

# Influence of chronic undernutrition and leptin on GOAT mRNA levels in rat stomach mucosa

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## Abstract

The most unique feature of ghrelin is the acyl-modification of a hydroxyl group of the Ser3 in the N-terminus. The Ser3 is commonly modified by *n*-octanoic acid in vertebrates being needed for its biological effects, at least in terms of feeding. Therefore, a critical question regarding the role of ghrelin was to characterize the mechanism involved in its acylation. The acyltransferase that catalyzes ghrelin octanoylation has been recently identified and named ghrelin *O*-acyltransferase (GOAT). The aim of this study was to clarify the physiological implications of GOAT in the regulation of energy balance, by assessing the effect of undernutrition, as well as fasting in adult male rats. We have determined GOAT mRNA expression levels by real time-PCR in the stomach mucosa. Our results show that chronic food restriction led to an increase in GOAT mRNA, particularly following long-term chronic malnutrition (21 days). Furthermore, following 48 h complete fasting, a situation with high-circulating ghrelin levels, we found similar mRNA expression of GOAT in fed and fasted rats; exogenous leptin administration markedly increase GOAT mRNA levels in the stomach mucosa of fasted rats. These findings suggest that increased GOAT mRNA levels may have a role in mediating the physiological responses to chronic undernutrition and could represent an adaptive response to prevent long-lasting alterations in energy balance and body weight homeostasis. Furthermore, our data also offer mechanistic insights into the reason why during fasting acylated ghrelin levels are not increased at a time when a marked increase in an orexigenic signal as important as acylated ghrelin will be expected.

*Journal of Molecular Endocrinology* (2008) **41**, 415–421

## Introduction

Ghrelin released from ghrelin-producing cells in the stomach circulates through the body and exhibits multiple biological actions in different tissues. Data gleaned over the last few years have shown that ghrelin is the most potent endogenous orexigenic peptide in both rodents and humans (Kojima *et al.* 1999, Tschöp *et al.* 2000, Wren *et al.* 2001, van der Lely *et al.* 2004, López *et al.* 2008a). Ghrelin's important physiological role of body weight and metabolic homeostasis derives from several facts, such as those indicating that a) ghrelin might be an important signal to prepare for meal initiation in humans and rodents (Cummings *et al.* 2001, Drazen *et al.* 2006); b) deletion of ghrelin (and/or its receptor) prevents high-fat diet-induced obesity, increased basal insulin level, enhanced glucose-stimulated insulin secretion, and improved peripheral insulin sensitivity; and c) ghrelin modulates key hypothalamic neuropeptide systems, such as agouti-related protein/neuropeptide Y and hypothalamic metabolic pathways, such as fatty acid synthesis and oxidation, regulating feeding (Broglia *et al.* 2004, Dezaki *et al.* 2004, 2006, Gauna *et al.* 2004, 2006, Iwakura *et al.* 2005, Granata *et al.* 2007, López *et al.* 2008a,b).

These effects suggest that ghrelin has unique actions on the handling of metabolic substrates in general and on key components of glucose homeostasis.

The most unique feature of the ghrelin structure is the acyl-modification of a hydroxyl group of the serine residue at position 3 of the N-terminal of ghrelin. The Ser3 is commonly modified by *n*-octanoic acid in vertebrates being needed for its biological effects, at least in terms of feeding (Gutierrez *et al.* 2008, Yang *et al.* 2008). Since the effects of ghrelin on GH secretion and food intake were initially only observed after administration of the acylated form, besides the fact that unacylated ghrelin (UAG) was unable to bind to the type 1a of ghrelin receptor (GHS-R) led initially to suggestions that UAG was devoid of biological activity (Kojima *et al.* 1999). However, it is currently clear that in some cases the biological effects of ghrelin are mediated by the acylated form properly, with UAG being inactive, whereas in other effects UAG can mimic ghrelin action or even exert antagonistic effects (Gauna *et al.* 2004, 2006, Dezaki *et al.* 2006, Granata *et al.* 2007). In this sense, it is now believed that the ratio acylated/UAG is particularly important in terms of glucose homeostasis. Thus, it has been found that endogenous ghrelin inhibits the glucose-induced insulin

