Ghrelin O-acyltransferase knockout mice show resistance to obesity when fed high-sucrose diet

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Abstract

Ghrelin is an appetite-stimulating hormone secreted from stomach. Since the discovery that acylation of the serine-3 residue by ghrelin O-acyltransferase (GOAT) is essential for exerting its functions, GOAT has been regarded as a therapeutic target for attenuating appetite, and thus for the treatment of obesity and diabetes. However, contrary to the expectations, GOAT-knockout (KO) mice have not shown meaningful body weight reduction, under high-fat diet. Here, in this study, we sought to determine whether GOAT has a role in body weight regulation and glucose metabolism with a focus on dietary sucrose, because macronutrient composition of diet is important for appetite regulation. We found that peripherally administered acylated-ghrelin, but not unacylated one, stimulated sucrose consumption in a two-bottle-drinking test. The role of acylated-ghrelin in sucrose preference was further supported by the finding that GOAT KO mice consumed less sucrose solution compared with WT littermates. Then, we investigated the effect of dietary composition of sucrose on food intake and body weight in GOAT KO and WT mice. As a result, when fed on high-fat diet, food intake and body weight were similar between GOAT KO and WT mice. However, when fed on high-fat, high-sucrose diet, GOAT KO mice showed significantly reduced food intake and marked resistance to obesity, leading to amelioration of glucose metabolism. These results suggest that blockade of acylated-ghrelin production offers therapeutic potential for obesity and metabolic disorders caused by overeating of palatable food.

Keywords

- ghrelin
- sucrose
- body weight
- insulin sensitivity
- food intake

Introduction

Ghrelin, a 28-amino-acid peptide hormone mainly produced by X/A-like cells of the stomach (Date et al. 2000), was originally identified as an endogenous ligand of the growth hormone secretagogue receptor type 1a (GHS-R1a) (Kojima et al. 1999). Octanoylation of the serine-3 residue by ghrelin O-acyltransferase (GOAT) is essential for its physiologic actions (Gutierrez et al. 2008, Yang et al. 2008). It discovers that administration of exogenous acylated-ghrelin stimulates food intake in humans (Wren et al. 2001, Neary et al. 2004) and rodents (Tschop et al. 2000,
Nakazato et al. 2001) and that plasma concentration of acylated-ghrelin rises immediately before meals (Cummings et al. 2001) suggest that acylated-ghrelin, GOAT and GHS-R1a may function as an appetite-controlling endocrine system in vivo. These findings raised expectations to an height that inhibiting GOAT activity would offer a therapeautic option to treat obesity and other metabolic disorders as well as GHS-R1a antagonism. However, this expectation has been challenged by findings from gene-knockout studies, which have shown that food intake and body weight were not reduced in ghrelin- or GOAT-deficient mice compared to WT mice given high-fat (HF) diet or standard chow (Sun et al. 2003, Wortley et al. 2004, Sato et al. 2008, Zhao et al. 2010). These phenotypes may not be explained by a compensation mechanism, because postnatal ablation of ghrelin-secreting cells has failed to demonstrate attenuated appetite for HF diet (McFarlane et al. 2014).

Food composition is important for studying the effect of acylated-ghrelin on appetite and weight control. As GOAT utilizes dietary fatty acid as a substrate for acylation-ghrelin, a diet containing a large amount of medium-chain triglycerides (MCT) given to mice led to a significant increase of acylated-ghrelin concentration in blood (Nishi et al. 2005). It is naturally expected that an increase in blood acylated-ghrelin may stimulate food intake and accelerate body weight gain compared to GOAT knockout (KO) mice, which have an undetectable concentration of acylated-ghrelin in blood. However, feeding MCT diet to GOAT KO mouse resulted in only a modest reduction of body weight (Kirchner et al. 2009). These previous investigations cast doubt upon the importance of the endogenous acylated-ghrelin level in appetite regulation and whole body weight control.

GHS-R1a is expressed in brain areas involved in ‘homeostatic feeding’, such as the hypothalamus and brainstem and also in ‘hedonic feeding’ (feeding linked to a rewarding system, not necessarily accompanied by caloric need), such as the ventral tegmental area and the nucleus accumbens (Nac) (Naleid et al. 2005, Abizada et al. 2006). A recent investigation has shown that peripherally and centrally administrated acylated-ghrelin increased consumption of palatable sucrose pellets (Skibicka et al. 2012). Another study has reported that administration of GHS-R1a antagonist resulted in reduced intake and preference of sucrose solution in a two-bottle-choice drinking test and operant self-administration test in rats (Landgren et al. 2011), suggesting that endogenous acylated-ghrelin level contributes to sucrose consumption. Although those findings suggested that the ghrelin/GOAT system has a close relationship with hedonic feeding (Davis et al. 2012), it has not been verified whether the change of preference of sucrose in mice lacking acylated-ghrelin signaling contributes to a long-term effect of sweetened food intake and progression to chronic diseases such as obesity and insulin resistance.

