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Growth Hormone-Releasing Hormone and Gonadotropin-Releasing Hormone Stimulate Nitric Oxide Production in 17β-Estradiol-Primed Rat Anterior Pituitary Cells

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It was reported that neuronal nitric oxide synthase (nNOS) was expressed only in gonadotrophs and folliculo-stellate cells in the anterior lobe of the pituitary gland. However, recent studies have demonstrated the occurrence of nNOS in the somatotrophs and lactotrophs. In the present study, we investigated effects of growth hormone-releasing hormone (GHRH), gonadotropin-releasing hormone (GnRH), and 17β-estradiol on nitric oxide (NO) release in cultured rat anterior pituitary cells in vitro. The NO_2^- level in the incubation medium of the rat anterior pituitary cells was dependent on the cell density. Pretreatment with 10 μ*M* 17β-estradiol resulted in an increase in medium NO_2^- level. GHRH and GnRH failed to change medium NO_2^- levels, but they elicited increases in medium NO_2^- levels in estrogen-treated cells. The GHRH-induced increase in NO_2^- level was inhibited by N^{ω}-nitro-L-arginine methyl ester, a NOS inhibitor. These findings suggest that GnRH and GHRH could activate nNOS in the gonadotrophs and the somatotrophs, respectively.

Key Words: Nitric oxide; 17β-estradiol; growth hormone-releasing hormone; gonadotropin-releasing hormone; pituitary.

Introduction

Nitric oxide (NO) exerts a broad spectrum of biological actions in physiology and pathophysiology *(1)*, including neurotransmission in the central and peripheral nervous systems *(2–4)*. NO indirectly modulates the secretion of anterior pituitary hormones through modulating the secretion of hypophysiotrophic hormones such as gonadotropin-releasing hormone (GnRH) *(5)*, corticotropin-releasing hormone *(6,7)*, and somatostatin *(8)* in the hypothalamus.

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There has been a line of evidence indicating implications of NO in regulating hormone secretion in the anterior pituitary gland. Sodium nitroprusside, a NO donor, inhibited secretagogue-induced secretion of growth hormone (GH) *(9)*, prolactin *(10)*, and luteinizing hormone (LH) *(11)* from rat pituitary cells in culture or from hemipituitary fragments in vitro. In addition, the secretion of these pituitary hormones was enhanced by nitric oxide synthase (NOS) inhibitors *(9–11)*, suggesting an inhibiting role of endogenous NO on hormone secretion in the adenohypophysis. It has also been reported that NO stimulates gonadotropin secretion through a cyclic guanosine monophosphate-independent mechanism *(12)*.

Initially, it was reported that immunohistochemical localization of neuronal NOS (nNOS) in rat anterior pituitary was negligible *(3)*. Subsequent immunohistochemical and *in situ* hybridization studies revealed restricted nNOS expression in gonadotrophs and folliculo-stellate cells *(11)*. There has been a line of evidence suggesting the occurrence of nNOS in the somatotrophs and lactotrophs. GH_3 cells, immortalized rat pituitary cells of somatomammotroph origin, express nNOS mRNA *(13)* and liberate NO *(14)*. Human pituitary adenoma cells, GH as well as gonadotropin producing, were reported to express nNOS mRNA and protein *(15)*. Finally, Kostic et al. *(16)* recently demonstrated the expression of nNOS in rat somatotrophs and lactotrophs using reverse transcriptase–polymerase chain reaction (RT-PCR). Qian et al. *(17)* also found that a small percentage of GH cells was positive for nNOS immunoreactivity in the rat pituitary gland.

However, the mechanisms that regulate nNOS activity in the somatotrophs at the cellular level remain to be fully elucidated. In the present study, we investigated effects of GH-releasing hormone (GHRH), GnRH, and 17β-estradiol on NO release in cultured rat anterior pituitary cells in vitro.

