Metabolic regulation of ghrelin O-acyl transferase (GOAT) expression in the mouse hypothalamus, pituitary, and stomach

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1. Introduction

The primary source of circulating ghrelin is the stomach, where proghrelin can be modified by the addition of an O-linked octanoyl side group added to its serine-3 residue, via the actions of ghrelin O-acyltransferase (GOAT), to produce acylated-proghrelin (Kojima et al., 1999; Ariyasu et al., 2001; Garg, 2007; Gonzalez et al., 2008; Gutierrez et al., 2008; Yang et al., 2008; Gomez et al., 2009; Sakata et al., 2009). Then, prohormone convertase 1/3 (PC1/3) can further process proghrelin (acylated or des-acylated) to generate the mature 28-amino acid peptide ghrelin (Garg, 2007). Only a small proportion of circulating ghrelin is acylated, while the remaining is in the unmodified or des-acylated form, where both forms have biological effects (van der Lely et al., 2004; Kojima and Kangawa, 2005). Acylated-ghrelin is the primary endogenous ligand for the growth hormone secretagogue receptor (GHS-R), and it is now recognized that the acyl-ghrelin/GHS-R system exerts actions on multiple tissues, many of them related to regulation of metabolic functions (for review, van der Lely et al., 2004). In particular, acylated-ghrelin works at the level of the hypothalamus as a potent orexigenic signal (Kojima and Kangawa, 2005), and at the level of the pituitary to modulate hormone release including augmentation of growth hormone (GH), adrenocorticotropic (ACTH) and prolactin release (Kojima and Kangawa, 2005; Gahete et al., 2009). In addition, acylated-ghrelin acts systemically to regulate glucose homeostasis and adiposity, where the deletion of the genes encoding ghrelin or its receptor prevents diet-induced obesity, improves insulin sens-
sitivity and enhances glucose-stimulated insulin secretion (Yada et al., 2008).

Although the GI track is considered the major source of acylated-ghrelin, other tissues express ghrelin, PC1/3 and GOAT (Gonzalez et al., 2008; Gutierrez et al., 2008; Yang et al., 2008) (for review, van der Lely et al., 2004). Therefore, it is possible that locally produced non-acylated-proghrelin might be converted to acylated-proghrelin within target tissues by locally active GOAT, to mediate tissue-specific effects. Local production of acyl-ghrelin is supported by a recent report demonstrating that acylated-ghrelin can be produced by a thyroid and a pituitary cell line that express ghrelin, PC1/3 and GOAT if n-octanoic acid is available as a substrate (Takahashi et al., 2009). Given the fact that hypothalamic and pituitary are targets for acylated-ghrelin, coupled with the fact that these tissues also express ghrelin (for review, van der Lely et al., 2004), GOAT (Gonzalez et al., 2008; Gutierrez et al., 2008; Yang et al., 2008) and PC1/3 (Dong and Day, 2002; Nillni, 2007), it is possible that local production of acylated-ghrelin, as well as that produced by the GI tract, may play an important role in regulating this neuroendocrine axis in response to metabolic signals.

Therefore, the goals of the current study were (1) to determine whether GOAT expression in the stomach correlates with circulating acylated-ghrelin levels in mice under conditions of metabolic stress (i.e., fasting, and obese mouse models), (2) to confirm if GOAT is expressed within the hypothalamus and pituitary and if expression levels are mediated by metabolic stress and (3) to determine whether primary regulatory factors for the pituitary-metabolic interface can directly regulate GOAT expression, by using primary mouse pituitary cell cultures as a model system.

