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Nitric Oxide Stimulates Growth Hormone Secretion from Human Fetal Pituitaries and Cultured Pituitary Adenomas

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Nitric oxide (NO), a highly reactive free radical, has been identified as a neurotransmitter in the central and peripheral nervous system. NO synthase (NOS) is the enzyme responsible for NO production from L-arginine and plays an important role in regulating the release of several hypothalamic peptides. In the pituitary, NO was found to increase growth hormone (GH) secretion in several in vitro and in vivo models. However, its role in human GH regulation is unknown. The aim of this study was to investigate the regulatory effects of NO on human GH and prolactin secretion using primary cell cultures of human fetal pituitaries and cultured hormone-secreting adenomas. Incubation of the human fetal pituitaries (21–24 wk gestation) in the presence of sodium nitroprusside (SNP; 1 m*M*), a NO donor, for 4 h resulted in a 50–75% increase in GH secretion, similar to the stimulatory effect evoked by growth hormone–releasing hormone (GHRH) (10 n*M*). However, fetal PRL secretion was not affected by SNP. GH release was also stimulated (40–70% increase) by SNP in 60% of the cultured GH-secreting adenomas studied. SNP-induced GH release was inhibited in both fetal and adenomatous cells by PTI0, a NO scavenger. The addition of cGMP (0.1–1 m*M*), the second messenger of multiple NO actions, enhanced fetal and adenomatous GH secretion by 55–95%. Neuronal NOS (nNOS) was expressed in normal (fetal and adult) human pituitary tissues and in GH-secreting adenomas. Examination of its functional expression using L-arginine (1 μ*M*) yielded a 35% increase in GH release from cultured GH-secreting adenoma. This response was blocked by a NOS inhibitor with high selectivity for the neuronal enzyme and by a guanylyl cyclase inhibitor. In conclusion, NO stimulates human GH in cultured fetal pituitaries and GH-secreting adenomas. Cyclic GMP is probably involved in this hormonal regulation.

Key Words: Fetal pituitary; growth hormone; nitric oxide; pituitary adenoma.

Introduction

Nitric oxide (NO) has been recognized as a neurotransmitter in the central and peripheral nervous system *(1)*. Evidence indicates that it serves as an intracellular and intercellular messenger in the control of different physiological events in the brain and peripheral tissues, including neuroendocrine regulation *(2,3)*. Neurons that contain NO synthase (NOS), the enzyme that catalyzes the NADPH-dependent production of NO and citrulline from L-arginine, play an important role in regulating the release of several hypothalamic peptides, namely, somatostatin *(4)*, corticotropin-releasing hormone *(5)*, gonadotropin-releasing hormone *(6)*, and dopamine *(7)*. In the pituitary, NOS has been identified in gonadotrophs and follicle-stellate cells *(8,9)*. NO-dependent soluble guanylyl cyclase, which is responsible for cGMP production, is expressed in the pituitary *(10)*, and NO increases pituitary cGMP concentration *(11)*. NO also exerts a direct effect on the secretion of the pituitary hormones, gonadotropin and ACTH *(8,12)*. Furthermore, rat models have shown that NO suppresses the release of prolactin (PRL) from pituitary glands *(13)* and that it is directly involved in the control of growth hormone (GH) secretion. However, whether the latter effects are stimulatory or inhibitory remains controversial. On the one hand, endogenous NO was found to inhibit basal GH release and TRH-stimulated GH release in rat GH3 cells *(14)* and to suppress GHRH-induced GH release in cultured rat pituitary cells *(15)*. On the other hand, others reported that NO stimulated rat GH secretion in vitro *(16)*, and that NO deprivation blunted GHRH-stimulated GH release both in vivo and in vitro *(17)*. A stimulatory function of NO in GHRH-induced GH secretion was also noted in beagles in vivo *(18)*. The mechanism of NO action also remains unclear. cGMP has been shown to stimulate GH secretion in rat pituitary cells *(19)*. However, both cGMP and guanylyl cyclase inhibitors failed to influence the stimulatory effect of NO on GH secretion in vitro, suggesting that the action of NO is mediated by a cGMP-independent mechanism *(16)*. Further studies are needed to

