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Nitric oxide synthase in the rat anterior pituitary gland and the role of nitric oxide in regulation of luteinizing hormone secretion

(gas/hormones/pulsatile secretion/autocrine/gonadectomy)

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ABSTRACT **By using immunohistochemistry and *in situ* hybridization, we have demonstrated that the nitric oxide (NO)-synthesizing enzyme NO synthase is present in gonadotrophs and in folliculo-stellate cells of the anterior pituitary gland of male and female rats. A marked increase in levels of NO synthase protein and mRNA was observed after gonadectomy. *In vitro* studies on dispersed anterior pituitary cells suggest that NO inhibits gonadotropin-releasing-hormone-stimulated luteinizing hormone release. An inhibitory effect of NO has also been shown on growth-hormone-releasing-hormone-stimulated release of growth hormone [Kato, M. (1992) *Endocrinology* 131, 2133–2138]. Thus these findings support a dual mechanism for NO in the control of anterior pituitary hormone secretion, an autocrine mediation of luteinizing hormone release on gonadotrophs, and a paracrine effect on growth hormone secretion involving folliculo-stellate cells closely related to somatotrophs. We speculate that NO may participate in producing the pulsatile secretion patterns of these two pituitary hormones.**

It is now well established that nitric oxide (NO), a free radical gas, is not only an endothelium-derived relaxing factor but also an inter- and intracellular messenger in many other biological systems (1–5). NO is formed from L-arginine by the enzyme NO synthase (NOS). This enzyme has been purified from rat brain (6, 22), and the gene for rat brain NOS has been cloned (7). Thus, by using immunohistochemistry and *in situ* hybridization, it has been possible to map the distribution of NOS in various tissues including the nervous system (refs. 8, 9, 23 and 24; see also ref. 10). Little is known about the cellular localization of NOS in the endocrine system, but there is information on possible functional roles for NO in hormonal regulation. For example, the cytokine interleukin 1 increases production of NO in insulin-producing cells (11, 12). At the pituitary level Kato (13) has shown that NO inhibits growth-hormone (GH)-releasing-hormone (GHRH)-stimulated GH secretion. With regard to localization of NOS in the pituitary gland, this enzyme has so far been demonstrated only in the posterior lobe, presumably representing nerve endings originating from the magnocellular hypothalamic neurosecretory neurons, which also contain NOS (8, 10).

In the present study, we have used *in situ* hybridization and immunohistochemistry utilizing light and electron microscopy to find evidence for NO synthesis in the anterior pituitary. In addition we have evaluated a role for NO in the release of luteinizing hormone (LH) from rat anterior pituitary cells in culture. The results demonstrate two distinct cell populations containing NOS, gonadotrophs and the non-hormone-producing folliculo-stellate cells, and provide evidence for an inhibitory role of NO in the stimulated release of LH.

MATERIALS AND METHODS

Immunofluorescence Histochemistry. Ten male and 10 female Sprague–Dawley rats (body weight, 200 g; Alab, Stockholm) were used. Five of each sex were gonadectomized 2 weeks before sacrifice. All rats were deeply anesthetized (sodium pentobarbital, 40 mg/kg, i.p.) and perfused transcardially with buffer followed by 4% (wt/vol) paraformaldehyde and 0.3% picric acid in 0.16 M sodium phosphate (13, 14) for 6 min. The pituitary was rapidly dissected out, immersed in the same fixative for 90 min, rinsed in 0.1 M sodium phosphate containing 10% (wt/vol) sucrose, cut in a cryostat, thaw-mounted on slides, and incubated overnight with rabbit antiserum to p56, a peptide corresponding to aa 845–864 of rat neuronal NOS, diluted 1:400 (25). After rinsing, sections were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies (1:80 dilution; Boehringer Mannheim Scandinavia), rinsed, mounted, examined in a Nikon fluorescence microscope equipped with proper filter combinations, and photographed with Kodak Tri-X film. For double-staining experiments, a mouse monoclonal antibody to human betaLH (diluted 1:1600; Immunotech, Luminy, France) was used, followed by rhodamine-conjugated donkey anti-mouse antibody (1:40 dilution; Jackson) with an incubation protocol as described above. For control, NOS antiserum preabsorbed with aa 845–864 of rat NOS at 10^{-6} M was used.

