GOAT: the master switch for the ghrelin system?
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Abstract
The ghrelin–ghrelin receptor system is one of the most important mechanisms regulating energy balance and metabolism. Among other actions, central and peripheral administration of ghrelin increases food intake and adiposity. During the last years, many efforts have been made in the investigation of the cellular and molecular mechanisms modulating the effects of ghrelin. One particularity of this peptide hormone is its acylation at serine-3 with an eight-carbon fatty acid (octanoate), which confers its biological activity. Recent reports have demonstrated that the ghrelin O-acyltransferase (GOAT) is the enzyme that catalyzes ghrelin octanoylation. Therefore, all questions concerning the posttranslational acylation of ghrelin are of great interest for the complete understanding of this system. In this review, we summarize the discovery and characterization of GOAT, and remark the importance of GOAT as a novel and potential target that regulates the biological actions of ghrelin, revealing several therapeutical possibilities for the treatment of the metabolic syndrome.

Introduction
Ghrelin’s discovery in 1999 by Kojima et al. (1) opened a new frontier in the understanding of energy homeostasis regulation since ghrelin is the only circulating peptide hormone that stimulates food intake and adiposity in humans (2) and rodents (3–5). Independent of its ability to stimulate GH secretion and besides the orexigenic and adipogenic effects of ghrelin, this peptide influences glucose (6) and lipid metabolism (7). Many other physiological roles of ghrelin have been described for a variety of tissues and organs, such as beneficial effects on cardiovascular and gastroenteropancreatic physiology (8, 9), modulation of the immune system (10), or improvement of memory and learning (11).

Ghrelin is mainly produced in the stomach from a distinct group of endocrine cells located within the gastric oxyntic mucosa (12). A certain amount of ghrelin has been observed along the gastrointestinal tract and pancreas (12, 13). The expression of ghrelin within the brain (14–16), testis (17, 18), pituitary (19), kidney (20), thyroid gland (21), and placenta (22) among others has also been reported.

Ghrelin, like many other peptide hormones, is generated from a precursor protein. Prepro-ghrelin contains 117 amino acids (23). After removing the signal sequence by cleavage of amino acid-23, the pro-ghrelin peptide sequence (94 amino acids) has an N-terminal glycine residue. The prohormone convertase PC1/3 then cleaves pro-ghrelin after arginine-28, generating the mature 28-amino acid ghrelin (24) (Fig. 1). Recent studies based on different cultured cell lines with different patterns of processing protease expression have demonstrated that not only PC1/3, but also both PC2 and furin can process pro-ghrelin to the 28-amino acid ghrelin (25). Therefore, ghrelin is the N-terminal fragment generated by cleavage of pro-ghrelin, whereas the C-terminal fragment is called ‘obestatin’. To date, there is no clear evidence supporting a role of obestatin in the regulation of energy balance (26–28), even though previous reports claimed that obestatin had an anorexigenic effect (29). Therefore, the potential role of the C-terminal fragment of pro-ghrelin remains unknown.

During posttranslational modification of the ghrelin precursor protein, serine-3 is acylated with an eight-carbon fatty acid (octanoate). Octanoylation of ghrelin is a specific modification that is required for ghrelin to bind to its receptor GHS-R1a and to exert most of its biological properties. Although ghrelin in its unacylated form (UAG) is believed to be the dominant form of ghrelin in the plasma, most of the biological actions
ascribed to ghrelin needs acylation (9) including GH secretion, feeding, insulin secretion, and adiposity. The enzyme responsible for the octanoylation of ghrelin is ghrelin O-acyltransferase (GOAT) (30, 31) that is also named membrane bound O-acyltransferase 4 (MBOAT4). Since the structural domains of GOAT are highly conserved among species and its unique substrate seems to be ghrelin, this enzyme became an excellent target to regulate ghrelin action. In this review, we recompile different aspects of GOAT such as its discovery, enzymological properties, gene regulation, and its potential physiological role as regulator of energy homeostasis.

**Relevance of ghrelin in human energy balance**

Circulating ghrelin levels are negatively associated with obesity (4, 32), whereas in states of anorexia and cachexia (33, 34) the secretion of ghrelin is increased. There are some clinical exceptions such as the patients who had lost weight after gastric bypass surgery (35) or the patients who had Prader–Willi syndrome, a genetic disease characterized by severe obesity and hyperphagia (36). In contrast to most forms of obesity, patients with Prader–Willi syndrome show massively elevated ghrelin levels (36). Furthermore, circulating ghrelin level is increased before meals suggesting its role in meal initiation in humans (37). Although circulating ghrelin levels are strongly regulated by nutritional status or obesity, it seems that mutations in the ghrelin or ghrelin receptor gene are not very common within obese patients. Specifically, ghrelin Arg-51-Gln, prepro-ghrelin, and prepro-ghrelin Leu-72-Met (38–40) have been found to be related with obesity.