Results

When the rat anterior pituitary cells were cultured at varying cell densities, the NO_2^- level in the incubation medium was dependent on the cell density (Fig. 1). NO_2^- was the major accumulated metabolite of NO and NO_3^- was negligible (data not shown). Neither GHRH nor GnRH changed

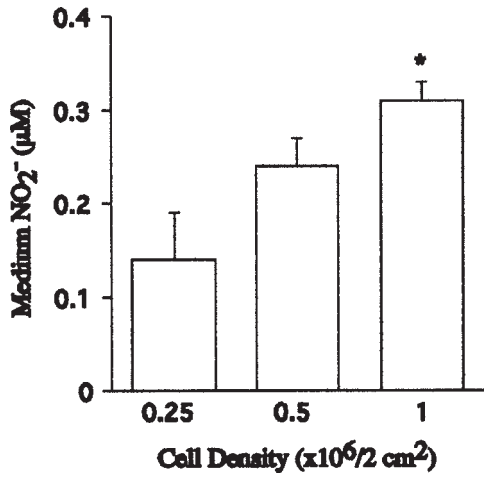

Fig. 1. Effect of cell density on medium NO_2^- levels in rat anterior pituitary cells. The cells were cultured at a density of 2.5×10^5, 5×10^5, or 10×10^5 cells/well (2 cm^2). Mean ± SE values of medium NO_2^- concentration are shown. *$p < 0.05$ versus 2.5×10^5 cells/well.

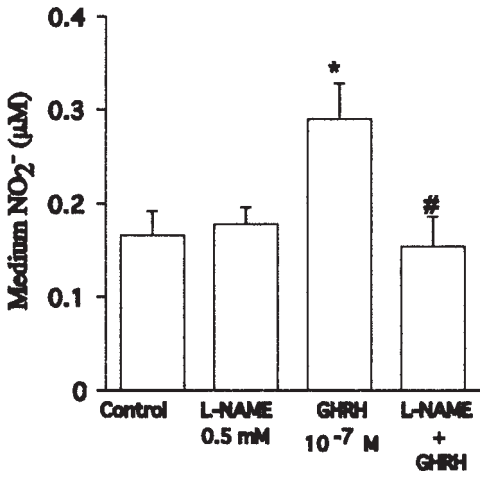

Fig. 3. Inhibition by L-NAME on NO_2^- accumulation induced by GHRH in rat anterior pituitary cells pretreated with 17β-estradiol. The cells were incubated in the presence of 10^{-7} *M* GHRH and 0.5 m*M* L-NAME. *$p < 0.05$ versus control; #$p < 0.05$ versus GHRH alone.

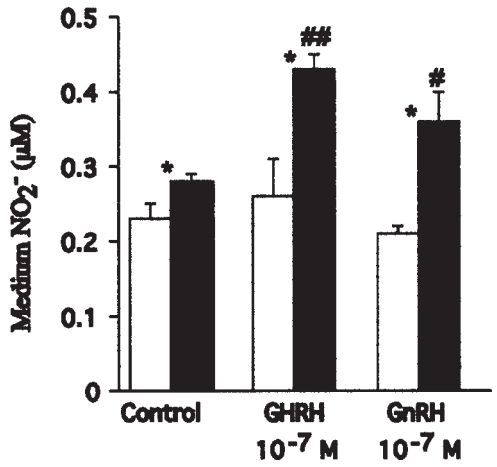

Fig. 2. Effects of 17β-estradiol, GnRH, and GHRH on medium NO_2^- levels in rat anterior pituitary cells pretreated with 10 μ*M* 17β-estradiol (solid bar) or vehicle (0.1% ethanol, open bar). The cells were incubated for 2 h in the presence of 10^{-7} *M* GHRH or GnRH. *$p < 0.05$ versus vehicle; #$p < 0.05$; and ##$p < 0.01$ versus control.

medium NO_2^- levels in the incubation medium. Pretreatment with 10 μ*M* 17β-estradiol resulted in an increase in medium NO_2^- level (Fig. 2). GHRH and GnRH elicited increases in medium NO_2^- levels in estrogen-treated cells (Fig. 2). The GHRH-induced increase in NO_2^- level was inhibited by N^{ω}-nitro-L-arginine methyl ester (L-NAME) (Fig. 3). L-NAME did not change the basal NO_2^- concentration (Fig. 3).

