Endocrine, *vol. 14, no. 1, 101–104, February 2001* 0969–711X/01/14:101–104/$11.00

Presence of Ghrelin in Normal and Adenomatous Human Pituitary

Márta Korbonits,[1] Masayasu Kojima,[2] Kenji Kangawa,[2] and Ashley B. Grossman[1]

[1]Department of Endocrinology, St. Bartholomew's Hospital, London, UK; and [2]Department of Biochemistry, National Cardiovascular Center Institute, Fujishirodau, Suita, Osaka, Japan

Recently, an endogenous ligand has been described for the growth hormone secretagogue receptor (GHS-R), named ghrelin. It was originally isolated from the stomach, but it is also present in the hypothalamus, where the highest concentration of GHS-R has been detected. It is well established that synthetic GHSs exert their effects on the growth hormone (GH) axis principally via the hypothalamus, although they are also able to stimulate GH release directly from the pituitary. We have previously demonstrated the presence of GHS-R mRNA expression in normal and abnormal human pituitary. We have therefore now investigated the expression of the newly recognized endogenous ligand in rat as well as in human pituitary. We readily detected ghrelin mRNA message in normal rat pituitary using reverse transcriptase polymerase chain reaction with published primers. We then designed primers to the corresponding region on the human ghrelin sequence and successfully detected mRNA message in normal human pituitary, as well as in somatotroph, lactotroph, corticotroph, thyrotroph, and nonfunctioning adenomas. We confirmed the expected polymerase chain reaction product by direct sequencing. In conclusion, we suggest that in addition to the probable hypothalamic effects of ghrelin, the peptide is synthesized locally within the pituitary gland, where it may influence the release of GH in an autocrine or paracrine manner.

Keywords: Ghrelin, growth hormone secretagogues, growth hormone secretagogue receptor; pituitary adenoma; reverse transcriptase polymerasechain reaction.

Introduction

The classic hypothalamo-pituitary growth hormone (GH) axis includes the hypothalamic hormones somatostatin and GH-releasing hormone (GHRH) as well as pituitary GH, and although GH has some direct effects on peripheral tissues, it acts mainly by stimulating the synthesis of insulin-like growth factor-1 in the liver as well as in peripheral tissues. Several other hypothalamic regulatory factors directly or indirectly influence the activity of the GH axis, and some of these are also expressed in the gastrointestinal system, forming the group of gut-brain peptides *(1)*.

Since 1982, a group of GH-releasing synthetic compounds has been developed; these were originally termed GH-releasing peptides but now are called GH secretagogues (GHSs) *(2)*. A specific G-protein-coupled receptor was later identified (*3*), suggesting the possible existence of an endogenous ligand. More recently, as the last step in this "reverse pharmacology" process, an endogenous ligand, ghrelin, has indeed been identified from the rat stomach *(4)*. It has also been shown to be present in the rat hypothalamus and, in particular in the arcuate nucleus, which plays a central role in the regulation of the GH axis. Because a number of primarily hypothalamic hormones are also expressed in the pituitary, we speculated that ghrelin also may be synthesized in the pituitary gland.

Results

This study showed that ghrelin mRNA was expressed in rat pituitary tissue, as well as in normal and adenomatous human pituitary tissue. Normal pituitaries included samples from patients with a wide age range (1 d to 54 yr), and all of them expressed ghrelin mRNA. Pituitary tumors arising from somatotroph, corticotroph, lactotroph, gonadotroph, and thyrotroph cells, as well as nonfunctioning adenomas, showed expression of ghrelin mRNA (**Fig. 1**).

Discussion

Ghrelin is a 28 amino acid peptide that has been isolated from stomach but is shown to be present in other tissues, including the hypothalamic arcuate nucleus *(5)*. It has been

Received June 2, 2000; Revised 2000; Accepted 2000.
Author to whom all correspondence and reprint requests should be addressed: Dr. Márta Korbonits, Department of Endocrinology, St. Bartholomew's Hospital, West Smithfield, London, UK EC1A 7BE. E-mail: m.korbonits@mds.qmw.ac.uk