The potent action of ghrelin in stimulating appetite in humans is being well reported (41), and its effects on insulin and glucose have also been extensively studied. Ghrelin levels seem to correlate negatively with insulin secretion (42). Treatment of healthy volunteers with ghrelin decreased insulin secretion, caused hyperglycemia (8, 43), and decreased insulin-stimulated glucose disposal, suggesting an impaired insulin sensitivity (44–46). However, some studies have shown controversial results indicating that ghrelin levels correlated positively with insulin sensitivity (47), or that plasma ghrelin concentrations were not regulated by glucose or insulin (48).

The correlation between fatty acids and ghrelin has also been studied, and different data were found.
For instance, it was reported that lipid infusion did not decrease circulating ghrelin levels in humans (49), and that free fatty acids decreased circulating ghrelin concentrations (50). Given the recent findings obtained in rodents (51), it is very likely that the type of fatty acids used in the different studies might explain these controversial data. By contrast, when exogenous ghrelin is administered in humans, lipolysis was stimulated (45, 46) and free fatty acids were increased (52).

Thus, ghrelin has a clinical significance because it is a good candidate for treatment of different disorders related with overnutrition, metabolism, and energy balance. The complete understanding of ghrelin system and signaling will be necessary to develop efficient treatments of these disorders.

Discovery of GOAT and its enzymological properties

In 2008, GOAT was identified and characterized simultaneously by two independent groups (30, 31). Previous studies made on Drosophila wingless gene and its mammalian homolog, Wnt, described that the enzyme porcupine is required for serine-209 acylation with palmitoleic acid and for the transport of Wnt3a from the endoplasmic reticulum (ER) (53–55). Porcupine possesses structural similarities to MBOAT (56). Based on these studies, the authors raised the question of whether or not a member of this family of MBOAT could mediate the acylation of ghrelin (30, 31).

To answer this question, Gutierrez et al. (30) performed gene-silencing experiments in a human medullary thyroid carcinoma cell line, a cell line that possesses the necessary enzymatic machinery for ghrelin acylation. Candidate sequences similar to known acyltransferases with homology to human transferases were used to screen for GOAT. By detecting both acyl and des-acyl forms of ghrelin by MALDI-TOF assays, they demonstrated that the N-terminal sequence is absolutely conserved in all vertebrates (23). Yang et al. (59) also suggest that the acylation of ghrelin occurs after the signal sequence is cleaved by a signal peptide peptidase because the addition of two extra amino acids (AS) at the N-terminus of pro-ghrelin reduces its ability to accept an octanoyl group.

In summary, different studies led to the conclusion that GOAT, a member of MBOAT family, is the unique enzyme that acylates pro-ghrelin at serine-3 in a process requiring four amino acids at the N-terminal.

GOAT mRNA expression and its regulation

The fact that ghrelin is uniquely acylated by GOAT has led to the investigation of the mRNA expression of this enzyme in tissues that produce ghrelin. In human tissues, there are elevated levels of GOAT transcripts in cDNA from species as different as mouse, rat, or zebrafish in HEK-293 cells successfully yielded octanoylated human ghrelin, thus indicating that GOAT is highly conserved across vertebrates (30).

Yang et al. (31) chose an entirely different approach to identify the enzyme that octanoylates ghrelin. Led by original work by Hofmann et al. (2000) (58) that suggested a conserved amino acid sequence for porcupine and a variety of MBOATs, they transfected the respective MBOAT species into three different murine endocrine cell lines (AT-20, INS-1, and MIN-6 cells), and used molecular tools and cellular biology strategies to identify and further characterize GOAT. Radiolabeled [3H]octanoate was used to confirm that GOAT modifies ghrelin with octanoate. Using site-directed mutagenesis, the predicted catalytic GOAT residues asparagine-307 and histidine-338 were substituted with alanine, resulting in an inability of the mutated GOAT to acylate ghrelin.