Electron Microscopic Immunocytochemistry. Three male and three female Sprague–Dawley rats (as above) were deeply anesthetized and perfused with buffer followed by 4% paraformaldehyde/0.2% picric acid/0.1% glutaraldehyde in 0.1 M sodium phosphate for 15 min. The pituitary gland was dissected out and post-fixed in the same fixative for 8 h. Vibratome sections were cut at 50 μm and subjected to freeze–thaw treatment. The sections were incubated in NOS antiserum (1:400 dilution; as above) for 36 h, followed by biotinylated goat anti-rabbit IgG (1:200 dilution) and then by avidin–biotin-peroxidase complex (1:100 dilution, Vector ABC kit). The sections were incubated for 15–25 min in 60 mg of 3,3′-diaminobenzidine/200 mg of glucose/40 mg of ammonium chloride/0.7 mg of glucose oxidase (Sigma, type VII) in 100 ml of phosphate-buffered saline (0.01 M sodium phosphate/0.15 M NaCl). The sections were postfixed in 1% osmium tetroxide for 30 min, dehydrated in ethanol, passed

Abbreviations: GH, growth hormone; GHRH, GH releasing hormone; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; MeArg, *N*-methyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; NOS-LI, NOS-like immunoreactivity; SNP, sodium nitroprusside.

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through propylene oxide, incubated in Epon 812, flat-embedded in Epon 812, cut on an LKB III Ultratome, and counterstained with uranyl acetate and lead citrate. Sections were examined in a JEOL-1200 electron microscope.

***In Situ* Hybridization.** Male (n = 35) and female (n = 20) Sprague–Dawley rats (as above) were used. Twenty-five male rats were castrated and decapitated 3 h, 12 h, 24 h, 4 days, and 14 days after surgery (n = 5 for each group). A