2. Material and methods

2.1. Animals and cell culture

All experimental procedures were approved by the Animal Care and Use Committee of the University of Cordoba, University of Illinois at Chicago and the Jesse Brown VA Medical Center. Mice were housed under standard conditions of light (12-h light, 12-h dark cycle; lights on at 07:00 h) and temperature (22–24 C), with free access to tap water and food (standard rodent chow; Labdiet, St. Louis, MO, USA; Catalog no. 5008; fat, 17 kcal%; carbohydrate, 56 kcal%; protein, 27 kcal%). Leptin-deficient ob/ob mice and their corresponding controls were purchased from Jackson Laboratories, while growth-hormone-releasing hormone (GHRH), somatostatin (SST) and neuropeptide Y (NPY) knockout mice were bred in house. Original breeders were obtained from Dr. Ute Hochgeschwender (SST-KO), Dr. Richard D. Palmer (NPY-KO) and Dr. Roberto Salvadori (GHRH-KO) as previously reported (Alba and Kineman, 2006; Luque et al., 2006, 2007b; Luque and Kineman, 2006, 2007). Mice were handled daily at least 1 week prior to euthanasia to acclimate them to personnel and handling procedures and were sacrificed by decapitation, without anesthesia, under fed conditions unless otherwise specified. Trunk blood was immediately mixed with MiniProtease inhibitor (Roche, Nutley, NJ) and placed on ice, centrifuged and plasma was stored at –80 C until analysis of total- and acylated-ghrelin by ELISA (Linco, St. Charles, MO) following the manufacturer’s instructions, including the addition of hydrochloric acid at a final concentration of 0.05N in order to prevent rapid des-acylation of ghrelin after the plasma collection. Tissues (stomachs, pituitary and hypothalamus) were immediately frozen in liquid nitrogen and stored at –80 C until further analysis of mRNA levels by quantitative real-time RT-PCR (qRT-PCR; see below).

2.2. Influence of metabolic stress on circulating total and acylated-ghrelin and stomach, pituitary and hypothalamic GOAT mRNA levels

For these studies, serum and tissues previously processed and analyzed for other endpoints were used, where each study is listed below followed by a brief summary of the experimental design. Fasting – Ten-week-old male C57Bl/6 mice were either fed ad libitum or fasted for 12 h (food removed at 19:00 h). 24 h (food removed at 07:00 h) (Luque et al., 2007c). An additional group of 8–12-week-old male C57Bl/6 mice were obtained from “Centro de Instrumentación Científica” (University of Granada, Spain) or from Jackson Laboratories (Bar Harbor, ME, USA) and were enzymatically dispensed (n = 3–5) into single and cultured (250,000 cells/well, 24-well plates) in serum containing α-MEM, as previously described (Luque et al., 2006; Luque and Kineman, 2006). After 24–48 h of culture, media was removed and wells were washed in serum-free media and subsequently treated with mouse acylated-ghrelin, desacyl-ghrelin, GHRH, NPY, insulin, GDP-1 (10 nM each); SST-14 (100 nM) and mouse leptin (10 ng/ml), for 24 h (Sigma, Saint Louis, MO or Phoenix, Burlingame, CA, USA), and then total cellular RNA was extracted for determination of GOAT mRNA levels by qRT-PCR (see below). In order to determine if the direct actions of GHRH, SST, NPY or leptin could be maintained in vivo expression of pituitary GHRH in total RNA of control and GHRH-deficient mice (GHRH-KO) (B6129PF1/J), SST-KO mice (C57Bl/6j), NPY-KO mice (129/sv), leptin-KO (ob/ob) C57Bl/6j and their respective littermate controls used in other studies (Alba and Salvadori, 2004; Park et al., 2005; Luque et al., 2006, 2007b; Luque and Kineman, 2006, 2007) were also evaluated from GOAT mRNA levels by qRT-PCR.