The observed octanoylation occurs before pro-ghrelin is transported to the Golgi where it is cleaved by protease convertase to form mature ghrelin (Fig. 1). These findings suggest that GOAT may be located in the membrane of the ER compartment and may mediate the translocation of the octanoyl-CoA from the cytosolic side to the ER lumen (31). Further studies are necessary to understand how this translocation occurs and if there are other proteins implicated in this process.

Within the same year (2008), Yang et al. published another work involving an in vitro biochemical assay and INS-1 cells to test the sequence requirements for substrate recognition by GOAT (59). By using mutagenesis studies, they demonstrated that the N-terminal glycine-1, serine-3, and phenylalanine-4 of ghrelin are crucial for GOAT recognition (59). These three amino acids in the ghrelin sequence are absolutely conserved in all vertebrates (23). Yang et al. (59) also suggest that the octanoylation of ghrelin occurs after the signal sequence is cleaved by a signal peptide peptidase because the addition of two extra amino acids (AS) at the N-terminus of pro-ghrelin reduces its ability to accept an octanoyl group.
stomach and pancreas, and very low levels in most other tissues (30). However, compared with the presence of ghrelin transcript, GOAT mRNA levels appear to be much lower. Similar results were obtained by Yang and colleagues in mouse tissues with low amounts also in small intestine, colon, and testis (31). Moreover, the analysis of GOAT mRNA in samples enriched with mouse gastric ghrelin cells has demonstrated a high expression of GOAT within ghrelin cells and a high correlation with ghrelin distribution (60). GOAT mRNA expression was also evaluated in rat tissues which contained a similar pattern of expression observed in human and mouse tissues (61).

In addition to the multiplicity of ghrelin actions affecting GH secretion, food intake, glucose homeostasis, cardiovascular, gastrointestinal and immune functions, it has been reported that ghrelin also plays an important role in bone formation (62) and regulation of chondrocyte metabolism in cartilage (63). Therefore, the expression of GOAT mRNA was also assessed in murine and human chondrocytes. GOAT gene expression was found in both murine and human chondrocytes, although its levels were lower than those observed in stomach tissue (64). GOAT mRNA expression is low during the first days of the chondrocyte differentiation, but its expression increases as differentiation progresses and follows the same pattern of expression of prepro-ghrelin (64). In this study, the researchers also investigated the influence of several hormone and drug treatments in murine and human chondrocyte cell lines. Only lipopolysaccharide produced a decrease in GOAT expression. However, neither GH nor ghrelin induced changes in enzyme mRNA expression in these experimental models (64).

In the case of energy balance, the effect of acute food deprivation and chronic malnutrition on Goat mRNA expression in stomach mucosa from normal male rats was evaluated (61). Despite the changes in body weight caused by chronic malnutrition, Goat mRNA levels remained stable. Goat and ghrelin mRNA levels increase significantly only after the animals lost a considerable amount of weight (day 21). When rats were fasted for 48 h, leptin levels decreased whereas prepro-ghrelin mRNA levels increased. This increase in prepro-ghrelin expression was also observed in fed rats treated with leptin, but not observed in fasted rats treated with leptin. Curiously, Goat mRNA expression remained unaltered in fed and fasted rats. Only fasted rats treated with leptin showed an increase in Goat mRNA expression (61).

Recent studies have shown that fasting C57BL/6 mice for 36 h significantly decreases the expression of gastric Goat mRNA. Plasma acyl ghrelin levels remained unchanged along the 36-h fasting period, while des-acyl ghrelin levels increased significantly. The fact that neither gastric Goat mRNA expression nor prepro-ghrelin mRNA expression are upregulated during 36 h fasting points toward the idea that the predominant physiological function of ghrelin may not necessarily, or at least not exclusively, be a hunger signal reflecting an empty stomach (51). Kirchner et al. also investigated the role of chronically enhanced food intake and obesity on the GOAT–ghrelin system. They showed that Goat and prepro-ghrelin mRNAs in the gastric tissue of ob/ob mice did not change in leptin-deficient mice when compared with lean littermates (51).

Overall, these studies indicate that even though ghrelin and Goat genes are similarly distributed in rodent tissues, the regulation of GOAT remains poorly understood. The analysis of Goat mRNA expression in animal models under different energy status, together with in vitro studies assaying the influence of several hormones such as GH, leptin, and ghrelin suggest a complex pattern of Goat expression. Further studies would be necessary to completely elucidate the factors that regulate its gene transcription and its correlation with protein and activity levels.

