Minireview

Ghrelin, a widespread hormone: insights into molecular and cellular regulation of its expression and mechanism of action

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Abstract The recent discovery of ghrelin, the endogenous ligand for the growth hormone secretagogue receptor, is the result of almost 25 years of research by many groups all around the world, and represents a milestone in our understanding of growth hormone secretion and energy homeostasis. This minireview is focused on recent studies on ghrelin, pointing out the cellular and molecular mechanisms involved in the gene expression of ghrelin since recent studies have unequivocally shown that ghrelin biological activity is dependent on a peculiar post-translational processing. Major interest in this peptide derived from the fact that, in addition to other effects, it is involved in the regulation of energy balance by inducing weight gain and reducing fat utilization. These activities are likely mediated by a CNS network of cells that is also modulated by other hormones such as leptin. Ghrelin has emerged as a premeal initiation factor that informs CNS about the status of the energy balance. The development of ghrelin analogs, agonists and antagonists, appears as a suitable approach for possible therapeutic intervention in a variety of disease states linked to alterations in body weight homeostasis.

Key words: Ghrelin; Metabolism; Hypothalamus; NPY; Body weight homeostasis

1. Ghrelin, the unique orexant gastrointestinal acylated peptide: isolation, identification and structures

Ghrelin was discovered at the end of 1999 [1] as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R); GHSs are a group of synthetic compounds with the ability to induce growth hormone secretion in all species tested to date [2]. Ghrelin identification represents a brilliant result on how to search endogenous ligands with the use of the orphan receptor strategy. In the last years research aimed to identify novel ligands using orphan G protein-coupled receptor (GPCR)-expressing cells have resulted in the discovery of several novel bioactive peptides such as orexins, prolactin releasing peptide and many others. Unlike other orphan receptors, GHS-R is known to bind artificial compounds with growth hormone secretagogue activity such as GHRP-6 or hexarelin, supplying a suitable positive control for the assay used to identify the endogenous ligand. In order to identify the ligand for GHS-R, a cultured cell line transfected and expressing the receptor was established and used to identify tissue extracts that produced a classical intracelullar Ca^{2+} increase. By screening several tissues, a very strong activity was unexpectedly found in stomach extracts. The active peptide was purified by gel filtration, ion exchange, and reverse liquid chromatography and its sequence determined by Edman degradation. To note, an expressed sequence tag clone, containing the coding region of the peptide, revealed that the third residue was a serine, which was then confirmed in cDNA clones encoding the peptide precursor isolated from a rat stomach cDNA library. Initially, a synthetic peptide based on the cDNA sequence was synthesized and compared to purified natural ghrelin. This comparison revealed that the synthetic peptide, unlike the purified protein, did not increase intracellular calcium, had a retention time in high-performance liquid chromatography (HPLC) shorter than that of natural peptide and, intriguingly, its molecular mass was 126 atomic mass units (amu) smaller than natural peptide. Further studies demonstrated that the difference in mass was due to an esterification of serine 3 with an *n*-octanoic acid. The mature 28-amino acid peptide is cleaved from its precursor preproghrelin and is characterized by a very peculiar structural modification, since the hydroxyl group of serine in position 3 is covalently acylated by an *n*-octanoic acid residue, although other types of acylations (10 carbon fatty acid group with and without insaturations) have been observed [1,3,4]. Although the acylation mechanism is not fully addressed, it is reasonable that it is necessary for crossing the blood–brain barrier; at any rate, this uniqueness has potential therapeutic implications for ghrelin blockade, since an antagonist to the putative transacylation enzyme that octanoylates ghrelin should inactivate this hormone in a very selective manner. This biochemical post-translational modification is the first observed in peptides isolated from natural sources and

