

Review Article

Recent Advances in Potential Clinical Application of Ghrelin in Obesity

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Ghrelin is the natural ligand of the growth hormone secretagogue receptor (GHS-R1a). Ghrelin is a 28 amino acid peptide possessing a unique acylation on the serine in position 3 catalyzed by ghrelin *O*-acyltransferase (GOAT). Ghrelin stimulates growth hormone secretion, but also appetite, food intake, weight gain, and gastric emptying. Ghrelin is involved in weight regulation, obesity, type 2 diabetes, and metabolic syndrome. Furthermore, a better understanding of ghrelin biology led to the identification of molecular targets modulating ghrelin levels and/or its biological effects: GOAT, ghrelin, and GHS-R1a. Furthermore, a recent discovery, showing the involvement of bitter taste receptor T2R in ghrelin secretion and/or synthesis and food intake, suggested that T2R could represent an additional interesting molecular target. Several classes of ghrelin-related pharmacological tools for the treatment of obesity have been or could be developed to modulate the identified molecular targets.

1. Introduction

Ghrelin, the natural ligand of the growth hormone secretagogue receptor (GHS-R1a) [1], is a potent stimulator of growth hormone secretion [2, 3]. Moreover, ghrelin is also an appetite-stimulating hormone inducing food intake and weight gain in human [4–6], and promoting gastric emptying [7]. Ghrelin is a 28 amino acid peptide predominantly produced and secreted into the blood stream by the endocrine stomach mucosal cells named “X/A like” in rat [8, 9] and P/D1 cells in humans [10]. Ghrelin has the particularity to be acylated on the serine in position 3 [1]. During the processing of preproghrelin, both ghrelin 1-28 and ghrelin 1-27 can result and then are subjected to the acylation of the hydroxyl group of Ser3 [11]. Acylation, a unique peptidic modification, is catalyzed by ghrelin *O*-acyltransferase, a member of the membrane-bound *O*-acyltransferase family, during the processing of the peptide [2, 3]. The most frequently acylation is with an octanoyl group (C8:0), and more rarely with a decanoyl (C10:0) or a decenoyl (C10:1) group [11]. Acylation of ghrelin can be increased by ingestion of either medium-chain fatty acids or medium-chain triacylglycerides [12].

Des-acyl ghrelin represents more than 90% of human plasma ghrelin immunoreactivity [13]. It remains presently uncertain if both ghrelin and des-acyl ghrelin, present in the stomach, are both secreted into the bloodstream via similar or different regulated pathway(s). In the rat stomach, ghrelin is deacylated by lysophospholipase I [14, 15], and degraded by N-terminal proteolysis [14, 16]. Shorter half-life of ghrelin compared to des-acyl ghrelin [17] and plasma ghrelin deacylation [16, 18] could account for the vast predominance of des-acyl ghrelin in the circulation. Human butyrylcholinesterase and other esterase(s), such as platelet-activating factor acetylhydrolase, and rat carboxylesterase are responsible for ghrelin desoctanoylation in these species [16, 19]. Interestingly, butyrylcholinesterase knockout mice fed with a normal standard 5% fat diet had normal body weight while mice fed with high-fat diet (11% fat) became obese. Butyrylcholinesterase was suggested to play a role in fat catabolism as the obese phenotype could not be explained by increased ghrelin, caloric intake, or decreased exercise [20]. The suggested participation of human paraoxonase in ghrelin deacylation [21] remains controversial [16]. Due to ghrelin degradation by serum, it is difficult to accurately

determine the ghrelin level and consequently its physiological and pathophysiological roles. In the circulation, des-acyl ghrelin is mostly present as a free peptide while the vast majority of acyl ghrelin is bound to larger molecules and in particular to lipoproteins [19, 21]. The presence of the acyl group is necessary for ghrelin interaction with triglyceride-rich lipoproteins and low-density lipoprotein but not high-density lipoproteins and very high-density lipoproteins. Besides, ghrelin interacts via its N- and C-terminal parts with high-density lipoproteins and very high-density lipoproteins. These data support the transport of acylated ghrelin by triglyceride-rich lipoproteins and of both ghrelin and des-acyl ghrelin by high-density lipoproteins and very high-density lipoproteins [19]. Modifications to lipoprotein levels in response to obesity may affect ghrelin transport as well as free ghrelin levels.