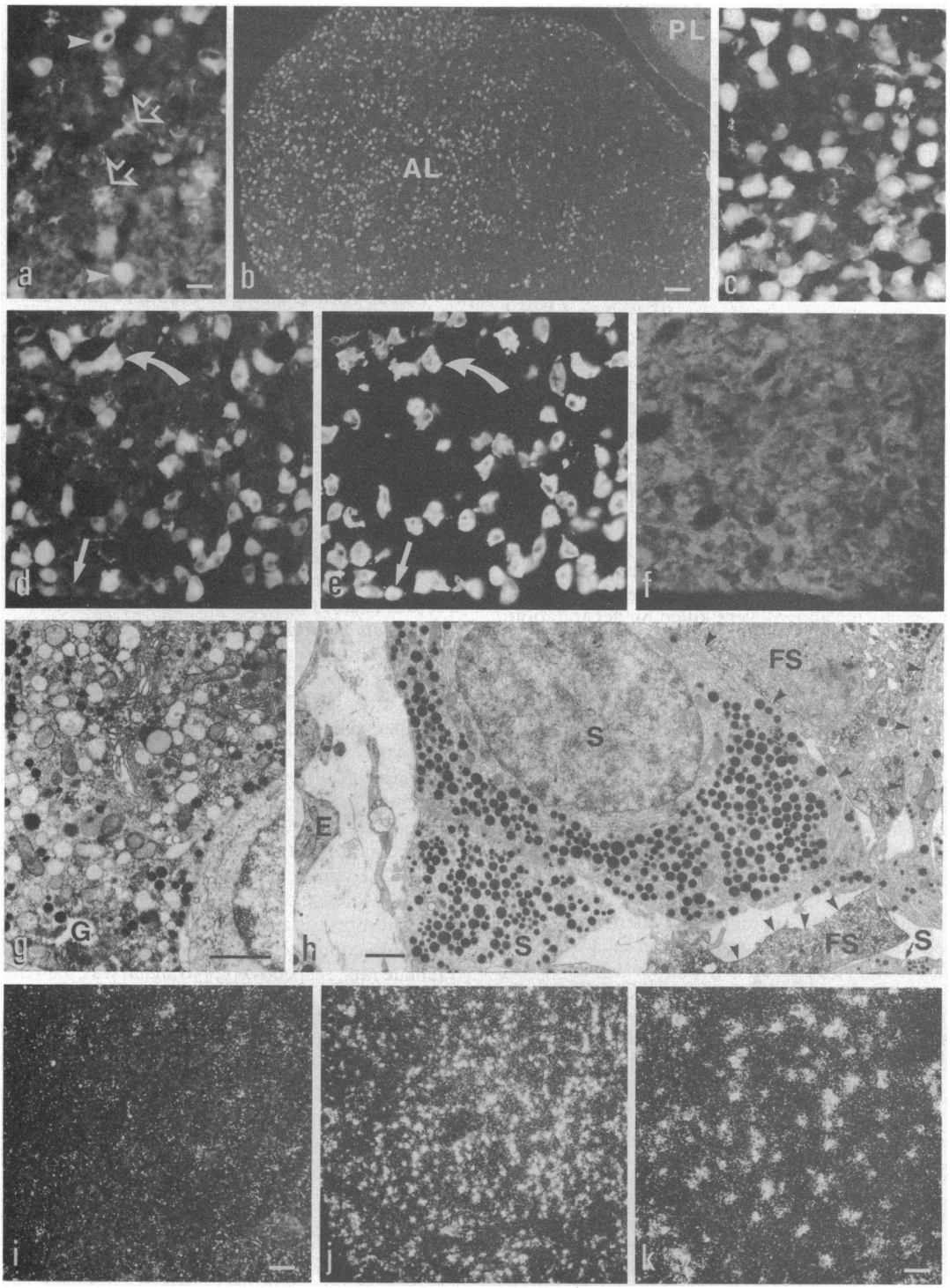

FIG. 1. Immunofluorescence micrographs (*a–f*), immunoelectron micrographs (*g* and *h*), and *in situ* hybridization autoradiographs (*i–k*) of normal male rat (*a* and *i*), castrated male rat (*b–f*, *j*, and *k*), and normal female rat (*g* and *h*) after incubation with antiserum to NOS (*a–d*, *g*, and *h*), LH (*e*), control serum (*f*), or hybridization with probe complementary to NOS mRNA (*i–k*). In normal rats, round glandular cells (arrowheads) and stellate-shaped cells (open arrows) are seen (*a*). After castration an increased number of NOS-positive cells are seen in the anterior lobe (AL) (*b–d*). Note fluorescent fibers in the posterior lobe (PL) (*b*). As shown by double-staining, virtually all NOS-positive cells are LH-positive (curved arrows), but some NOS-negative LH-positive cells can also be seen (straight arrows) (*d* and *e*). No fluorescent cells are seen after incubation with antigen-absorbed NOS antiserum (*f*). NOS-LI is seen in the cytoplasm of a gonadotroph (G) (*g*). Two somatotrophs (S) are seen surrounded by NOS-positive folliculo-stellate cells (FS) (*h*). Arrowheads point to cell membrane of these FS cells (*h*). Note endothelial cells (E) surrounding a blood vessel (*h*). A weak hybridization signal is seen in control rats (*i*), whereas a marked increase in NOS mRNA levels is seen after castration (*j* and *k*). (Bars: *a*, *c–f*, 25 μm; *b*, and *j*, 200 μm; *g* and *h*, 1 μm; *i* and *k*, 50 μm.)