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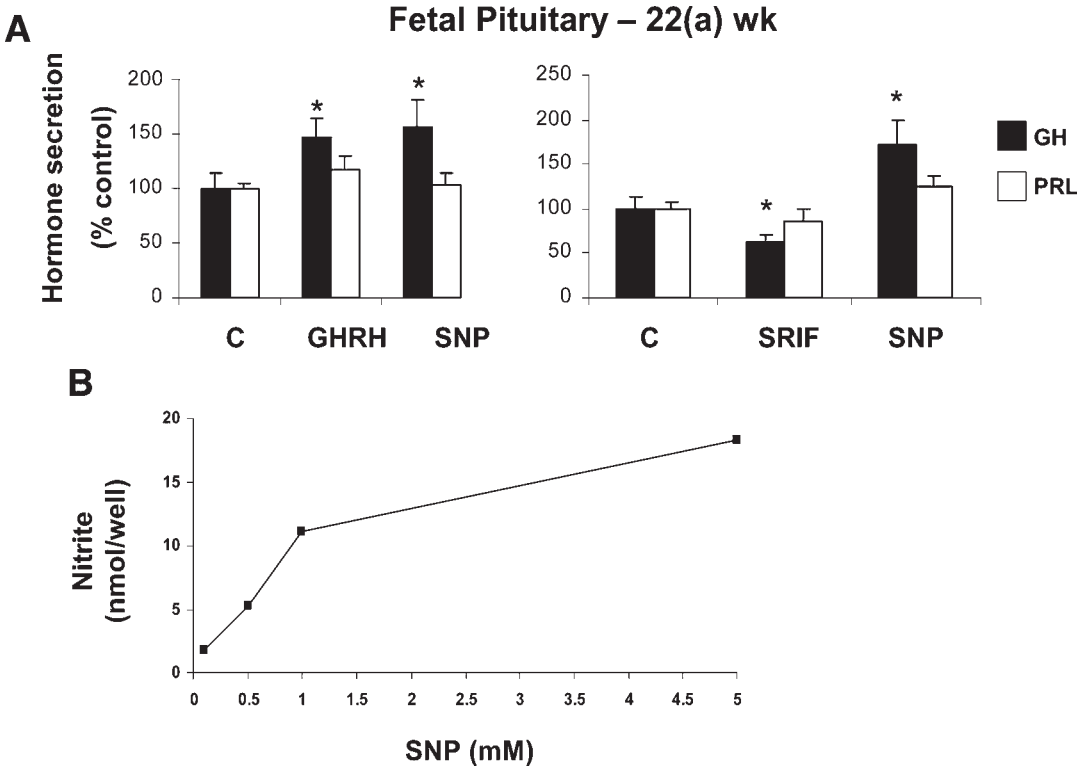

Fig. 1. (**A**) Human GH (black bars) and PRL (white bars) secretion from pituitary cell cultures (5×10^4 cells/well) derived from a fetal [22(a) wk gestation] specimen treated with SNP (1 m*M*), GHRH (10 n*M*), or somatostatin (SRIF; 10 n*M*) for 4 h in serum-free defined medium. Control wells (C) were treated with vehicle solution. Each bar represents mean (±SEM) GH or PRL release in six wells compared to control wells (100%). *$p < 0.05$ vs untreated wells. (**B**) Nitrite levels determinations in medium of SNP-treated wells. Serum-free defined medium was incubated at 37°C for 4 h in the presence of SNP (0.1–5 m*M*). Nitrite levels were determined as described in Materials and Methods.

determine the exact regulatory effects of NO on GH secretion and the mediatory signal transduction pathways.

To help fill this gap, we studied the regulatory effects of NO on human GH and PRL secretion, directly at the pituitary level, using primary cultures of human fetal pituitary cells and cultured GH- and PRL-secreting adenomas. These models were also used to investigate whether cGMP serves as a potential mediator of these physiological effects.

Results

Effects of NO Donor on Human GH and PRL Secretion

The incubation of cultured human fetal pituitary (22 wk gestation, 22a) for 4 h in the presence of sodium nitroprusside (SNP; 1 m*M*), which spontaneously releases NO (nitrite level in medium, 11 nmol/well; Fig. 1B) resulted in a 50–75% increase in GH secretion (Fig. 1A), comparable to the stimulatory effect evoked by GHRH (10 n*M*; Fig. 1A). GH release was also significantly stimulated (40–70% increase) by NO (SNP; 1 m*M*) in five of the eight cultured GH-cell adenomas studied (Fig. 2A). A time-dependent SNP-stimulated GH accumulation was shown in cultured GH adenoma cells as well (Fig. 2B). PRL secretion was not affected by NO, either in fetal pituitaries (Fig. 1A) or in cultured adenomas (data not shown). To confirm that the GH-stimulatory effect of SNP was due to NO release, we added PTI0, a NO scavenger. In both the fetal pituitary (22 wk gestation, 22b) and cultured adenoma cells, PTI0 (10–100 μ*M*) abrogated the stimulatory effect of SNP on GH release (Fig. 3), supporting the role of NO in stimulating GH release from pituitary cells in response to SNP.