Administration of ghrelin to rats leads to stimulation of food intake and decrease of energy expenditure, accounting for body weight increase [6, 22–25]. Intravenous ghrelin administration in humans also increases appetite and stimulates food intake [6]. Plasma ghrelin levels are negatively correlated with BMI and fluctuate in a compensatory manner to body weight variations [26]. Indeed, plasma ghrelin level is increased in anorexia nervosa and cachexia, and decreased in obesity [27]. Ghrelin levels decrease with weight gain resulting from overfeeding [28], pregnancy [29], olanzapine treatment [30], or high fat diet [31]. Central ghrelin administration to rats submitted to high fat diet does not result in greater food intake, while increased adiposity in white adipose tissue occurs [32]. In white adipose tissue, ghrelin stimulates the gene expression of lipogenic enzymes such as stearyl CoA desaturase, acetyl CoA carboxylase, and fatty acid synthase. These data suggest that central ghrelin simultaneously regulates food intake and adipose tissue metabolism through distinct mechanisms [32].

Acute feeding response appears to be mediated by GHS-R1a [33]. Chronic weight gain effect of ghrelin may be modulated by both GHS-R1a [34] as well as an as yet unidentified receptor for ghrelin as both ghrelin and a ghrelin antagonist induced body weight gain [35]. Nevertheless, further studies would be required to clarify this issue.

Des-acyl ghrelin has been recently taken into consideration as a modulator of food intake that could act through an as yet unidentified receptor [36, 37]. However, des-acyl ghrelin appears to have controversial effects on food intake [36, 37]. Indeed, GOAT knockout mice displayed reduced fat mass despite increased des-acyl ghrelin levels [38]. The identification of speculated des-acyl ghrelin receptors could deeply increase our knowledge on the mechanisms and actions sites of this peptide.

High plasma ghrelin levels have been reported in patients with Prader-Willi syndrome (PWS), a genetic disorder characterized by mental retardation and hyperphagia leading to severe obesity [39, 40]. In this disorder, ghrelin may be responsible, at least partially, for the insatiable appetite and the obesity of the patients.

From the molecular biological point of view, it is interesting to note that both ghrelin and its receptor (GHSR) genes are located on chromosome 3 in regions that have been

linked to obesity [41, 42]. Polymorphisms of both ghrelin and its receptor GHSR1a have been studied in obesity [43–52]. However, further studies are required to assess unambiguously the functional significance of these mutations in the pathogenesis of obesity.

Due to the observed association between plasma ghrelin levels and insulin levels as well as insulin resistance [53–56], it was suggested that inhibition of ghrelin secretion and/or of GHS-R1a could be a useful treatment and/or prevention for type 2 diabetes. In this respect, data from numerous studies evaluating the therapeutic implications of ghrelin on glucose-insulin homeostasis have been recently reviewed [57, 58].

The involvement of ghrelin in obesity led to the development of several ghrelin-related pharmacological tools for the treatment of obesity. The present review focuses on the recent advances made in potential clinical applications of ghrelin in obesity.