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is postulated to be crucial for the biological activity of the peptide, although acyl modification has been seen in G proteins and receptors, which are conjugated to myristoyl acid (C14) or palmitoyl acid (C16) [5]. Intriguingly, short fragments encompassing the first four to five residues of ghrelin (with intact acylated serine) were able to activate signal transduction of ghrelin receptor (GHS-R type a). In addition, a mechanism of alternative splicing of the ghrelin gene originates an analogous peptide except for the glutamine in position 14 that is missing [6]. This deletion results from the use of the CAG codon, which encodes Gln14 as a splicing signal. Thus, two types of active ghrelin peptide are produced in rat stomach, ghrelin and des-Gln^{14}-ghrelin. The activity of both ghrelins is the same. However, des-Gln^{14}-ghrelin is only present in low amounts in the stomach, indicating that ghrelin is the major active form. Finally, the testis-specific expression of another species of the ghrelin gene transcript in mice, called ghrelin gene-derived transcript (GGDT) has been reported. GGDT is not expressed in the stomach, only in testis, and the sequence acting as the unique exon is located at intron 3 of the ghrelin gene, indicating that it is generated by alternative usage of this intron as a testis-specific exon, for yielding a 12 amino acid peptide [7].

2. Tissue distribution and regulation

Ghrelin is produced prevalently in the stomach by the X/A-like cells within the oxyntic glands of the gastric fundus mucosa [8], although minor amounts are present elsewhere in the body. The placenta [9], testis [10], kidney [11], pituitary [12], small intestine [13], pancreas [14], lymphocytes [15], brain [16], lung [17] and ovary [18,19] also express significant levels of ghrelin. At any rate, two-thirds of plasma ghrelin levels comes from the stomach, and at least one-third from the small intestine [20]. The ubiquitous expression of ghrelin in a host of tissues is suggestive of local paracrine and/or autocrine actions. Thus, it has been found that ghrelin regulates testicular steroidogenesis and testosterone secretion by the Leydig cells [21]. In addition, ghrelin inhibits the expression of stem cell factor (SCF, the major stimulator of germ cell development) in staged rat seminiferous tubules ([22] and M. Tena-Sampere, personal unpublished communication). In keeping with an important paracrine role for ghrelin in gonadal development, it has been shown that ghrelin inhibits development of mouse pre-implantation embryos [23], while it is noticeable that SCF had been found to be involved in mouse blastocyst implantation. For instance, ghrelin has been found in placenta [9], an organ that contain all the main regulatory elements of the somatotropic axis, i.e. GH, GHRH, somatostatin and IGF-1; in spite of the existance of a specific profile of expression of ghrelin mRNA throughout pregnancy, the physiological function of ghrelin in this organ is not fully known at present, although very recently it has been reported that ghrelin is involved in the decidualization of human endometrial stromal cells [24]. Ghrelin expression is up-regulated in rodents after fasting, hypoglycemia or leptin administration [25], and it is strongly increased by chronic undernutrition in animal models [26], suggesting that ghrelin expression and secretion are enhanced in situations of negative energy balance. In addition, in experimental conditions of negative energy balance, such as streptozotocin-induced diabetes in rats, preproghrelin mRNA expression and ghrelin secretion are strongly enhanced [27]. Caloric intake or chronically positive energy balance suppress ghrelin expression and secretion. Intriguingly, in pregnancy, a hypermetabolic condition characterized by increase in maternal body fat and weight, gastric ghrelin mRNA, as well as plasmatic levels, do not change. These data indicate that increased food intake in pregnancy is unlikely to be mediated by increased ghrelin gene expression. In contrast, but in keeping with data obtained in non-pregnant animals, food restriction during pregnancy also led to increased ghrelin gene expression, suggesting a sort of adaptive response to prevent the long-lasting alterations associated with undernutrition [26]. Plasma ghrelin levels are specifically modulated also in experimental models of inflammatory cachexia as well as in patients with rheumatoid arthritis, a degenerative systemic inflammation often associated with alterations in body weight homeostasis. Surprisingly, in experimental arthritis in rats, there is a compensatory variation of ghrelin levels that relates to body weight adjustments. Plasma ghrelin levels decrease during the first part of the chronic inflammation together with body weight, while a recovery of ghrelin levels, together with an increase in body weight, in the latter stage suggests an adaptive response and may represent a compensatory mechanism under catabolic conditions. It is noteworthy that, in rheumatoid arthritis patients, a chronic imbalance in ghrelin levels has been observed, suggesting that this gastric hormone may participate, together with other factors, in alterations of metabolic status during inflammatory stress (O. Gualillo, personal unplished communication). In addition, no gender-based differences in circulating ghrelin have been shown in humans [28] and similarly, gender does not alter ghrelin mRNA expression in rats [29].