further group ($n = 5$) was castrated and received testosterone substitution with a single dose (1.7 mg) of Triolandren (CIBA Pharmaceutical). Five additional rats were sham-operated and served as controls. Ten female rats were ovariectomized and decapitated 4 and 14 days after surgery. Five female rats received estrogen substitution [3.0 μg of Ovex B (Organon)/100 g for 14 days; 1.0 mg of Gestapuran (Lövens, Ballerup, Denmark)/100 g for the last 8 days)] and were decapitated 14 days after ovariectomy. Five additional ovariectomized female rats were sham-operated and served as controls. After decapitation, the pituitary was rapidly dissected out, frozen, and cut in a cryostat (Microm, Heidelberg, Germany) at a 14-μm thickness, thaw-mounted onto Probe-On slides (Fisher Scientific), and stored until use. Our hybridization procedure followed published protocols (15, 16). The oligonucleotide probe for rat brain NOS was complementary to the mRNA coding for aa 151–164 (7) and was labeled at the 3′ end with deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate (NEN) using terminal deoxynucleotidyltransferase (IBI). The labeled probe was purified and dithiothreitol was added to a final concentration of 10 mM. Specific activity obtained was 1–4 $\times$ 10^6 dpm/ng of oligonucleotide. Sections were hybridized for 16–18 h at 42°C with 10^6 cpm of labeled probe per 100 μl of a hybridization solution. After hybridization, the slides were rinsed, dehydrated, dried, and apposed to Amersham β-max film plus a ^{14}C plastic standard for quantification, which was carried out as described in detail elsewhere (15). Subsequently, the slides were dipped in NTB2 nuclear-track emulsion (Kodak), exposed, developed, fixed, mounted, and analyzed in a Nikon Microphot-FX microscope.

Pituitary Cell Dispersion and Culture Procedures. Male Sprague–Dawley rats (as above) were killed by decapitation, and the adenohypophysis was rapidly removed and enzymatically dispersed (17). The cells were plated on multi-well plates (1–2 $\times$ 10^5 cells per ml per well), whereby 24 adenohypophyses typically yielded 144 incubation wells. The culture medium consisted of Dulbecco's modified Eagle's medium supplemented with fresh serum. Cells were counted in a Burker's chamber; the trypan blue dye exclusion technique was applied to assure >95% viability of the dispersed cells used. On the third day, fresh Krebs–Ringer bicarbonate medium (containing 1% bovine serum albumin), test substances, and Trasylol (2×10^{-5} M) were added in a total volume of 1 ml per well. Control and test substances were kept at pH 7.4 and incubated in eight equivalent wells for 4 h. One hundred microliters was aspirated from each well after 2 and 4 h, diluted in 900 μl of buffer, and stored at −20°C. Synthetic gonadotropin-releasing hormone (GnRH) was a kind gift from Hoechst Pharmaceuticals. *N*-Methyl-L-arginine (MeArg) and sodium nitroprussid (NP) were obtained from Sigma. LH was determined using a RIA kit (Amersham) as described (18).

RESULTS

Immunohistochemistry. The immunofluorescence analysis revealed two populations of NOS-positive cells in the anterior pituitary in normal male and female rats, one made up of round cells with a diameter of ≈20 μm distributed all over the gland, but especially laterally (Fig. 1 *a* and *b*). The second type was small cells with multiple processes (Fig. 1*a*). Fourteen days after castration, there was an increase in the intensity of NOS-like immunoreactivity (NOS-LI) in the endocrine cells in both sexes, and these cells also appeared more numerous than in controls (Fig. 1*b*; cf. Fig. 1 *c* with *a*). Double-staining experiments revealed that all NOS-positive cells were LH-immunoreactive, and vice versa, that most LH-positive cells were also NOS-positive (cf. Fig. 1 *d* with *e*), although the enzyme levels varied between different cells (Fig. 1*d*). The electron microscopic analysis confirmed the fluorescence histochemical results. Thus, NOS-LI was present in cells with the typical appearance of gonadotrophs (19) (Fig. 1*g*), as described in previous electron microscopic studies (19). In addition, folliculo-stellate cells (19) were also NOS-positive (Fig. 1*h*). They were often seen in close contact to somatotrophs (Fig. 1*h*) (19), which by themselves never exhibited NOS-LI. The immunoreactivity described above could not be observed after incubation with control serum (Fig. 1*f*).