2. Ghrelin O-Acyltransferase: A Pharmacological Target to Decrease Acylated Ghrelin Levels

Ghrelin O-acyltransferase (GOAT), identified as being an orphan membrane-bound O-acyltransferase (MBOAT), catalyzes the addition of an octanoyl group on the serine in position 3 [2, 3] (Figure 1). Ghrelin octanoylation is essential for its recognition by GHS-R1a. Two distinct scientific approaches led to the identification of GOAT. The first approach was based on gene-silencing experiments targeting a candidate gene encoding an uncharacterized protein containing structural motifs reminiscent of the MBOAT acyltransferase family in the human medullary thyroid carcinoma cells expressing octanoylated ghrelin [2]. The authenticity of the human gene identified was verified using RT-PCR and 5' RACE reactions, and the predicted protein encoded by the gene was named GOAT. The functional activity of GOAT, the octanoylation of ghrelin, was demonstrated by transient transfections of the GOAT cDNA. GOAT was also shown to acylate ghrelin with fatty acid ranging from C7 to C12. Furthermore, additional experiments revealed the importance of the conserved MBOAT-histidine residue in position 338 of GOAT to its acylation activity [2]. The second approach was based on the transfection of rat insulinoma Ins-1 cells with ghrelin cDNA and subsequently of one at a time, of the sixteen MBOAT cDNAs [3]. Only one MBOAT cDNA led to acylated ghrelin production. Furthermore, mutation of either serine in position 3 of ghrelin or of the conserved MBOAT-histidine residue in position 338 of GOAT abolished ghrelin acylation. GOAT exhibits some specificity for medium chain fatty acids like octanoate and proceeds to ghrelin octanoylation before its translocation to the Golgi where it is cleaved by prohormone convertase 1/3 to form mature ghrelin. This suggested that GOAT is located in the endoplasmic reticulum [3].

GOAT is a highly hydrophobic protein with eight postulated membrane-spanning helices presenting a high degree of sequence conservation across vertebrates. GOAT is coexpressed with acyl ghrelin in ghrelin-expressing tissues [59].

