NO (10),

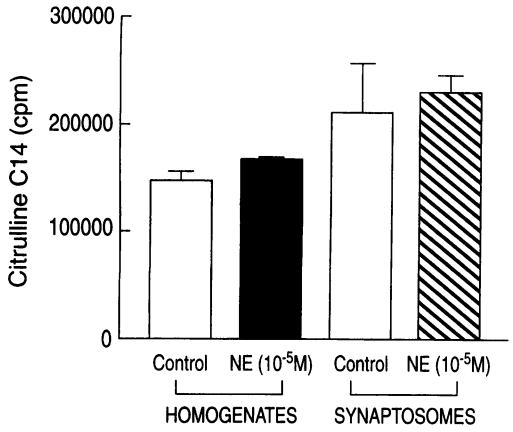

FIG. 2. Effect of adding NE to the homogenate of the MBH explants, or to a crude synaptosomal preparation made from MBHs, on the activity of NOS estimated by the [^{14}C]citrulline method. In this and subsequent figures, the height of the column represents the mean and the vertical lines one standard error of the mean. There were six samples in each mean.

to MBH explants and evaluated NOS activity. At the same time we measured the effect of NP on LHRH release. Indeed, we obtained a bell-shaped dose-response curve of LHRH release with a peak response induced by 600 μM NP and reductions below the peak with greater concentrations of NP (Fig. 4). In an earlier experiment (not shown), there was a significant, dose-related decrease in LHRH release below the peak with these higher NP concentrations. NOS activity was unaffected by low concentrations of NP, but declined at concentrations at and above the peak of LHRH release (Fig. 5). In contrast to NE, which was ineffective when added to MBH homogenates, NP suppressed NOS activity even more effectively when incubated with the MBH homogenates (Fig. 6).

The Effect of cGMP on the Activity of NOS. NO activates soluble guanylate cyclase in the MBH, leading to the production and liberation of cGMP (10), which mediates many of the actions of NO. It occurred to us that this cGMP might diffuse to the NOergic neurons and alter the activity of the enzyme. Indeed, when added to MBH explants, concentrations of cGMP at $\leq 10^{-2}$ M that released LHRH (5) also reduced the activity of NOS (Fig. 7). As in the case of NP, cGMP was also effective, although less so, in decreasing NOS activity when incubated with MBH homogenates (Fig. 8). By contrast, 8-bromo cAMP (10^{-2}–10^{-6} M) had no effect on NOS activity in either tissue or homogenate (data not shown).

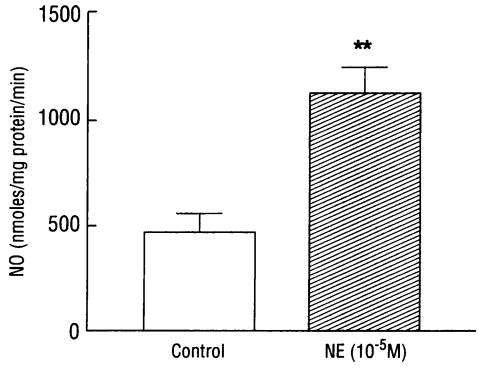

FIG. 3. Effect of incubation of MBH explants with NE on highly purified NOS activity as measured in terms of nmoles/miligram protein/min. **P < 0.01 versus activity from control tissue that was incubated with diluent.

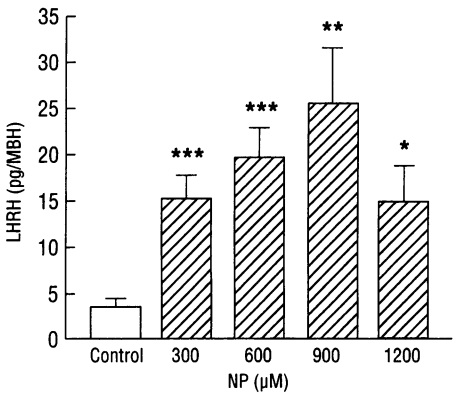

FIG. 4. Dose-response curve of NP on LHRH release from MBH explants. ***P < 0.005 versus control; **P < 0.01 versus control; *P < 0.02 versus control.

DISCUSSION

Our results indicate that the original method of Bredt and Snyder (4) in which [^{14}C]arginine is incubated with the tissue leads to incomplete tissue uptake of arginine and inconstant effects of NE on the production of labeled citrulline, an index of the tissue content of NOS. When [^{14}C]arginine was added to the tissue homogenate, followed by incubation for 30 min, much greater activity of the enzyme was found, which was blocked by nitroarginine methyl ester, an inhibitor of the enzyme. NOS activity could be significantly increased by NE. Because the activity was not altered by NE added to the homogenate, or to synaptosomes, and was still present after purification of NOS, we conclude that the enzyme content of the tissue was increased by NE during the 30 min incubation, probably by increased synthesis of NOS.