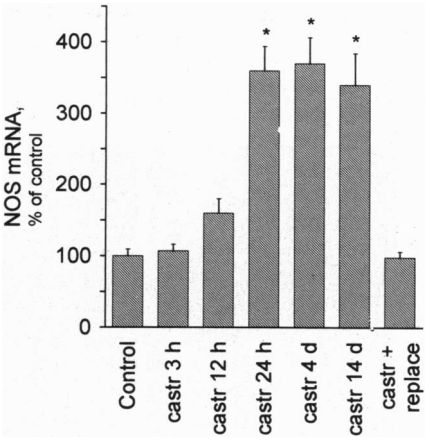

FIG. 2. Histogram showing the effect of castration (castr) and of testosterone replacement on NOS mRNA levels 3 h, 12 h, 24 h, 4 days, and 14 days after surgery. Results are expressed as percentage of control (mean ± SD; $P < 0.05$; ANOVA and Dunnet's test).

***In Situ* Hybridization.** After hybridization with the NOS probe, a weak signal was seen on an x-ray film over the anterior lobe of the pituitary gland of male and female rats (Fig. 1*i*). Castration (Fig. 1 *j* and *k*) and ovariectomy, respectively, resulted in markedly increased NOS mRNA levels. The quantitative evaluation (Fig. 2) revealed that in the male rat a small, but not significant, increase was seen 12 h after surgery, whereas at 3 h levels were still similar to sham-operated rats. After 24 h there was a >3-fold increase in mRNA levels, and this remained elevated for 14 days, the longest period studied (Fig. 2). Also in the female rats, there was a clear but less strong increase at 4 and 14 days after ovariectomy (Fig. 3). Substitution treatment with testosterone (Fig. 2) or estrogen (Fig. 3) completely prevented these increases in male and female rats, respectively. No signal was seen over the anterior

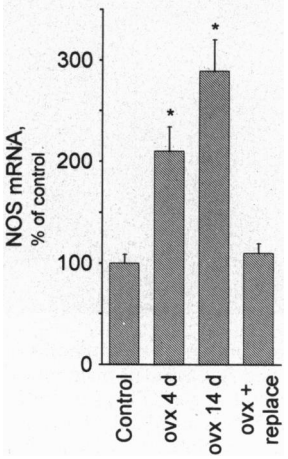

FIG. 3. Histogram showing the effect of ovariectomy (ovx) and estrogen replacement on NOS mRNA levels 4 and 14 days after surgery. Results are expressed as percentage of control (mean ± SD; $P < 0.05$; ANOVA and Dunnet's test).

pituitary after hybridization with labeled NOS probe with added NOS mRNA (100-fold excess).

Release Studies. Addition of 10^{-5} to 10^{-4} M MeArg, a competitive inhibitor of NOS, did not influence basic LH release from dispersed cells after 4 h in culture. GnRH at 10^{-6} M caused a 2.5-fold increase in LH secretion, which was significantly augmented in the presence of 10^{-5} M MeArg (Fig. 4). SNP, a NO donor, in doses from 5×10^{-5} to 5×10^{-4} M did not significantly affect LH release, although there was a tendency toward a reduction at the lower doses tested (Fig. 5). However, at higher doses (5×10^{-3} to 5×10^{-2} M), SNP stimulated LH release (data not shown). SNP at 5×10^{-4} M significantly inhibited the GnRH-induced release, whereas lower doses showed a small but not significant inhibition. At the highest dose tested (10^{-2} M), SNP caused a marked increase in GnRH-stimulated secretion (data not shown).

DISCUSSION

The present combined immunohistochemical, *in situ* hybridization, and biochemical studies provide evidence for NOS synthesis in two cell populations of the anterior pituitary gland, gonadotrophs and folliculo-stellate cells, and for an inhibitory effect of NO on stimulated release of LH. The follicular-stellate cells do not produce any known hormones themselves (19) but may indirectly influence GH secretion (see below).