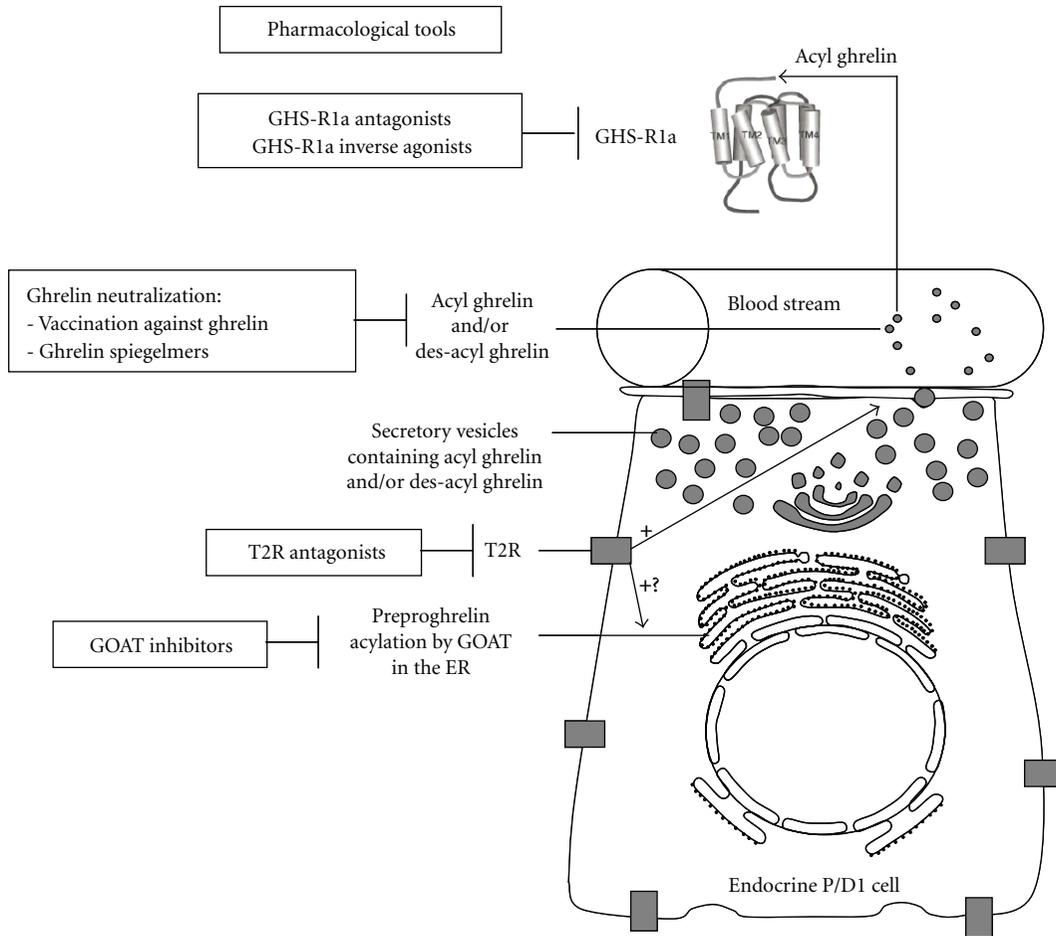


FIGURE 1: Synthesis, processing, and release of ghrelin by the human endocrine stomach P/D1 cells. Synthesis of ghrelin and acylation by GOAT occur in the endoplasmic reticulum (ER). Both acyl and des-acyl ghrelin are then secreted into the blood stream by fusion of secretory vesicles with the plasma membrane. Ghrelin secretion and/or acylation are upregulated following T2R activation. After secretion of acyl and/or des-acyl ghrelin, acyl ghrelin binds to specific GHS-R1a receptors. Pharmacological tools tried or currently developed are indicated and targeted GOAT, T2R, ghrelin, and GHS-R1a.

GOAT displays a preference for hexanoyl-CoA over octanoyl-CoA as an acyl donor [60]. However, the precise mechanism leading to the entry of acyls-CoA into the endoplasmic reticulum lumen remains unknown. One hypothesis is that GOAT could possibly bind acyl-CoA and, due to its hydrophobic properties, allow the acylation of ghrelin in the endoplasmic reticulum lumen.

An *in vitro* biochemical assay for GOAT activity [3] revealed the importance of proper recognition of several amino acids in proghrelin (glycine-1, serine-3, and phenylalanine-4) for GOAT activity [61].

Fasting and satiation could modulate the activity of GOAT as ghrelin levels rise before meals [4, 62] and decrease with food intake [5]. Moreover, long-term fasting inhibits ghrelin acylation but not total ghrelin secretion whereas feeding suppresses both acyl and des-acyl ghrelin [63]. However, the effect of fasting and feeding on GOAT mRNA levels remain unclear [38, 64]. Experimental evidences showed that GOAT is a leptin-regulated gene [38]. Increased GOAT mRNA levels in response to long-term chronic malnutrition

[64] could represent the underlying mechanism responsible for increased acylated ghrelin levels in anorexia nervosa [26].

Dietary lipids are critical for the activation of GOAT, and consequently ghrelin acylation. Indeed, GOAT knock-out mice submitted to a diet containing 10% medium-chain triglyceride exhibited lower body weight that can be explained by lower fat mass compared to wild-type mice [38]. In addition, GOAT transgenic mice only fed with a medium-chain triglycerides supplementation produced large amounts of acyl ghrelin [38].

An essential function of ghrelin could be the maintenance of viability during periods of famine. This hypothesis is supported by the data showing that wild-type and GOAT knock-out mice submitted to 60% calorie-restricted diet displayed 30% and 75% body weight loss, respectively [65].

Much work remains to be done to fully understand how GOAT fits into the control of energy homeostasis. However, measurement of both GOAT protein levels and GOAT activity will be crucial to determine its gene expression and functional regulation. Indeed, GOAT knock-out mice represent

a valuable tool to determine the physiological consequences of a specific deficiency in acylated ghrelin.

Recently, genetic variation of GOAT was suggested to be involved in the etiology of anorexia nervosa [66]. It would be interesting to determine if genetic variation of GOAT might also be linked to obesity. If this proves to be the case, personalized medicine targeting GOAT could be envisioned as a novel therapeutic approach for the treatment of obesity.

