A BRIEF COMMUNICATION

Central Effects of Ghrelin on Serum Growth Hormone and Morphology of Pituitary Somatotropes in Rats

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Ghrelin, an endogenous ligand for the growth hormone (GH) secretagogue receptor, was originally purified from rat stomach; subsequently, ghrelin neurons were found in the arcuate nuclei of rats. Central effects of the peptide on GH release, however, remain to be clarified. The aim of the present study was to determine the morphologic features of GH-producing pituicytes and serum GH concentration after central administration of ghrelin. Five injections of rat ghrelin or phosphate-buffered saline (PBS; $n = 10$ rats/group) were given every 24 hrs (1 μg of ghrelin in 5 μl of PBS) into the lateral cerebral ventricle of male rats. Significant ($P < 0.05$) increases in absolute and relative pituitary weights occurred in ghrelin-treated rats versus controls (58% and 41%, respectively). Morphometric parameters (i.e., the volume of GH cells, volume of their nuclei, and volume density) all significantly ($P < 0.05$) increased by 17%, 18%, and 19%, respectively, in the ghrelin-treated group versus controls. Terminal serum concentration of GH was significantly ($P < 0.05$) increased by 15% with ghrelin treatment. The results clearly document that daily nanomolar doses of ghrelin into the lateral cerebral ventricle stimulate GH cell proliferation and promote GH release. Thus, achieving pharmacologic control of central ghrelin receptors is a promising modality to modulate the actions of GH. Exp Biol Med 231:1610–1615, 2006

Key words: ghrelin; cerebroventricular injections; pituitary; GH cells; rats; morphology

Introduction

Pituitary growth hormone (GH) secretion is regulated by at least two hypothalamic hormones. The GH-releasing hormone (GHRH) is a potent stimulator of GH release from somatotropes in the anterior pituitary, whereas somatostatin inhibits GH secretion (1). Since the discovery of met-enkephalin–induced GH secretion from the anterior pituitary (2), small synthetic peptide and nonpeptide molecules, called GH secretagogues (GHSs), have been developed. The GHSs stimulate GH release *in vivo* and *in vitro* via a mechanism distinct from GHRH (3–5). In addition, an endogenous peptide ligand for the GHS receptor 1a, ghrelin, has been purified from the rat stomach and subsequently cloned (6). Human ghrelin is a 28-amino-acid, acylated peptide, and it differs from rat ghrelin by only two amino acids at positions 11 and 12. Ghrelin is widely expressed in different rat and human tissues, including the pituitary, hypothalamus, bowel, kidney, heart, pancreas, and testis, suggesting both endocrine and paracrine effects (7). Originally, however, ghrelin was recognized as an important component in the regulation of GH release and appeared to be a third "physiological" regulator of GH release (8). Intravenously administrated ghrelin stimulated GH release in rats (9–11). Furthermore, ghrelin-positive neurons were found in the arcuate nucleus of the rat hypothalamus in an

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Table 1. Effects of Intracerebroventricularly Applied Ghrelin on Body Weight and on Absolute and Relative Pituitary Weight in Adult Male Rats[a]

Experimental group	Body weight (g)	Absolute pituitary weight (mg)	Relative pituitary weight (mg/100 ml)
Control	196.6 ± 21.4	5.8 ± 1.5	2.7 ± 0.9
Ghrelin	222.0 ± 27.3 (+13%)	9.2 ± 1.2* (+58%)	3.8 ± 0.3* (+41%)

[a] Values are presented as the mean ± SD ($n = 10$).*$P < 0.05$ versus control.

immunohistochemical study (6), suggesting that ghrelin of central origin also affects GH release. Differing experimental conditions and routes of administration, however, have produced various results with regard to GH release (see "Discussion").