2.4. RNA isolation and reverse transcription (RT)

Tissues and cell cultures were processed for recovery of total RNA using the Absolutely RNA RT-PCR Miniprep Kit with Deoxynucleobinucleotide treatment (Stratagene, La Jolla, CA) as previously described (Luque et al., 2006, 2007a; Luque and Kineman, 2006, 2007; Kineman et al., 2007). Specific primer sequences, GenBank accession numbers and productsizes for mouse GOAT, ghrelin and cyclophilin used in this study were as follows: GOAT (Mboat4; forward 5′-AATGTTGAAGGACCAAGTGGAC-3′ and reverse 5′-CAGGACACACGAGAGAAAGG-3′, NM_00126314, 120 bp); ghrelin (forward 5′-TTACA AAGCCAACCTAAGA-3′ and reverse 5′-AACCTGAGAGAGGATCTAGGAG-3′, NM_021488, 128 bp) and cyclophilin (forward 5′-TGCTCTGAGAGGCTGAAGA-3′ and reverse 5′-TCTCAGC CAGTGAATGCT-3′, NM_008907, 109 bp). Primers were selected using Primer 3 software (Rozen and Skaltsky, 2000) with selection parameters set to identify primer sets that: (1) span an intron (when possible), (2) differ by no more than 1°C in annealing temperature, (3) are at least 20 bp in length, (4) have a GC content between 45 and 55%, but (5) exclude primers that may form primer-dimers. Sequences of selected primers were used in BLAST (NCBI) searches to check for potential homology to sequences other than the designated target. Initial screening of primer efficiency using real-time detection was performed by amplifying 2-fold dilutions of RT products, where optimal efficiency was demonstrated by a difference of one cycle threshold between dilutions and a clear melting peak. Amplifications were then column-purified (QIAGEN, Valencia, CA) and sequenced to confirm target specificity. Initial screening of primer efficiency using real-time detection was performed by amplifying 2-fold dilutions of RT products, where optimal efficiency was demonstrated by a difference of one cycle threshold between dilutions and a clear melting peak. Amplifications were then column-purified (QIAGEN, Valencia, CA) and sequenced to confirm target specificity. Initial screening of primer efficiency using real-time detection was performed by amplifying 2-fold dilutions of RT products, where optimal efficiency was demonstrated by a difference of one cycle threshold between dilutions and a clear melting peak. Amplifications were then column-purified (QIAGEN, Valencia, CA) and sequenced to confirm target specificity.
a no DNA control were run on each plate to control for genomic DNA contamination and to monitor potential exogenous contamination, respectively. Also, to control for variations in the amount of RNA used in the RT reaction and the efficiency of the RT reaction, mRNA copy number of the transcript of interest was adjusted by the mRNA copy number of cyclophilin A (used as a housekeeping gene), where cyclophilin A mRNA levels did not significantly vary between experimental groups, within tissue type or treatment group (data not shown).

2.6. Data presentation and statistical analysis

Samples from all groups within an experiment were processed at the same time. The effects of fasting and leptin replacement were assessed by one-way ANOVA followed by a Newman–Keuls test for multiple comparisons, while the effects of obesity, genotype and the in vitro effects of acylated-ghrelin, desacyl-ghrelin, GHRR, SST, NPY, leptin, insulin and IGF-I were assessed by Student’s t-test. p < 0.05 was considered significant. All data are expressed as means ± SEM. The in vivo effects of fasting/obesity/genotype were obtained from a minimum of 5 animals per group. Results from in vitro studies were obtained from 3 to 7 separate, independent experiments (3–5 wells/treatment) carried out in different days and with different cells preparation. Endpoints displaying heterogeneity of variance were log transformed prior to analysis. All statistical analyses were performed using the GB-STAT software package (Dynamic Microsystems, Inc., Silver Spring, MD, USA).

3. Results and discussion

3.1. Quantification of GOAT mRNA levels in mouse stomachs, pituitaries and hypothalami

Using qRT-PCR, we found that GOAT mRNA levels were 5-fold greater in the stomach as compared to the pituitary and hypothalamus (Table 1). These results are consistent with other reports showing relative expression levels of GOAT in both human (Gutierrez et al., 2008) and rodent (Gonzalez et al., 2008; Yang et al., 2008; Sakata et al., 2009) tissues. Interestingly, the expression level of GOAT transcripts in the mouse tissues analyzed in the current study (Table 1) paralleled the mRNA levels of ghrelin in the same tissues (stomach >> pituitary = hypothalamus), as previously reported (Kineman et al., 2007).

