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The Expression of the Growth Hormone Secretagogue Receptor Ligand Ghrelin in Normal and Abnormal Human Pituitary and Other Neuroendocrine Tumors*

MÁRTA KORBONITS, STEPHEN A. BUSTIN, MASAYASU KOJIMA, SUZANNE JORDAN, ERIC F. ADAMS, DAVID G. LOWE, KENJI KANGAWA, AND ASHLEY B. GROSSMAN

Department of Endocrinology (M.K., A.B.G.), Academic Department of Surgery (S.A.B.), and Department of Histopathology (S.J., D.G.L.), St. Bartholomew's and the Royal London School of Medicine and Dentistry, London, United Kingdom EC1A 7BE; Department of Biochemistry, National Cardiovascular Center Institute (M.K., K.K.), Suita, Osaka 565-8565, Japan; and Pharmaceutical Sciences Institute (E.F.A.), Aston University, Birmingham B4 7ET, United Kingdom

ABSTRACT

Ghrelin is a recently identified endogenous ligand of the GH secretagogue (GHS) receptor. It was originally isolated from the stomach, but has also been shown to be present in the rat hypothalamus. It is a 28-amino acid peptide with an unusual octanoylated serine 3 at the N-terminal end of the molecule, which is crucial for its biological activity. Synthetic GHSs stimulate GH release via both the hypothalamus and the pituitary, and the GHS receptor (GHS-R) has been shown by us and others to be present in the pituitary. We investigated whether ghrelin messenger ribonucleic acid (mRNA) and peptide are present in the normal human hypothalamus and in normal and adenomatous human pituitary.

RNA was extracted from pituitary tissue removed at autopsy and transsphenoidal surgery (n = 62), and ghrelin and GHS-R type 1a and 1b mRNA levels were investigated using real-time RT-PCR. Both ghrelin and GHS-R mRNA were detected in all samples. Corticotroph tumors showed significantly less expression of ghrelin mRNA, whereas GHS-R mRNA levels were similar to those in normal pituitary tissue. Gonadotroph tumors showed a particularly low level of expression of GHS-R mRNA. Immunohistochemistry, using a polyclonal antibody against the C-terminal end of the ghrelin molecule, revealed positive staining in the homolog of the arcuate nucleus in the human hypothalamus and in both normal and abnormal human pituitary. Pituitary tumor ghrelin peptide content was demonstrated using two separate RIA reactions for the N-terminal and C-terminal ends of the molecule. Both forms were present in normal and abnormal pituitaries, with 5 ± 2.5% octanoylated (active) ghrelin (mean ± SD) present as a percentage of the total. We suggest that the presence of ghrelin mRNA and peptide in the pituitary implies that the locally synthesized hormone may have an autocrine/paracrine modulatory effect on pituitary hormone release. (*J Clin Endocrinol Metab* **86:** 881–887, 2001)

THE REGULATION OF GH release from the pituitary is influenced by a number of hypothalamic, pituitary and circulating factors. GHRH and somatostatin are the two classical hypothalamic stimulatory and inhibitory regulators of pulsatile GH release, but free fatty acids, acetylcholine, amino acids, opiates, glucocorticoids, and other agents also have direct or indirect effects on GH release (1). In 1977, a group of synthetic small peptides were developed (2), originally derived from Met-enkephalin, which showed GH-releasing ability *in vitro* and *in vivo* (3) via both the pituitary and the hypothalamus, and in 1996 it was revealed that they acted via a specific G protein-coupled receptor: the GH secretagogue receptor (GHS-R) (4). Recently, an endogenous ligand has been described for this receptor and has been named ghrelin (5). This is a 28-amino acid peptide with an octanoyl group on the third N-terminal amino acid serine that appears to be essential for its biological activity. A splice variant of ghrelin, with 27 amino acids, has also been isolated and has been shown to have similar biological activity (6). Ghrelin was originally isolated from the stomach, but it is also expressed in a number of other tissues, including the hypothalamus, where the highest concentration of the GHS receptor (GHS-R) has been described (4). Synthetic GHSs exert their *in vivo* effects on the GH axis principally via the hypothalamus, although they are also able to stimulate GH release directly from the pituitary. We and others have previously demonstrated the presence of GHS-R messenger ribonucleic acid (mRNA) expression in normal and abnormal human pituitaries (7–12). We therefore investigated the expression of the newly recognized endogenous ligand in normal human hypothalamus and in normal and abnormal human pituitaries.

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Address all correspondence and requests for reprints to: Ashley Grossman, M.D., Department of Endocrinology, St. Bartholomew's Hospital, West Smithfield, London, United Kingdom EC1A 7BE. E-mail: a.b.grossmann@mds.qmw.ac.uk.

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Materials and Methods

Tissue samples

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 morphological and immunocytochemical data. All patients with Cushing's disease were treated with cortisol-lowering drugs for 6–8 weeks before surgery to produce mean serum cortisol levels within the normal range. Patients were routinely treated with hydrocortisone (100 mg, im) with their

premedication before surgery. Normal human pituitaries (n = 7) were also collected at autopsy (4–24 h postmortem) from patients with no evidence of endocrine abnormality. A total of 55 pituitary adenomas were studied for ghrelin mRNA expression: 22 somatotroph adenomas, 4 lactotroph adenomas, 12 nonfunctioning pituitary adenomas (NFPAs), 12 corticotroph tumors, and 5 FSH-secreting adenomas (Table 1A). Several pituitaries were also extracted for C- and N-terminal ghrelin measurement: 3 somatotroph adenomas, 2 lactotroph adenomas, 6 NFPAs, 1 corticotroph tumor, 1 FSH-secreting adenoma, 1 TSH-secreting adenoma, and a normal pituitary collected at autopsy. In addition, nonpituitary neuroendocrine tumors removed at surgery were studied: 2 ACTH-secreting ectopic tumors (of the thymus and pancreas), 3 pancreatic insulinomas, and a pancreatic gastrinoma (Table 1B). A normal human stomach biopsy sample was removed at gastroscopy, and a