release via the GHS-R1a, as demonstrated by the marked increase of insulin response to glucose after deletion of the ghrelin gene (Dezaki *et al.* 2006). Moreover, ablation of the ghrelin gene improves glucose tolerance, insulin secretion, and insulin sensitivity in genetically leptin-deficient (*ob/ob*) obese mice (Sun *et al.* 2006). Administration of exogenous ghrelin suppresses further insulin secretion, and it worsens insulin sensitivity and glucose tolerance after a meal or a glucose load (Broglia *et al.* 2004). UAG administration neither has effects on glucose-induced insulin release in a perfused pancreas model, nor induces significant changes in systemic fasting levels of insulin and glucose *in vivo* (Gauna *et al.* 2004, 2006, Dezaki *et al.* 2006, Granata *et al.* 2007). However, UAG increases insulin release *in vitro* by insulinoma cell lines exposed to high glucose concentrations (Gauna *et al.* 2006), and overexpression of (endogenous) UAG in pancreatic islets improves the insulin sensitivity to an i.p. glucose load in mice (Granata *et al.* 2007). Moreover, when coadministered with ghrelin, UAG completely prevents the ghrelin-induced increase in circulating glucose levels and worsens insulin sensitivity (Broglia *et al.* 2004, Iwakura *et al.* 2005). These and other effects (Korbonits *et al.* 2004, van der Lely *et al.* 2004, Gualillo *et al.* 2006) illustrate the relevance of the ghrelin/UAG ratio and led to the hypothesis that targeting of the enzyme responsible for ghrelin acylation might be of therapeutic interest for those pathological conditions characterized by insulin resistance and impaired insulin release.

The acyltransferase that catalyzes ghrelin octanoylation has recently been identified and named ghrelin O-acyltransferase (GOAT; Hofmann 2000, Gutierrez *et al.* 2008, Yang *et al.* 2008), also called membrane-bound O-acyl transferase 4 (MBOAT4). All biochemically characterized members of this superfamily are enzymes transferring organic acids, typically long-chain fatty acids, to hydroxyl groups in membrane-embedded substrates. All of them have several membrane-spanning regions, typically between eight and ten, and share a region of detectable sequence similarity. Structural domains of GOAT are conserved across vertebrates, including lower vertebrates such as zebrafish. Curiously, zebrafish GOAT, which shows ~60% amino acid homology with the human counterpart, is able to acylate human ghrelin, which suggest a highly conserved evolutive function of this enzyme (Hofmann 2000). The discovery of GOAT brings important questions and raises several therapeutic possibilities. First, drugs inhibiting GOAT might be able to prevent diet-induced obesity and might be an effective therapy for type 2 diabetes, increasing insulin secretion and enhancing peripheral insulin sensitivity. Furthermore, it is believed that research on GOAT will offer new insights into the pathophysiology of energy homeostasis, and might lead to the identification of further therapeutic targets. The aim of this study was to clarify the physiological implications of GOAT in the regulation of

energy balance, by assessing its mRNA levels in two different experimental paradigms, namely the effect of acute (fasting) and chronic (21 days) undernutrition in normal male rats. Furthermore, we evaluated the effect of leptin on GOAT expression in fed and fasted rats.

## Materials and methods

### Animals

Male Sprague–Dawley rats (200–250 g) were housed in air-conditioned rooms (22–24 °C) under a 12:12 h light/darkness cycle and fed standard rat chow and water *ad libitum*. Animals were killed by decapitation. Tissues were collected and frozen at –80 °C until they were used. All the animal procedures were conducted according to the principles approved by the Santiago de Compostela Medical School Animal Care Research Committee.

### Screening in rat tissues

The distribution of GOAT and the other MBOATs predicted in rat (MBOAT 2 and 5) was analyzed in several tissues of adult male rats. Animals were killed by decapitation and all tissues were rapidly snapped frozen on dry ice.

### Effect of acute food restriction and leptin

The effect of fasting on stomach mucosa GOAT and ghrelin mRNA expression was analyzed. Adult males were subjected to food deprivation for 48 h, and stomach mucosa was collected at the end of the fasting period. In addition, the effects of leptin administration upon GOAT and ghrelin mRNA levels were evaluated. One group of animals that received the leptin treatment was fed *ad libitum* and the other group was fasted during the last 48 h of treatment. In this setting, rats were injected intraperitoneally with vehicle or recombinant leptin (Sigma) at a dose of 200 µg/rat every 12 h, for 2 days (López *et al.* 2008a) We used eight animals per group.

