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Gastric Peptides and their Regulation of Hunger and Satiety

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Abstract

Ingestion of food affects the secretion of hormones from specialized endocrine cells scattered within the intestinal mucosa. Upon release, these hormones mostly decrease food intake by signaling information to the brain. Although enteroendocrine cells in the small intestine were thought to represent the predominant gut-brain regulators of food intake, recent advances also established a major role for gastric hormones in these regulatory pathways. First and foremost, the gastric endocrine X/A-like cell was in the focus of many studies due to the production of ghrelin which is until now the only known orexigenic hormone that is peripherally produced and centrally acting. Although X/A-cells were initially thought to only release one hormone that stimulates food intake, this view has changed with the identification of additional peptide products also derived from this cell, namely desacyl ghrelin, obestatin and nesfatin-1. Desacyl ghrelin may play a counter-regulatory role to the food intake stimulatory effect of ghrelin. The same property was suggested for obestatin; however, this hypothesis could not be confirmed in numerous subsequent studies. Moreover, the description of the stomach as the major source of the novel anorexigenic hormone nesfatin-1 derived from the NUCB2 gene further corroborated the assumption that the gastric X/A-like cell is not only a stimulator but also an inhibitor of feeding thereby acting as so far unique dual regulator of food intake located in a logistically important place where the gastrointestinal tract has initial contact with food.

Keywords

brain-gut axis; desacyl ghrelin; food intake; ghrelin; nesfatin-1; NUCB2; obesity; obestatin; X/A-like cell

Introduction

Specialized enteroendocrine cells located within the mucosa of the small but also the large intestine [1] were established as main regulators of food intake and intestinal functions due to the production and release of peptide hormones [2]. However, also the stomach contains distinct endocrine cells, namely gastrin-releasing cells (G cells), somatostatin-producing cells (D cells >20% of gastric oxyntic endocrine cells in humans, 5-10% in rats), enterochromaffin-like cells producing histamine (ECL, 30% in human and 65% in the rat) and in low abundance serotonin-containing enterochromaffin (EC) cells [1]. These cells' products were mainly implicated in the regulation of acid production and secretion and motility. Moreover, a fifth endocrine cell type was described scattered in the gastric oxyntic

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mucosa and was named P/D_1 cell in man and X/A-like cell in rats [1]. These cells account for 20-30% of the oxyntic endocrine cells thereby representing the second most abundant gastric endocrine cell type [1]. The function of these cells, unknown for a long time, was unraveled with the discovery of ghrelin in 1999 [3] identified as the only peripherally produced and centrally acting orexigenic peptide hormone [4]. This landmark discovery drew attention to this cell type in the context of regulating food intake and body weight. Subsequently, additional peptide products have been identified in this cell type including desacyl ghrelin and *n*-decanoyl ghrelin [4, 5], obestatin [6] and nucleobindin2/nesfatin-1 (NUCB2/nesfatin-1) [7]. Several studies were conducted to characterize these hormones with regard to their effects on food intake as recently reviewed for desacyl ghrelin [8], nesfatin-1 [9] and obestatin [10]. Overall, these studies point towards an anorexigenic effect of these additional peptide hormones thereby highlighting a unique role for the gastric X/A-like cell as dual regulator of food intake.

Peptide products of the gastric endocrine X/A-like cell

Ghrelin and desacyl ghrelin

More than a decade ago Kangawa and Kojima discovered the endogenous ligand of the long time orphan receptor, growth hormone (GH) secretagogue receptor 1a (GHS-R1a), which was named ghrelin [3, 11•]. The peptide ghrelin consists of 28 amino acids with a unique octanoyl fatty acid modification on the serine-3 residue that increases lipophilicity [3]. This modification is essential for binding to the GHS-R1a [3, 12], which was later renamed ghrelin receptor (GRLN-R) [13]. Subsequent structure-function studies established that the full length peptide is not necessary to stimulate the GRLN-R and identified the first five N-terminal amino acids containing the serine-3 fatty acid modification as the active core required to activate the receptor [14]. Recently, another form of ghrelin, called *n*-decanoyl ghrelin, was identified in rodents and shown to be the major circulating form of ghrelin in mice [5]. Interestingly, it was also shown that food derived medium chain fatty acids can be used as direct source for the acylation of ghrelin [14].