TABLE 1a. Clinical details of patients with pituitary tumors

Diagnosis	Age (yr)	Sex	Size	Immunohistochemistry	Real-time PCR copy no./μg RNA			Duplex PCR GHS-R1a/GAPDH ratio
					Ghrelin	GHS-R1a	GHS-R1b	
Somatotroph	53	M	Macro	GH	9.2×10^5	4.9×10^{7a}	1.7×10^{6a}	N/A
Somatotroph	26	M	Macro	GH, TSH, αSU	2.0×10^5	1.2×10^8	1.9×10^7	1.16
Somatotroph	34	M	Macro	GH	4.5×10^4	2.6×10^7	1.3×10^7	1.5
Somatotroph	29	F	Macro	GH, PRL, LH	1.7×10^6	3.6×10^7	1.8×10^7	1.2
Somatotroph	44	M	Macro	GH, PRL, LH, FSH, αSU	7.4×10^5	2.2×10^7	2.3×10^7	1.4
Somatotroph	22	M	Macro	GH	7.9×10^4	1.4×10^7	7.2×10^6	1.03
Somatotroph	24	F	Macro	GH, (PRL, αSU)	5.4×10^4	3.3×10^7	7.3×10^6	1.18
Somatotroph	30	M	Macro	GH, PRL	8.0×10^5	3.0×10^7	1.8×10^7	N/A
Somatotroph		M	Macro	GH, PRL	7.9×10^4	2.5×10^{5a}	4.3×10^{5a}	N/A
Somatotroph	61	M	Macro	GH	5.4×10^5	5.4×10^{6a}	3.2×10^{5a}	N/A
Somatotroph	47	M	Macro	GH	2.6×10^5	2.2×10^{6a}	3.1×10^{5a}	N/A
Somatotroph	61	F	Macro	GH, PRL	8.5×10^5	5.3×10^{6a}	1.7×10^{6a}	N/A
Somatotroph	64	F	Micro	GH	7.8×10^4	2.0×10^5	3.4×10^{5a}	N/A
Somatotroph	34	M	Macro	GH, (LH, TSH)	3.5×10^5	7.8×10^{6a}	8.0×10^{5a}	N/A
Somatotroph	40	M	Micro	GH, LH, FSH	3.3×10^5	4.5×10^{6a}	4.1×10^{5a}	N/A
Somatotroph	28	F	Macro	GH	3.8×10^5	2.6×10^{6a}	6.6×10^{5a}	N/A
Somatotroph	66	M	Micro	GH	2.8×10^5	3.8×10^{6a}	9.5×10^{5a}	N/A
Somatotroph	44	M	Macro	GH	4.6×10^5	1.8×10^{6a}	1.0×10^{6a}	N/A
Somatotroph	66	M	Macro	GH	1.4×10^5	9.9×10^{5a}	3.0×10^{6a}	N/A
Somatotroph	44	F	Macro	GH	5.4×10^5	7.4×10^{6a}	3.0×10^{6a}	N/A
Somatotroph	48	M	Macro	GH	1.0×10^6	1.8×10^{7a}	1.4×10^{6a}	N/A
Somatotroph	52	F	Macro	GH	9.8×10^5	6.9×10^{6a}	9.4×10^{5a}	N/A
Corticotroph	28	F	Micro	ACTH	6.0×10^4	6.5×10^{2a}	0.0	N/A
Corticotroph	50	F	Micro	ACTH	0.0	6.0×10^6	0.0	N/A
Corticotroph	25	F	Micro	ACTH	8.1×10^5	2.4×10^{6a}	4.8×10^6	N/A
Corticotroph	25	F	Micro	ACTH	5.6×10^4	6.8×10^7	6.3×10^6	1.1
Corticotroph	28	F	Macro	ACTH	1.4E+02	6.2×10^6	0.0	0
Corticotroph	69	F	Macro	ACTH	6.2×10^4	5.2×10^7	2.2×10^6	0.56
Corticotroph	26	F	Macro	ACTH	3.3×10^3	3.4×10^5	1.0×10^7	0.24
Corticotroph	60	F	Macro	ACTH	1.9×10^4	2.0×10^7	2.9×10^6	0.53
Corticotroph	64	F	Macro	ACTH	1.40×10^5	1.10×10^8	4.70×10^6	0.25
Corticotroph	49	M	Macro	ACTH	2.5×10^3	1.1×10^6	1.1×10^7	0.58
Corticotroph	31	M	Macro	ACTH	1.2×10^4	2.9×10^6	1.6×10^5	0.1
Corticotroph	74	F	Macro	FSH, (αSU)	1.0×10^5	4.4×10^6	6.8×10^6	0.6
Gonadotroph	74	M	Macro	All negative	4.7×10^5	1.1×10^5	0.0	0
Gonadotroph	59	M	Macro	FSH, αSU, βHCG	1.1×10^5	1.3×10^4	0.0	N/A
Gonadotroph	16	M	Macro	(αSU)	6.8×10^4	5.0×10^5	2.1×10^5	N/A
Gonadotroph	73	M	Macro	LH, FSH, αSU	3.2×10^5	2.0×10^5	0.0	1.13
Gonadotroph	64	M	Macro	All negative	2.5×10^5	1.3×10^5	0.0	0
NFPA	38	M	Macro	(LH, αSU)	1.8×10^5	6.8×10^4	0.0	0
NFPA	80	F	Macro	LH, FSH, αSU	3.1×10^5	6.2×10^5	0.0	0
NFPA	70	F	Macro	All negative	4.2×10^5	1.4×10^7	N/A	0
NFPA	82	M	Macro	All negative	5.6×10^4	6.3×10^7	9.3×10^6	2.2
NFPA	68	M	Macro	All negative	3.5×10^5	3.1×10^5	0.0	0
NFPA	41	M	Macro	LH, h-CGβ	1.7×10^6	1.2×10^5	0.0	0
NFPA	66	F	Macro	All negative	4.6×10^4	4.4×10^4	0.0	N/A
NFPA	50	M	Macro	All negative	6.0×10^5	1.8×10^{7a}	2.6×10^7	N/A
NFPA	55	F	Macro	All negative	9.6×10^5	3.0×10^{3a}	0.0	N/A
NFPA	61	F	Macro	All negative	8.8×10^3	1.8×10^{5a}	4.3×10^3	N/A
NFPA	60	F	Macro	All negative	3.6×10^5	6.3×10^4	1.1×10^4	N/A
NFPA	50	M	Macro	PRL, TSH, (GH)	6.8×10^6	$4.6E+01^a$	0.0	N/A
Lactotroph	36	M	Macro	PRL	1.0×10^5	6.3×10^7	2.2×10^7	1.2
Lactotroph		M	Macro	PRL, αSU	1.6×10^4	5.0×10^4	0.0	0.33
Lactotroph	23	F	Micro	PRL	4.4×10^4	2.2×10^5	0.0	0.1
Lactotroph	61	M	Macro	PRL	9.1×10^5	3.9×10^{6a}	1.3×10^6	N/A