### Effect of chronic food restriction on stomach mucosa GOAT and ghrelin mRNA expression

Male rats were randomly assigned on day 1 to one of two dietary groups as previously described (Gualillo *et al.* 2002): rats fed *ad libitum* and a restricted group of rats fed with 30% of the amount of food that *ad libitum* rats ate the previous day. Male rats at different days of restriction were killed and stomach mucosa samples were collected at 8, 12, 16, and 21 days of food restriction. Tissues were frozen at –80 °C until processing. We used eight animals per experimental group.

## Plasma leptin levels

Plasma leptin levels were measured by RIA as previously described (Gualillo *et al.* 2002, López *et al.* 2008a) using reagents provided in commercial kits (Rat leptin RIA, Linco Research Inc., St Charles, MO, USA).

## RNA isolation and real time quantitative RT-PCR

Total RNA was isolated from rat tissues using TRIzol (Invitrogen, Life Technologies), according to the manufacturer's recommendations. First-strand cDNA was synthesized from 2 µg total RNA by random priming RT. The resulting cDNA was subjected to PCR amplification (Nogueiras *et al.* 2004a, López *et al.* 2006, Caminos *et al.* 2007, Vázquez *et al.* 2008), using sense and antisense primers specific for the rat GOAT, MBOAT 2 and 5 mRNAs (Table 1). The mRNA levels of GOAT and ghrelin in stomach mucosa were studied by using real time-PCR (TaqMan; Applied Biosystems; Foster City, CA, USA) by using specific primers and probes (Table 1) as described previously. All reactions were carried out using the following cycling parameters: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. For the analysis, the input value of GOAT and ghrelin were standardized to the 18S value for the sample group and were expressed compared with the average value for the control group. We used eight rats per experimental group.

To verify the identity of amplified cDNAs, PCR products were electrophoresed on a 1.5% agarose gel; they yielded DNA fragments of the expected length for all specific genes mRNAs. Primers spanned an intron,

providing a control for potential amplification of genomic DNA. PCR products were sequenced to ensure the correct amplified cDNAs (DNA and Protein Sequencing Service, University of Valencia, Spain). Hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) for rat was used as a control housekeeping gene (Nogueiras *et al.* 2004a, López *et al.* 2006, Caminos *et al.* 2007, Vázquez *et al.* 2008).

## Statistical analysis

Data were expressed as mean ± S.E.M. Statistic significance was determined by Student's *t*-test (when two groups were compared) or ANOVA and *post hoc* Bonferroni test (when more than two groups were compared).  $P < 0.05$  was considered significant.

## Results

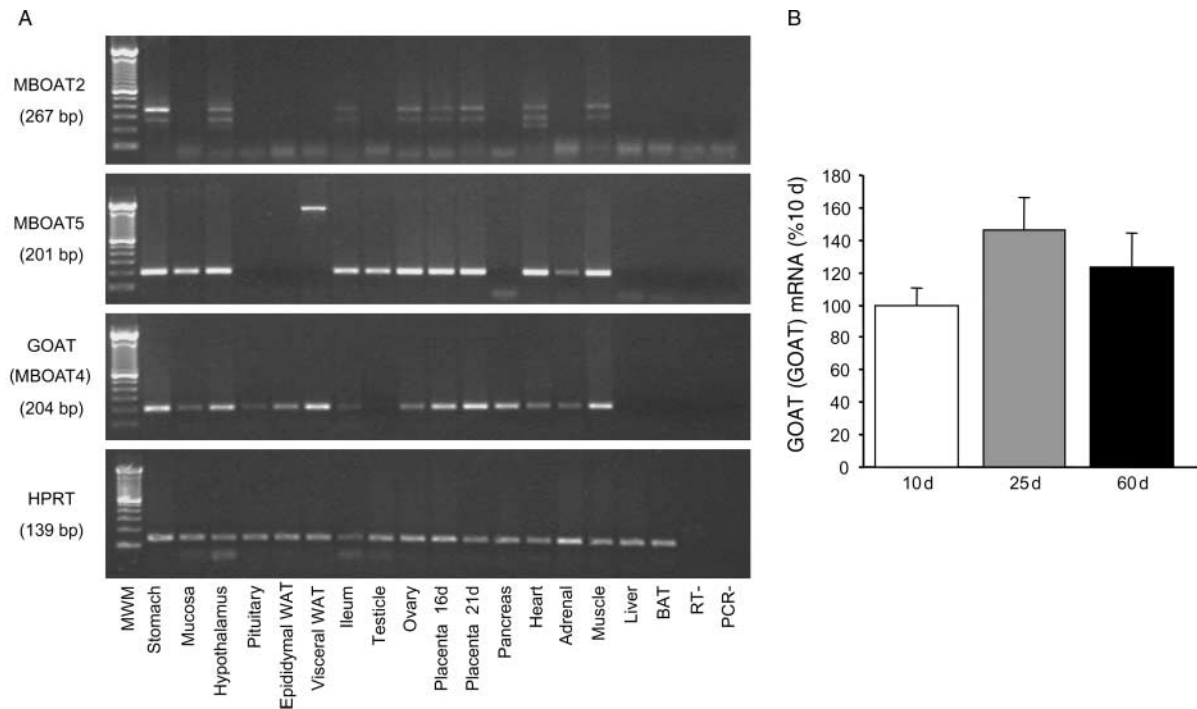
### GOAT mRNA expression in different tissues

