Brazilian Journal of Medical and Biological Research (2006) 39: 1003-1011
ISSN 0100-879X Review

Novel mechanisms of growth hormone regulation: growth hormone-releasing peptides and ghrelin

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Abstract

Growth hormone secretion is classically modulated by two hypothalamic hormones, growth hormone-releasing hormone and somatostatin. A third pathway was proposed in the last decade, which involves the growth hormone secretagogues. Ghrelin is a novel acylated peptide which is produced mainly by the stomach. It is also synthesized in the hypothalamus and is present in several other tissues. This endogenous growth hormone secretagogue was discovered by reverse pharmacology when a group of synthetic growth hormone-releasing compounds was initially produced, leading to the isolation of an orphan receptor and, finally, to its endogenous ligand. Ghrelin binds to an active receptor to increase growth hormone release and food intake. It is still not known how hypothalamic and circulating ghrelin is involved in the control of growth hormone release. Endogenous ghrelin might act to amplify the basic pattern of growth hormone secretion, optimizing somatotroph responsiveness to growth hormone-releasing hormone. It may activate multiple interdependent intracellular pathways at the somatotroph, involving protein kinase C, protein kinase A and extracellular calcium systems. However, since ghrelin has a greater ability to release growth hormone *in vivo*, its main site of action is the hypothalamus. In the current review we summarize the available data on the: a) discovery of this peptide, b) mechanisms of action of growth hormone secretagogues and ghrelin and possible physiological role on growth hormone modulation, and c) regulation of growth hormone release in man after intravenous administration of these peptides.

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Research supported by FAPESP (Nos. 97/07689-0, 02/03508-0, and 02/14118-9) and CNPq (No. 300550/87-1). A.-M.J. Lengyel is a Senior Scientist of CNPq.

Received July 20, 2005
Accepted May 29, 2006

Key words
- Growth hormone
- Growth hormone secretagogues
- Ghrelin
- Somatostatin
- Hypothalamus

Introduction

In 1982, before the identification of growth hormone-releasing hormone (GHRH), Bowers et al. (1) discovered a new group of synthetic substances with growth hormone (GH)-releasing ability (for a review, see Ref. 2). These compounds were developed from the met-enkephalin molecule by theoretical conformational energy calculations, computer modelling, peptide chemical modifications, and studies of biological activity. Initially these small peptides were able to release GH weakly. Further chemical modifications led to the development of more potent compounds, including peptides such as

GH-releasing peptide-6 (GHRP-6), GHRP-2, hexarelin, and non-peptides such as MK-0677, which could be administered orally (2). In the last decade several studies were performed with these growth hormone secretagogues (GHS), especially with GHRP-6, and the results suggested a possible role for these peptides in GH modulation (3). It was demonstrated that GHS release GH by different mechanisms than those stimulated by GHRH (for reviews, see Refs. 2,3). Furthermore, these peptides act through different receptors than those of GHRH, somatostatin or opioid peptides. In 1996, Howard et al. (4) cloned the GHS receptor (GHS-R), which was mainly located in the anterior pituitary and in the brain, both in hypothalamic and non-hypothalamic areas. In 1999, Kojima et al. (5) discovered the endogenous ligand for these orphan receptors in the stomach, and this new hormone was designated ghrelin (from *ghre*, the Indo-European root of the word "grow"). Ghrelin is also present in small amounts in the hypothalamus and induces GH release in a quite potent manner (5,6). This peptide is a new member of the brain-gut peptide family, and is also involved in the control of appetite, an effect apparently independent of GH release (for reviews, see Refs. 7,8). Ghrelin might have other actions, which are currently being investigated (7,8). The discovery of ghrelin is an excellent example of reverse pharmacology, in which a new hormone was isolated starting from the chemical synthesis of compounds such as GHS, which then led to the discovery of the endogenous orphan receptor and finally to the isolation of its natural ligand.