Regulation of ghrelin acylation

In vitro experiments performed by Yang et al. (2008b) (59) showed that octanoylated pentapeptides inhibited ghrelin acylation. They incubated membranes that express mouse GOAT with several pentapeptides, and found that the first five amino acids of the ghrelin sequence with the C-terminus region amidated (GSSFL-NH2) can be used as a substrate for the enzyme GOAT nearly as well as for pro-ghrelin. This inhibition may occur primarily by competition where the peptide reaches the binding site of GOAT and does not necessarily serve as a substrate. The results observed in this study are consistent with the idea that pro-ghrelin remains bound to the enzyme (59). It remains to be determined, though, whether the inhibitory effects of these pentapeptides bear any relevance in vivo.

Most of the in vivo studies assessing the physiological role of the GOAT–ghrelin system are focused on its actions as a regulator of energy balance. Before GOAT was characterized, it was reported that dietary lipids can directly influence ghrelin acylation (65). There is new evidence supporting the idea that the acylation of ghrelin by GOAT is regulated by nutrient availability and depends on specific dietary lipids as acylation substrates (51). First, to test the hypothesis that regulation of acylated ghrelin (AG) production and secretion is dependent on medium-chain fatty acid (MCFA) substrate availability, C57BL/6 mice were fed a diet rich in glycerol triheptanoate (a medium-chain triglyceride (MCT) containing heptanoic acid, which is not synthesized de novo in mice). It was found that ghrelin was acylated with heptanoic acid and was more abundant than octanoylated ghrelin in stomach tissue and blood samples. Secondly, to investigate whether incoming dietary lipids have an impact on GOAT activity, UAG and AG were measured in mice during
different time points throughout the light and darkness cycles. Blood concentrations of AG increased significantly 2 h after the beginning of the dark phase when mice eat actively. Thirdly, it was observed that Goat knockout mice were resistant to high-fat diet (HFD)-induced obesity when fed a diet rich in MCT. Although no differences in body composition were found in GOAT-deficient mice fed on HFD, GOAT-null mice fed a diet rich in MCT showed lower fat mass, which was probably due to higher energy expenditure in the light cycle (51). Finally, transgenic mice overexpressing human ghrelin and human GOAT in liver were generated. Curiously, these mice lacked octanoyl-modified human ghrelin in circulation, which is likely due to the deficit of MCFAs in liver under normal dietary conditions. For this reason, it was necessary to supply the diet with triglyceryl octanole. The transgenic mice on this diet showed high concentrations of human UAG and AG. Despite the higher body weight and fat mass in mice overexpressing ghrelin and GOAT, the food intake did not differ compared with WT mice. The energy expenditure was significantly lower in transgenic mice during light and darkness phases suggesting that these transgenic mice oxidize less fat than WT. Two weeks after being switched to a regular chow diet, the differences in body weight between transgenic and WT mice disappeared, indicating that ghrelin acylation is dependent on dietary lipids (51). More studies investigating lipid metabolism are required to understand the importance of dietary lipids versus endogenous lipids on ghrelin acylation.

According to the published data, it is reasonable to hypothesize that the synthesis of a ghrelin transcript and the secretion of AG constitute two independent processes. The GOAT–ghrelin system is only activated when certain fatty foods are consumed to inform the brain about food availability, whereas regulation of ghrelin transcript synthesis may be mediated by different inputs.

This idea is supported by recent studies performed in humans (66). The profile of total ghrelin levels is well established: ghrelin is secreted before a meal and suppressed after a meal. During long-term fasting periods, the amount of AG decreases, but the total ghrelin circulating levels remain unchanged because UAG levels increase (66). This means that the long-term fasting regulates ghrelin activity by inhibition of ghrelin acylation, whereas ghrelin synthesis still continues to occur. Thus, this would explain why the increased appetite only occurs during short-term fasting. These results obtained in humans are consistent with those observed in rats, where the proportion of UAG markedly increases after 48 h of fasting (3).

Another study based on Goat −/− null mice reveals the physiological importance of AG during calorie restriction (67). Under 60% calorie-restricted diet, both WT and Goat −/− mice lost body weight and fat mass, and blood glucose levels decrease equally. However, after 4 days of calorie restriction, WT mice stabilize blood glucose levels, whereas Goat −/− mice are not able to maintain blood glucose within a physiological range and levels continue to decline over time (67). Hypoglycemia in Goat −/− mice is associated with a decrease in GH plasma levels. Thus with this work the authors suggested that one essential function of AG is the elevation of GH levels during severe calorie restriction to preserve sufficient blood glucose and survival (67). However, it cannot be ruled out that the metabolic effects of ghrelin per se might also explain those findings.