Desacyl ghrelin lacking the octanoyl group represents the major form of ghrelin in the circulation [15] although recent advances in improving blood processing for labile peptides changed the acyl/desacyl ghrelin ratio from 1:55 [16] to 1:5 [17•]. In contrast to ghrelin and *n*-decanoyl ghrelin, desacyl ghrelin does not bind to the GRLN-R [3] and the receptor of desacyl ghrelin is yet to be identified.

Innovative advancements came in 2008 when two independent groups identified the enzyme involved in catalyzing the acyl modification as the fourth member of membrane-bound *O*-acyltransferases (MBOATs) which was named ghrelin-*O*-acyltransferase (GOAT) [18, 19]. GOAT not only catalyzes the attachment of the octanoyl but also the decanoyl group resulting in octanoyl and decanoyl ghrelin respectively [19]. This acylation is assumed to occur before proghrelin is transported into the Golgi apparatus [18]. However, besides gastric GOAT mRNA and protein expression [20, 21], GOAT protein was also detected in the circulation of rats and mice [21] leading to the speculation about an extracellular acylation of ghrelin. This finding is yet to be confirmed in humans although first pilot data also show the occurrence of GOAT protein in human plasma (unpublished observations). Moreover, GOAT mRNA expression was shown to be widely present in human tissues including oesophagus, stomach, small intestine, colon, pancreas, liver, gallbladder, bile duct, spleen, kidney, cortex of adrenal gland, myocardium, lung, skeletal muscle, fat tissue, thyroid gland, testis, prostate, ovary, placenta and pituitary gland [22] suggesting possible additional functions of GOAT. A recent study suggested that GOAT plays a critical role in bile acid reabsorption [23].

Obestatin

Obestatin is a 23 amino acid peptide also derived from the ghrelin gene and was first described in 2005 based on a computer predicted splicing and post-translational processing site [6]. Like ghrelin, obestatin is expressed in rodent gastric X/A-like cells [24] and human endocrine P/D1 cells [25, 26]. This peptide was thought to act in opposition to ghrelin by antagonizing ghrelin's orexigenic effect and reducing body weight [6, 27].

NUCB2/Nesfatin-1

Recently, another peptide hormone has been identified in rat X/A-like cells, namely nucleobindin2 (NUCB2)/nesfatin-1 [7]. Nesfatin-1, an 82 amino acid peptide, was first described in the rat hypothalamus as peptide product derived from NUCB2 [28]. NUCB2/nesfatin-1 is widely expressed in the rat brain [29-31] but also in the periphery including the pancreas, liver and white (subcutaneous and visceral fat) as well as brown (interscapular fat) adipose tissue [32]. Interestingly, NUCB2 mRNA expression is 10-times higher in the rat gastric mucosa than in the brain giving rise to a predominant production of nesfatin-1 in the stomach [7]. Phenotypical characterization in rats showed that nesfatin-1 immunoreactivity colocalized with ghrelin within the same gastric endocrine cells with a different subcellular cytoplasmatic localization in distinct and different pools of vesicles [7]. In addition, the expression of prohormone convertase (PC) 1/3 in X/A-like cells [18] also supports the coexpression of ghrelin and nesfatin-1 in one cell type as this hormone is required for the maturation of both peptide hormones [18, 33]. The colocalization of ghrelin and nesfatin-1 was recently also confirmed in the anterior intestine of goldfish [34] highlighting the phylogenetic stability of this expression pattern which gives rise to its physiological importance. Whether this finding can be translated into humans warrants further investigation. However, first pilot data in human stomach also show a colocalization of nesfatin-1 and ghrelin immunoreactivity in endocrine P/D_1 cells (unpublished observation).