Pharmacological tools have been developed to target the inhibition of GOAT (Figure 1). Indeed, a pentapeptide, corresponding to the first five N-terminal amino acids of ghrelin with its C-terminal end amidated competitively inhibited GOAT activity through an end-product inhibition mechanism. The inhibition of GOAT is better achieved when pentapeptides contain an octanoyl group linked to serine-3 by an amide linkage [3]. Moreover, GOAT was also inhibited by peptide-based bisubstrate analog, GO-CoA-Tat, in cultured cells, as well as in mice [67]. The design of this bisubstrate analog was based on the theory that GOAT could use a ternary complex mechanism to proceed to the linkage of octanoyl-CoA to ghrelin. The intraperitoneal administration led to reduced weight gain and improved glucose tolerance in wild-type mice but not in ghrelin knock-out mice [67]. Even though GO-CoA-Tat presents some limitations as a peptide-based drug, it is likely that future synthetic derivatizations will maximize its pharmacological properties.

In conclusion, GOAT represents an extremely promising candidate for the development of antiobesity and/or antidiabetes drugs. Indeed, it is the unique enzyme responsible for ghrelin acylation and its modulation would only affect the physiological process of ghrelin acylation.

3. Neutralization of Ghrelin

Vaccination against ghrelin represents a strategy to block the effects of ghrelin (Figure 1). Rats immunized with ghrelin hapten immunoconjugates led to the production of antibodies specifically directed against acylated ghrelin, and reduced body weight gain with preferential reduction of fat mass concomitant to decreased feeding efficiency [68]. The human relevance of using vaccination against ghrelin remains uncertain. Indeed, phase I/II a trial using CYT 009-Ghr Qb vaccine, from Cytos Biotechnology AG, demonstrated no weight-loss effect in obese humans despite efficient antibody response.

High-affinity antiacyl ghrelin specific monoclonal antibodies specifically bind acyl ghrelin, dose-dependently inhibits GHS-R1a activation in vitro, and block ghrelin-induced food intake in mice in vivo [69].

Neutralization of ghrelin was also achieved using spiegelmers, antisense polyethylene glycol-modified L-oligonucleotides capable of specifically binding a target molecule (Figure 1). The spiegelmer NOX-B11-2 decreased food intake and body weight in diet-induced obese mice [70–72]. Another spiegelmer, NOX-B11-3 exerted a long-lasting action on the inhibition of ghrelin-induced GH release in rats [73], but did not block the fasting-induced neuronal activation in the hypothalamic arcuate nucleus [74]. The neutralization of circulating ghrelin by spiegelmers may be

useful to treat diseases associated with high ghrelin levels such as PWS characterized by severe obesity. Pfizer Inc. has taken over further development of the NOX-B11 spiegelmers originally developed by NOXXON Pharma AG.

In conclusion, the therapeutic usefulness of vaccination against ghrelin and the use of ghrelin spiegelmers in the treatment of obesity remain to be proven.

4. GHS-R1a: A Pharmacological Target to Antagonize Ghrelin-Induced Responses

4.1. GHS-R1a Antagonists. The inhibition of ghrelin signaling represents an attractive target for pharmacological treatment of type 2 diabetes, obesity, particularly PWS, and metabolic syndrome. Consequently, several classes of GHS-R1a antagonists have been developed (Figure 1).

[D-Lys⁻³]GHRP-6, a peptide GHS-R1a antagonist, decreased food intake in lean and obese mice, and reduced weight gain [70, 75].

Piperidine-substituted quinazolinone derivatives were identified as a novel class of small GHS-R1a antagonists molecules [76]. Phenyl or phenoxy groups are optimal substituents at position 6 of the quinazolinone core, and the replacement of phenyl groups in position 2 by small alkyl substituents were proven to be beneficial [76]. YIL-781, a piperidine-substituted quinazolinone derivative acting as a potent GHS-R1a antagonist, improved glucose-stimulated insulin secretion and reduced food intake and weight loss in diet-induced obese mice [77].