Human Pituitary nNOS Expression

RT-PCR analysis was performed to further examine the expression of nNOS in human pituitary cells. nNOS (also referred to as brain NOS), one of three NOS isoforms, is highly expressed in neuronal cells and other tissues and is activated by calmodulin *(20)*. Total RNA was extracted from normal adult and fetal (21 wk gestation) pituitaries and from GH-secreting pituitary adenomas. RNA was subjected to RT, followed by PCR amplification for nNOS cDNA. Results indicated that nNOS was expressed in normal and fetal pituitary tissue, and in two GH-secreting adenomas (Fig. 4A; 546 bp product). The negative controls (in the absence of reverse transcriptase) were free of PCR products. The specificity of PCR was indicated by digestion with *Hinf*I restriction enzyme, which resulted in the expected 320- and 226-bp DNA products (Fig. 4B). In addition, Western blot-

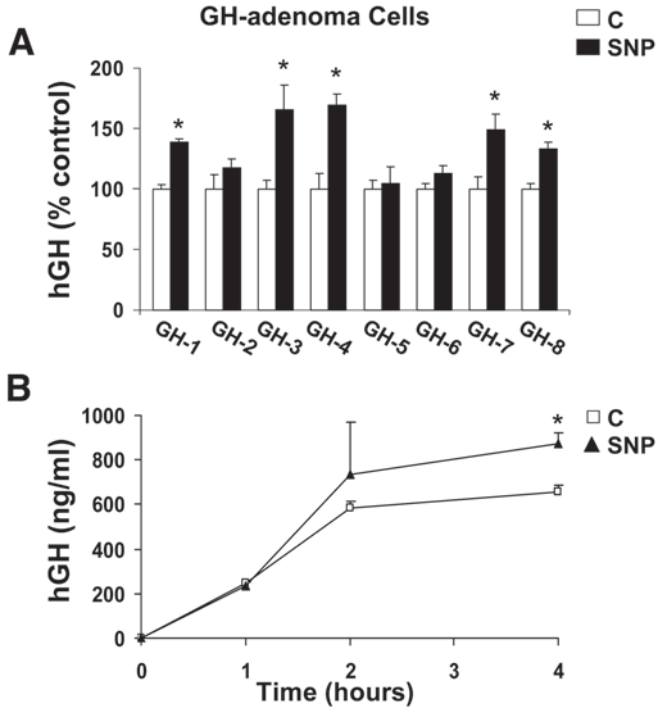

Fig. 2. (**A**) Human GH release from eight cultured GH-secreting adenomas (GH-1–8) treated with SNP (1 m*M*) for 4 h. Each pair of bars represents an experiment done in a different GH-secreting adenoma. Mean GH secretion (±SEM) in 6–8 wells treated with SNP (black bars) was compared to 6–8 control wells (C, 100%; white bars) in each experiment. (**B**) A time course (0–4 h) of GH accumulation in medium of control cells and cells treated with SNP. GH-secreting adenoma cells were either left untreated or incubated with SNP (1 m*M*). Medium aliquots were collected at the indicated time intervals for hormone measurements. *$p < 0.05$ vs untreated wells.

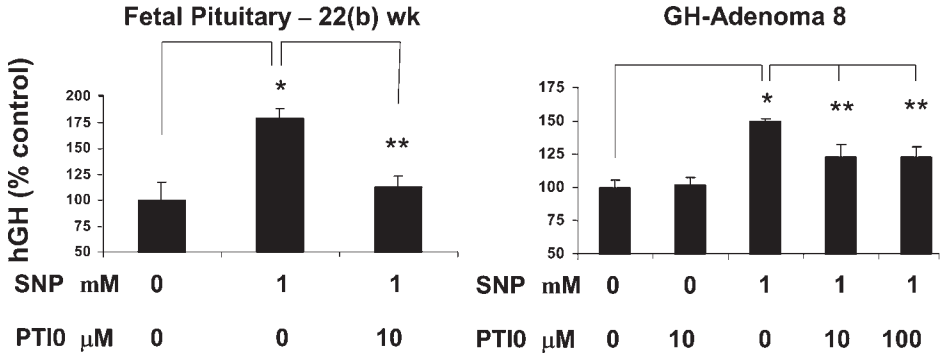

Fig. 3. Inhibition of human SNP-induced GH stimulation (for 4 h) in cultured fetal pituitary [22(b) wk gestation] and GH-secreting adenoma cells (GH-8) by PTI0, a NO scavenger. Control wells were treated with vehicle solution. Each bar represents mean (±SEM) GH release in six wells, compared to control wells (100%). *$p < 0.05$ vs untreated wells. **$p < 0.05$ vs cells treated with SNP.