Regulation of peptide release

Ghrelin and desacyl ghrelin

The major source of circulating ghrelin is the stomach as indicated by a marked decrease of plasma ghrelin levels following gastrectomy [35]. Other, although quantitatively less important, sources of peripheral ghrelin encompass the small and large intestine [4], pancreas [36] and other organs such as kidney, liver, testis and adipose tissue [37, 38]. Blood levels of ghrelin are well established to be regulated by feeding status with a rise before food intake and a fall quickly thereafter [39, 40]. Recent studies also indicate the cephalic phase initiated by presenting pictures of food increases circulating ghrelin levels in humans [41]. In addition, fasting also results in an increase of circulating ghrelin levels associated with an increased gastric production and release of this peptide [42••-44]. Likewise, gastric GOAT mRNA expression and circulating GOAT protein concentration is increased under conditions of food restriction [21, 45] likely to further stimulate the acylation of ghrelin. Besides these short term influences on ghrelin, long term alterations of metabolic homeostasis also affect blood ghrelin levels with an increase under conditions of decreased body weight such as cachexia and anorexia and a marked decrease in obese subjects [46-48]. As ghrelin and desacyl ghrelin are derived from the same precursor, for a long time desacyl ghrelin was thought to merely represent the inactivated ghrelin peptide. However, recent studies provide evidence that both peptide forms are subject to differential regulation. In particular, the reduction of gastric pH results in the release of desacyl ghrelin but not ghrelin [49]. In addition, various stressors (injection of lipopolysaccharide as immunological stressor or abdominal surgery as physical stressor) influence the acyl/desacyl ghrelin ratio due to a rapid decline of ghrelin [50, 51]. Modification of dietary nutrition was also recently reported to influence deacyl ghrelin. High fish oil increased the circulating

levels compared to standard high fat diet while high fat butter reduced it compared to other groups indicative that different levels of fatty acid saturation in the composition of high fat food influences fasting levels of desacyl ghrelin [52]. Although the underlying mechanisms remain to be fully characterized, a reduction of acylation due to a decrease in GOAT expression observed under these conditions [50] has been hypothesized. On a general note, one has to keep in mind that although the regulation of ghrelin under different metabolic and other conditions has been extensively described, the underlying mechanisms controlling these alterations of expression and release on a cellular basis are not well characterized. This is largely due to the fact that the low quantities of X/A-like cells hamper the enrichment to pure cell preparations and immortalized ghrelin producing MGN3-1 tumor cell lines [53] only allow a limited proportion of extrapolation to native X/A-like cells. This was recently shown by the demonstration that dopamine stimulates ghrelin release from MGN3-1 cells [54], while *in vivo* increased levels of dopamine in the circulation [55] or in the gastric mucosa [56] did not alter fasting ghrelin levels in rats. Animal models with fluorescent dye coupled to the ghrelin promoter allow to enrich ghrelin producing cells to higher purity as in the recent use of green fluorescent dye [57••]. With this approach the expression of GRP120 on isolated ghrelin cells was demonstrated along with the interaction of long chain free fatty acids with this receptor to reduce ghrelin release [57••].

Ghrelin binds to the GRLN-R widely expressed in the periphery (e.g. abdominal organs, vagal afferents, myenteric neurons, adipose tissue and pituitary) and the brain [37, 58-60]. After stimulation by ghrelin, the GRLN-R desensitizes through endocytosis *via* clathrin-coated pits [61] to prevent an overstimulation. Since ghrelin is the only known peripherally produced and centrally acting stimulator of food intake several studies attempted to block feeding and body weight gain using ghrelin antagonists. However, although initial data were promising none of the candidates moved towards clinical trials likely owing to the fact that the GRLN-R displays a high constitutive activity [62] even in the presence of antagonists. Based on these observations, focusing on the development of inverse agonists with high stability in blood and slow blood clearance as well as large diffusion in tissues [63•] may be a promising approach. Unlike ghrelin, desacyl ghrelin is unable to bind to the GRLN-R; hence the receptor for this peptide remains to be established. Since desacyl ghrelin exerts actions on sites where the GRLN-R is not expressed such as stimulation of insulin release from INS-1E cells [64] or inhibition of proliferation of breast cancer cells [65] the expression of a distinct desacyl ghrelin receptor is strongly suspected but is yet to be discovered.