The present study was designed to evaluate the effect of daily nanomolar doses of ghrelin by the intracerebroventricular (ICV) route in freely moving, male rats over a period of 5 days and to assess the terminal blood level of GH as well as changes in the immunocytochemical appearance and quantitative morphology of somatotropes of the pituitary gland. The hypothesis to be tested was that small, repetitive doses of centrally administered ghrelin elevates baseline serum GH and, thus, should yield evidence of pituitary changes in GH-producing cells.

Materials and Methods

The present study was performed on adult male Wistar rats (body wt, 200 ± 20 g) bred at the Institute of Biomedical Research "Galenika" in Belgrade, Serbia. They were kept in individual metabolic cages under a 12:12-hr light:dark cycle at 22°C ± 2°C and were accustomed to daily handling. They were fed a balanced diet for laboratory rats (prepared by D.D. Veterinarski zavod Subotica, Subotica, Serbia). Food and water were available to rats *ad libitum*.

Animal Preparation. Surgical procedures were performed under ether anesthesia (aether ad narcosis Ph. Iug. III.; produced by Lek, Ljubljana, Slovenia). The rats were implanted with a headset that was used later for ICV injections. A minimum recovery time of 5 days was permitted before the onset of experiments. The headset consisted of a silastic-sealed, 20-gauge cannula (12) implanted into a lateral cerebral ventricle 1 mm posterior and 1.5 mm lateral to the bregma and 3 mm below the cortical surface. A small, stainless steel anchor screw was placed at a remote site on the skull. The cannula and screw were cemented to the skull with dental acrylic (Simgal; Galenika, Belgrade, Serbia).

Animal Treatment. After recovery, rats were divided into two groups, with each group containing 10 animals. The first group consisted of rats that were ICV treated with 1 μg of rat ghrelin (lot no. C-et-004; Global Peptide Services, LLC, Ft. Collins, CO) dissolved in 5 μl of phosphate-buffered saline (PBS) every 24 hrs during five consecutive days. This dose was based on the results of previous studies (13) and pilot experiments in our laboratory. The second group was a control group comprised of rats treated in the same manner, except that the control rats received only 5 μl of PBS. All animals were sacrificed by decapitation during deep anesthesia 2 hrs after the last injection. Experimental protocols were approved by the local Animal Care Committee and conformed to the recommendations given in the Guide for the Care and Use of Laboratory Animals.

Light Microscopy and Immunocytochemistry. Pituitary glands were excised, fixed in Bouin's solution for 48 hr, and embedded in paraffin. Serial sections (thickness, 5 μm) were deparaffinized in xylol and serial alcohol. Pituitary hormones were localized by the peroxidase-antiperoxidase complex (PAP) method (14). Endogenous peroxidase activity was blocked by the incubation in 9 m*M* hydrogen peroxide in methanol for 30 mins at ambient temperature. Before application of specific primary antisera, nonspecific background staining was minimized by incubation of the sections with nonimmune porcine serum diluted with PBS (pH 7.4) for 60 mins. Sections were then overlaid with the appropriate dilutions of the specific primary antibodies (hGH-antisera; Dako A/S, Glostrup, Denmark) for 24 h at 4°C. After washing in PBS, sections were incubated for another 60 mins with the second antibody-swine-antirabbit IgG for 45 mins, rinsed again with PBS for 10 mins, and then incubated with rabbit PAP serum for 45 mins. Antibody localization was visualized by incubating the sections in Tris-HCl–buffered saline (0.05 *M*, pH 7.4) supplemented with 3,3-diaminobenzidine tetrachloride (DAB; Serva, Heidelberg, Germany) and 9 m*M* hydrogen peroxide. Slides were thoroughly washed under running tap water, counterstained with hematoxylin, and mounted in Canada balsam (Alkaliod, Skopje, Macedonia). Control sections were incubated without primary antisera or by substituting nonimmune rabbit serum for the primary antiserum. Digital images were made on a DM RB Photomicroscope Olympus BX51 (Olympus, Tokyo, Japan) with a DP70 digital camera (Olympus) for acquisition and analysis of the images.