Discussion

In an early report, Ceccatelli et al. *(11)* demonstrated that the pituitary nNOS mRNA and protein levels were increased by gonadectomy in rats of both sexes. The increase in nNOS level in the gonadotrophs induced by gonadectomy was blocked by substitution with sex steroids *(11)*. Subsequent studies revealed that GnRH might play a major role in the induction of gonadotroph nNOS. GnRH antagonists inhibited upregulation of nNOS mRNA and protein induced by castration *(18,19)*. Administration of a long-acting GnRH agonist elicited an increase in nNOS level in intact male rats *(18)*. In addition, Lozach et al. *(20)* found a GnRH-dependent increase in pituitary nNOS immunoreactivity and NADPH-diaphorase activity during proestrus, suggesting physiological significance of nNOS induction by GnRH. In contrast to the established role in nNOS gene expression, there has been limited information regarding activation of nNOS enzyme activity by GnRH. In this study, we found that GnRH increased medium nitrite levels in rat pituitary cell culture after an incubation period of 2 h. In an in vivo study, Garrel et al. *(18)* observed an increase in pituitary nNOS levels only 8 h but not until 4 h after an injection of long-acting GnRH agonist. Thus, it is possible to speculate that GnRH increased NO release by activating the pituitary nNOS enzyme activity without induction of nNOS protein in this study. Another point to be noted is that GnRH was effective in increasing NO release only when pituitary cells were preincubated with 17β-estradiol. Priming with estrogen may be necessary for the GnRH action on nNOS.

Direct effect of estrogen on nNOS expression and activity in the anterior pituitary cells remains to be fully elucidated. In female rats, nNOS immunoreactivity was colocalized with LH, whereas nNOS was mainly expressed in folliculo-stellate cells and in only a small number of gonadotrophs in male rats *(21)*. This sexual dimorphism in the distribution pattern of nNOS in the pituitary gland suggests such a stim-

ulatory role of estrogen on gonadotroph nNOS expression as was seen in the brain *(22,23)* and peripheral tissues *(22)*. On the other hand, it was reported that treatment with estradiol for 8 wk resulted in a decrease in pituitary nNOS mRNA and protein levels in female rats in vivo *(17)*. In the present study, we found that estrogen pretreatment resulted in an increase in medium NO_2^- level and a response to GnRH. It should be noted that estrogen could also enhance pituitary nNOS enzyme activity without inducing nNOS expression. It was reported that 17β-estradiol enhanced nNOS activity in the cytosol fraction of rabbit cerebellum and the purified enzyme through Ca^{2+}–calmodulin *(24)*. Subsequently, it was demonstrated that tamoxifen, an antiestrogen, inhibited the activation of rat brain nNOS by calmodulin *(25)*. Further studies are necessary to clarify physiological significance, gender differences and the precise mechanisms involved in the estrogen-induced increase in NO production in the pituitary cells.

An interesting finding in this report is that GHRH increased NO production in estrogen-primed rat anterior pituitary cells. The effect of GHRH was abolished by L-NAME, a NOS inhibitor. As in the case of GnRH, GHRH was ineffective in the cells without estrogen pretreatment. This observation is similar to that of other investigators *(16)*. Although nNOS was initially reported to be present exclusively in gonadotrophs and folliculo-stellate cells, there has been a line of evidence suggesting the occurrence of nNOS in the somatotrophs and lactotrophs *(16)*. Considering the presence of nNOS in the somatorophs, it is possible that stimulation by GHRH results in activation of somatotroph nNOS and, subsequently, the activated enzyme catalyzes NO production. Alternatively, intrapituitary paracrine factors released from the somatotrophs upon GHRH stimulation could act on the gonadotrophs or the folliculo-stellate cells to activate nNOS in these cells. However, we cannot provide any evidence that such mechanisms could operate under our experimental conditions in which the dispersed cells were cultured in sparse density and the normal structure of the pituitary gland was not preserved.