Obestatin

Unlike ghrelin, plasma levels of obestatin do not change with metabolic status [6, 66], a finding that does not support the proposed role as negative modulator of food intake. In the study describing the discovery of obestatin, the authors reported the GPR39, a seven transmembrane domain G protein-coupled receptor, to mediate obestatin's effects [6]. The same group also recently described expression and functional relevance of this receptor in gastrointestinal and adipose tissues to mediate the actions of obestatin [67]. However, various independent groups of investigators did not confirm these findings and showed that obestatin does not bind to the GPR39 [68-71] resulting in the conclusion that obestatin is not the endogenous ligand of the GPR39.

NUCB2/Nesfatin-1

As expected for a negative modulator of feeding, plasma levels of NUCB2/nesfatin-1 are affected by the feeding status with a decline during fasting and a restoration of levels upon refeeding [7]. As gastric NUCB2 mRNA expression is also decreased during fasting [7, 72], the lower circulating levels of NUCB2/nesfatin-1 may result from reduced gastric

production and release. A recent study indicates that NUCB2/nesfatin-1 is directly regulated by the mammalian target of rapamycin (mTOR) as the inhibition of gastric mTOR signaling by rapamycin blunted the expression of gastric NUCB2/nesfatin-1 at the gene and protein levels *in vivo* and *in vitro* suggesting a direct regulatory effect of mTOR on NUCB2/nesfatin-1 [72]. Interestingly, these short term changes of circulating NUCB2/nesfatin-1 concentrations in response to feeding and fasting were not observed in healthy human subjects [73, 74]. However, under conditions of body mass index (BMI) alterations, plasma NUCB2/nesfatin-1 levels were lower in anorexic [75] and higher in obese patients [76, 77] which is opposite to the results observed for ghrelin before. This increased expression in obese could be directly related to the increased amount of white adipose tissue where expression of NUCB2 was recently reported [76]. Contrasting with our increasing knowledge on nesfatin-1's regulation and effects [9, 78], the receptor mediating those actions is still unknown. A previous study suggested the mediation by a G-protein-coupled receptor due to the fact that nesfatin-1 increased [Ca^{2+}] in isolated cultured hypothalamic cells which was linked with protein kinase A signaling [29]. The isolation of the receptor and description of its expression sites will represent a big leap forward in our understanding of the physiology of NUCB2/nesfatin-1.

Effects on short term food intake and long term energy homeostasis

Ghrelin and desacyl ghrelin

Ghrelin's food intake stimulating effects have been early on described in animals [79, 80] and humans [81], an effect inhibited by GRLN-R antagonists [82]. In addition, mice lacking the GRLN-R do not respond with increased food ingestion following injection of ghrelin [83, 84] highlighting the role of this receptor in the mediation of ghrelin's orexigenic effect. Similar to ghrelin's effects, also *n*-decanoyl ghrelin stimulates feeding matching the binding affinity to the GRLN-R [5]. It became apparent over the past few years that ghrelin is not only involved in the short term regulation of food intake but also in the long term mediation of energy homeostasis thereby influencing body weight as also reflected in the negative correlation of ghrelin with BMI. When ghrelin is infused chronically, body weight gain is increased due to stimulated food intake but also increased fat storage and reduced mobilization of fat [46, 85, 86]. This is likely due to an increase in enzymes involved in the mediation of fat storage such as lipoprotein lipase, stearoyl-CoA desaturase-1, acetyl-CoA carboxylase alpha and fatty acid synthase along with a decrease in mRNA expression of an enzyme promoting fat oxidation, namely carnitine palmitoyl transferase-1 alpha [87]. Interestingly, these expression levels are reversed under conditions of ghrelin depletion in mice lacking ghrelin [87] supporting a physiological role of ghrelin in mediating alterations of these pathways. In addition to ghrelin, also GOAT is suggested to play a regulatory role as mice lacking GOAT displayed a lower body weight compared to wild type littermates under conditions of high fat diet feeding whereas when fed normal chow no differences were apparent [88••]. Moreover, in GOAT knock-out (KO) mice fed food enriched with medium-chain triglycerides, fat mass was decreased [88••] resulting in the hypothesis that GOAT can act as endogenous lipid sensor.