Morphometry. Measurements were performed on sections from the widest portion of the pituitary gland, and immunocytochemically labelled GH cells were analyzed by the M_{42} test system (15). The standard methods and formulae described by Weibel and Gomez (16) were used for the calculations of the cell and nuclear volumes.

The mean value for each rat was computed from 50 fields from the three widest sections ($n = 150$ measurements/rat). The measurements were taken by an investigator who was unaware of the animal's treatment history.

Hormone Assay. Blood samples were collected immediately after each animal was killed, and separated serum was stored at −20°C until assayed. Serum concentrations of GH in control and experimental rats were measured using the Delfia method (hGH-Delfia kits; LKB, Turku, Finland). This radioimmunoassay has an intraassay variability of 3%–4%, an interassay variability of 6%–7%, and a sensitivity of 30 ng/l.

Statistical Analyses. Biochemical and morphometric data obtained from each rat were averaged per experimental group, and the standard deviation of the mean (SD) was calculated. The Student's t test was used to evaluate differences between the two groups, and a probability value of 5% or less was considered to be statistically significant.

Results

Data summarizing the body weight and the absolute and relative weights of the pituitary glands of the control and ghrelin-treated groups appear in Table 1. A non-significant ($P > 0.05$) increase in body weight (13%) was observed in ghrelin-treated rats versus controls. Absolute and relative pituitary weights were significantly ($P < 0.05$) increased in peptide-treated animals by 58% and 41%, respectively, compared with the controls.

Immunocytochemically identified GH cells in control rat pituitaries ranged from ovoidal to pyramidal in shape, with a centrally located spherical nucleus. The GH cells usually were situated along sinusoids. These cells were intensely stained (Fig. 1A). In the rats treated with ghrelin, neither the shape nor the localization of GH immunoreactive cells was significantly changed compared with the controls, but their staining properties were significantly changed. Small, specific secretory granules were distributed mainly at the periphery of the cytoplasm (Fig. 1B).

The three morphometric parameters measured in the present study (i.e., the volume of the GH cells, their nuclear volume, and the volume density) are depicted in Figure 2. As Figure 2 illustrates, these three morphometric parameters increased by 17%, 18%, and 19%, respectively, in male rats treated with ICV ghrelin versus controls, and the differences were statistically significant ($P < 0.05$).

Serum concentration of GH was significantly increased ($P < 0.05$) by 15% after ghrelin treatment compared with the concentration in control rats (Fig. 3).

Discussion

The present study demonstrated that the ICV ghrelin regimen over a 5-day period clearly stimulated GH release. The biochemical data (i.e., increased serum GH concentration 2 hrs after the last injection) were consistent with stimulatory changes observed by quantitative morphology of somatotropes from the pituitaries of the same rats. Thus, ghrelin might play an important role in the regulation of GH secretion. This effect could result from a direct action of ghrelin or by interaction with other GH-releasing peptides, other neurohumoral control mechanisms, or both.

The present results also demonstrate statistically significant changes in morphometric parameters of the pituitary gland. Ghrelin changed both absolute and, more importantly, relative pituitary weight, suggesting hypertrophic as well as hyperplasic effects of the centrally applied peptide. Other immunohistochemical features of GH cells support the opinion that hypertrophic and hyperplasic changes are related primarily to GH cells, because GH total cell volumes were significantly increased (17%) compared with controls. Significant increases (18%) in GH cell nuclear volumes suggested higher ghrelin-induced GH cell nuclear activity, indicative of increased cell function when the 5-day experiment was ended. Finally, relative volume density of GH cells showed that after prolonged treatment with ghrelin, GH cells significantly increased in number (19%) compared to other pituitary cell types.