The current study was aimed to evaluate the concept that inhibiting enzymatic activity of GOAT may ameliorate chronic metabolic disorders. To test this, we compared amount of food intake and body weight gain between GOAT KO and WT mice fed on several types of high-calorie diets (HF, MCT and high sucrose). We also performed glucose and insulin tolerance test (ITT) to investigate whether insulin resistance elicited by high-calorie diet load was ameliorated in GOAT KO mice.

Materials and methods
Animals and housing
All the animals were maintained on 12 h artificial light:12 h artificial darkness cycles and housed in individual cages. The studies were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility. The experimental protocols were reviewed and approved by our Animal Care and Use Committee.

Generation of GOAT-deficient mice
To generate GOAT-deficient mice, a targeting vector for homologous recombination on mouse embryonic stem (ES) cells was constructed (Fig. 1A). The 5' and 3' flanking DNA sequences of exon 1 of the mouse membrane bound O-acyltransferase domain containing 4 (Mboat4) gene were subcloned by PCR from the bacterial artificial chromosome (BAC) clone RP23-99B10 (Life Technologies). These sequences were ligated with a PGK-Neo cassette (Ishida et al. 2003), so as to replace Mboat4 exon 1. A DT-A cassette (Yagi et al. 1993) was inserted at the 3' terminus of the vector. The targeting vector was then linearized and transduced into mouse ES cells derived from C57BL/6 strain (CMTI-2, Millipore, Darmstadt, Germany) by electroporation using Nucleofector kits for mouse embryonic fibroblasts (Millipore) treated with mitomycin C (Nacalai tesque) in knockout DMEM with 15% knockout serum replacement, 5% fetal bovine serum embryonic stem cell-qualified, 1×MEM non-essential amino acids solution,
55 nM 2-mercaptoethanol (these were from Life Technologies), 1.7 \times 10^3 unit/ml mouse leukemia inhibitory factor, 1 ES cell qualified nucleosides (these were from Millipore), and the appropriate antibiotics. The recombinant ES cell clones were selected for a week with 200 ng/ml of G418 (Geneticin; Life Technologies). The ES clones considered to be recombinant clones by PCR amplifying the 5' flanking sequence (forward 5'-acaccaaggatagttggtgcttc-3', reverse 5'-agagctccctggcgaattcggtac-3') were picked out and stocked. Homologous recombination was confirmed by southern hybridization using a digoxigenin-conjugated probe to out of the 3' flanking sequence and Neo (PCR DIG Probe Synthesis Kit, Roche Diagnostics) (Fig. 1B). Chimera mice were generated by the aggregation method with an eight-cell stage embryo from ICR mice (CLEA, Tokyo, Japan) as described elsewhere (Andras et al. 2003). After confirmation of germ-line transmission, homozygous knockout mice were generated by inbreeding of heterozygous mice. Disappearance of Mboat4 gene expression in the knockout mice was confirmed by quantitative RT-PCR utilizing a 7500 Fast real-time PCR system (Applied Biosystems) and a One Step SYBR Prime-Script PLUS RT-PCR Kit (Takara Bio, Shiga, Japan) as directed. Primers used were as follows: Mboat4 forward 5'-gacagtgggcctcacattcag-3' and reverse 5'-gtggaaaaggtgtcacaagatgc-3', glyceraldehyde-3-phosphate dehydrogenase (Gapdh) forward 5'-aaatggtgaaggtcggtgtg-3' and reverse 5'-tgaaggggtcgttgatgg-3',

**Two-bottle-drinking test**

After habituation of free access to two bottles of 5% (w/v) sucrose (Wako, Osaka, Japan) and water for 1 week, twenty one C57BL/6 mice were assigned to one of three groups (n=7/group) based on consumption of sucrose, food intake and body weight, so as to randomize the animals. At the group assignment, sucrose consumption (5% sucrose intake/day: ml) was 15.9 ± 1.9 ml (saline group), 15.8 ± 1.7 ml (acylated-ghrelin), 16.4 ± 2.0 ml (unacylated-ghrelin), food intake (g/day) was 2.3 ± 0.1 g
mice fasted overnight were anaesthetized by isoflurane and sacrificed. Mesenteric fat was carefully isolated and weighed.