Ghrelin released from gastric X/A cells to stimulate feeding can act directly on food intake regulatory brain nuclei after crossing the blood-brain barrier [89, 90] or mediate its orexigenic action indirectly *via* the vagus nerve [91, 92]. Earlier studies showed that rats with subdiaphragmatic or gastric vagotomy do not respond with increased food intake following intravenous injection of ghrelin [91]. In contrast, another group reported that the orexigenic stimulus exerted by intraperitoneally injected ghrelin is still observed in rats after elective subdiaphragmatic vagal deafferentation [93]. These discrepant results could be explained by different surgical techniques, different doses of ghrelin used and also different routes of administration but add to the debate about the predominant route of orexigenic

action of circulating ghrelin. In addition to its predominant source in the periphery, ghrelin is also produced in the brain in neurons adjacent to the third ventricle [94] and in the arcuate nucleus of the hypothalamus [95], a key brain center integrating food intake regulatory signals [96]. Ghrelin containing neurons in these nuclei are connected to other cells containing the orexigenic brain transmitters, neuropeptide Y (NPY) and agouti-related peptide (AgRP) [94, 97]. It has been shown early on that peripheral [98] as well as central [99] injection of ghrelin activates NPY neurons in the arcuate nucleus suggesting a downstream mediation of ghrelin's orexigenic effect by these peptides. This was corroborated by pharmacological (anti-NPY and anti-AgRP antibodies) [100] as well as genetic (NPY and AgRP knockout) [101] approaches showing that inhibiting both, NPY and AgRP signaling blocks ghrelin's orexigenic effects whereas single blockade of either one had no effect most likely related to a compensatory action.

While desacyl ghrelin was initially thought to represent an inactive or inactivated peptide, several sets of evidence now point towards a food intake modulatory action of this peptide although the data are less compelling compared to ghrelin [8]. Several studies reported a reduction of food intake following peripheral [102] and central (intracerebroventricular) injection [103], while one study described an increased food intake after intracerebroventricular injection of a low dose of desacyl ghrelin in rats or mice [104]. Furthermore, another study did not observe any food intake modulatory effects when desacyl ghrelin was injected alone but when co-injected with ghrelin, desacyl ghrelin abolished ghrelin's orexigenic effect [105]. This observation led to the speculation of a counterbalancing role of desacyl ghrelin in the regulation of food intake. Similar to ghrelin, also desacyl ghrelin has been suggested to play a role in long term control of energy and body weight homeostasis since mice over-expressing desacyl ghrelin display a smaller body size and less white adipose tissue mass [106] resulting in reduced body weight [107] compared to wild type controls. Despite the fact that these findings give rise to a reduction of lipid accumulation by desacyl ghrelin, recent *in vitro* data indicated a stimulation of intracellular lipid accumulation in human adipocytes by desacyl ghrelin [108]. These conflicting results may be due to methodological differences (*in vitro versus* whole animals) but may also point towards species differences (human *versus* mouse) and warrant further investigations.

Obestatin

The authors discovering obestatin reported that this peptide is a physiological anorexigenic mediator of feeding counteracting ghrelin's orexigenic effect [6]. However, very few subsequent studies were able to partially reproduce these data whereas, by far, most studies did not observe any modulatory effect on food intake, body weight (for review see [66]) or ghrelin's orexigenic effect [109]. Based on this converging evidence obestatin is not considered to play a role in the regulation of hunger and satiety and the physiological role of this peptide is yet to be determined.