Since it has been previously reported that the enzyme PC1/3 which is required for the conversion of preprohormones, including pre-proghrelin to its 28 amino acid form, is also expressed in the pituitary and hypothalamus (Dong and Day, 2002; Nillini, 2007), as well as the GI tract (Macrò et al., 1996), it is possible that the source of acylated-ghrelin within the pituitary and hypothalamus may include locally produced ghrelin, in addition to that found in the circulation. Therefore, the current study compared the impact of metabolic stress (fasting and obesity) on circulating ghrelin (acylated and total) levels, as well as GOAT and ghrelin mRNA levels in the stomach, pituitary and hypothalamus. Specifically, Fig. 1 illustrates the impact of fasting, diet-induced obesity (DIO) and obesity caused by leptin deficiency (ob/ob mice) on circulating acylated- and total-ghrelin levels and stomach GOAT mRNA levels (panels A, B and C, respectively), as well as pituitary and hypothalamic GOAT mRNA levels (panels D, E and F, respectively). It should be noted that, in some cases we have previously published the regulation of stomach, pituitary and hypothalamic ghrelin mRNA levels in these model systems (Kineman et al., 2007; Luque et al., 2007a,c). These results are summarized in Fig. 1 as the relative changes in ghrelin mRNA levels (△, +, ↓), as compared to controls, as shown below each graph of tissue-specific GOAT expression.

3.2. Effects of nutrient deprivation on GOAT/ghrelin axis

As shown in Fig. 1, panel A, fasting did not alter circulating levels of total-ghrelin or stomach ghrelin expression, but did increase circulating acylated-ghrelin (12 and 24 h) and stomach GOAT mRNA levels (24 h). The impact of 24 h fasting on stomach GOAT mRNA levels was confirmed in tissues taken from an independent set of male mice from another study (Kineman et al., 2007), using two different primer sets for amplification of the GOAT transcripts (supplemental Fig. 1). These results differ from those of a recent report by Kirchner et al. (2009) showing that fasting (12, 24 and 36 h) significantly increased total-ghrelin levels and tended to increase acylated-ghrelin levels, but unexpectedly suppressed stomach GOAT mRNA in C57Bl/6 male mice. It is possible that such differences may be related to the time of food withdrawal, time of sample collection, type of standard rodent chow provided and/or the method of euthanasia which were not clearly defined in that study. In fact, Kirchner et al. (2009) observed a diurnal pattern of circulating ghrelin and stomach ghrelin/GOAT mRNA levels, as well as an association between stomach GOAT expression and the type of dietary lipids supplied. It is also possible that the differences observed are related to the analytical techniques applied, where circulating ghrelin levels were assessed by commercial assays in the current study, but in the case of Kirchner et al. (2009), were assessed by MALDI-TOF mass spectrometry of immunoprecipitated ghrelin. Finally, with respect to GOAT mRNA levels, although both the current study and that of Kirchner et al. (2009) used real-time RT-PCR, the primer sets used and the characteristics and properties of the primers were not the same (length of primers, GC content, etc.). Nonetheless, our current observations are consistent with other reports showing a clear rise in acylated-ghrelin levels in fasted humans and other species (for review: Casanueva and Dieguez, 2002; Gottero et al., 2004; Kojima and Kangawa, 2005; Williams and Cummings, 2005; Cummings, 2006), including mice (Perreault et al., 2004; Luque et al., 2006, 2007c; Zizzari et al., 2007), as well as an increase in stomach GOAT mRNA levels in rats subjected to 21 days of caloric-restriction (Gonzalez et al., 2008). However, it should be noted that like Kirchner et al., Liu et al. failed to detect a rise in circulating acylated-ghrelin in fasted humans (Liu et al., 2008). It should also be emphasized that in the current study the fasting-induced rise in acylated-ghrelin was observed at 12 and 24 h, but began to fall after 48 h, while the fasting-induced rise in GOAT mRNA was only observed at 24 h. These results suggest that an increase in GOAT expression levels in the stomach may only in part contribute to the rise in circulating acyl-ghrelin, where enhanced GOAT activity and/or substrate availability may precede that process. However, after 48 h of fasting circulating acylated-ghrelin levels and GOAT mRNA levels did not differ from controls (Fig. 1, panel A), where 48 h of fasting in a mouse represent a severe catabolic state (Luque et al., 2007c). This observation is consistent with reports showing that the proportion of circulating acylated-ghrelin falls after long-term fasting in humans (61.5 h-fasting) (Liu et al., 2008) and rats (48 h-fasting) (Toshinai et al., 2001; Gonzalez et al., 2008) and with the recent observation indicating that 48 h of fasting did not significantly alter stomach GOAT mRNA expression in rats (Gonzalez et al., 2008; Takahashi et al., 2009). Taken together our current results suggest that changes in stomach GOAT expression may contribute in part to fasting-induced changes in circulating acylated-ghrelin levels, a hypothesis previously put forth by other laboratories (Gonzalez et al., 2008; Gualillo et al., 2008). However, given the
Fig. 1. Circulating total- and acylated-ghrelin and stomach, hypothalamus and pituitary GOAT mRNA levels in: mice fed ad libitum or fasted for 12-, 24-, and 48-h (panels A and D), mice fed a low-fat diet (LFD control group) or high-fat diet (HFD obese group) (panels B and E) and lean controls (+/+) and obese (ob/ob) mice (panels C and F). Values are shown as mean ± SEM (n = 5–8 mice/group) and expressed as percent of the respective control group (set at 100%). Group means that do not share a common letter (a and b) significantly differ (p < 0.05; panels A and D). Asterisks (*p < 0.05, **p < 0.01) indicate values that significantly differ from lean control LFD or+/+ values. The tissue-specific regulation of ghrelin mRNA levels in these mice models as previously reported (Kineman et al., 2007; Luque et al., 2007a,c) are shown below each graph of tissue-specific GOAT expression and are provided as the relative changes (=, ↑, ↓) as compared to controls.