The present results appear to be comparable to those of other related studies. Two independent groups (8, 9) demonstrated that a single ICV dose of ghrelin to rats increased the plasma GH concentration in a dose-dependent manner, peaking 15–20 mins after administration and returning to baseline 60 mins later, which suggests that ghrelin was acting on its receptors in the hypothalamic-pituitary axis to directly stimulate GH release. Data from Wren *et al.* (17) support these conclusions, but with much higher ICV doses of ghrelin (10 μg/rat). Date *et al.* (9) also showed that a continuous ICV administration of ghrelin for 12 days increased the plasma GH concentration measured on Day 6 but did not sustain this high GH concentration on Day 12, although the GH level was still higher on Day 12 compared with the baseline on Day 1.

A number of studies have suggested that the pattern of peripheral ghrelin administration is important for GH effects. For example, 7-day intermittent intravenous infusions (every 3 hrs) of ghrelin augmented GH secretion, whereas continuous infusion suppressed GH secretory episodes (18, 19). Intravenous administration of 3–12 nmol/kg of ghrelin in freely moving rats caused a dose-dependent increase in GH release (9–11). Ghrelin also was reasonably effective *in vitro* in a dose-dependant manner (6). This study by Kojima *et al.* showed a specific effect of ghrelin on GH release, because the other pituitary hormones were unaffected. The effects of ghrelin *in vivo,* however, appear to be much stronger than the *in vitro* effect and to be more potent in humans than in animals (20).

The potency of ghrelin, as measured through its GH-releasing ability, was higher than that of GHRH and comparable to that of synthetic GHS (21). Results of several studies also have suggested that the important component of the GH stimulatory effect of ghrelin is at the hypothalamic