NUCB2/Nesfatin-1

The discovery of NUCB2/nesfatin-1 in the hypothalamus was paired with the description of the reduction of food intake after single injection and a blunted body weight gain after continuous third ventricular infusion of nesfatin-1 [28]. These effects are likely to present a physiological action of this peptide and not merely a pharmacological phenomenon as blockade of endogenous NUCB2/nesfatin-1 signaling by the use of a 3rd ventricle injection of an antisense oligonucleotide resulted in a stimulation of food intake and an increased body weight gain in rats [28]. This anorexigenic action upon central administration was also shown in several other studies by independent groups in rats [110, 111] and subsequently also in mice [112, 113] and goldfish [114]. The reduction of dark phase food intake was due

to the stimulation of satiation (indicated by a reduction of meal size) and satiety (indicated by a decrease in meal frequency and prolongation of inter-meal intervals) as recently shown in mice using an automated episodic food intake monitoring device [113]. One has to note that nesfatin-1 selectively inhibits dark phase food intake in *ad libitum* fed animals [28, 110, 115, 116] whereas results were not robust during the light phase under fasting conditions [110, 115]. This observation may point towards the interaction with other brain transmitters specifically activated during the dark photoperiod. The anorexigenic action was also observed following intracerebroventricular injection of mid segment nesfatin-$1_{30\text{-}59}$ in mice giving rise to the active core of the peptide able to activate the yet unknown receptor [117]. After establishing the effects on food intake several studies investigated the brain site of nesfatin-1's anorexigenic action. While after injection in cerebrospinal fluid at the level of the hypothalamus the effect on dark phase food intake was delayed in onset with a maximum reduction occurring during the third hour post injection [110], the effect was already observed during the first hour when the peptide was injected into the hindbrain at the level of the cisterna magna or the fourth ventricle [110]. Interestingly, while the corticotropin releasing factor receptor 2 (CRF_2) antagonist, $astressin_2$-B blocked the anorexigenic effect of nesfatin-1 injected at the level of the hypothalamus, its injection into the hindbrain did not influence nesfatin-1's anorexic effect indicative of different forebrain *versus* hindbrain downstream signaling pathways [110]. Besides CRF_2 signaling, the melanocortin and oxytocin pathways seem to be involved in nesfatin-1 food intake inhibitory action as injection of the melanocortin 3/4 receptor antagonist, SHU9119 [28, 115] and the oxytocin antagonist, ornithine vasotocin [111, 116] blocked the nesfatin-1-induced anorexigenic effect. Convergent reports also established that nesfatin-1's action is independent of leptin [28, 116, 118] which is an important feature in the context of an anti-obesity target since leptin sensitivity is reduced under conditions of increased fat mass.

Contrasting with consistent evidence from numerous studies reporting the anorexigenic effect of centrally injected nesfatin-1, although NUCB2/nesfatin-1 is more abundant in the stomach than in the brain [7] the effects of nesfatin-1 on food intake are not well established. Similar to the central effect, the mid segment nesfatin-$1_{30\text{-}59}$ also reduces food intake in mice when injected intraperitoneally although higher doses are needed [119]. This effect is likely to be vagally mediated as nesfatin-1 activates the Ca^{2+} influx in primary cultured nodose ganglion neurons *in vitro* [120] and the anorexigenic effect is not observed in animals pretreated with the neurotoxin capsaicin [119]. Similar to the study in rodents, also in goldfish only high doses of nesfatin-1 injected peripherally reduced food intake [114] suggesting that the major site of nesfatin-1 action is within the brain.

Summary & Conclusion

While gastric X/A-like cells were long thought to be exclusively involved in the stimulation of food intake due to the production of the only known peripherally produced and centrally acting orexigenic peptide hormone ghrelin, recent studies highlighted a function as anorexigenic modulator as well due to the production of desacyl ghrelin and nesfatin-1 in the same cells. Although our knowledge on the effects of these peptides markedly increased especially over the last three years. several important nuts have to be cracked over the next few years, especially the identification of the receptors for desacyl ghrelin and nesfatin-1 as well as the subcellular regulatory mechanisms controlling synthesis and release of the peptides produced in X/A-like cells.

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