Macro, Pituitary adenoma diameter greater than 10 mm on MRI; micro, pituitary adenoma diameter less than 10 mm on MRI; αSU, α-subunit. Hormone names in *parentheses* suggest low levels of hormone staining. N/A, Not available. For details regarding the data for GHS-R1a/GAPDH ratio, please refer to Ref. 7.

[a] These values were not included in the statistical analysis because they were obtained with a slightly different assay protocol.

TABLE 1b. Clinical details of patients with nonpituitary tumors

Diagnosis	Age (yr)	Sex	Immunocytochemistry	Real-time PCR copy number/μg RNA			Duplex PCR GHS-R1a/ GAPDH ratio
				Ghrelin	GHS-R1a	GHS-R1b	
Ectopic ACTH-secreting tumor thymus	55	M	ACTH, chromogranin	5.6E + 05	1.1E + 04	0.0	Min
Ectopic ACTH-secreting tumor pancreas, metastatic	66	F	ACTH, chromogranin	1.8E + 04	5.3E + 05	0.0	Min
Gastrinoma, metastatic, MEN 1	42	F	Gastrin, chromogranin, glucagon, PPP, (insulin)	2.8E + 03	5.2E + 04	0.0	0.1
Insulinoma MEN 1	26	F	Insulin, VIP	5.7E + 04	2.3E + 06	2.8E + 04	Min
Insulinoma, metastatic	57	M	Insulin	1.4E + 06	1.6E + 04	0.0	Min
Insulinoma	69	M	Insulin, chromogranin, (somatostatin)	8.7E + 05	2.1E + 04	0.0	0.18

MEN 1, Multiple endocrine neoplasia type 1; PPP, pancreatic polypeptide; VIP, vasoactive intestinal polypeptide.

TABLE 2. Primers and probes used in PCR reactions

	Probe	Forward primer	Reverse primer	Amplicon size (bp)
Ghrelin (GenBank accession no. AB029434)	TCCGGTTCAACGCCCCCTTTG	GGGCAGAGGATGAACTGGAA	CCTGGCTGTGCTGCTGGTA	90
GHS-R 1a (GenBank accession no. U60179)	AGGGACCAGAACCACAAGCAAACCG	TCGTGGGTGCCTCGCT	CACCACTACAGCCAGCATTTTC	65
GHS-R 1b (GenBank accession no. U60181)	AGGGACCAGAACCACAAGCAAACCG	TCGTGGGTGCCTCGCT	GCTGAGACCCACCCAGCA	66
GAPDH (GenBank accession no. J04038)	CAAGCTTCCCGTTCTCAGCC	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226

normal human hypothalamus was collected at autopsy. All studies received institutional ethical approval.

RNA preparation

Total RNA was prepared using the SV isolation kit (Promega Corp., Southampton, UK), which includes a deoxyribonuclease step. RNA was quantified by spectrophotometry (Cecil CE5501 Computing Double Beam UV Spectrophotometer, Cecil Instruments Ltd., Cambridge, UK). RNA was diluted to 100 ng/μL for use in the RT-PCR assay and was stored at −50 C.

Primers and probes

RT-PCR primers and probes were designed for human ghrelin, GHS-R types 1a and 1b, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using Primer Express software (PE Applied Biosystems, Warrington, UK) based on the sequence data of the genes available in GenBank (Table 2). In each case, the primers were designed to cross exon-intron boundaries. The probes were labeled with a fluorescent dye (6-carboxy-fluorescein) and a quencher dye (6-carboxy-tetramethylrodamine). Primers and probes were purchased from PE Applied Biosystems.

Quantitative RT-PCR

The assay relies on the nucleolytic activity of the polymerase displacing and cleaving any probe annealed to the amplicon after target amplification (13). After target amplification, the probe anneals to the amplicon and is displaced and cleaved between the reporter and the quencher dyes by the nucleolytic activity of the polymerase. The amount of product resulting in detectable fluorescence at any given cycle within the exponential phase of the PCR is proportional to the initial number of the template copies. The number of PCR cycles (threshold cycles, C_t) needed to detect the amplicons is therefore a direct measure of the template concentration (Fig. 1). We used a one-enzyme/one-tube reaction protocol as previously described (14), except that the RT step was reduced to 15 min. In short, the RT-PCR reaction conditions were the following: 25 μL reaction mixture including 1 μL RNA template were heated for 15 min at 50 C in the presence of 0.01 U/μL AmpErase UNG (PE Applied Biosystems). After RT for 15 min at 60 C, the reaction mixture was denatured at 92 C for 5 min followed by 40 cycles of PCR at 92 C for 20 s and at 62 C for 1 min in each cycle in the presence of the 6-carboxy-fluorescein-labeled oligonucleotide probe. The RT-PCR reactions were performed, recorded, and analyzed by using the ABI7700 Prism Sequence Detection System (PE Applied Biosystems).

Absolute mRNA copy number quantitation

Quantitation of mRNA samples was carried out by relating the PCR threshold cycle obtained from tissue samples to amplicon-specific standard curves (15). Serial dilutions of the single stranded sense oligodeoxynucleotide amplicons were carried out in duplicate from 1×10^8 molecules to 10 molecules and used in triplicate RT-PCR reactions. Standard curves were obtained by plotting the log [calculated copy number] against the threshold cycle. The log copy numbers (N) of unknown samples were calculated from the regression line according to the formula: $\log N = (C_T - b)/m$, where C_T is the threshold cycle, b is the *y*-intercept, and m is the slope of the standard curve line. As normalization to the GAPDH housekeeping gene is inaccurate, mRNA expression levels are presented as the mRNA copy number per μg total RNA. Any copy number under 3000 copies/μg total RNA was assumed to be due to illegitimate transcription (16, 17).