ting was applied and nNOS protein expression was detected in two large GH-secreting adenomas (Fig. 4C).

Functional Expression of NOS in Human Pituitary Cells

Next, we sought to determine if intracellular-formed NO affects GH secretion. The application of L-arginine (1 μ*M*), a precursor of endogenous NO, increased GH release from cultured GH-cell adenoma by 35% (Fig. 5). The response was abrogated by the addition of 7-nitroindazole, which selectively inhibits nNOS, the enzyme responsible for NO production from L-arginine (Fig. 5). This finding suggests that NOS mediates the stimulatory effects of L-arginine on human GH.

Involvement of the Guanylyl Cyclase/cGMP Pathway in NO-Induced GH Secretion

NO stimulates soluble guanylyl cyclase, which, in turn, generates cGMP, the classical second messenger for a wide range of NO cellular actions. We found that 8-Br-cGMP (1 m*M*), a permeable analog of cGMP, enhanced GH secretion by 55–95% in both human fetal pituitary cultures (23 and 24 wk gestation; Fig. 6A) and cultured GH-secreting adenomas (Fig. 6B). A time-dependent cGMP-induced GH accumulation in adenoma cells is shown in Fig. 6C. The addition of a soluble guanylyl cyclase inhibitor, NS2028, abolished the stimulatory effect of L-arginine on GH in GH-adenoma cells (Fig. 6D), thereby supporting the mediatory role of

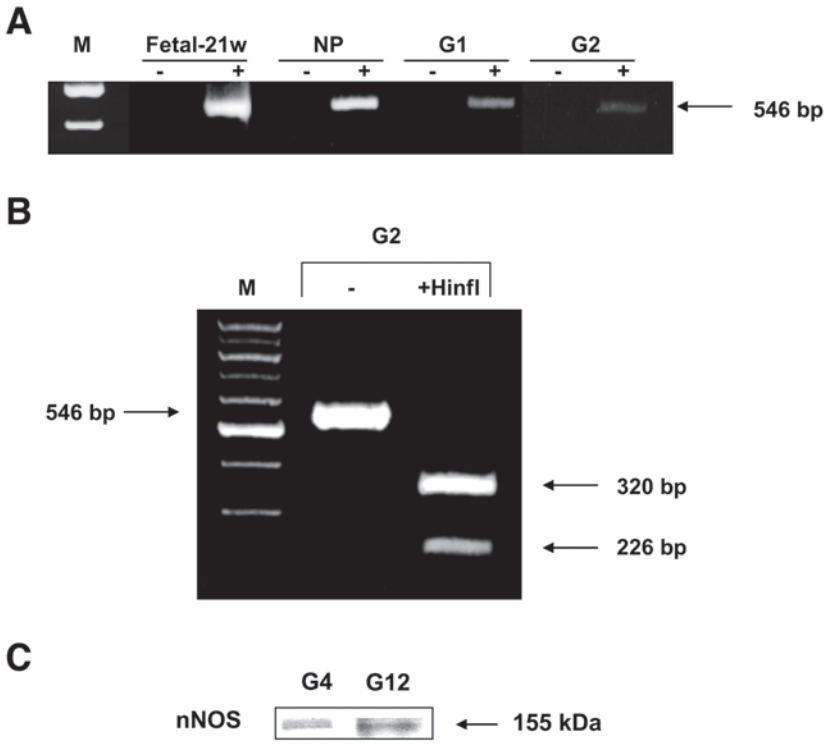

Fig. 4. (**A**) Neuronal NOS mRNA expression in human pituitary tissues. Human nNOS mRNA expression was studied by RT-PCR in fetal pituitary tissue (21 wk), two GH-secreting pituitary adenomas (G1, G2), and normal pituitary (NP). The expected PCR products of nNOS (546 bp) are depicted in all tissues studied (– indicates the absence of reverse transcriptase). (**B**) PCR products were digested by *Hinf*I to verify PCR specificity. M, ladder. (**C**) Human nNOS protein expression was studied by immunoblot assay. Extracts (50 μg) of large GH-secreting pituitary adenomas (G4, G12) were resolved by SDS-PAGE and immunoblotted with polyclonal antibody raised against neuronal NOS, as described in Materials and Methods. The expected 155-kDa nNOS protein is depicted in the GH-adenomas studied.