Growth hormone secretagogue receptor

In 1996 Howard et al. (4) cloned the GHS-R, which belongs to the G-protein family. It has seven transmembrane-spanning segments and three intracellular and extracellular loops. There are two subtypes of receptors, GHS-R1a, which is active, and GHS-R1b, a shorter isoform, which apparently does not have biological activity (4). Other subtypes might also exist. The human GHS-R1a has 366 amino acids and is highly conserved in evolution. It is located in the anterior pituitary and in the hypothalamus, and in other areas of the brain, such as hippocampus and substantia nigra (4). GHS-R1a was found in several areas of the hypothalamus, including the arcuate, ventromedial and paraventricular nuclei (2,4). Because of its location it has been suggested that GHS-R might modulate biological rhythms, mood, memory, learning, and appetite (2). In the pituitary GHS-R was detected exclusively in somatotrophs by immunocytochemistry (2). In knockout mice for GHS-R1a ghrelin is unable to increase GH release or food intake, which indicates that this type of receptor is involved in both actions of ghrelin (9). It was also shown that GHS-R1a is present in other tissues such as pancreas, heart, adrenal gland, and the thyroid (10). It is interesting that GHS-R1b has a widespread distribution in peripheral tissues but its function is still unknown (10). As mentioned previously, it is likely that other receptor subtypes might also exist.

Ghrelin: overview

When different tissue extracts were added to an experimental system with cells expressing human GHS-R1a, Kojima et al. (5) surprisingly found a major increase in intracellular calcium concentrations with the addition of stomach extracts. These investigators isolated a 28-amino acid peptide with a fatty acid chain modification (n-octanoic acid), in the serine 3 residue. This hydrophobic compound, which is the first known natural bioactive peptide modified by an acyl acid, was called ghrelin. An intriguing finding was the lack of structural similarity between ghrelin and the GHS, such as GHRP-

6 (5). The post-translational fatty acid chain modification (n-octanoyl residue) is essential for some of its biological activity, including GH release and appetite stimulation. Shorter fragments, with the first four to five residues, but with intact acylated serine, are also able to activate signal transduction of GHS-R1a *in vitro* (7). Non-acylated ghrelin, which is the main circulating form, might have non-endocrine actions (5). This latter peptide is mainly secreted from the stomach as its circulating levels are reduced by 80% after gastrectomy or gastric bypass in humans (11). It was recently shown that ghrelin crosses the blood brain barrier, and this transport occurs in both directions, from blood to brain and from the central nervous system to blood (12). It has also been shown that the acyl residue is important for this transport (12). The gene that encodes ghrelin was also identified (5) and is located on chromosome 3 in man. This gene encodes a precursor of 117 amino acids, with an 82% homology within species (5). In the stomach, two isoforms of prepro-ghrelin mRNA are produced by the same gene by alternative splicing (5). One encodes the ghrelin precursor while the other encodes des-Gln^{14} ghrelin precursor, which lacks glutamine at position 14 (5). This latter peptide has 27 amino acids and is biologically active, but is present in small amounts. Therefore, the main active form is ghrelin. Ghrelin is located in the submucosal layer of the stomach fundus, in endocrine oxyntic cells (X/A) which are near the capillaries and not in contact with the lumen, and also, at lower concentrations, in the gastrointestinal tract (7). Both ghrelin and its mRNA are present in the arcuate nucleus of the hypothalamus and in the pituitary gland (5,10). Both n-octanoyl-modified, which is the major form, and des-acyl ghrelin were recently identified in the rat hypothalamus. At the pituitary level ghrelin might act in autocrine or paracrine manner. Recently, it has been shown that ghrelin is expressed in lactotrophs, somatotrophs and thyrotrophs, cells which are dependent on Pit-1 gene expression for differentiation (8). Interestingly, ghrelin is capable of modulating Pit-1 transcription. In peripheral tissues ghrelin has a widespread distribution and has been found in the kidney, placenta, lung, ovary, and testis, among others, but its physiological role in these locations remains to be elucidated (10). Since the distribution of the biologically active receptor (GHS-R1a) is not the same as the peptide, it is likely that other receptor subtypes might exist (10). Considerable amounts of ghrelin are present in blood and this peptide has several actions apart from GH regulation (7,8). Ghrelin enhances food consumption by activation of NPY/AGRP (agouti-related protein) neurons in the hypothalamus, while leptin has the opposite effect (7,8). Ghrelin is able to increase GH release both in animals and in man, and also induces prolactin, ACTH, cortisol, and aldosterone secretion *in vivo* (5,6,13,14). Ghrelin causes a slight increase in glucose levels and a reduction of circulating insulin (14). The discovery of ghrelin reinforced the concept of a third pathway of GH regulation (2,3,7,8). However, the physiological role of this potent endogenous GHRP remains to be determined.