Quality standards for RT-PCR

All serial dilutions were carried out in duplicate. The reactions to generate standard curves were repeated twice, each time in triplicate. All clinical samples were tested in duplicate. As spectrophotometric analysis alone may not accurately reflect either the quality of isolated nucleic acids or its performance in a subsequent RT-PCR analysis, amplification of GAPDH mRNA was used as a standard for the quality of the RNA samples investigated. Samples negative for GAPDH were excluded from quantification. Two no-template controls were included with every amplification run; one was prepared before opening all the tubes and dispensing the various reagents, and the other was prepared at the end of the experiment. This allowed us to monitor any contamination arising during the handling of the reagents. Contamination of ACTH- and FSH-secreting tumors and NFPAs by somato-, lacto-, or thyrotroph cells of nontumorous tissue was excluded by confirming undetectable expression of the Pit-1 gene, as described previously (7, 18).

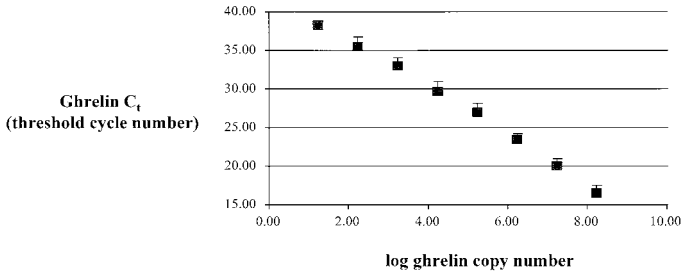

FIG. 1. Standard curve for ghrelin. The threshold cycle number is plotted against the amount of standard added to the reaction. An unknown sample copy number could be calculated using the regression line according to the formula: $\log N = (C_T - b)/m$, where C_T is the threshold cycle, b is the *y*-intercept, and m is the slope of the standard curve line.

Protein extraction

Pituitary samples were boiled in 5 mL water for 5 min. After cooling the samples on ice, AcOH and HCl were added to final concentrations of 1 N and 20 mmol/L, respectively. After homogenizing by Polytron (Brinkmann Instruments, Inc., Westbury, NY), the supernatant was collected, and the precipitate was extracted again. Both fractions were applied to a Sep-Pak Plus column (Waters Corp., Milford, MA). After lyophilization, the samples underwent two RIA reactions (see below).

Ghrelin RIA

Tissue ghrelin content was determined by two separate RIA reactions. The first one uses a polyclonal rabbit antibody (no. 3–7) against the C-terminal part of the molecule [[Cys]ghrelin-(13–28)]; it, therefore, recognizes both the octanoylated (active) form of ghrelin and the non-octanoylated (inactive) form of both human and rat ghrelin (19). The second RIA reaction uses another polyclonal rabbit antibody (no. 4–4), which was raised against the N-terminal of the ghrelin molecule [rat ghrelin-(1–11)] with *n*-octanoyl modification at serine 3) and recognizes the octanoylated form of the peptide in both human and rat. Ghrelin peptide without the *n*-octanoyl modification (des-acyl ghrelin) is not recognized by this antibody. [^{125}I]Tyr-ghrelin-(13–28) and [^{125}I]Tyr^{29}-ghrelin were used as tracers in the C- and N-terminal RIAs, respectively. The minimal detectable concentration is 10 fmol/tube for the C-terminal assay and 0.5 fmol/tube for the N-terminal RIA.

Immunohistochemistry

Paraffin sections (3 μm) of human stomach, hypothalamus, and pituitary samples were air-dried, then placed in a 60 C oven overnight. Sections were dewaxed in xylene, followed by immersion into a solution of 750 μL 30% hydrogen peroxide and 50 mL methanol for 10 min to block endogenous peroxide. Sections were rehydrated in tap water, ready for antigen retrieval. Sections required heat-mediated antigen retrieval treatment (20). Sections were superheated for 4 min in citrate buffer 0.01 mol/L, pH 6.0, then placed into tap water immediately to avoid drying of sections. The sections were then transferred to phosphate-buffered saline (PBS) before immunostaining. Immunohistochemistry was performed using a standard avidin-biotin complex (ABC) method. The primary antibody (no. 3–7, see above) was placed on sections at a 1:5000 dilution for 40 min at room temperature in a wet chamber. Sections were washed in PBS and then incubated in a biotinylated antirabbit second layer for 30 min. Sections were again washed in PBS and then incubated in the ABC (Vectastain Elite ABC peroxide kit PK6200 Vector Laboratories, Inc., Peterborough, UK) for 20 min. After three washes in PBS, sections were visualized with activated 3′,3′-diaminobenzidene-tetrahydrochloride solution (Kentec DAB tablets 4170, Biostat, Stockport, UK) for 10 min; this resulted in a brown end-product. Sections were counterstained with Gill's hematoxylin and dehydrated through graded alcohol before mounting in DPX. Specificity of the ghrelin staining was assessed initially by preabsorption of the antibody with the full-length human ghrelin peptide; this completely abolished ghrelin staining.

Immunostaining of other hormones

Pituitary hormone stains were performed to establish the histological hormone phenotype of the tumor samples. GH, ACTH, PRL, TSH, LH, FSH, gastrin, chromogranin, glucagon, insulin, and hCGβ antibodies were supplied by DAKO Corp. (Oxford, UK), whereas pancreatic polypeptide and vasoactive intestinal polypeptide antibodies were supplied by Novacastra (Peterborough, UK). Sections were incubated overnight in the primary antibody at 4 C. The standard ABC protocol was followed thereafter. For pituitary staining a normal human pituitary was used as a positive control; the hormone antibody was omitted and replaced by mouse Ig as a negative control.

Statistical analysis

The data were analyzed by a nonparametric ANOVA test, the Kruskal-Wallis test, using the Arcus Quickstat Biomedical version 1.2 (Buchan I, Addison Wesley Longman Ltd., Cambridge, UK). Significance was taken at $P < 0.05$.