3. Mechanism of action

Ghrelin was purified using the receptor for the synthetic GH-secretagogue MK-0677 (so called GHS-R1a), so it is obvious that ghrelin must operate through it. There is an ongoing controversy about whether the cloned secretagogue receptor is 'the' receptor or just 'one of the' receptors for that family of compounds. Differences in the binding activities of the different peptidic (GHRP-6, ghrelin) and non-peptidyl (MK-0677) molecules have been reported [30]. Further support for the existence of several receptors derives from detailed studies using hexarelin as tracer. However, the fact that KO mice for the cloned GHS-R are unresponsive to ghrelin in terms of GH secretion and food intake indicates that at least these two functions are mediated by GHS-R type 1a [31]. Although there is little doubt that the effects of GHRH and ghrelin on the somatotroph are mediated through different receptors, the possible interaction among both receptors is of great interest and has recently been studied. It was reported that activation of the GHS receptor alone had no effect on cAMP production, while the coactivation of the GHS and GHRH receptors produced a cAMP response approximately twice that observed after activation of the GHRH receptor alone. This potentiated response is dose-dependent with respect to both GHRH and GHS, it also depends on the expression of both receptors, and was observed with a variety of peptide and non-peptide GHS compounds as well as with ghrelin. Pharmacological inhibition of signaling molecules associated with GHS-receptor activation, including G protein βγ-subunits, phospholipase C, and protein kinase C

(PKC), had no effect on GHS potentiation of GHRH-induced cAMP production. Importantly, the potentiation appears to be selective for the GHRH receptor. Treatment of cells with forskolin elevated cAMP levels, but these levels were not further increased by GHS-receptor activation. Similarly, activation of two receptors homologous to the GHRH receptor, the vasoactive intestinal peptide and secretin receptors, increased cAMP levels, but these levels were not further increased by GHS-receptor activation [30]. Based on these findings, it is possible that direct interactions between GHRH and GHS receptors may explain the observed effects on signal transduction.

4. Biological action

Ghrelin possesses a strongly dose-related growth hormone-releasing effect both in vitro and in vivo; although initially its GH secretagogue action was likely specific in cultured pituitary cells, ghrelin administration in vivo also affects adrenocorticotropic hormone, cortisol, prolactin, and aldosterone levels [32–34]. Intriguingly, co-administration of ghrelin and GHRH resulted in a significant synergistic effect on GH secretion but not on ACTH or prolactin secretion. The presence of GHS-R in the somatotrophs [2], together with the fact that ghrelin increases in vitro GH secretion, leaves little doubt that ghrelin acts directly at the pituitary level. Nevertheless, the fact that ghrelin is much more potent in vivo than in vitro, and that the GH response to ghrelin is impaired in patients with hypothalamus–pituitary disconnection, indicates that, in terms of GH secretion, its major role is exerted at the hypothalamic level [35]. However, it is possible that ghrelin exerts other effects on somatotroph cell function independently of its effects on GH secretion. In this regard, it has been shown that ghrelin influences the expression of the transcription factor Pit-1. [36]. This factor is transcribed in a highly restricted manner in the anterior pituitary gland, and is responsible for the somatotroph cell-specific expression of the GH gene. By a combination of Northern and Western blot analyses, it was found that ghrelin elicits a time- and dose-dependent activation of Pit-1 expression in monolayer cultures of infant rat anterior pituitary cells. The effect was blocked by pretreatment with actimomycin D but not by cycloheximide, suggesting that this action was due to direct transcriptional activation of Pit-1. Further assessment of the responsive regions of the Pit-1 promoter showed that the effect of ghrelin takes place in a sequence that contains two cAMP-responsive elements (CREs) and that both of them are needed to induce the transcriptional activation of this gene. Although the transducing pathways that mediate this effect of ghrelin are not yet fully understood, preliminary evidence suggests that it is dependent on PKC, mitogen-activated protein kinase (MAPK) and protein kinase A (PKA) activation [37,38]. Taking into account the important role played by Pit-1 in somatotroph cell differentiation and cell proliferation, these data indicate that ghrelin, in addition to its effects on GH secretion, may play an important role in the physiological control and physiopathological alterations related to somatotroph cell function. Besides the strong GH-releasing activity, ghrelin has other significant actions, including: (1) orexigenic action coupled with control of energy homeostasis, (2) control of acid secretion and gastric motility [38], (3) influences on pancreatic activity [39,40], (4) influences on sleep [41], (5) cardiovascular actions [42], and (6) antiproliferative effects on several cell lineages [43]. In spite of the plethora of physiological actions exerted by ghrelin, here we focus prevalently on the role of ghrelin as endogenous feeding signal and its relationships with the sophisticated system controlling food intake and body weight homeostasis.