only other report examining this relationship in mice gave different results (Kirchner et al., 2009), and the fact that there are many potential levels of GOAT regulation (transcriptional, translational, enzyme activity level, substrate availability), it will be important for other laboratories to validate and confirm these findings.

3.3. Effects of obesity on GOAT/ghrelin axis

As shown in Fig. 1, panel B, diet-induced obesity tended (50% reduction, p = 0.13) to suppress total-ghrelin levels in mice without altering acylated-ghrelin levels, consistent with a significant decline in stomach ghrelin expression, while GOAT mRNA levels
remained unchanged. In contrast, obesity as a result of leptin deficiency (ob/ob mice; Fig. 1, panel C) led to a significant decline in circulating acylated-ghrelin levels (and a downward trend in total-ghrelin levels), changes which were accompanied by a significant decrease in stomach ghrelin but not GOAT mRNA levels, similar to DIO mice. This is the first report to explore the impact of DIO on stomach GOAT and ghrelin expression, while a previous study showed similar results for stomach GOAT and ghrelin mRNA levels in ob/ob mice (Kirchner et al., 2009). In addition, others have reported a significant decline in total- or desacyl-ghrelin in DIO (Ueno et al., 2007) and obese Ay (Nonogaki et al., 2006) mice and a reduction in total- and acylated-ghrelin levels in obese humans (Casanueva and Dieguez, 2002; Gottero et al., 2004; Ghigo et al., 2005; Kojima and Kangawa, 2005), where DIO-induced alterations in acylated-ghrelin may be dependent on the time of day sampled (Perreault et al., 2004). Taken together these results indicate that the decline in circulating total-ghrelin levels in the obese state could be in part due to a decline in stomach ghrelin gene expression, however alterations in circulating acylated-ghrelin may be related to factors independent of GOAT expression, such as the rate of degradation of acylated-ghrelin, GOAT enzyme activity levels or substrate (ghrelin or fatty acid) availability.

3.4. Impact of metabolic stress (fasting and obesity) on hypothalamic and pituitary GOAT expression

As shown in Fig. 1, panel D, fasting also enhanced expression of GOAT at both the hypothalamic [at 48 h \( (p = 0.0016) \)] and pituitary [24h \( (p = 0.017) \) and 12 h \( (p = 0.051) \)] level. Interestingly, it should be noted that the timing of the GOAT mRNA rise was different from that observed in the stomach (Fig. 1, panel A). Conversely, DIO (Fig. 1, panel E) and obesity caused by leptin deficiency (Fig. 1, panel F) did not alter hypothalamic GOAT expression, but did result in a significant decline in pituitary GOAT mRNA levels. As in the stomach, hypothalamic and pituitary GOAT expression did not always parallel local changes in ghrelin expression. Taken together, these results suggest that local production (ghrelin gene expression) and/or modification (via GOAT gene expression) of ghrelin may contribute to the regulation of hypothalamic and pituitary function independent of circulating ghrelin levels produced by the stomach which would be supported by a recent study indicating that various cell lines, including the mouse pituitary cell line AtT20, can produce acylated-ghrelin when both ghrelin and GOAT are present in the cells and when substrate (fatty acid) is available in the culture medium (Takahashi et al., 2009).