Results

Ghrelin expression

Ghrelin was expressed in normal pituitary and in adenomatous pituitary tissue at both the mRNA and peptide level (Figs. 2 and 3). Using real-time PCR we detected 2.5×10^3 to 7×10^6 copies of ghrelin mRNA molecules/μg total RNA, with a significant difference among the different pituitary tissue types ($P = 0.0042$). Specifically, corticotroph tumors showed a significantly lower level of mRNA expression compared with normal pituitary (Fig. 2), with one sample showing a completely absent expression of ghrelin mRNA. In patients with acromegaly we studied the relationship of circulating GH levels before transsphenoidal surgery and the level of ghrelin mRNA expression in the tumors, but found no significant correlation. Ectopic ACTH-secreting tumors and insulinomas expressed ghrelin at copy numbers of 2×10^4 to 5×10^5 and 6×10^4 to 1×10^6, respectively, whereas the gastrinoma expressed low levels of ghrelin mRNA (3×10^3).

We detected very strong positive ghrelin immunostaining in human stomach tissue, both at 1:5,000 and 1:10,000 ghrelin antibody concentrations (Fig. 3, *upper left panel*). Ghrelin peptide was also detected in human hypothalamus in the arcuate (infundibular) nucleus, whereas the other hypothalamic nuclei did not stain positively (Fig. 3, *upper right* and *lower left panels*). Normal pituitary also stained positively for ghrelin, although only weakly, in some, but not all, cells (Fig. 3, *lower right panel*). Positive staining was also seen in a somatotroph adenoma, an NFPA, and a prolactinoma, whereas a corticotroph adenoma was negative. Human stomach was used as a positive control, and full-length human ghrelin pretreatment blocked staining in both pituitary and hypothalamus.

A subset of pituitary samples was extracted and assayed

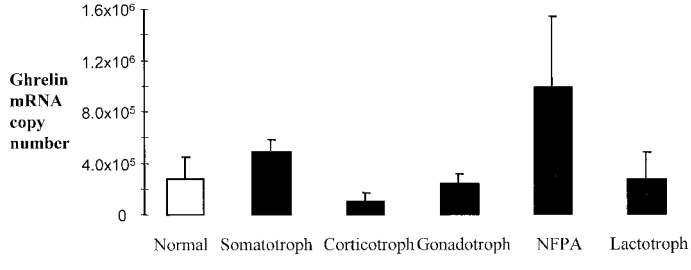

FIG. 2. Ghrelin mRNA expression in pituitary samples (mean ± SEM).

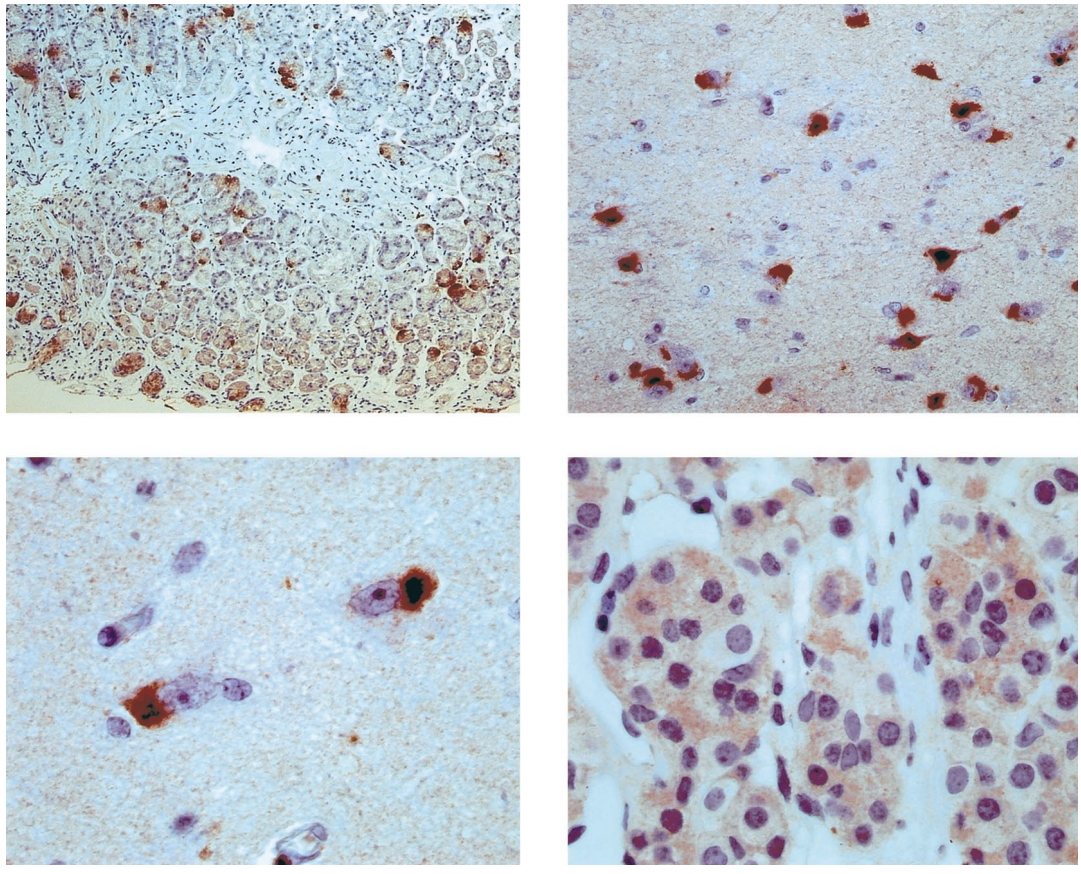

FIG. 3. Ghrelin immunostaining. The nuclei are counterstained with Gill's hematoxylin. *Upper left panel*, Intense cytoplasmic ghrelin immunostaining (*brown*) in human stomach tissue. Magnification, ×100. *Upper right panel*, Intense cytoplasmic ghrelin immunostaining in human hypothalamic neurons in the arcuate nucleus. Magnification, ×400. *Lower left panel*, Two ghrelin-containing cell in the arcuate nucleus with intense granular staining suggestive of vesicular storage. Magnification, ×1000. *Lower right panel*, Weak cytoplasmic ghrelin immunostaining in normal human pituitary with occasional cells showing negative staining. Magnification, ×1000.