Expression of the mRNA encoding GOAT, MBOAT2 and MBOAT5 in different tissues was evaluated by RT-PCR using specific primers (Fig. 1A). These analyses demonstrate a specific pattern of expression of the message of the GOAT gene in several tissues in comparison to the other two members of the family, MBOAT2 and MBOAT5. Our data are in general agreement with other reports showing a similar pattern of GOAT mRNA expression in both rodent and human tissues (Gutierrez *et al.* 2008, Yang *et al.* 2008). Whether some discrepancies such as the fact that

**Table 1** Primers and probes for classic PCR and real time-PCR analysis

Primer/probe	Sequence	Product (bp)	Genebank accession number
GOAT Fw	5'-GGCCGGAGCTTTTCTCTCT-3'	72	NM_001107317
GOAT Rv	5'-AAAGGCAGTACGTTACAGGGAAG-3'		
GOAT Pb	FAM 5'-TGCCGGCTGTGCTGTTCTTACAACA-3'-TAMRA		
GOAT Fw (Classic PCR)	5'-TGAAAGCCGACTATCTGATCCA-3'	204	
MBOAT 2 Fw	5'-TGGCATTTCCTACTGCATCATG-3'	267	NM_001108016.1
MBOAT 2 Rv	5'-CCCTCGATGAAAGCAATGTAGTCT-3'		
MBOAT 5 Fw	5'-TGAAGGGACAGCTGACTGACGTA-3'	201	NM_001012189.1
MBOAT 5 Rv	5'-ATTTGCCCCAGATCAGCATGT-3'		
Ghrelin Fw	5'-GAGCCCAGAGCACCAGAAAG-3'	70	AB029433.1
Ghrelin Rv	5'-GCTCGTGGCTGCAGTTTAGC-3'		
Ghrelin Pb	FAM 5'-CCAGCAGAGAAAGGAATCCAAGAAGCCA-3'-TAMRA		
18S Fw	5'-CGGCTACCATCAAGGAA-3'	186	M11188
18S Rv	5'-GCTGGAATTACCGCGGCT-3'		
18S Pb	FAM 5'-GACGGCAAGTCTGGTGCCAGCA-3'-TAMRA		
HPRT1Fw	5'-CAGTCCCAGCGTCGTGATTA-3'	139	NM_012583
HPRT1Rv	5'-AGCAAGTCTTTCAGTCCTGTC-3'		

MBOAT, membrane bound O-acyl transferase.



**Figure 1** (A) Representative RT-PCR assay of the mRNA expression levels of GOAT (GOAT: membrane bound *O*-acyltransferase 4), MBOAT2 and 5 in the described tissues. Negative (water) controls are shown. A 100 bp molecular weight marker (MWM) was used. Stomach, total stomach; mucosa, stomach mucosa; WAT, white adipose tissue; BAT, brown adipose tissue. (B) GOAT mRNA (mean  $\pm$  S.E.M.) expression in the stomach mucosa of male rats at different ages (10, 25, and 60 days old).