3.5. Direct regulation of GOAT mRNA levels by ghrelin, desacyl-ghrelin, GHRH, SST, leptin, NPY, insulin and IGF-I in mouse primary pituitary cell cultures

Given the dynamic changes in pituitary GOAT expression in both fasting (up-regulation; Fig. 1, panel D) and obesity (down-regulation; Fig. 1, panels E and F), we used primary mouse pituitary cell cultures as a model system to explore which central or systemic factors might mediate these changes. First, to confirm that mouse primary pituitary cells maintain differentiated function (i.e. GOAT expression) after dispersion and culture, the absolute mRNA levels (copy numbers/0.05 µg total RNA) of GOAT were compared between whole tissue extracts and extracts prepared from pituitary cultures 24 h after incubation in serum-free media, and the results are shown in Table 1. Transcript levels did not significantly vary between in vivo and in vitro samples, indicating that the cell preparation and culture conditions were appropriate to maintain GOAT expression. Based on reports from our laboratory and others showing that the levels and/or actions of ghrelin, GHRH and NPY are up-regulated in fasting (Henry et al., 2001; Park et al., 2005; Luque et al., 2006, 2007c; Kirchner et al., 2009) and down-regulated in obesity (Tannenbaum et al., 1990; Ahmad et al., 1993; Maccario et al., 1999; Lin et al., 2000; Perreault et al., 2004; Luque and Kineman, 2006; Nonogaki et al., 2006; Luque et al., 2007a) while those for SST, leptin, insulin and IGF-I are down-regulated in fasting (Henry et al., 2001; Frystyk, 2004; Park et al., 2005; Luque et al., 2006, 2007c; Kirchner et al., 2009) and up-regulated in obesity (Zhou et al., 1997; Frystyk, 2004; Luque and Kineman, 2006; Luque et al., 2007b, 2008), we incubated primary pituitary cell cultures with these hormones for 24 h and measured the impact on GOAT mRNA levels. As shown in Fig. 2, top panel, acylated-ghrelin clearly stimulated GOAT expression compared to vehicle-treated controls (set at 100%). In order to confirm that this effect on GOAT mRNA levels was directly exerted by acylated-ghrelin and not by desacyl-ghrelin (as result of a des-octanoylation of acylated-ghrelin in the pituitary cell cultures after 24 h of incubation), pituitary cell cultures were treated with desacyl-ghrelin for 24 h. The results clearly indicate that treatment with desacyl-ghrelin did not alter GOAT mRNA levels. In addition, as shown in Fig. 2 (top panel), GHRH and leptin stimulated and
that pituitary and stomach GOAT mRNA levels were greater than consistent with the report of Gonzalez et al. (2008) showing that was examined in the ob/ob model (Luque et al., 2007a), we observed also interesting to note that when the impact of leptin replacement IGF-I had no effect, compared to vehicle-treated controls (set at native ghrelin/0.05/H9262.

itary, because our laboratory has identified a spliced mRNA ghrelin expression observed in response to metabolic extremes. Intrigu-

ation in response to metabolic stress. Although much work remains to be done to fully understand how GOAT fits into the control of energy homeostasis, our results reinforce the contention that GOAT/acylated-ghrelin system could operate as a novel molec-

ular conduit for the integration of energy balance, metabolism and pituitary function.

SST inhibited pituitary GOAT expression, while NPY, insulin and IGF-I had no effect, compared to vehicle-treated controls (set at 100%). In order to determine if these direct actions might also be important in maintaining pituitary GOAT expression in vivo, we examined pituitary expression of GOAT in available mouse mod-

eLS that lack GHRH, SST, NPY and leptin. Accordingly, we found an opposite effect of GHRH-KO, SST-KO and leptin-KO on pituitary GOAT expression, as compared to that observed after in vitro treat-