Mechanism of action and physiological role of GHS and ghrelin on GH release

GHS and ghrelin act at both hypothalamic and pituitary levels to modulate GH secretion (for reviews, see Refs. 3,7,8). These peptides directly activate the GHS-R in pituitary cells *in vitro* to stimulate GH release (5). When GHRH is associated with GHS or ghrelin *in vitro*, an additive response is observed in most studies. However, when these peptides are administered together with GHRH *in vivo*, a synergistic effect on GH release is observed, which indicates different mechanisms of action of GHS and GHRH and suggests a main hypothalamic site of

action of GHS (6,14-16). Moreover, in hypothalamic pituitary disconnection there is a lack of GH release after GHRP-6 or ghrelin, both in animals and in man (17,18). It has been shown that an intact GHRH system is necessary for these actions to occur. The administration of antibodies against GHRH decreases both GH pulsatility and GH responsiveness to ghrelin and GHS in rats (19). GHS-induced GH release is also inhibited by a GHRH antagonist (20). In the *lit/lit* mouse, which has a GHRH receptor mutation, GHS do not increase GH release, but there is an enhancement of hypothalamic *c-fos* expression, which is a marker of neuronal activity (21). The GH response to GHS is blunted in humans with GHRH receptor mutations, but the ACTH- and prolactin-releasing effects are preserved, suggesting that they are mediated by the hypothalamus (22). The arcuate nucleus is the main target of ghrelin action, where ghrelin may bind and activate the GHS-R. It has been shown that GHS and ghrelin act centrally by increasing electrical activity and *c-fos* expression in a subpopulation of cells in the arcuate nucleus, some of which are GHRH-producing neurons (21). Moreover, one fourth of these GHRH neurons express the GHS-R, suggesting a direct effect of GHS on these cells (19) (Figure 1).

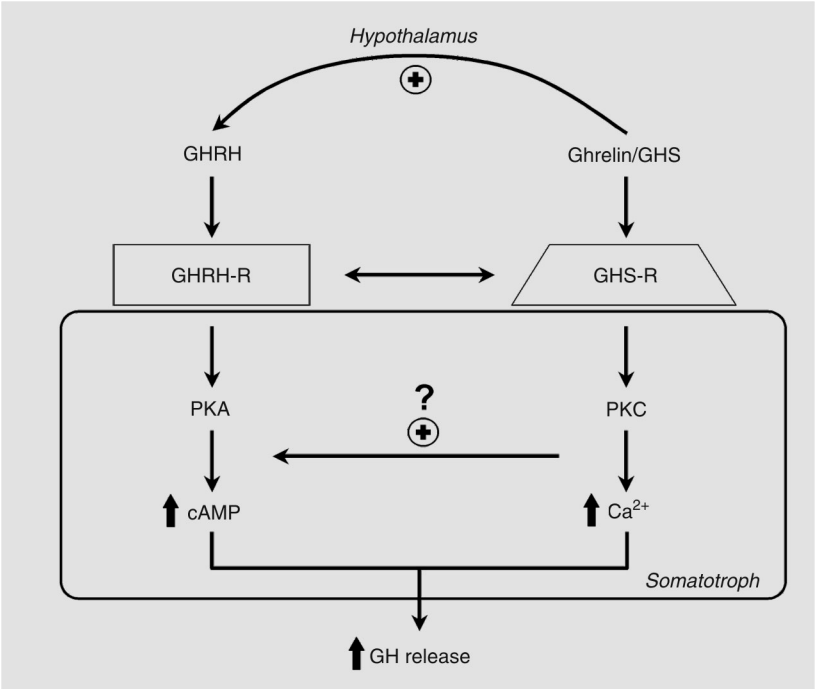

Figure 1. Proposed model of the interactions between GHRH and ghrelin/GHS at the hypothalamic and pituitary level. GHRH = growth hormone-releasing hormone; GHS = growth hormone secretagogues; PKA = protein kinase A; PKC = protein kinase C. Taken in part from ref. 20, with permission.

Ghrelin also increases GHRH release from hypothalamic tissue *in vitro*, but this was not observed with GHS (23,24). It has also been shown that GHS increase GHRH release into the pituitary portal system in sheep (25). GHS and ghrelin did not alter hypothalamic somatostatin release in most *in vivo* and *in vitro* studies (23-25). However, GHS act as functional somatostatin antagonists (19,26). GHS cause depolarization of the somatotroph and an increase in the number of cells secreting GH, while somatostatin has the opposite effect (26).