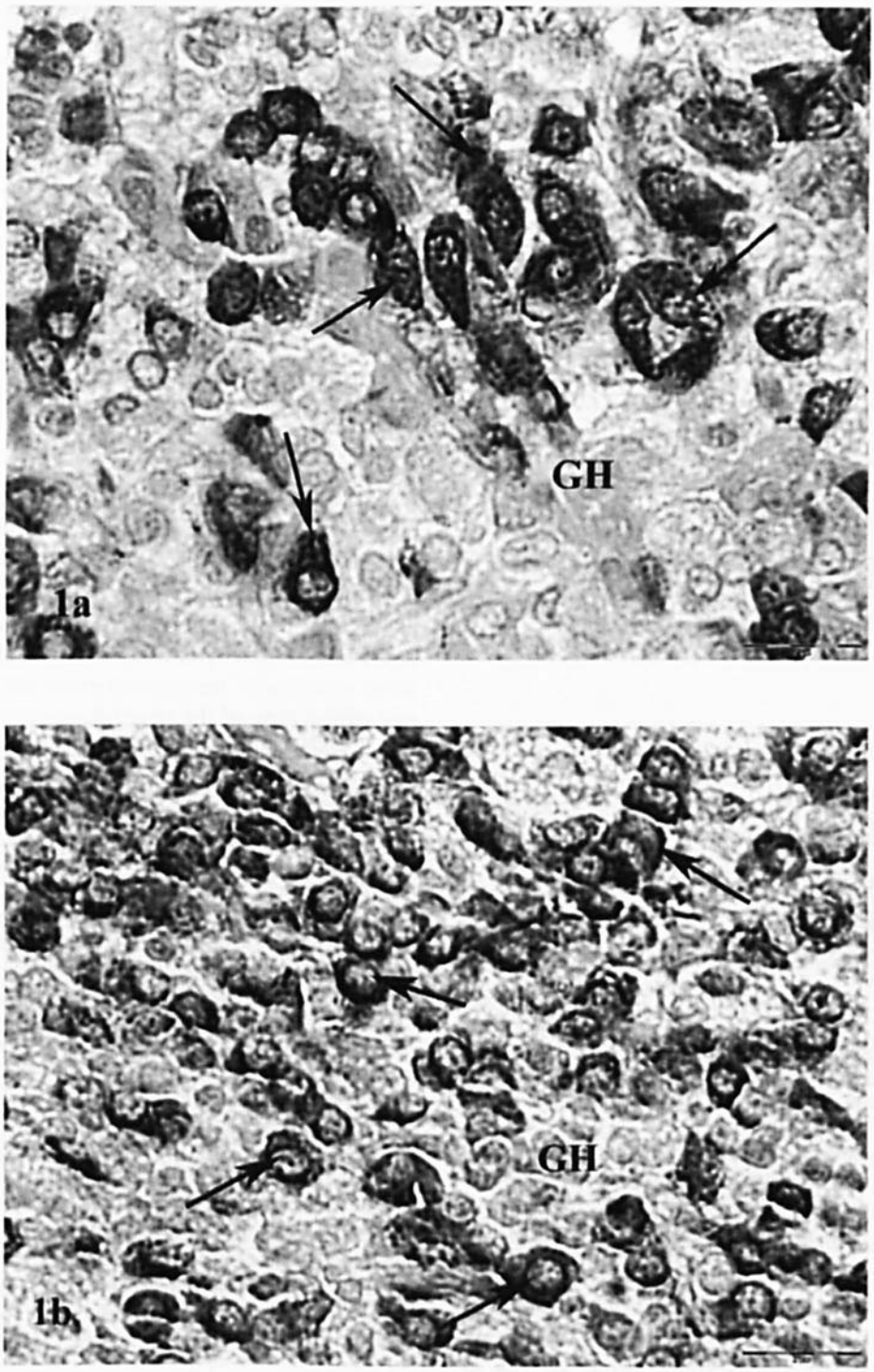

Figure 1. Typical appearance of immunohistochemically labeled GH cells (arrows). (A) Control rats. (B) Ghrelin-treated rats (PAP). Bar, 10 μm. Color figure available on-line.

level and that, for ghrelin to be operative on GH secretion, the normal functioning of the GHRH neurons/receptor also is necessary (22, 23). Ghrelin increases the activity of cells in the arcuate nucleus, including NPY, GHRH, and POMC cells (7).

It should be pointed out that some studies, unlike our own and those cited above, have shown no effects of ghrelin on GH release. The reasons for such disparity are uncertain. Kamegai *et al.* (13) did not observe stimulation of GH cells after ICV administration of ghrelin (1 μg/rat) either after a single dose or after six doses every 12 hrs. Also, Tamura *et al.* (8) found significant ghrelin-induced GH secretion with higher, supraphysiologic doses. To our knowledge, no significant correlation between GH and ghrelin levels has, as