GOAT mRNA was detected in testis and liver (Yang *et al.* 2008) in contrast to our study are related to the use of different animal species remains to be established.

### GOAT mRNA levels in rat stomach

Since the stomach is the most relevant source of acylated ghrelin, we assessed in greater detail GOAT mRNA levels in this tissue. Also since the ghrelin-expressing cells are located in the mucosa, we assessed GOAT mRNA levels in samples extracted from this source. Furthermore, taking into consideration that ghrelin mRNA levels can be influenced by the age of the animals (Gualillo *et al.* 2001, Cortelazzi *et al.* 2003), we first assessed GOAT mRNA levels in samples from male rats of different ages. We found that GOAT mRNA levels were relatively stable at all the ages studied (Fig. 1B).

### Influence of chronic food deprivation on GOAT mRNA expression in the stomach mucosa of rat

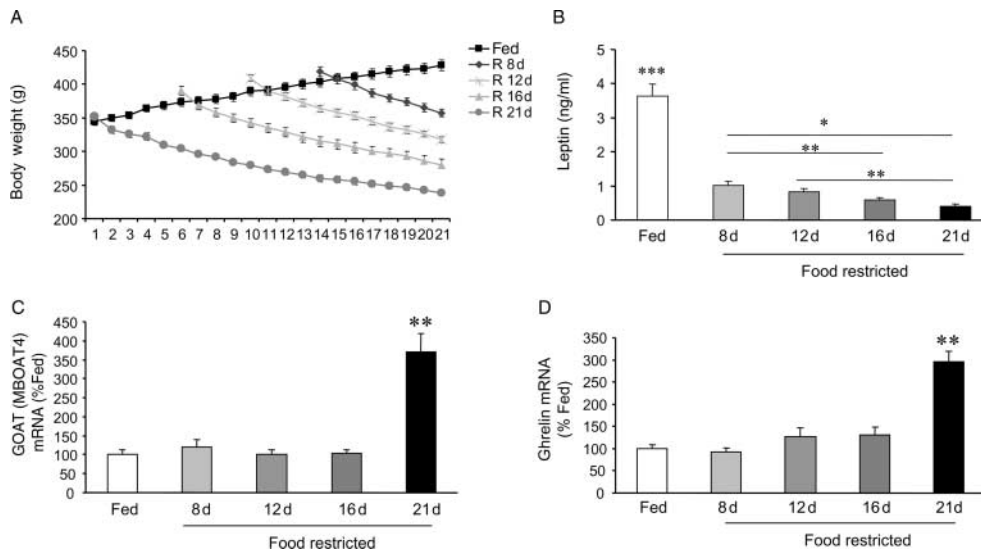
It is well known that chronic food deprivation leads to marked changes in stomach-derived preproghrelin mRNA levels and circulating acylated- and non-acylated-ghrelin levels. Following a protocol of partial food deprivation the rats started to lose weight as

expected (Fig. 2A). A parallel decrease in leptin levels was also observed (Fig. 2B). Despite the changes in body weight, GOAT mRNA levels remained fairly stable until the animals lost a considerable amount of body weight (day 21) where there was a marked and concomitant increase in both GOAT (Fig. 2C) and ghrelin (Fig. 2D) mRNA levels.

### Influence of fasting and leptin on GOAT mRNA expression in the stomach mucosa of rat

In the light of the previous findings of decreased leptin levels and increased GOAT mRNA, we decided to assess the influence of complete fasting, which lead to a rapid decrease in body weight (Fig. 3A) and leptin levels (Fed (mean  $\pm$  S.E.M.):  $3.38 \pm 0.38$  ng/ml versus Fast 48 h (mean  $\pm$  S.E.M.):  $0.7 \pm 0.11$  ng/ml;  $P < 0.001$ ) with and without exogenous leptin replacement, on GOAT mRNA expression in the stomach mucosa of rats. As shown in Fig. 3B, stomach mucosa GOAT mRNA levels were unchanged in 48 h-fasted rats, compared with control *ad libitum* group. Administration of exogenous leptin failed to modify GOAT mRNA levels in fed rats but markedly increased its expression in fasted rats (Fig. 3B). As expected preproghrelin mRNA levels were increased in fasted rats in comparison with *ad libitum* fed animals (Fig. 3C). Also preproghrelin levels were