**Energy expenditure**

Seven-week-old WT (n = 8) and GOAT KO mice (n = 8) were fed on MCT + sucrose diet for 1 week, and habituated to metabolic chambers for 5 days. Then, oxygen consumption (VO₂) and carbon dioxide production (VCO₂) were measured over 40-min intervals by mass spectrometry (ARCO-1000A, Arco System, Kashiwa, Japan) for 24 h beginning from 1000 h. Respiratory quotient (RQ = VCO₂/VO₂) and energy expenditure (3.815 + 1.232 × RQ) were determined. At the same time, locomotor activity was measured by using an infrared light beam system (Neuroscience, Co., Ltd, Japan). Mice had free access to water and food (MCT + sucrose diet) during the test. The gas analyzer was initially calibrated by using a certified gas mixture and atmospheric air.

**OGTT and ITT**

For OGTT, mice (n = 10/group) were fasted overnight, and blood samples were obtained from the tail vein to measure baseline glucose levels. Then, glucose (0.5 g/kg body weight) was administered orally, and blood samples were collected at each time point and centrifuged at 20,400 g to obtain plasma samples. Plasma glucose level was measured using an automatic analysis instrument (Hitachi) and insulin concentration was measured by using ELISA kit (mouse insulin ELISA kit, Shionogi, Osaka, Japan) (Imai et al. 2015).

For ITT, mice had fasted for 4 h, and blood samples were obtained followed by i.p. injection with 0.5 U/kg of insulin (Humalin; Eli Lilly, Inc., Indianapolis, IN, USA). Samplings from the tail vein and plasma glucose measurement were carried out as mentioned above.

### Table 1  Dietary composition

<table>
<thead>
<tr>
<th>Formula (kcal %)</th>
<th>HF (D12492)</th>
<th>MCT (D12331)</th>
<th>HF + sucrose (D12445)</th>
<th>MCT + sucrose (D12327)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>20</td>
<td>16</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>7</td>
<td>13</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Maltodextrin 10</td>
<td>13</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lard</td>
<td>60</td>
<td>0</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>Coconut oil</td>
<td>0</td>
<td>58</td>
<td>0</td>
<td>40</td>
</tr>
</tbody>
</table>

HF, high-fat; MCT, medium-chain triglycerides.
Statistical analysis

All experimental results were expressed as mean ± S.E.M. Statistical comparisons of the two-bottle-drinking test were performed using a 2 way ANOVA. The area under the curve for blood glucose and insulin level during OGTT, and ITT, mesenteric fat mass, locomotor activity, respiratory quotient, and energy expenditure data were analyzed by the Student’s t-test. Body weight and food intake data were analyzed by mixed model with repeated measures. P value below 0.05 was considered to be statistically significant.

Results

Effects of acylated- and unacylated-ghrelin on sucrose consumption

Two-bottle-drinking test showed acylated-ghrelin administered in mice stimulated sucrose consumption, while unacylated-ghrelin did not (Fig. 2A).

Voluntary saccharin and sucrose consumption test in GOAT KO mice

To test whether endogenous acylated-ghrelin signaling is involved in the consumption of sucrose, GOAT KO mice and WT control mice were subjected to the two-bottle-drinking test. As shown in Fig. 2B, GOAT KO mice consumed less sucrose than WT mice (P<0.05) and took in more water instead. In contrast, there was no significant difference in saccharin intake between WT and KO mice (P=1.00) (Fig. 2C), suggesting that reduced intake of sucrose is not due to indifference to sweet taste.

Food intake, body weight, mesenteric fat mass and energy expenditure

As GOAT KO mice showed reduced consumption of sucrose under voluntary access condition compared to WT mice, we hypothesized that feeding sucrose-rich diet to GOAT KO mice may lead to reduction of food intake. WT and KO mice were fed on one of the diets listed in Table 1, and body weight and food intake were monitored for 12 weeks. As shown in Fig. 3A and B, mice fed HF diet did not reveal any significant difference in body weight and food intake weight between WT and KO mice after 12-week feeding (body weight: WT, 49.4±0.9 g, KO, 48.8±2.1 g, P=0.24; total food intake: WT, 1323±26 kcal, KO, 1343±42 kcal, P=0.19). Figure 3D and E shows that KO mice fed MCT diet exhibited only a slight reduction of body weight and food intake compared to WT mice (body weight: WT, 46.9±1.6 g, KO, 44.2±1.7 g, P=0.96; total food intake: WT, 1320±35 kcal, KO, 1254±31 kcal, P<0.05). On the other hand, feeding sucrose-rich diet resulted in a notable decrease in food intake and strong
resistance to obesity in KO mice (body weight: WT, 45.5 ± 1.2 g, KO, 39.6 ± 1.8 g, P<0.01; total food intake: WT, 1218 ± 22 kcal, KO, 1130 ± 30 kcal, P<0.01) (Fig. 3G and H), and the difference in body weight and food intake was even more marked with a sucrose-rich MCT diet (body weight: WT, 46.5 ± 0.9 g, KO, 38.0 ± 1.3 g, P<0.01; total food intake: WT, 1251 ± 16 kcal, KO, 1124 ± 26 kcal, P<0.01) (Fig. 3J and K). Consistent with body weight reduction, mesenteric fat mass was significantly lower in GOAT KO mice on a sucrose-rich diet (Fig. 3I and L). As shown in Fig. 4A and B, GOAT KO mice fed on sucrose-rich MCT diet exhibited slightly, but significantly higher food intake: WT, 1251 ± 16 kcal, KO, 1124 ± 26 kcal, P<0.01) (Fig. 3J and K). Consistent with body weight reduction, mesenteric fat mass was significantly lower in GOAT KO mice on a sucrose-rich diet (Fig. 3I and L). As shown in Fig. 4A and B, GOAT KO mice fed on sucrose-rich MCT diet exhibited slightly, but significantly higher