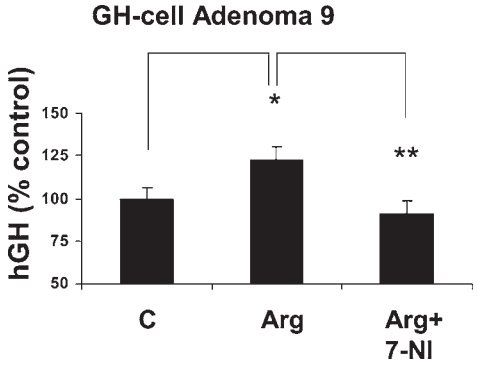

Fig. 5. Suppression of L-arginine (Arg; 1 μ*M* for 4 h)-induced GH stimulation in cultured GH-secreting adenoma (GH-9) by co-incubation with 7-nitroindazole (7-NI; 0.1 m*M*), a specific nNOS inhibitor. Control wells (C) were treated with vehicle solution. Each bar represents mean (+SEM) GH release in six wells compared to control wells (100%). *$p < 0.05$ vs untreated wells. **$p < 0.05$ vs cells treated with L-arginine.

guanylyl cyclase/cGMP in NO-induced GH stimulation in hormone-secreting adenomas.

Discussion

This study shows that NO and its mediator, cGMP, stimulate human GH release in cultured human fetal pituitaries and GH-secreting adenomas. This biological effect has been shown before in cultures of rat pituitaries, where NO increased GH secretion *(16)*. NO is also involved in regulation of GH secretion from pituitary somatotrophs of the goldfish *(21)*, where NO donors and cGMP analogs increase GH release from pituitary cultures *(21)*, and NO scavengers abolished the GH response to gonadotropin-releasing hormone *(22)*. In accordance with these in vitro studies, GH release in rats was studied by microinjecting an inhibitor of NOS into the third ventricle *(23)*. This manipulation dramatically lowered plasma GH. In beagles, infusion of L-arginine resulted in GH enhancement, and NOS inhibitor blunted GHRH- or GH secretagogue-induced GH release *(18)*. In contrast, systemic administration of NOS blocker (NAME) to adult male rats did not change GH and PRL serum levels *(24)*. In humans, L-arginine stimulated GH secretion in vivo, but NO donors did not *(25,26)*. Moreover, infusion of a NOS inhibitor did not influence L-arginine-stimulated GH secretion *(27)*. Unlike normal subjects, acromegalic patients treated with a NO donor showed an increase in GH levels *(28)*, and in accordance with our results, the incubation of cultured GH-secreting tumors in the presence of SNP increased GH release in vitro *(28,29)*. Interestingly however, only 60% of our cultured GH-secreting adenomas responded to NO administration, probably because of impaired down-