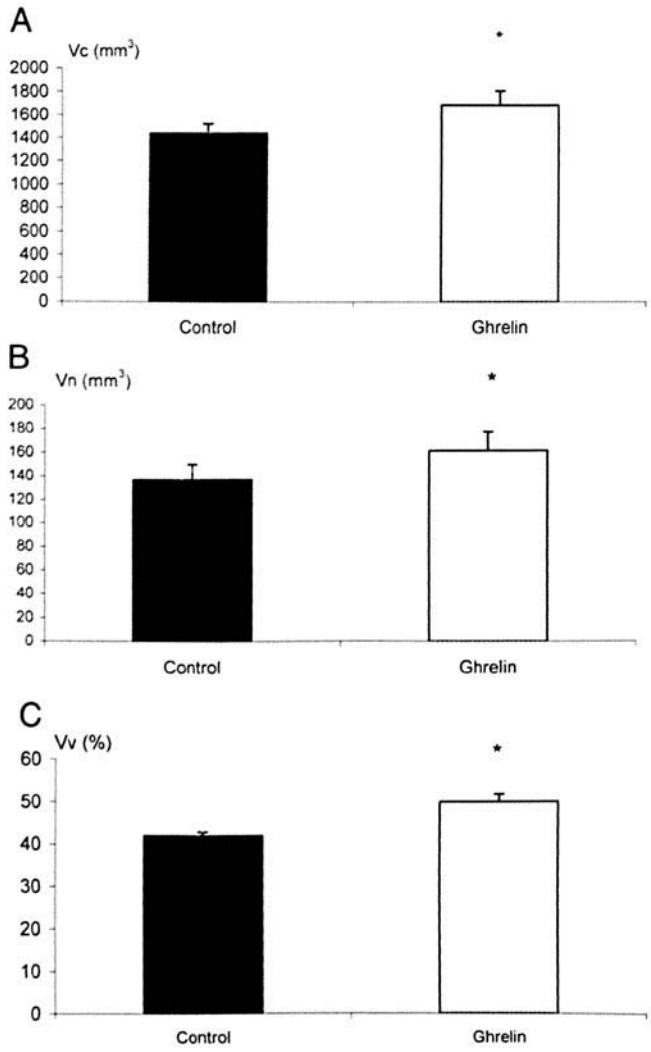

Figure 2. (A) Cellular volume (μm^3) of the immunoreactive GH cells. (B) Nuclear volume (μm^3) of GH cells. (C) Relative volume density (%) of GH cells expressed as a percentage of the total gland tissue. All values are presented as the mean ± SD ($n = 10$ animals/group). An asterisk indicates a significant ($P < 0.05$) difference versus control.

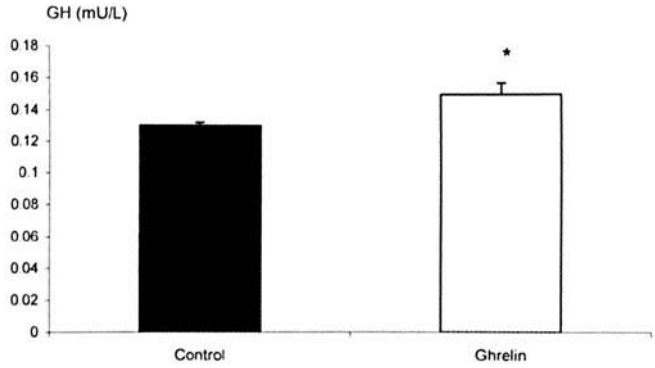

Figure 3. Serum concentration of GH in adult male rats. Values are presented as the mean ± SD ($n = 10$ animals/group). An asterisk indicates a significant ($P < 0.05$) difference versus control.

yet, been observed in either rats (24) or humans (25), and this fact does not support the concept that ghrelin is an essential regulator of GH physiology. Studies with GHS-receptor knockout models have suggested only a moderate effect of ghrelin in physiologic GH regulation (26, 27), but some recent studies have demonstrated strong activity of ghrelin on the regulation of pulsatile GH secretion (28).

The half-life of circulating ghrelin is approximately 30 mins (26), and the half-life of circulating rat GH is approximately 10 mins (29). Thus, we conclude that the low doses of ICV ghrelin over the present 5-day protocol that increased serum GH concentration 2 hrs after the last treatment likely reflect a central action of the peptide.

King (30) and Cummings *et al.* (31) have reviewed and discussed the multiple "short-term signal hormones," including ghrelin, cholecystokinin, peptide YY, and GLP-1, on central mechanisms controlling eating behavior and energy metabolism. More information, however, is necessary to understand when each of these systems operates physiologically. Guillemin (32), in his Starling Lecture, noted that "[t]he ultimate physiological control of the secretion of GH is by now multi-level and not totally clear." More specifically, he stated that "an ultimate and satisfactory explanation of the inter-relationships between GHRH, somatostatin and ghrelin is still to be explored."

In summary, the present study showed that repetitive central ghrelin administration increased serum GH levels and augmented pituitary GH cell volumes, GH cell nuclear volumes, as well as relative volume density. Thus, we support the opinion that ghrelin acts centrally as a stimulus to GH release. Development of pharmacologic agents to selectively alter ghrelin receptors may thus be a modality to influence GH physiology. Additional work will be necessary to determine whether ghrelin contributes to GH pulsatility and to define the exact role of ghrelin not only in GH physiology but also in the etiology of different pituitary-related diseases, including obesity or other disorders of energy metabolism.

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