TABLE 3. Ghrelin content of pituitary samples

	C-Terminal RIA (pmol/g)	N-Terminal RIA (pmol/g)	N-Terminal as % of C-Terminal
Somatotroph tumor	0.88	0.03	3.94
Somatotroph tumor	1.40	0.07	4.83
Somatotroph tumor	2.07	0.05	2.63
NFPA	2.78	0.10	3.48
NFPA	3.59	0.43	12.01
NFPA	0.42	0.04	8.88
NFPA	0.57	0.03	4.98
NFPA	0.52	0.04	6.92
NFPA	0.90	0.02	2.47
Lactotroph tumor	1.62	0.06	3.64
Lactotroph tumor	1.90	N/A	N/A
Corticotroph tumor	1.60	0.06	3.44
Gonadotroph tumor	1.50	0.06	4.22
Thyrotroph tumor	2.81	0.20	7.22
Normal pituitary	2.10	0.18	8.38

N/A, Not available.

for ghrelin content with two RIAs (Table 3). Ghrelin peptide was present in normal and adenomatous pituitary tissue, in both octanoylated and des-octanoylated forms. The C-terminal RIA (which measures both octanoylated and des-octanoylated ghrelin together) showed an average of 1.64 ± 0.93 (mean ± SD) pmol/g tissue peptide content, whereas the

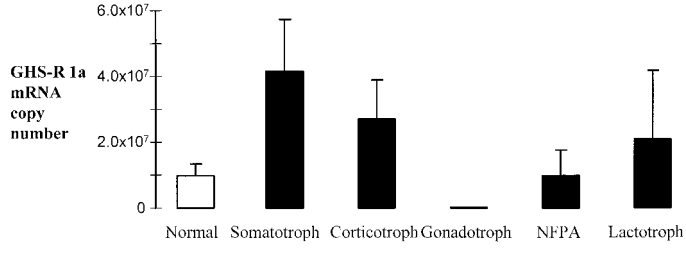

FIG. 4. GHS-R type 1a mRNA expression in pituitary tumors (mean ± SEM).

N-terminal assay (which measures octanoylated ghrelin alone) showed 0.10 ± 0.11 pmol/g peptide content, suggesting that 5.5 ± 2.8% of the total ghrelin content in these samples is octanoylated, *i.e.* biologically active.

GHS receptor expression

GHS-R 1a expression was detected in all pituitary samples, with significant differences between the different tumor types ($P = 0.0057$; Fig. 4). Somatotroph tumors showed the highest level of expression of GHS-R type 1a, whereas FSH-omas showed only a very low level of expression (Fig. 4). Corticotroph tumors expressed similar amounts of GHS-R type 1a as normal pituitaries; however, the ratio of GHS-R to

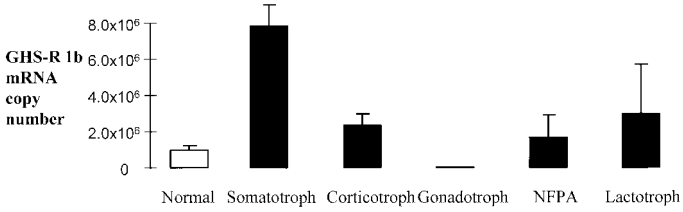

FIG. 5. GHS-R type 1b mRNA expression in pituitary tumors (mean ± SEM).

ghrelin expression was about 60 times higher in this tumor type compared with other tumor samples, significantly different from the ratio in normal pituitary and other tumor types. All of the nonpituitary neuroendocrine tumors expressed GHS-R type 1a. Previously, we reported GHS-R type 1a expression in pituitary and neuroendocrine tumors using a semiquantitative duplex RT-PCR comparing GHS-R expression to the housekeeping gene GAPDH (7). When we compared our current quantitative results with our previous data on a subset of samples (n = 30), there was a close correlation between the two datasets (Spearman correlation coefficient = 0.76; $P < 0.001$).

GHS-R type 1b mRNA was also detected in pituitary samples, with significant differences between the different groups. All normal pituitaries and somatotroph adenomas showed expression of GHS-R type 1b, but somatotroph adenomas had a significantly higher GHS-R type 1b mRNA copy number compared with normal subjects ($P = 0.02$; Fig. 5). Two of the 12 corticotroph, 3 of the 5 gonadotroph, 6 of the 12 nonfunctioning, and 2 of the 4 lactotroph adenomas were negative for GHS-R type 1b mRNA expression (Fig. 5). None of the neuroendocrine tumors expressed GHS-R type 1b, other than one insulinoma.

The absolute amounts of ghrelin and GHS-R type 1a and 1b mRNA did not correlate in the samples when analyzed as a single group or according to the different tumor subtypes.

Discussion

This study showed that ghrelin mRNA and peptide are expressed in normal and adenomatous human pituitary tissue. We used real-time quantitative PCR to detect ghrelin mRNA levels in pituitary and other neuroendocrine tumors and compared the expression with that of the GHS-R. Normal pituitaries included samples from patients over a wide age range (1 day to 54 yr), all of whom expressed ghrelin mRNA. Pituitary tumors arising from somatotroph, corticotroph, lactotroph, and gonadotroph cells as well as nonfunctioning adenomas also showed expression of ghrelin mRNA. We demonstrated that ghrelin expression is relatively low in corticotroph adenomas and is relatively high in somatotroph adenomas compared with that in normal tissue. The present quantitative measurements suggest that somatotroph tumors also have high levels of GHS-R expression. In addition, our finding of a particularly high ratio of receptor to ghrelin expression in corticotroph tumors may in part relate to up-regulation of GHS-R by glucocorticoids, as recently demonstrated in the rat hypothalamus (21, 22).