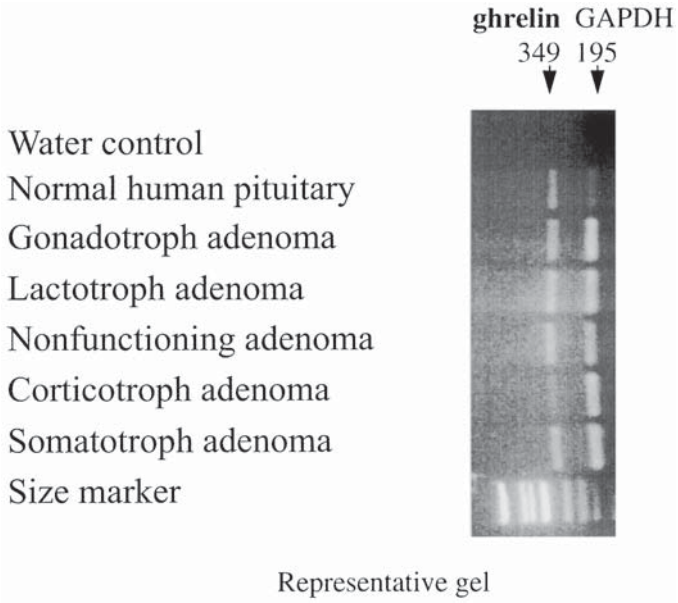

Fig. 1. Representative ethidium bromide-stained 2% agarose gel showing the expression of human ghrelin and the "house-keeping" gene GAPDH expression in normal and tumorous pituitary adenomas.

shown to specifically activate the GHS receptor (GHS-R) and stimulate GH release in vitro while having no effect on adrenocorticotropic hormone (ACTH), prolactin, follicle-stimulating hormone (FSH), luteinizing hormone, or thyroid-stimulating hormone secretion. It has also been shown to stimulate GH release from anesthetized rats in vivo. Ghrelin has an *n*-octanoyl group on the third amino acid, which appears to be necessary for biologic activity. Ghrelin is highly conserved between species, with rat and human ghrelin differing only by two amino acids *(5)*. Earlier in vitro and in vivo studies using synthetic GHSs suggested that they exert their effects primarily via the hypothalamus *( )*. However, GHSs also stimulate GH release directly from isolated rat or human pituitary *(7,8)*. We and others have shown the expression of GHS-R mRNA in normal human pituitary as well as in a variety of pituitary tumors *(6,9–12)*. A ligand arising from the hypothalamus might reach these pituitary receptors via the portal system; if the ligand is synthesized elsewhere, it may have access via the circulation. Locally synthesized ghrelin could have a direct effect on pituitary GHS-R. We shown the presence of mRNA synthesized in the pituitary: if protein synthesis also occurs, then local ghrelin might modulate the effect of hypothalamic regulators of GH release. There have been a number of examples in which hypothalamic peptides have been found to be synthesized in the pituitary including GHRH, corticotropin-releasing hormone, thyrotropin-releasing hormone, luteinizing hormone–releasing hormone, somatostatin, urocortin, and leptin *(13-30)*. It has been suggested that they may have a role in maintaining basal hormone synthesis and hormone output from the pituitary, and in maintaining optimal responsiveness of pituitary cells to their specific pulsatile releasing hormone arising from the hypothalamus *(18,19)*, but precise functions remain unclear.

In summary, we suggest that in addition to the probable hypothalamic effects of ghrelin, since we have shown that the peptide may be synthesized locally within the pituitary gland, it may have direct paracrine and autocrine effects on the release of GH.

Materials and Methods

Tissues

Rat pituitary was obtained from 200-g adult male Wistar rats. Human pituitary adenomas were obtained at the time of transsphenoidal surgery. The tumor type was determined on the basis of clinical and biochemical findings before surgery and morphologic and immunocytochemical data. Normal human pituitaries ($n = 4$) were collected at autopsy (4–24 h postmortem) from patients with no evidence of endocrine abnormality. A total of 22 pituitary adenomas was studied: 5 somatotroph adenomas, 2 lactotroph adenomas, 8 nonfunctioning pituitary adenomas, 4 corticotroph tumors, and 3 FSH-secreting adenomas.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted using a Promega SV RNA extraction kit (Promega, Southampton, UK), which includes a DNase treatment step. cDNA was manufactured from each sample using Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLVRT) (Life Technologies, Paisley, Renfrewshire, UK). The reaction mixture for each tube consisted of 10 μL of 5× first strand buffer, 2 μL of 0.1 *M* dithiothreitol (DTT) (buffer and DTT supplied with M-MLVRT), 2.5 μL of 20 μ*M* dNTP (Promega), 0.25 μL of 20

Table 1
Primer Sequences

Gene	Sense primer	Antisense primer	Product size (bp)
Human ghrelin[a]	5' CTGAGCCCTGAACACCAGAGA 3'	5' AGTTGCTGCAGAAGCAAGCGAA 3'	349
Rat ghrelin[a]	5' TTGAGCCCAGAGCACCAGAAA 3'	5' AGTTGCAGAGGAGGCAGAAGCT 3'	347
PIT-1[b]	5' AGTGCTGCCGAGTGTCTACCA 3'	5' TTTCTTTTCCTTTCATTTGCT 3'	560
GAPDH	5' CCATGGAGAAGGCTGGGG 3'	5' CAAAGTTGTCATGGATGACC 3'	195

[a]Human ghrelin primers correspond to the published rat ghrelin primers (*4*).
[b]Primers published by Haddad et al. (*5*).