In summary, it seems that dietary lipids are critical for the activation of GOAT and are therefore necessary for ghrelin acylation, suggesting that the ghrelin-GOAT system informs the brain of the presence of dietary calories. Furthermore, this system seems to have a physiological relevance for maintaining glucose homeostasis and GH levels under 60% calorie restriction. Importantly, the secretion of AG is a process that occurs independently of ghrelin synthesis.

**Physiological importance of GOAT and open questions**

Since ghrelin has a metabolic, endocrine, and clinical significance, the complete understanding of the ghrelin–GOAT system is an important mechanism involved in the pathogenesis of obesity and diabetes. Based on new data, the classical belief that ghrelin is just a hunger signal is being reconsidered. Although Kirchner et al. (51) provide valuable information to understand the conversion of UAG to AG (active form), there are still many open questions regarding the UAG form. Several studies suggest that UAG may regulate food intake in a GHS-R1a-independent manner (68–70), and it was proposed that UAG plays an important role in pancreatic function (71). Does UAG have a complementary action to AG? Furthermore, there are some variants of ghrelin such as decanoyl and des-Gln14 ghrelin resulting from alternative splicing of the ghrelin gene that may exhibit similar physiological functions to AG (72). Do they have physiological importance? The use of specific blockers for the different isoforms of ghrelin might serve to answer this question. In that direction, some studies have already been performed. The neutralization of bioactive ghrelin (acylated) by a synthetic oligonucleotide NOX-B11 leads to inhibition of ghrelin’s secretory effects in the CN (73), and ameliorates obesity in diet-induced obese mice (74). Indeed, the specific blockade of each different ghrelin isoform is methodologically complicated since several of these isoforms share the same nucleotide and amino acid sequences. To elucidate the specific actions of those ghrelin isoforms will be crucial for the complete understanding of the ghrelin signaling.
On the other hand, the discovery of GOAT has important implications in terms of developing drugs to target the acylation process and, consequently, the physiological effects of AG. GOAT is a good candidate because: i) it is the unique enzyme that acylates ghrelin in a highly conserved manner, ii) it is known that ghrelin only binds and activates its receptor GHSR1a when acylated, and iii) its inhibition or stimulation would not affect physiological processes other than ghrelin acylation. However, up to date, the methodologies for studying the role of GOAT are very limited. For instance, the measurement of GOAT protein levels and activity assays will be important to corroborate if gene expression (this is the only one measurement that all the studies concerning GOAT have used so far) correlates with ‘functional’ GOAT, and therefore, with the amount of AG. To get a specific antibody for GOAT will also allow us to investigate its tissue and cellular distribution. It would be also interesting to determine if the lower expression of GOAT mRNA compared with its substrate (ghrelin) has a physiological significance in regulating ghrelin acylation. GOAT knockout and GOAT transgenic mice have been generated (51) and are indeed important tools for understanding the physiological relevance of GOAT. However, genetic mice models allowing interruption or overexpression of GOAT in adulthood in specific tissues would help us to understand whether this is the rate-limiting step in ghrelin acylation or whether there are other mechanisms compensating – at least partially – the actions of GOAT. Further, if dietary lipids constitute the main source of MCFAs for ghrelin acylation, ghrelin activity could potentially be modulated through dietary intervention. Ultimately, a better understanding of GOAT regulation and especially the development of pharmacological GOAT inhibitors or activators will be crucial for understanding the true function of the GOAT–ghrelin system and its potential as a pharmacologically treatable target.

Finally, although GOAT may be the most important enzyme for the acylation of ghrelin, we must not forget that other enzymes playing a crucial role in the secretion of AG may exist. In this sense, it is important to point out that the secretion of AG is a process that occurs independently of ghrelin synthesis. Therefore, the investigation of several mechanisms that are likely independently affecting AG is also crucial. These mechanisms include the secretion rates of the bioactive ghrelin molecule, other enzymes deactivating or degrading circulating ghrelin, and the amount of expression of ghrelin receptor(s) in target tissue and receptor sensitivity to the level of AG.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

A R is a recipient of a contract, Isabel Barreto funded by Xunta de Galicia. This work has been supported by grants from Ministerio de Educación y Ciencia (R N: RYC-2008-02219 and SAF2009-070349, and European Union (R N: Health-F2-2008-223713; ‘Reprobesity’). CIBER de Fisiopatología de la Obesidad y Nutrición is an initiative of ISCIII.

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