We also showed that ghrelin mRNA is transcribed into peptide, as cytoplasmic ghrelin immunoreactivity was seen in normal and abnormal human pituitary tissue. Initially, we detected ghrelin in human stomach. We also observed ghrelin immunostaining in the human hypothalamus; specifically, many neurons in the human homolog of the arcuate nucleus showed strong cytoplasmic staining, primarily in a granular pattern suggestive of vesicular storage, whereas other hypothalamic nuclei did not contain ghrelin-positive cells. The arcuate nucleus has been identified as a site with one of the highest concentrations of the GHS-R (21, 22), and it is thus possible that locally produced ghrelin might activate these receptors (23). Ghrelin immunoreactivity was present in both normal and certain adenomatous pituitary samples. In the normal pituitary, weak cytoplasmic staining was seen in the majority of the cells; however, some pituitary cells clearly showed no ghrelin positivity. Somatotroph, lactotroph, and nonfunctioning adenomas also showed weak ghrelin staining, whereas corticotroph adenomas showed negative staining. This correlates with our findings at the mRNA level, where, again, corticotroph adenomas expressed the lowest level of ghrelin mRNA. As the antibody used for the immunohistochemistry recognizes both octanoylated and des-octanoylated ghrelin, 15 normal and adenomatous pituitary samples underwent protein extraction and RIA reactions for both octanoylated and des-octanoylated ghrelin. The data suggest that 5% of the total ghrelin content of the pituitary samples is in an active from.

Ghrelin is a 28-amino acid peptide that has been isolated from the rat stomach, but has also been shown to be present in other tissues in the rat, including the hypothalamic arcuate nucleus (24). It has been shown to specifically activate GHS-R and stimulate GH release *in vitro*, but has no direct effect on ACTH, PRL, FSH, LH, or TSH secretion. It has also been shown to stimulate GH release from anesthetized rats. Ghrelin has an *n*-octanoyl group on the third amino acid, which appears to be necessary for biological activity. Ghrelin is highly conserved between species; rat and human ghrelins differ only by two amino acids (24). Earlier *in vitro* and *in vivo* studies using synthetic GHSs suggested that they exert their effects primarily via the hypothalamus (25, 26). However, GHSs also stimulate GH release directly from isolated rat or human pituitary (3, 27). We and others have shown the expression of GHS-R mRNA in normal human pituitary and in a variety of pituitary tumors (7–10, 12). The endogenous ligand, ghrelin, arising from the hypothalamus might reach these pituitary receptors via the portal system; if the ligand is synthesized elsewhere, however, it may also have access to the peripheral circulation. Locally synthesized ghrelin could also have a direct paracrine or autocrine effect via pituitary GHS-R.

Comparing our current data on GHS-R expression with our earlier data on the same samples using duplex RT-PCR (7), we found a significant correlation between the two datasets. However, although a number of samples showed no expression even at high PCR cycle numbers with the earlier method, using the real-time PCR method we were able to detect a low level of mRNA expression in the same samples. It is known that the latter method is more sensitive and especially provides a more reliable and reproducible quantitative mRNA expression data (17). The lack of an ideal housekeeping gene (with the same level of expression in all tissue types at all circumstances) renders the assessment of duplex PCR results more difficult (17).

Nevertheless, the results of the two different techniques showed a good correlation in our hands.

For many years the pituitary gland has been considered to consist of different highly specialized cell types, each producing a specific hormone and each responding to specific hypothalamic hypophysiotropic hormones and peripheral hormones. However, today there is a large body of evidence supporting the hypothesis of paracrine/autocrine regulation in the pituitary (for reviews, see Refs. 28–30). Pituitary hormones themselves can have local effects, but classical hypothalamic hormones, such as GHRH, CRH, TRH, LHRH, somatostatin, urocortin, and even leptin, as well as cytokines have been shown to be synthesized in the pituitary and to modulate pituitary function (31–38). It has been suggested that they may play a role in supporting basal hormone synthesis and/or hormone output from the pituitary and in maintaining the optimal responsiveness of pituitary cells to their specific pulsatile releasing hormone arising from the hypothalamus (36, 37); however, their precise functions remain unclear. It now seems likely that ghrelin may also play a role in such activity, although the specific functional importance of this peptide in the pituitary and any possible role in tumorigenesis must await functional analysis.

In summary, we have demonstrated the presence of ghrelin mRNA and peptide in the human pituitary, and we speculate that in addition to the probable hypothalamic effects of ghrelin, locally produced ghrelin in the pituitary gland may have direct paracrine and/or autocrine effects on pituitary function.