ment with the corresponding hormone (Fig. 2, bottom panel). It is also interesting to note that when the impact of leptin replacement was examined in the ob/ob model (Luque et al., 2007a), we observed that pituitary and stomach GOAT mRNA levels were greater than that observed in pair-fed controls (supplemental Fig. 2), which is consistent with the report of Gonzalez et al. (2008) showing that administration of exogenous leptin markedly increased expression levels of GOAT in the stomach of fasted rats. Taken together these results indicate that acylated–ghrelin may in fact regulate its own production at the level of the pituitary by increasing the expression of GOAT. In addition, these results clearly demonstrate that GHRH, SST and leptin are key regulators of pituitary GOAT expression, an observation which may in part explain changes in pituitary GOAT expression observed in response to metabolic extremes. Intrigu-

ingly, the substrate of GOAT activity in the mouse pituitary may not be limited to native ghrelin being locally produced within the pituitary, because our laboratory has identified a spliced mRNAGHrelin variant containing exon2, intron2 and exon3, but lacking exon1, 4 and 5 (In2-ghrelin variant; Kineman et al., 2007) which is expressed at higher levels than the native ghrelin transcript in the pituitary (139 ± 24 copies for In2-ghrelin variant versus 18 ± 3 copies for native ghrelin/0.05 μg RNA). If this variant is translated, it would encode a protein that would include the first 36 amino acid of the pre-proghrelin, where residues 24–28 represent the first five amino acids of mature ghrelin and thus contain the Ser-3 acylation site. It is interesting to note that when we compared the impact of metabolic stress (fasting and DIO) or GHRH, SST and leptin deficiency on pitui-

tary expression of GOAT, native ghrelin and In2-ghrelin, the levels of GOAT expression paralleled those of In2-ghrelin variant but not native ghrelin, in all cases (summarized in Table 2, and details presented in supplemental Fig. 3). Based on these observations it seems reasonable to propose that the In2-ghrelin variant could be a primary substrate for GOAT in the anterior pituitary gland.

Although more experiments are required to fully elucidate the physiologic significance of our findings in the pituitary, our data give credence to the possibility that local production and/or modification of ghrelin (des-acylated to acylated via GOAT gene expression) may contribute to the fine regulation of pituitary func-

tion in response to metabolic stress. For example, circulating GH levels are known to rise in response to fasting in the mouse, and this is associated with an increase in pituitary expression of GH, GHRH-R and GHS-R and a decline in SST receptor expression (rat and mouse; Luque et al., 2007a, 2008). The acute rise in systemic acylated-ghrelin, in combination with an increased sensitivity of the pituitary to ghrelin and GHRH actions, might initiate local pro-
duction of acylated-ghrelin by augmentation of ghrelin and GOAT expression, thereby acting as a positive ultraslow feedback loop to enhance or facilitate GH release. In contrast, GH output is sup-
pressed in obesity, an effect which is believed to be due in part to enhanced SST tone (for review see Luque et al., 2008) and a decline in pituitary of GHRH-R, GHS-R and GH (Luque and Kineman, 2006; Luque et al., 2008). In this context, our data would support the idea that part of the negative effects of SST on somatotrope function could include direct suppression of GOAT expression and a decline of locally produced acylated-ghrelin, further contributing to the decline in GH release observed with weight gain.

In summary, we report herein a series of novel analyses on the regulation of the mouse GOAT/ghrelin system at the circulating-stomach–pituitary–hypothalamic levels by energy sta-
tus and relevant metabolic cues in the mouse. By applying a combination of studies involving in vivo models (fasting, obesity and knockout mice) and primary pituitary cell cultures, our results further characterize the impact of changes in body energy stores on the expression of GOAT in different tissues and define the role of key regulators, such as acylated-ghrelin itself, GHRH, SST and lep-
tin on the control of GOAT expression in the mouse. The fact that GOAT mRNA levels are oppositely regulated in extreme metabolic states (fasting, DIO) and its regulation is tissue-dependent suggests that this enzyme may be of biological relevance in coordinating the neuroendocrine response to metabolic stress. Although much work remains to be done to fully understand how GOAT fits into the control of energy homeostasis, our results reinforce the contention that GOAT/acylated-ghrelin system could operate as a novel molec-

ular conduit for the integration of energy balance, metabolism and pituitary function.

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**Appendix A. Supplementary data**


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