**Figure 2** (A) Body weight, (B) plasma leptin levels, (C) GOAT, and (D) ghrelin mRNA expression in the stomach mucosa of fed and food restricted rats at the described times (8, 12, 16, and 21 days). Data are expressed as mean  $\pm$  S.E.M. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  between the labeled groups. In the case of B, \*\*\* $P < 0.001$  fed versus all the food restricted groups (8, 12, and 16 days). In the case of C and D, \*\* $P < 0.01$  restricted 21 days versus the rest of the described groups (8, 12, and 16 days).

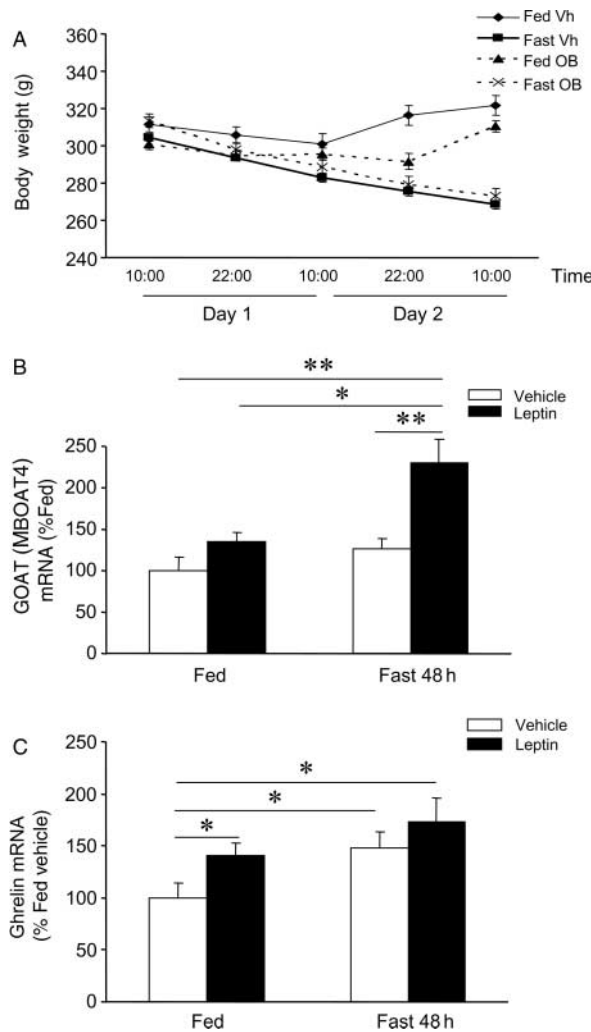
increased in fed rats after leptin treatment, but no further increase was observed in fasted rats treated with leptin (Fig. 3C). These data confirm that fasting leads to increased availability of preproghrelin mRNA that is further increased by leptin in fed animals while during fasting very low-leptin levels prevent increased GOAT mRNA expression.

## Discussion

The discovery of GOAT appears as a breakthrough in the understanding of ghrelin processing (Hofmann 2000, Gutierrez *et al.* 2008, Yang *et al.* 2008). Furthermore, its role of ghrelin acylation will help to understand the regulation of food intake and the pathophysiology of different clinical entities associated with changes in body weight and composition (van der Lely *et al.* 2004, López *et al.* 2007). It is well established that total circulating ghrelin levels are markedly influenced by body weight. Thus, the highest circulating levels are usually found in patients with the lower BMI, i.e. patients with anorexia nervosa, and on the contrary obese people exhibit lower ghrelin levels than normal controls (Gualillo *et al.* 2006). Noteworthy, the hyperghrelinemia in anorectic patients is caused at least partly by increased secretion of acylated ghrelin (Nakai *et al.* 2003) implying that chronic food restriction leads to a shift in the ratio of acylated/non-acylated ghrelin. On a theoretical basis, the higher proportion of acylated ghrelin could be due to increased expression

of the putative enzyme involved in ghrelin acylation or decreased activity of the enzymes, namely hepatic esterases, involved in ghrelin deacylation (Gualillo *et al.* 2006). To shed some light on this issue we assessed the influence of chronic undernutrition on GOAT mRNA levels in rat stomach mucosa. Our data show that GOAT mRNA levels are relatively stable during periods of decreased food intake. However, once the animals reach a marked weight loss, GOAT mRNA levels are markedly increased. These findings suggest that increased GOAT levels may be the mechanism underlying increased acylated ghrelin levels in chronic undernutrition (Nakai *et al.* 2003) and may represent an adaptive response to prevent long-lasting alterations in energy balance and body weight homeostasis.