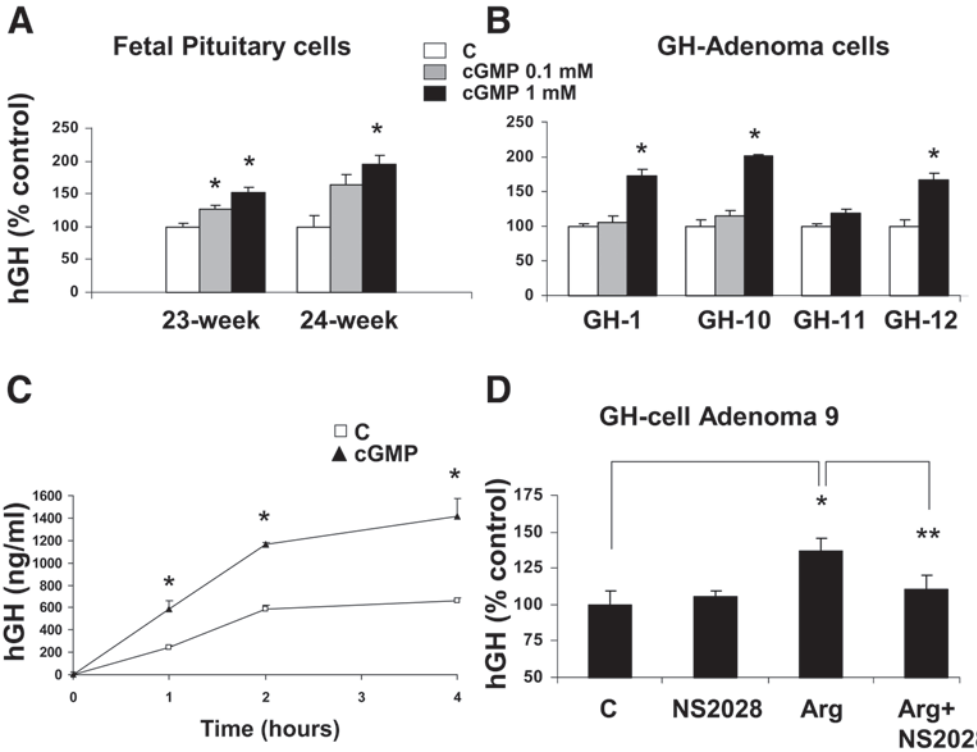

Fig. 6. Human GH stimulation in two cultured fetal pituitaries (**A**; 23 and 24 wk gestation) and four cultured GH-secreting adenomas (**B**; GH-1, 10–12) treated with 8-Br-24 cGMP, a permeable analog of cGMP (0.1, 1 m*M*) for 6 h. Control wells (C) were treated with vehicle solution. (**C**) A time-dependent (0–4 h) cGMP-induced GH accumulation in cultured GH-adenoma cells. GH-secreting adenoma cells were either left untreated or incubated with cGMP (1 m*M*). Medium aliquots for GH measurements were collected at the indicated time intervals. (**D**) L-arginine (Arg; 1 μ*M*)-induced GH stimulation in a cultured GH-secreting adenoma (GH-9) is abolished by co-incubation with NS2028 (0.1 m*M*), a guanylyl cyclase inhibitor. Each bar represents mean (+SEM) GH release in six wells compared to control wells (100%). $*p < 0.05$ vs untreated wells. $**p < 0.05$ vs cells treated with L-arginine.

stream signal processing (guanylyl cyclase/cGMP). It is noteworthy that other NO donors that release high nitrite levels were found to inhibit GH secretion by adenoma cells *(29)*.

Controversies also exist regarding NO regulation of PRL release. Our results show that NO plays no role in human fetal or adenoma PRL secretion, in agreement with a previous study in cultured hormone-secreting tumors *(28)*. However, others reported an inhibitory effect of NO donors on PRL secretion in rat hemipituitaries *(13)* and dispersed cells *(30)*. By contrast, one group found that activation of the NO pathway induces PRL release from dispersed rat pituitaries *(31)*. These discrepancies may be explained by differences in study design, namely, in vivo vs in vitro, use of hemipituitaries vs dispersed monolayer, and use of different species. Recently, Velardez et al. *(32)* reported cell death in rat anterior pituitaries after long-term exposure to NO. They suggested that 6 h of NO treatment is sufficient to trigger irreversible changes and commit the cells to apoptosis. Thus, the nitrite levels released by different NO donors and the exposure time may also account for the controversies in literature.

Although soluble guanylyl cyclase/cGMP was traditionally considered the only definitive signaling cascade of NO *(33)*, researchers now suggest that NO actions may occur independently of cGMP, and that some of them may be mediated by reactive nitrogen species, such as S-nitrosylation *(34)*. These assumptions were supported by a recent study in rat posterior pituitary nerve terminals *(35)*. At this point, the mechanism underlying the effects of NO in pituitary cells is still unclear. While our results indicate a crucial role of guanylyl cyclase/cGMP in the stimulatory effect of NO on human GH secretion, in agreement with previous studies in goldfish *(23)* and dispersed rat *(19)* anterior pituitary cells, they disagree with studies showing no involvement of cGMP in NO-stimulated GH secretion in rat hemipituitaries *(16)*, as well as NO inhibition of PRL *(30)*.