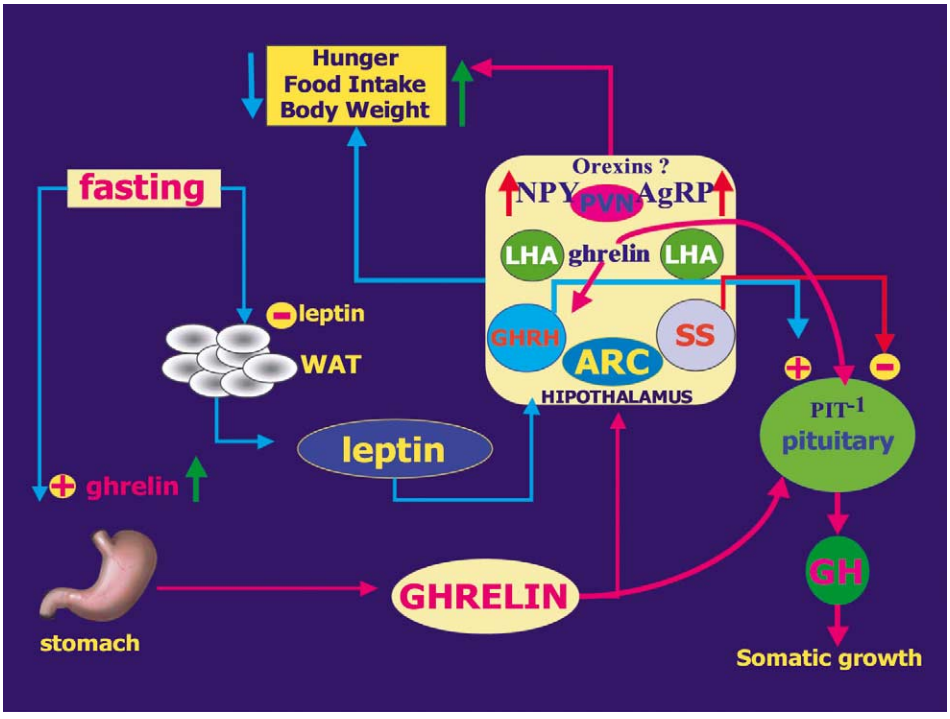

Fig. 1. Diagrammatic representation of the central and peripheral circuits that influence ghrelin expression and mechanism of action.