We believe that this increase in NOS content does not occur *in vivo* because the pulsatile release of NE activates α_1 adrenergic receptors, leading to a transient increase in intracellular free calcium combining reversibly with calmodulin to activate the enzyme (5). Thus, with the cessation of the pulse of NE, a decrease in intracellular calcium concentration occurs and the calcium-calmodulin dissociates from the enzyme and no increase in synthesis of NOS occurs. However, with time *in vitro*, as in our experiments, in the presence of continued activation by NE, the quantity of enzyme increases. Further studies are needed to determine if this is the correct mechanism. The results probably reflect a very rapid induction of synthesis of neural NOS; however, there is no precedent for such a rapid effect on synthesis. In the case of the inducible NOS, it takes hours to observe an induction of synthesis by lipopolysaccharide or cytokines (10–12).

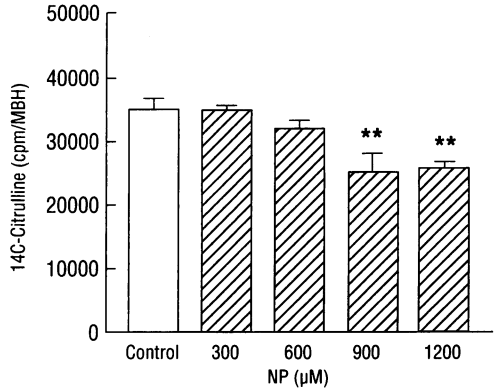

FIG. 5. Effect of NP on NOS activity of incubated MBH explants. **P < 0.01 versus control.

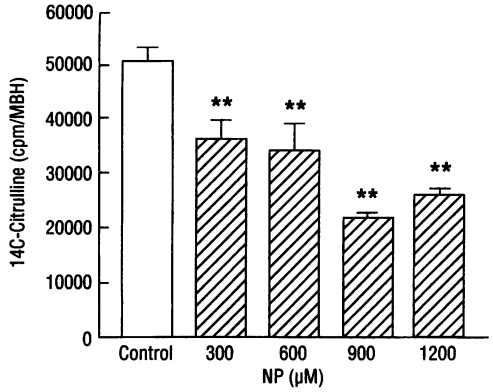

FIG. 6. Effect of NP added directly to MBH homogenates on NOS activity measured by the modified citrulline method. **P < 0.01 versus control. The NOS activity in homogenates incubated with 900 or 1200 μM NP was significantly less (P < 0.05) than the activity in samples incubated with 300 or 600 μM NP.

We used the method to show that NE activates the enzyme via activation of α_1 receptors, that this activation can be prevented by the α_1 receptor blocker, prazosine (5), that alcohol has no effect on the activity of the enzyme (5), even though it blocks the release of LHRH, and that the mechanism of the ethanol-induced blockade is via inhibition of the activation of cyclooxygenase by NO (5).

We have discovered that there are several feedbacks that inhibit the activity of NOS (13, 14). As shown here, high concentrations of NP, a releaser of NO, can block the activity of the enzyme. Therefore, NO itself, acting as a free radical, probably by changing the conformation of the heme group in NOS, inhibits NOS activity. We speculate that this may not be a factor in physiological conditions, but that it could play a role in situations of high release of NO from glial elements activated during CNS infections by cytokines to form inducible NOS (12). The high concentrations of NO produced could then suppress the release of NO from the NOergic neurons and lead to suppression of LHRH release, followed by decreased LH release and gonadal steroid production. Since LHRH also induces mating behavior, another result could be decreased libido (15). These events could occur in CNS infections, such as AIDS.

Finally, we have previously shown that activation of the LHRH neurons by NO leads to the release of cGMP into the medium (5) that could be taken up by the NOergic neurons and suppress enzyme activity. Indeed, we report here that concentrations of cGMP that induced LHRH release (5) acted during incubation of MBH explants or even in the tissue homogenate

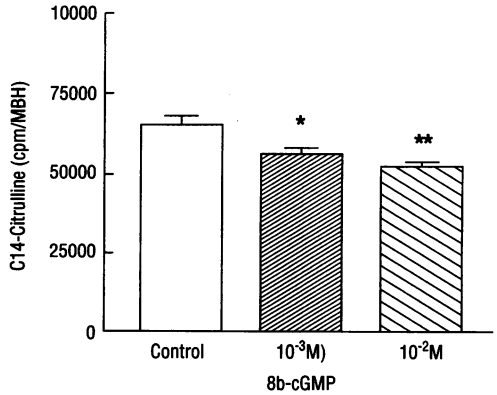

FIG. 7. Effect of cGMP incubated with MBH explants on the NOS activity measured by the modified citrulline method. *P < 0.05 versus control; **P < 0.01 versus control.