In the analysis of the effect of NO on LH secretion, the NOS inhibitor MeArg markedly potentiated GnRH-induced LH secretion, and the NO donor SNP significantly reduced the GnRH-stimulated LH secretion, whereas no effects were seen on basal secretion. These findings raise the possibility that NO may be a signal to terminate LH release, and NO may thus participate in producing the pulsatile secretion pattern characteristic of LH secretion (20). Interestingly, in the case of LH secretion, our studies strongly suggest that it is the gonadotroph itself that is responsible for NO production, since NOS mRNA was present in a discrete subpopulation of pituitary cells and since double-staining immunohistochemistry revealed that NOS-LI and LH were found in the same cells. This is interesting in view of our findings of a marked upregulation of NOS mRNA after castration and ovariectomy in male and female rats, respectively. It may be speculated that this upregulation is a response to the marked increases in LH secretion seen in gonadectomized animals.

A recent *in vitro* study by Kato (13) has demonstrated that MeArg potentiates the GHRH-stimulated GH secretion without affecting basal GH secretion, findings supported by experiments with the NO donor SNP. Thus, NO in the pituitary gland inhibits GHRH-induced, but not basal, release of GH (13). The present study provides some information on the origin of the NO involved in the control of GH secretion. Thus, whereas NO involved in LH secretion seems to originate in the gonadotrophs themselves, the somatotrophs do not seem to produce NO. Instead the folliculo-stellate cells are NOS-positive. Furthermore, as shown in the electron microscopic analysis, these cells have processes coming into close contact with the somatotrophs. Thus NO released from these cells may inhibit secretion from somatotrophs. In agreement, Kato (13) has demonstrated that hemoglobin, which is known to trap NO in the extracellular space, potentiates the GHRH-stimulated GH secretion.

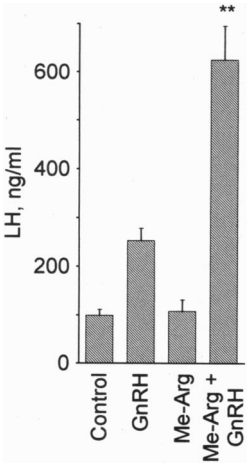

FIG. 4. Effect of MeArg (10^{-5} M) and MeArg in combination with GnRH (10^{-6} M) on LH secretion from rat anterior pituitary cells after 4 h in culture. Bars represent the mean ± SD from two experiments with $n = 8$ in each experiment. **, $P < 0.02$ (Mann–Whitney test).

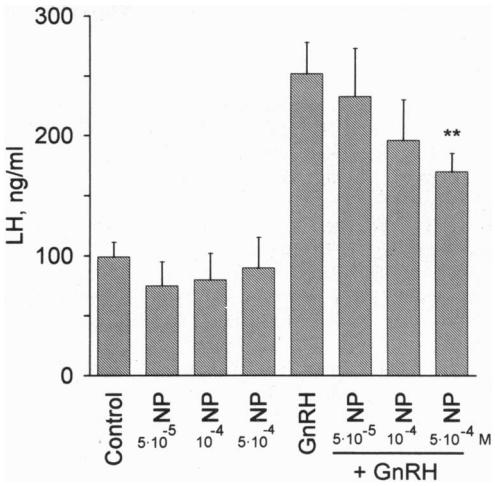

FIG. 5. Effect of SNP (5×10^{-5} to 5×10^{-4} M) and SNP (as indicated) combined with GNRH (10^{-6} M) on LH release from rat anterior pituitary cells after 4 h in culture. Bars represent the mean ± SD from two experiments with $n = 8$ in each experiment. **, $P < 0.02$ (Mann–Whitney test).

Interestingly, interstitial cells have been shown to be involved in NO-induced responses in a different tissue. Thus, Publicover *et al.* (21) have proposed that NO released from enteric inhibitory nerves may trigger an increase in intracellular calcium concentration in nearby interstitial cells. In turn, this results in increased NO synthesis and NO release from these interstitial cells, which may lead to NO-induced inhibition of smooth muscle cells. The latter, thus, reminds us of the situation with NO synthesis in folliculo-stellate cells, closely related to the somatotrophs in the adenohypophysis. Thus our results support a dual mechanism of action of NO in the control of anterior pituitary hormone secretion: it regulates LH secretion via an autocrine action and GH secretion in a paracrine fashion.

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