Some GHS analogs carrying a trisubstituted 1,2,4-triazole structure, such as JMV2866 and JMV2844, behaved as GHS-R1a antagonists [78, 79]. Recently, additional new GHS-R1a antagonists of global similar structure have been identified using homogenous time-resolved fluorescence-based assay screening [80].

Optimization of piperazine-bisamide analogs synthesis led to potent GHS-R1a antagonists. One of these analogs featured especially high potency as well as other interesting pharmacological properties, and inhibited GH release ex vivo [81].

Several carbonylhydrazide derivatives were identified as being potent and selective GHS-R1a antagonists [82]. Among these compounds, GSK1614343 was shown to be a potent competitive antagonist of rat GHS-R1a [83]. Unexpectedly, GSK1614343 produced an increase in food intake and body weight in both rats and dogs [84].

BIM-28163 was identified as a ghrelin antagonist blocking ghrelin-induced GH secretion [85]. However, chronic administration of GHS-R1a antagonist unexpectedly induced body weight gain [85].

Other GHS-R1a analogs developed to treat weight disorders, including obesity, are also still considered as preclinical compounds (TZP-301, from Tranzyme Pharma, and EX-1350, from Elixir Pharmaceuticals) [86].

In conclusions, several classes of GHS-R1a antagonists have been identified and could represent an interesting pharmacological tools for the treatment of obesity as well as type 2 diabetes and metabolic syndrome. However, long-term animal and human studies still remain necessary to

appropriately evaluate the beneficial properties of ghrelin antagonists in the context of obesity.

4.2. GHS-R1a Inverse Agonists. The high constitutive activity of GHS-R1a suggested that inverse GHS-R1a agonists, decreasing its constitutive activity, may be useful for the treatment of obesity [87, 88]. Long fasting induced, in the hypothalamus, increased GHS-R1a expression and concomitant signaling causing higher appetite and decreased energy expenditure. Therefore, reduction of the GHS-R1a constitutive activity by an inverse agonist could increase the sensitivity to anorexigenic hormones like leptin or PYY, and prevent food intake between meals [89].

[D-Arg¹, D-Phe⁵, D-Trp^{7,9}, Leu¹¹] substance P was identified as an inverse agonist on GHS-R1a [90].

In conclusion, GHR-R1a inverse agonists represent interesting pharmacological tool to inhibit GHS-R1a activity (Figure 1). However, additional studies evaluating the long-term use of the compounds in animal models are necessary to elucidate their usefulness in the treatment of obesity and related diseases in humans.

5. New Potential Pharmacological Target to Decrease Ghrelin Secretion

Very recently, gavage of bitter taste receptor (T2R) agonists was shown to increase plasma acyl ghrelin in mice through the stimulation of α -gustducin, the α -subunit of a trimeric G-protein complex involved in taste signal transduction [91]. Immunofluorescence studies revealed that the stomach endocrine cells expressing ghrelin displayed up to 90–95% colocalization with α -gustducin. Furthermore, gavage of T2R-agonists increased food intake in wild-type mice but not in α -gustducin or GHS-R1a knock out mice [91]. It is presently unclear if the transduction pathways induced following T2R activation could affect ghrelin acylation by GOAT and/or ghrelin release.

In conclusion, T2R could represent a new interesting pharmacological target to modulate ghrelin secretion (Figure 1). Furthermore, the potential use of T2R antagonists for the treatment of obesity remains to be evaluated.

6. General Conclusions

The involvement of ghrelin in obesity and the better understanding of ghrelin biology have led to the identification of pharmacological targets and the development of pharmacological compounds for the treatment of obesity and related diseases. So far, pharmacological compounds have been designed to target GOAT, ghrelin, and GHS-R1a. Very recently, it has been suggested that T2R could also represent an interesting target in the context of ghrelin and the treatment of obesity.

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