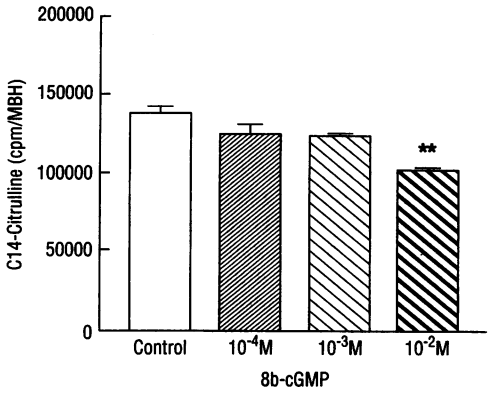

FIG. 8. Effect of cGMP added to the homogenate of MBH explants on the activity of NOS measured by the citrulline method. **P < 0.01 versus control.

to suppress the enzyme, indicating a direct inhibition of NOS. Whether the concentration of cGMP is high enough during physiological conditions to inhibit NOS remains to be determined but, as in the case of NO, the increased release of NO during CNS infections may elevate cGMP concentrations sufficiently to block NOS.

There is a very complex interplay between NE neurons, the NOergic neurons, and the LHRH neurons to initiate and terminate the pulsatile release of LHRH, which leads to the pulsatile release of LH in the intact animal (Fig. 9). Pulsatile release of NE from its terminals in the MBH activates α_1-adrenergic receptors on the NOergic neuron, leading to an increase in intracellular free calcium that combines with calmodulin to activate NOS. The NO released diffuses to the LHRH neuron where it has two effects, one is to activate soluble guanylate cyclase causing the generation of cGMP. This increases intracellular free calcium which activates phospholipase A_2 (PLA_2). PLA_2 then provides increased arachidonate from membrane phospholipids that is converted into prostaglandin E_2 (PGE_2) by NO-induced activation of consti-

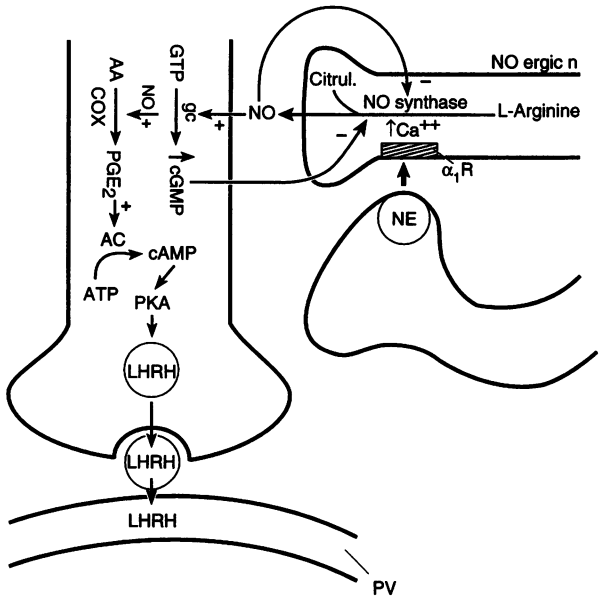

FIG. 9. Diagram of the inhibitory actions of NO and cGMP on NOS. NOn, nitricoxidergic neuron; NE, norepinephine; α_1r, α_1 receptor; Citrul, citrulline; NO, nitric oxide; GTP, guanosine triphosphate; GC, guanylate cyclase; cGMP, cyclic guanosine monophosphate; AA, arachidonic acid; COX, cyclooxygenase$_1$; PGE_2, prostaglandin E_2; AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate, PKA, protein kinase A; PV, portal vessel.

tutive cyclooxygenase leading to generation of PGE_2. PGE_2 in turn activates adenylate cyclase, leading to generation of cAMP, activation of protein kinase A, and extrusion of LHRH secretory granules into the hypophyseal portal vessels to stimulate the release of LH (5).

Additionally, the NO released also activates γ-aminobutyric acid neurons that then block the response of the LHRH terminals to NO, providing a negative feed-forward inhibition to terminate pulsatile LHRH release (13). The LHRH pulse is also terminated by the blockade of dopamine and NE release induced by the released NO diffusing to the terminals of these neurons, to terminate the pulse in a negative ultra-short-loop feedback (14). Finally, at least under conditions of high release of either NO or cGMP, these act within the NOergic neuron to suppress the activity of NOS.

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