GHRH is the classical human GH stimulator. Its agonistic effects at the pituitary are mediated through adenylyl cyclase activation and intracellular release of cAMP, which also increases intracellular Ca^{2+} influx, through voltage-gated Ca^{2+} channels. Ghrelin, the other potent natural human GH secretagogue *(36)*, requires a functional hypothalamic–pituitary GHRH system to act as a GH stimulator *(37)*. Specifically, it amplifies the GHRH-induced increase in cAMP and thereby potentiates GH release. The addition of a NO donor to perfused pituitary cells was found to lead to soluble guanylyl cyclase induction and rapid cGMP release,

and probably, to GH secretion *(10)*. These findings, taken together with our results suggesting a role for NO and cGMP in GH regulation in cultured human pituitary tissues, support the concept that GH release is controlled by different hypothalamic physiological systems (i.e., GHRH, ghrelin, somatostatin), and regulated by different parallel signaling pathways, including adenylyl cyclase–cAMP, and guanylyl cyclase–cGMP activation. cGMP regulates its biological effects through several downstream pathways, including protein phosphorylation by cGMP-dependent protein kinase G, cGMP-regulated phosphodiesterase, and cGMP-gated calcium influx *(33)*. Phosphodiesterases allow cross-talk between cGMP and cAMP signaling mechanisms. These different pathways can modify each other and, thus, may have additive or even synergistic effects on GH release via intracellular cross-talk between their components downstream.

In conclusion, this study shows for the first time that NO and its mediator, cGMP, can stimulate human GH release from cultured human fetal pituitaries. This biological effect was reported before in cultures of rat pituitaries *(16)*, but not in vitro in normal human pituitaries or in vivo in normal subjects *(25–28)*. Our findings in cultured GH-cell adenomas are also in accordance with previous in vitro *(28, 29)* and in vivo studies *(28)*. The action of NO in regulating GH in normal human pituitary cells in culture is probably mediated by guanylyl cyclase activation and a cGMP-dependent pathway.

Materials and Methods

NO Modulators

Sodium nitroprusside (SNP, 1 m*M*; Sigma Chemicals Co., St. Louis, MO) was used as the NO donor. L-arginine, a NOS substrate (1 μ*M*; Sigma), was used to generate NO, and 8-bromo-cGMP (8-Br-cGMP, 0.1, 1 m*M*; Calbiochem, San Diego, CA), was used as a permeable cGMP analog. PTI0 (10–100 μ*M*), a NO scavenger, NS 2028 (0.1 m*M*), a guanylyl cyclase inhibitor, and 7-nitroindazole (7-NI; 0.1 m*M*), a specific neuronal NOS (nNOS) inhibitor were all provided by Calbiochem. GH-releasing hormone (GHRH) and somatostatin-14 (SRIF) were purchased from Sigma.

Pituitary Tissues

Human pituitary tissues from fetuses of 21–24 wk gestation (male and female) were harvested from pathologic specimens within 0.5–2 h of therapeutic pregnancy terminations. All studies in human fetal pituitaries were performed in accordance with the guidelines of the National Advisory Board on Ethics in Reproduction *(38)*, and written informed consent was obtained from the pregnant subjects. Pituitary adenoma specimens were obtained during transsphenoidal surgical resections, in accordance with methods approved by our local institutional review board. Appropriate consent was provided by the patients (Table 1). Normal adult pituitary tissue was obtained during postmortem examinations.

Table 1
Clinical Characteristics of Patients Harboring GH-Secreting Pituitary Adenomas

Tissue no.	Age (yr)/gender	Size (mm)
GH-1	M	Macroadenoma
GH-2	39/M	20
GH-3	60/F	12
GH-4	43/F	Large macroadenoma
GH-5	59/M	9
GH-6	M	6
GH-7	58/F	11
GH-8	52/F	12
GH-9	50/M	5
GH-10	32/M	10
GH-11	70/M	15
GH-12	49/M	Large macroadenoma

Pituitary RNA Extraction

Normal human adult and fetal pituitaries and pituitary adenomas were harvested and kept at –70°C for RNA extraction. After homogenization, total RNA was extracted using guanidium isothiocyanate–phenol–chloroform (TRizol; Invitrogen Inc., Carlsbad, CA), and aliquots of RNA were electrophoresed through TBE gel to confirm RNA integrity.