A model for the action of GHS/ghrelin has been proposed which involves: 1) a functional antagonism of somatostatin; 2) activation of GHRH producing neurons in the arcuate nucleus, leading to an increase in GHRH release; 3) amplification of the effect of GHRH at the somatotroph level (2). At the pituitary level, GHS/ghrelin and GHRH bind to different receptors, and there is evidence of cross-talk between them (for a review, see Ref. 7). These peptides also activate different intracellular transduction pathways at the somatotroph level. GHRH stimulates intracellular cyclic AMP and protein kinase A mechanisms, while GHRP-6 activates protein kinase C, via inositol triphosphate signal transduction, with an increase in intracellular calcium concentrations (2,4) (Figure 1). Interestingly, it has been recently shown in pigs that ghrelin is able to stimulate multiple, interdependent, intracellular pathways at the somatotroph level, involving protein kinase A, C and extracellular calcium systems, with a broader effect than that of most GHS, but similar to that reported for GHRP-2 in this species (27). These data also

suggest the possibility of cross-talk between these transduction pathways. However, the physiological role of these potent stimulators of GH release is not clear.

A controversial issue is whether circulating ghrelin has a role in pituitary GH secretion. It is also unknown how the hypothalamic peptide participates in GH modulation. In the rat, ghrelin secretion occurs in a pulsatile manner, with no correlation with GH pulses, but in association with feeding and sleeping cycles (28). Also, circulating ghrelin levels were similar during the GH peak and trough periods in the rat (29). Ghrelin immunoneutralization did not alter GH pulsatility, while GHRH antibodies completely blocked endogenous pulsatile GH release (30). In humans, the administration of a GHRH antagonist strongly inhibited 24-h GH secretion, but failed to affect circulating ghrelin levels (31). However, in rats, intracerebroventricular or peripheral administration of GHS-R1a antagonists attenuated spontaneous GH secretion, basically by a decrease in pulse amplitude and mean GH levels (32-34). Interestingly, a missense mutation in the GHS-R, which severely impaired ghrelin binding, was associated with a case of familial short stature (35). It has also been shown in healthy volunteers that circulating ghrelin is related to GH pulses, suggesting that ghrelin participates in the pulsatile regulation of GH secretion or that the two hormones are regulated in parallel (36). Therefore, endogenous ghrelin might amplify the basic pattern of GH secretion (32-34). This peptide may also have a physiological role in GH release by optimizing somatotroph responsiveness to GHRH (37). Nevertheless, recent studies with ghrelin knockout animals failed to show a major effect on GH regulation (38). In contrast to predictions, these animals were not anorexic dwarfs (38). However, reduced GH and IGF-I levels were observed in transgenic models with decreased GHS-R mRNA expression in the arcuate nucleus (39). Also, GHS-R knockout mice had lower body weight and IGF-I values (40). These effects were only moderate, which is intriguing, since these peptides are quite potent GH stimulators. It has been suggested that the role of ghrelin in GH secretion might become more relevant during states of negative energy balance (30). However, further studies are necessary to elucidate the physiological role of these peptides in GH secretion.

Regulation of GH secretion by GHRP-6 and ghrelin in man

GHRP-6 and ghrelin increase GH release in a dose-dependent manner both *in vivo* and *in vitro* in several species, including man (1,5,6,13,16). The GH-releasing activity of ghrelin is similar to that of GHRH *in vitro* (5). However, in man, *iv* ghrelin administration at a dose of 1 µg/kg increases GH release in a potent manner and this response is higher than that obtained with GHRH, hexarelin and GHRP-6 (13,14,41). This effect is not specific since there is also an increase in prolactin, ACTH, cortisol, and aldosterone (14). Insulin values decrease and glucose levels increase after *iv* administration of this peptide (14). The latter effects and the aldosterone stimulation are not seen with other GHS. When ghrelin or GHRP-6 is administered together with GHRH a synergistic effect is seen, but with ghrelin this is better observed with low doses of this peptide (0.08 and 0.2 µg/kg) (14,16). It has been demon-