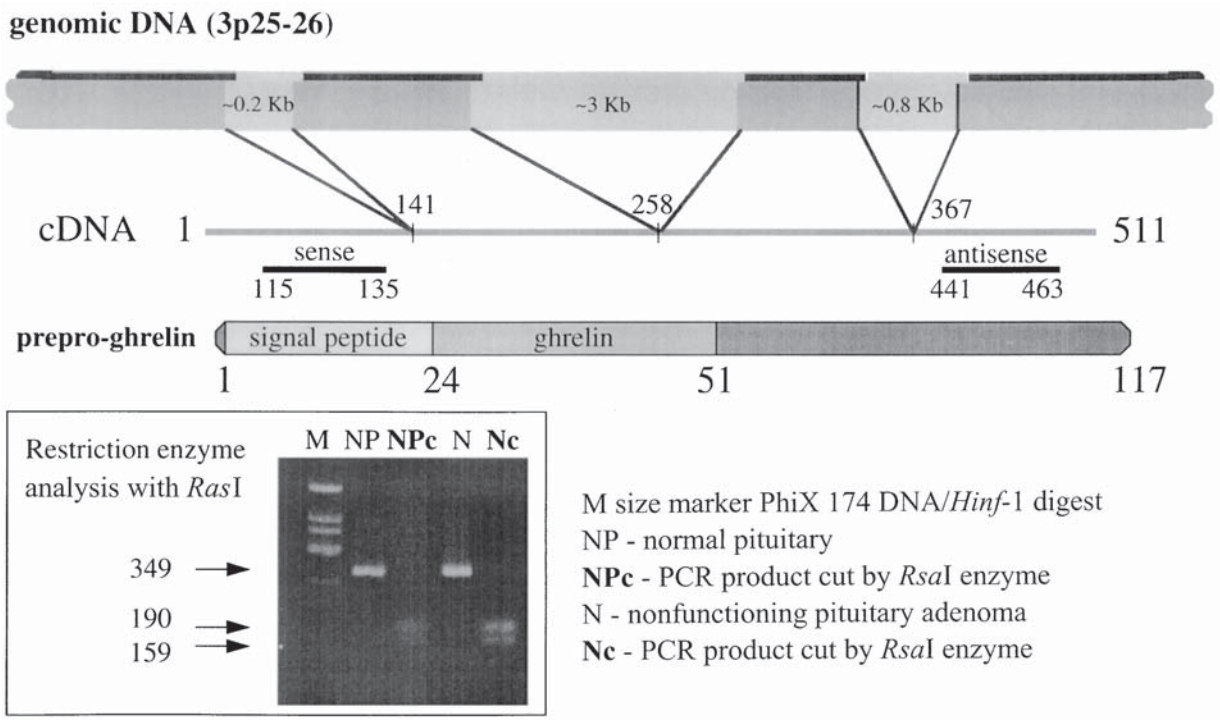

Fig. 2. Structure of the human ghrelin gene showing introns in the genomic DNA and the position of the applied primers. Restriction enzyme analysis of the PCR product of a normal human pituitary and of a non-functioning adenoma with the Ras I enzyme showing the expected 190 and 159 bp products.

mg/mL random hexamers (Boehringer Mannheim GmbH, Mannheim, Germany), 1 µL of 200 U/mL M-MLVRT, 0.1 µL of rRNasin (Promega), RNA stock solution containing 5 µg of RNA (heated to 65°C for 10 min), and water to a final volume of 50 µL. The program for the thermal cycler (Hybaid Omnigene; Hybaid, Teddington, Middlesex, UK) was 25ºC for 10 min, 37ºC for 60 min, and 92ºC for 10 min. The integrity of mRNA from each specimen was verified by reverse transcriptase polymerase chain reaction (RT-PCR) for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank accession no. M33197). RT-PCRs with omission of RT and with water replacing template were used as negative controls. The PCR was performed using primers spanning one or more introns of the genes studied to allow for exclusion of genomic DNA contamination (**Table 1**). Primers for rat ghrelin *(4)* (GenBank accession no. AB029433) and for a corresponding region on the human ghrelin gene (GenBank accession no. AB029434) gave rise to a product of 347 and 349 bp, respectively. The PCR products were analyzed by restriction enzyme analysis (**Fig. 2**), and the product obtained from human tissue also underwent direct sequencing, confirming the expected product.