RT-PCR

Reverse transcription (RT) followed by PCR amplification was performed to detect nNOS mRNA expression in normal and adenomatous pituitary tissues. RNA was treated with deoxyribonuclease before the RT reaction to eliminate contaminating genomic DNA. RNA was then used in a 20-μL RT reaction containing oligo$(dT)_{16}$ as a primer and Super Script II (Life Technologies, Carlsbad, CA). RT reactions were incubated at 42°C for 50 min and then 70°C for 15 min. Samples were also incubated without RT as negative controls.The resulting cDNA and negative controls were used for subsequent PCR amplification of nNOS in the presence of 2 m*M* $MgCl_2$ and 5 U Taq DNA polymerase (Bioline**,** Randolph, MA). Amplifications were carried out for 35 cycles, with an initial denaturation step at 95°C for 5 min and a final 7-min extension step at 72°C. Each cycle consisted of three steps (1 min each): denaturation at 94°C, annealing at 55°C, and elongation at 72°C. The primer set used was 5'-CCCTTCAGTGGCTGGTACAT (nucleotides 2447–2466, exon 10) and 3'-TGCCTGTCTCTGTGGCATAG [nucleotides 2974–2993, exon 14 *(39)*; GenBank accession no. U17327 for the human nNOS mRNA]. A 546-bp PCR product was generated, and it was digested by *Hinf*I (New England Biolabs, Beverly, MA) to 320- and 226-bp restriction products and visualized with ethidium bromide after electrophoresis on 2% agarose gel.

Protein Extraction and Western Blot Analysis

Frozen tissue specimens were minced in liquid nitrogen followed by sonication in RIPA buffer (50 m*M* Tris-HCl

pH 7.4, 150 m*M* NaCl, 1 m*M* EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitors. Subsequent to incubation for 30 min in 4°C, samples were centrifuged (15,000*g*, 15 min, 4°C) and protein concentrations of supernatants were determined by Bradford assay (Bio-Rad Laboratories, Inc., Carlsbad, CA, USA). Protein extracts (50 µg) were loaded on 10% SDS-PAGE, transferred to nitrocellulose membrane, and blocked in 2% BSA. The membrane was then incubated with polyclonal anti-neuronal NOS (BD Transduction Laboratories, Lexington, KY), subsequently incubated with peroxidase conjugated anti-rabbit Envision reagent (DakoCytomation, Glostrup, Denmark), and finally developed using Super Signal chemiluminescent substrate (Pierce, Rockford, IL).

Human Fetal Pituitary and Pituitary Adenoma Cell Cultures

Fetal pituitary and tumor specimens were treated similarly and washed in low-glucose DMEM supplemented with 0.3% BSA, 2 m*M* glutamine, and antibiotics, then minced and enzymatically dissociated using 0.35% collagenase and 0.1% hyaluronidase (both from Sigma) for 45–60 min. Cell suspensions were filtered through 80 µm nylon mesh (Millipore, Bedford, MA) and resuspended in low-glucose DMEM supplemented with 10% FBS**,** 2 m*M* glutamine, and antibiotics. For primary cultures, approx 5×10^4 cells were seeded in 48-well tissue culture plates (Costar, Cambridge, MA) in 0.5 mL medium, and incubated for 72–96 h in a humidified atmosphere of 95% air/5% CO_2, at 37°C. The medium was then changed to serum-free defined (SFD) low-glucose DMEM containing 0.2% BSA, 120 n*M* transferrin, 100 n*M* hydrocortisone, 0.6 n*M* triiodothyronine, 5 U/L insulin, 3 n*M* glucagon, 50 n*M* parathyroid hormone, 2 m*M* glutamine, 15 n*M* EGF, and antibiotics. The cells were treated for 4 h with the indicated peptides and NO modifiers. A single pituitary specimen (either fetal or adenoma) was divided and plated onto 60–80 wells, depending on its size. In each experiment, 6–8 wells served as controls (treated with vehicle solution) and groups of 6–8 wells were treated as indicated. The medium was then collected and stored at −20°C for later hormone measurements.

Nitrite Measurement

Serum-free defined low-glucose DMEM was incubated at 37°C for 4 h in the presence of SNP (0.1–5 m*M*). Equal volumes of samples and Greiss reagent (Sigma) were mixed and incubated at room temperature for 10 min *(10)*. The mixtures were then quantified spectrophotometrically at 550 nm. Nitrite concentrations were determined using a standard curve of increasing concentrations of sodium nitrite (Sigma; 0–40 µmol/L).

Hormone Assays

Human GH was measured by RIA (Diagnostic Products Corp., Los Angeles, CA). Intra- and interassay precisions were 1.5% and 3.4%, respectively. Human PRL was measured by IRMA (Diagnostic Products). Intra- and interassay precisions were 2.0% and 3.6%, respectively.

Statistical Analysis

Results are presented as the mean ± SEM. As absolute hormonal levels differed among fetal specimens and among adenoma specimens, hormonal data were expressed as percentage of control (100%). Data were analyzed by one-way ANOVA, and p values < 0.05 were considered significant.

Acknowledgments

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