5. Ghrelin as orexigenic factor: Molecular and cellular pathways

Apart from its role in the regulation of somatotrophic cell function and growth hormone secretion, several studies have unequivocally shown that ghrelin is strongly involved in the regulation of energy homeostasis. These studies, in agreement with previous reports on the orexigenic effects of synthetic ghrelin analogs, received a powerful boost after the report that, in rodents, ghrelin stimulates food intake while reducing fat depot utilization [44]. These findings have been confirmed by other groups, indicating the involvement of this new hormone in the regulation of energy balance (Fig. 1). Ghrelin stimulates food intake in rodents, as well as in humans, by an activity that occurs through mechanisms other than those implicated in growth hormone regulation [45,46]. Ghrelin is able to elicit its orexigenic effect when administered by any route, either centrally or peripherally, a relevant observation considering that other orexigenic peptides are devoid of action via the periphery. Ghrelin increases the expression of mRNA for Agouti-related protein (AgRP) and neuropeptide Y (NPY) [47] and triggers the expression of immediate early-response genes in the medial arcuate nucleus of the hypothalamus, an area rich in NPY and AgRP neurons [48–50]. Intriguingly, synthetic ghrelin analogs are able to induce potent orexigenic effects in NPY null mice (NPY−/−) arguing for a major role of AgRP [51]. In addition, competitive blockade of AGRP action by melanocortin-receptor agonist MT-II prevented ghrelin analog-induced weight gain in Npy(−/−) mice, suggesting that chronic peripheral treatment with a ghrelin receptor agonist induces a positive energy balance leading to fat gain also in the absence of NPY and that these effects could be mediated in part by AGRP. The influences of ghrelin in the hypothalamus are likely to be opposite to those exerted by leptin [52–55]. In addition, very recently Yamanaka et al., [56] have shown that ghrelin directly activates a population of isolated orexin neurons by depolarization with increases in action potential frequency. Circulating ghrelin, as well as ghrelin-containing neurons, may in part mediate activation of orexin neurons such as that occurring during food restriction. Induction of food intake by ghrelin, which counteracts reduction in body weight, may be mediated in part by orexins, which also induce food intake pharmacologically. The standard hormonal model of ghrelin action asserts that circulating ghrelin, derived prevalently from the gastrointestinal tract, accesses the arcuate nucleus of the hypothalamus through a leaky blood–brain barrier at that location, and increases food intake by activating NPY/AgRP neurons. Recent evidence has indicated that at least part of the effect of ghrelin on energy homeostasis in the hypothalamus might be of intrinsic origin. Cowley et al. showed that ghrelin is expressed in a previously uncharacterized group of neurons in the hypothalamus. These neurons lie in the space between the lateral, arcuate, ventromedial, dorsomedial and paraventricular hypothalamic nuclei, and they send projections to several of these nuclei as well as outside the hypothalamus [57]. Intriguingly, this area overlaps with the projections from the suprachiasmatic nucleus, which might allow the production of ghrelin to be directly modulated by the circadian clock. Recent findings give a clue to what hypothalamic ghrelin does: it is likely that ghrelin-positive neurons are closely apposed to the axon terminals of NPY neurons, raising the possibility that ghrelin is able to presynaptically modulate the release of NPY and GABA (γ-aminobutyric acid), by increasing the activity of the NPY-containing neurons and hyperpolarizing pro-opiomelanocortin (POMC)-containing neurons in the arcuate. It is clear that the demonstration of an endogenous ghrelin system in the hypothalamus might shed light on several obscure aspects that have been associated with the concept that ghrelin from the stomach acts on hypothalamic nuclei to regulate energy homeostasis. Clearly, if more work confirms the function of hypothalamic ghrelin, it will represent a very important step in our understanding of how such complex interactions between the brain and the gastrointestinal system regulate food intake and energy expenditure.

6. Conclusions

Ghrelin is a 28 amino acid peptide, predominantly produced by the stomach, showing a unique structure with an *n*-octanoyl ester at its third serine residue, which is essential for its potent stimulatory activity on somatotroph secretion. In fact, it has been demonstrated that ghrelin specifically stimulates GH secretion from both rat pituitary cells in culture and rats in vivo. It displays strong growth hormone-releasing activity mediated by the hypothalamus–pituitary GH secretagogue receptors that have been found to be specific for a family of synthetic, orally active GH secretagogues. The discovery of ghrelin brings us to a new understanding of the regulation of GH secretion. However, ghrelin is much more than simply a natural GH secretagogue. It also acts on other central and peripheral receptors and exhibits other actions, including stimulation of lactotroph and corticotroph secretion, orexigenic, influences gastroenteropancreatic functions, and has metabolic, cardiovascular and antiproliferative effects. Knowledge of the whole spectrum of the biochemical mechanisms involved in ghrelin synthesis and secretion will provide new understanding of the cell biology related to post-translational regulation of gene expression. This could help in the design of novel analogs, acting as agonists or antagonists, which in turn could become candidate drugs for the treatment of different pathophysiological conditions associated with altered energy balance such as obesity, or cachexia-associated clinical entities.

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