For the PCR we used 5 µL of cDNA, 1.25 µL of 20 m*M* human ghrelin primers (supplied by Genosys), 0.5 µL of 20 m*M* deoxynucleotides, 0.125 µL of 20 m*M* GAPDH primers, QiaBuffer containing 1.5 mmol/L of $MgCl_2$, 0.125 U of *QiaHotstart* (Qiagen), and 5 µL of Q solution in a 25-µL reaction. Thirty-two cycles were performed at 94ºC for 1 min, 57ºC for 1 min, and 72ºC for 1 min after a denaturing cycle of 95ºC for 15 min. For the Pit-1 gene reaction, we used 0.25 µL of 20 m*M* primers; and 30 cycles were per-

formed at 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 45 s after a denaturing cycle of 95ºC for 3 min using Promega Taq enzyme (Promega). A final extension cycle of 10 min at 72ºC was used. The PCR products were run on ethidium bromide–stained 2% agarose gels.

Contamination of ACTH- and FSH-secreting tumors and nonfunctioning pituitary adenomas by somato-, lacto-, or thyrotroph cells of nontumorous tissue was excluded by confirming undetectable expression of the Pit-1 gene *(21,22).*

References

1. Dieguez, C., Page, M. D., and Scanlon, M. F. (1988). *Clin. Endocrinol. (Ox)* **28**, 109–143.
2. Korbonits, M. and Grossman, A. B. (1995). *Trends Endocrinol. Metab.* **6**, 43–49.
3. Howard, A. D., Feighner, S. D., Cully, D. F., et al. (1996). *Science* **273,** 974–977.
4. Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999). *Nature* **402**, 656–660.
5. Honda, K., Bailey, A. R. T., Bull, P. M., MacDonald, L. P., Dickson, S. L., and Leng, G. (1999). *Neuroscience* **90**, 875–883.
6. Smith, R. G., Van der Ploeg, L. H. T., Howard, A. D., et al. (1997). *Endocr. Rev.* **18**, 621–645.
7. Bowers, C. Y., Momany, F. A., Reynolds, G. A., and Hong, A. (1984). *Endocrinology* **114**, 1537–1545.
8. Adams, E. F., Buchfelder, M., Lei, T., et al. (1996). *In: Pituitary adenomas: From basic research to diagnosis and therapy* von Werder, K. and Fahlbusch, R. (eds.). pp. 43–47.Elsevier: Amsterdam.
9. deKeyzer, Y., Lenne, F., and Bertagna, X. (1997). *Eur. J. Endocrinol.* **137**, 715–718.
10. Adams, E. F., Huang, B., Buchfelder, M., et al. (1998). *J. Clin. Endocrinol. Metab.* **83**, 638–642.
11. Skinner, M. M., Nass, R., Lopes, B., Laws, E. R., and Thorner, M. O. (1998). *J. Clin. Endocrinol. Metab.* **83**, 4314–4320.
12. Shimon, I., Yan, X., and Melmed, S. (1998). *J. Clin. Endocrinol. Metab.* **83**, 174–178.
13. Thapar, K., Kovacs, K., Stefaneanu, L., et al. (1997). *Am. J. Pathol.* **151**, 769–784.
14. Levy, L., Bourdais, J., Mouhieddine, B., et al. (1993). *J. Clin. Endocrinol. Metab.* **76**, 85–90.
15. Lechan, R. M. and Jackson, I. M. D. (1982). *Endocrinology* **111**, 56–65.
16. Corchero, J., Fuentes, J. A., and Manzanares, J. (1999). *Life Sci.* **64**, 905–911.
17. Luo, L. G. and Jackson, I. M. (1998). *Peptides* **19**, 1295–1302.
18. Krsmanovic, L. Y., Martinez-Fuentes, A. J., Arora, K. K., et al. (2000). *Endocrinology* **141**, 1187–1195.
19. Wong, M. L., al-Shekhlee, A., Bongiorno, P. B., et al. (1996). *Mol Psychiatry* **1**, 307–312.
20. Morash, B., Li, A., Murphy, P. R., Wilkinson, M., and Ur, E. (1999). *Endocrinology* **140**, 5995–5998.
21. Haddad, G., Penabad, J. L., Bashey, H. M., et al. (1994). *J. Clin. Endocrinol. Metab.* **79**, 1399–1403.
22. Korbonits, M., Jacobs, R. A., Aylwin, S. J. B., et al. (1998). *J. Clin. Endocrinol. Metab.* **83**, 3624–3630.