In contrast to chronic undernutrition, data gleaned recently have shown that after a few days of complete fasting, the proportion of acylated ghrelin falls (Liu *et al.* 2008). Thus, under these conditions, the balance between ghrelin and des-acyl ghrelin may be changed by the regulation of ghrelin acylation. Toshinai *et al.* (2001) reported that in the stomach of rats fasted for 48 h, the ratio of des-octanoylated ghrelin to *n*-octanoylated ghrelin markedly increased after fasting. Based on that, it was postulated that complete fasting regulates ghrelin activity by a mechanism that inhibits the addition of the acyl group as ghrelin is synthesized. Since this event is dependent on GOAT an obvious possibility was that fasting prevents GOAT expression. Our data show that this may not well be the case since GOAT mRNA levels were not decreased in 48 h-fasted rats in contrast to



**Figure 3** (A) Body weight, (B) GOAT, and (C) ghrelin mRNA expression in the stomach mucosa of fed and fasted rats for 48 h treated with vehicle or leptin (OB). Data are expressed as mean  $\pm$  S.E.M. \* $P < 0.05$  and \*\* $P < 0.01$  between the labeled groups.

preproghrelin, which were elevated as expected. Despite the fact that GOAT mRNA levels were not influenced by fasting, and the associated low-leptin levels, we decided to assess the influence of exogenous leptin in GOAT mRNA levels, since it is well established that leptin plays an opposite functional role of ghrelin in food intake and that leptin regulates key components of the ghrelin system such as the ghrelin receptor GHS-R1a (Nogueiras *et al.* 2004b, López *et al.* 2007). We found that exogenously administered leptin to fasted rats markedly increased GOAT mRNA levels. These data indicate that during fasting low-leptin levels prevent an increase in GOAT mRNA levels and therefore GOAT can be added to the list of leptin-regulated genes (López *et al.* 2007) at least under this specific condition. These data also resemble previous results regarding the

neuroendocrine effects of leptin which are only uncovered under fasting conditions (Carro *et al.* 1997, López *et al.* 2000).

Overall, our data show that chronic undernutrition markedly increased the expression of GOAT mRNA levels in stomach mucosa. Since this is the major source of circulating ghrelin levels (Korbonits *et al.* 2004, van der Lely *et al.* 2004, Gualillo *et al.* 2006), our results offer a mechanistic explanation of the increased acylated-ghrelin levels observed in patients with severe malnutrition (Nakai *et al.* 2003). On the other hand, our data showing that leptin administration in 48 h-fasted rats markedly increased GOAT mRNA levels indicate that low-leptin levels in this setting prevents an increase GOAT mRNA levels. This evidence also offers mechanistic insights into the reason why in this particular paradigm acylated ghrelin levels are not increased at a time when a marked increase in an orexigenic signal, as important as acylated ghrelin, will be expected. Further work on how central stomach GOAT is regulated could provide another important step in unraveling the effect of ghrelin on feeding and glucose homeostasis control, which will improve our understanding of obesity and its treatment.

## Declaration of interest

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

## Funding

This work has been supported by grants from Xunta de Galicia (CD and ML: GRC2006/66), Fondo Investigaciones Sanitarias (ML: PI061700), Spanish Ministry of Education and Science (MEC; CD: BFU2005), Mútua Madrileña (CD and ML) and European Union (CD: LSHM-CT-2003-503041, Diabesity <http://www.euroidiabesity.org>). CIBER de Fisiopatología de la Obesidad y Nutrición is an initiative of ISCIII.

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Received in final form 22 September 2008

Accepted 3 October 2008

Made available online as an Accepted Preprint 3 October 2008