In neuronal *(26)* and endothelial cells *(27)*, a transmitter-induced increase in intracellular Ca^{2+} is the key process in activating constitutive NOS. This is also the case in pituitary cells. We have demonstrated that mobilization of intracellular Ca^{2+} by thyrotropin-releasing hormone stimulation activates Ca^{2+}-dependent NOS in GH_3 cells *(14)*. GnRH activates phosphoinositidase C, and generated inositol-triphosphate is responsible for the mobilization of intracellular Ca^{2+} *(28)*. GHRH induces an increase in intracellular Ca^{2+} resulting from Ca^{2+} entry through L-type Ca^{2+} channels in a cAMP-dependent fashion in the somatotrophs *(29)*. Taken together, it is plausible that an increase in the intracellular Ca^{2+} results in activation of nNOS in the gonadotrophs and the somatotrophs. In conclusion, GnRH and GHRH elicited increases in NO production in estradiol-primed rat anterior pituitary cells in vitro. It was suggested that GnRH and GHRH activated nNOS through an increase in intracellular Ca^{2+} level in the gonadotrophs and the somatotrophs, respectively.

Materials and Methods

Experiments were approved by the Ethical Committee for Animal Studies at Shimane Medical University. Rat anterior pituitary glands were obtained by rapid decapitation from Wistar strain male rats weighing 180–220 g (Japan Crea Co., Tokyo, Japan). Rat anterior pituitary cells were dispersed and cultured as previously described *(30)*. Briefly, the tissues were minced by fine scissors in ice-cold phosphate-buffered saline (PBS) and the cells were dispersed in PBS containing 0.25% trypsin at 37ºC for 20 min by gentle stirring in a spinner flask. The dispersed cells were collected, filtered through 40-mm nylon mesh, and washed three times with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), penicillin (100 IU/mL), and streptomycin (100 µg/mL). The cells were cultured in 24-well dishes for 24 h under humidified atmosphere of 5% CO_2–95% air at 37ºC at a density of 5×10^5 cells/2 cm^2 in 1 mL serum-containing medium unless otherwise mentioned. Then, the medium was aspirated and the cells were further cultured in serum-free medium containing 0.1% BSA for 24 h in the presence or absence of 10 µ*M* 17β-estradiol (Sigma, St. Louis, MO). 17β-Estradiol was first dissolved in ethanol and then diluted with the medium. The final concentration of ethanol was 0.1%.

After aspirating the culture medium, the cells were washed three times with 1 mL of 140 m*M* NaCl, 4.7 m*M* KCl, 1.0 m*M* $CaCl_2$, 1.2 m*M* $MgCl_2$, 1.2 m*M* KH_2PO_4, 11 m*M* glucose, and 5 m*M* HEPES, pH 7.4 (Krebs–HEPES buffer), and were preincubated in Krebs–HEPES buffer for 30 min. After the preincubation, the cells were incubated in fresh Krebs–HEPES buffer containing 0.1 µ*M* GHRH (Sumitomo Pharmaceutical Co., Osaka, Japan), 0.1 µ*M* GnRH (Tanabe Pharmaceutical Co., Osaka, Japan), or 0.5 m*M* L-NAME (Sigma, St. Louis, MO) for 2 h. The dose of the peptides was chosen to see the maximal effects of the peptides. L-NAME was first dissolved in ethanol and then diluted with the medium. The final concentration of ethanol was 0.1%. GHRH and GnRH were dissolved with Krebs–HEPES buffer. The incubations were kept at 37ºC and under humidified atmosphere of 5% CO_2–95% air. The medium samples were aspirated and were submitted to determination of NO_2^- and NO_3^- concentrations. Krebs–HEPES buffer was prepared with high-performance liquid chromatography (HPLC)-grade distilled water to minimize contamination of NO_2^- and NO_3^-.

NO_2^- and NO_3^- concentrations were determined by a reverse-phase HPLC system combined with a cadmium-reducing column, reaction with Griess reagent, and a spectrophotometer (NOD-10, Eicom, Kyoto, Japan) as previously described (*14*). The minimal detectable concentration for NO_2^- and NO_3^- was 0.039 µ*M*. The intraassaay and interassay coefficients of variance were less than 5% and 8%, respectively.

All of the experimental data were expressed as mean ± SE. Statistical differences were estimated with analysis of variance in combination with Duncan's new multiple-range test. A p-value less than 0.05 was considered significant.

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