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References

1. **Dieguez C, Page MD, Scanlon MF.** 1988 Growth hormone neuroregulation and its alterations in disease states. Clin Endocrinol (Oxf). 28:109–143.
2. **Momany FA, Bowers CY, Reynolds GA, Chang D, Hong A, Newlander K.** 1981 Design, synthesis and biological activity of peptides which release growth hormone *in vitro*. Endocrinology. 108:31–39.
3. **Bowers CY, Momany FA, Reynolds GA, Hong A.** 1984 On the *in vitro* and *in vivo* activity of a new synthetic heptapeptide that acts on the pituitary to specifically release growth hormone. Endocrinology. 114:1537–1545.
4. **Howard AD, Feighner SD, Cully DF, et al.** 1996 A receptor in pituitary and hypothalamus that functions in growth hormone release. Science. 273:974–977.
5. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K.** 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 402:656–660.
6. **Hosoda H, Kojima M, Matsuo H, Kangawa K.** 2000 Purification and characterisation of rat des-Glu^{14}-ghrelin, a second endogenous ligand for growth hormone secretagogue receptor. J Biol Chem. 275:21995–22000.
7. **Korbonits M, Jacobs RA, Aylwin SJB, et al.** 1998 Expression of the growth hormone secretagogue receptor in pituitary adenomas and other neuroendocrine tumors. J Clin Endocrinol Metab. 83:3624–3630.
8. **deKeyzer Y, Lenne F, Bertagna X.** 1997 Widespread transcription of the growth hormone-releasing peptide receptor gene in neuroendocrine human tumors. Eur J Endocrinol. 137:715–718.
9. **Shimon I, Yan X, Melmed S.** 1998 Human fetal pituitary expresses functional growth hormone-releasing peptide receptors. J Clin Endocrinol Metab. 83:174–178.
10. **Adams EF, Huang B, Buchfelder M, et al.** 1998 Presence of growth hormone secretagogue receptor messenger ribonucleic acid in human pituitary tumors and rat GH(3) cells. J Clin Endocrinol Metab. 83:638–642.
11. **Nielsen S, Mellemkjaer S, Rasmussen LM, et al.** 1998 Gene transcription of receptors for growth hormone-releasing peptide and somatostatin in human pituitary adenomas. J Clin Endocrinol Metab. 83:2997–3000.
12. **Skinner MM, Nass R, Lopes B, Laws ER, Thorner MO.** 1998 Growth hormone secretagogue receptor expression in human pituitary tumors. J Clin Endocrinol Metab. 83:4314–4320.
13. **Heid CA, Stevens J, Livak KJ, Williams PM.** 1996 Real time quantitative PCR. Genome Res. 6:986–994.
14. **Bustin SA, Gyselman VG, Williams NS, Dorudi S.** 1999 Detection of cytokeratins 19/20 and guanylyl cyclase C in peripheral blood of colorectal cancer patients. Br J Cancer. 79:1813–1820.
15. **Li SR, Gyselman VG, Lalude O, Dorudi S, Bustin SA.** 2000 Transcription of the inositol polyphosphatase 1-phosphatase gene (INPP1) is upregulated in human colorectal cancer. Mol Med Today. 27:322–327.
16. **Chelly J, Concordet JP, Kaplan JC, Kahn A.** 1989 Illegitimate transcription: transcription of any gene in any cell type. Proc Natl Acad Sci USA. 86:2617–2621.
17. **Bustin SA.** 2000 Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol. 25:169–173.
18. **Haddad G, Penabad JL, Bashey HM, et al.** 1994 Expression of activin/inhibin subunit messenger ribonucleic acids by gonadotroph adenomas. J Clin Endocrinol Metab. 79:1399–1403.
19. **Hosoda H, Kojima M, Matsuo H, Kangawa K.** Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptides in gastrointestinal tissues. Biochem Biophys Res Commun. In press.
20. **Norton AJ, Jordan S, Yeomans P.** 1994 Brief, high-temperature heat denaturation (pressure cooking): a simple and effective method of antigen retrieval for routinely processed tissues. J Pathol. 73:371–379.
21. **Thomas GB, Bennett PA, Carmignac DF, Robinson ICAF.** 2000 Glucocorticoid regulation of growth hormone (GH) secretagogue-induced growth responses and GH secretagogue receptor expression in the rat. Growth Horm IGF Res. 10:45–52.
22. **Tamura H, Kamegai J, Sugihara H, Kineman RD, Frohman LA, Wakabayashi I.** 2000 Glucocorticoids regulate pituitary growth hormone secretagogue receptor gene expression. J Neuroendocrinol. 12:481–485.
23. **Bennett PA, Thomas GB, Howard AD, et al.** 1997 Hypothalamic growth hormone secretagogue-receptor (GHS-R) expression is regulated by growth hormone in the rat. Endocrinology. 138:4552–4557.
24. **Honda K, Bailey ART, Bull PM, MacDonald LP, Dickson SL, Leng G.** 1999 An electrophysiological and morphological investigation of the projections of growth hormone-releasing peptide-6-responsive neurons in the rat arcuate nucleus to the median eminence and to the paraventricular nucleus. Neuroscience. 90:875–883.
25. **Korbonits M, Grossman AB.** 1995 Growth hormone-releasing peptide and its analogues; novel stimuli to growth hormone release. Trends Endocrinol Metab. 6:43–49.
26. **Smith RG, Van der Ploeg LHT, Howard AD, et al.** 1997 Peptidomimetic regulation of growth hormone secretion. Endocr Rev. 18:621–645.
27. **Adams EF, Buchfelder M, Lei T, et al.** 1996 In vitro responses of GH-secreting tumours with and without gsp oncogenes to octreotide, GHRH and growth hormone-releasing peptide. In: von Werder K, Fahlbusch R, eds. Pituitary adenomas: from basic research to diagnosis and therapy. Amsterdam: Elsevier; 43–47.
28. **Denef C.** 1994 Paracrine mechanisms in the pituitary. In: Imura H, ed. The pituitary gland, 2nd Ed. New York: Raven Press; 351–378.
29. **Denef C.** 1998 Autocrine/paracrine intermediates in hormonal action and modulation of cellular responses to hormones. In: Conn M, ed. Handbook of physiology: cellular endocrinology. New York: Oxford University Press; 461–514.
30. **Jones TH, Brown BL, Dobson PRM.** 1990 Paracrine control of anterior pituitary hormone secretion. J Endocrinol. 127:5–13.
31. **Thapar K, Kovacs K, Stefaneanu L, et al.** 1997 Overexpression of the growth-hormone-releasing hormone gene in acromegaly-associated pituitary tumors: an event associated with neoplastic progression and aggressive behavior. Am J Pathol. 151:769–784.
32. **Levy L, Bourdais J, Mouhieddine B, et al.** 1993 Presence and characterization of the somatostatin precursor in normal human pituitaries and in growth hormone secreting adenomas. J Clin Endocrinol Metab. 76:85–90.
33. **Lechan RM, Jackson IMD.** 1982 Immunhistochemical localisation of thyrotropin-releasing hormone in the rat hypothalamus and pituitary. Endocrinology. 111:56–65.
34. **Corchero J, Fuentes JA, Manzanares J.** 1999 Chronic treatment with CP-55,940 regulates corticotropin releasing factor and proopiomelanocortin gene expression in the hypothalamus and pituitary gland of the rat. Life Sci. 64:905–911.
35. **Luo LG, Jackson IM.** 1998 Antisense oligomers of cfos and cjun block glucocorticoid stimulation of thyrotropin-releasing hormone (TRH) gene expression in cultured anterior pituitary cells. Peptides. 19:1295–1302.
36. **Krsmanovic LY, Martinez-Fuentes AJ, Arora KK, et al.** 2000 Local regulation of gonadotroph function by pituitary gonadotropin-releasing hormone. Endocrinology. 141:1187–1195.
37. **Wong ML, al-Shekhlee A, Bongiorno PB, et al.** 1996 Localization of urocortin messenger RNA in rat brain and pituitary. Mol Psychiatry. 1:307–312.
38. **Morash B, Li A, Murphy PR, Wilkinson M, Ur E.** 1999 Leptin gene expression in the brain and pituitary gland. Endocrinology. 140:5995–5998.