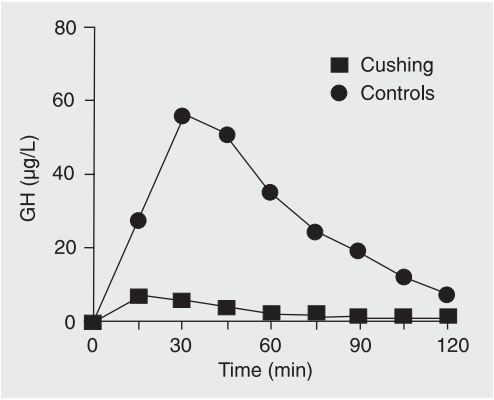

Figure 2. Serum growth hormone (GH) concentration after ghrelin administration (1 µg/kg, *iv*) in patients with Cushing's disease (N = 12) and in controls (N = 9) (from Ref. 58, with permission).

strated that the combined administration of GHRP-6 and GHRH is an excellent test for the diagnosis of GH deficiency in adults. There are no gender differences in the GH responsiveness to GHRP-6 and ghrelin (3, 15,42), but an age-related decrease of the response has been reported for both peptides (3,15,42). Obese subjects have blunted GH responses to GHRP-6 and ghrelin (43,44). Recently, a 55% decrease in ghrelin-induced GH release was shown in women with visceral adiposity and a body mass index of 36.2 kg/m^2 (44). There is a highly reproducible response to GHS in normal subjects studied on different occasions, different from that observed for GHRH. Hyperglycemia, free fatty acids and somatostatin decrease GHRP-6 and the ghrelin-induced GH response (45,46). Arginine was not able to alter GH responsiveness to ghrelin (47).

The effect of cholinergic agonists and antagonists on ghrelin-induced GH release is controversial. Pyridostigmine failed to modify GH responsiveness to both ghrelin and GHRP-6 (15,48). However, atropine blunted this response, but pirenzepine, a muscarinic receptor antagonist, was not able to alter GH release after ghrelin (48). These latter compounds only blunt the GH response to GHRP-6, while they completely abolish the GH response to GHRH (15). Glucocorticoid and GH administration, which probably enhances hypothalamic somatostatin release, only attenuates the GH response to GHS (49,50).

In patients with Cushing's disease, a blunted GH response to both GHRP-6 and ghrelin has been reported by us and by others (51-54) (Figure 2). In these patients GHS- and ghrelin-induced ACTH and cortisol release is enhanced (53-55), which could be due to a direct action of these peptides on the GHS-R in the corticotroph adenoma or, perhaps, activation of hypothalamic arginine vasopressin and also, to some extent, corticotrophin-releasing hormone pathways (7). Interestingly, chronic glucocorticoid administration does not interfere with GHRP-6-induced GH release (52). This might indicate, as suggested previously, that the time of exposure to hypercortisolism determines the GH response to these peptides. In adrenal insufficiency a 72-h withdrawal of glucocorticoid replacement therapy does not influence the GH responsiveness to GHRP-6 (56). In hyperthyroidism we have observed a decrease in GH responsiveness to GHRH while GHRP-6-induced GH release was maintained, which could suggest that thyroid hormones interfere with GHRH-releasing mechanisms, with preservation of GHRP-6-activated pathways (57). Interestingly, we have recently shown that there is a decrease in the GH response to ghrelin in these patients, suggesting that thyroid hormones interfere with additional pathways of GH release activated by ghrelin (58). In type 1 diabetes mellitus the GH response to GHRP-6 and hexarelin is either normal or enhanced, demonstrating that hyperglycemia is unable to decrease the GH release induced by these peptides, differently than in normal subjects (59). It has recently been shown that ghrelin-induced GH release is decreased in anorexia nervosa, which is an unexpected finding because these patients have high GH levels and enhanced responses to GHRH and GHS (60). The possibility that GH-releasing substances, especially the orally active compounds, could represent an alternative treatment in GH-deficient states has received considerable attention. However, these substances have failed to show benefit over GH therapy, although they would be considered more physiological since they induce endogenous pulsatile GH release.

Conclusion

Ghrelin is a new hormone secreted from the stomach to the circulation, but is also produced in the hypothalamus and other tissues, with both endocrine and paracrine effects. Its acyl modification is essential for its

biological effects of enhancement of GH release and stimulation of food intake. Several questions remain to be answered concerning the roles of circulating and hypothalamic ghrelin in GH secretion. The available data suggest that ghrelin might have a physiological role in pulsatile GH release, but further studies are necessary to clarify its precise role in GH modulation.

Acknowledgments

The author thanks the following colleagues from Escola Paulista de Medicina: the postgraduate students who contributed to this study, Prof. Dr. José G.H. Vieira for constant laboratory support, and Aparecida Filomena Machado and Walkiria Miranda for assistance with the assays.

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