**Figure 3**
Body weight, food intake and mesenteric fat mass. WT and GOAT KO mice fed high-fat (HF) diet (A), medium-chain triglyceride (MCT) diet (D), HF + sucrose diet (G), and MCT + sucrose diet (J) were weighted for 12 weeks. Food intake was measured every week, and the columns of food intake show total calorie consumption for 12 weeks (B, E, H, and K). After WT and GOAT KO mice were fed diets for 15 weeks, mesenteric fat mass was dissected and weighted (C, F, I, and L). Data are presented as mean ± S.E.M. *P<0.05 **P<0.01 between WT and GOAT KO mice. NS, not significant.
Figure 4
Energy expenditure, respiratory quotient (RQ), and locomotor activity. Energy expenditure (A), respiratory quotient (C), and locomotor activity (E) were measured for a period of 24 h in WT (n=8) and GOAT KO mice (n=8) fed on an MCT+sucrose diet. Average energy expenditure (B) and RQ (D) were calculated in the light and dark periods. Data are presented as mean ± S.E.M. *P<0.05 **P<0.01 between WT and GOAT KO mice. NS, not significant.

OGTT and ITT

To investigate whether GOAT KO mice show improvement in glucose homeostasis accompanied by reduction of body weight, the OGTT was performed 12 weeks after the initiation of diet load. While GOAT KO mice with HF or MCT diet did not show significant improvement in glucose metabolism compared to WT mice (Fig. 5A and C), glucose concentration was considerably reduced in GOAT KO mice fed sucrose-rich HF and MCT diets (Fig. 5E and G). In addition, plasma insulin concentration during the OGTT was dramatically decreased (Fig. 5F and H), indicating that GOAT KO mice showed increased insulin sensitivity. Moreover, ITT revealed that GOAT KO mice exhibited improved insulin response (Fig. 6C and D). Importantly, KO mice with HF or MCT diet tend to show reduced insulin concentration in OGTT (Fig. 5B and D) and exhibited a slight improvement in insulin response in ITT compared to WT mice (Fig. 6B), although body weight was not significantly reduced. These results suggest that increased insulin sensitivity in GOAT KO mice may occur partly independently of weight loss.

Discussion

The study presented herein demonstrated that GOAT KO mice were protected from obesity on a HF, high-sucrose diet, implying that sucrose content in diet may have a significant impact on food intake behaviour elicited by the ghrelin/GOAT pathway in mice. This is a novel finding because other reports have repeatedly expressed the view that the ghrelin system may not play a major role in appetite regulation. Most of the reports that did not demonstrate body weight reduction in mice with deleted ghrelin signal utilized HF diet-induced obesity model (Wortley et al. 2004, Zhao et al. 2010). Alternatively, Kirchner et al. (2009) showed that GOAT KO mice with MCT diet exhibited a slight resistance to obesity. Similarly, the present results showed no significant weight loss in GOAT KO mice on HF diet compared to WT mice, and...
only a modest decrease in body weight in GOAT KO mice with MCT diet. A significant reduction in food intake were observed in GOAT KO mice with MCT diet, which may be explained by the effect of increased acylated-ghrelin level on food intake in WT mice. Confirming results reported previously, the current study has added significant evidence that GOAT KO mice showed marked resistance to diet-induced obesity, improved insulin sensitivity and reduced adiposity when fed HF and high-sucrose diet. Interestingly, attenuation of body weight in GOAT KO mice was more remarkable when MCT was added to the high sucrose diet, possibly because availability of high amount of middle-chain fatty acid as a substrate led to enhanced formation of acylated-ghrelin in WT mice.

These results indicate that an endogenous ghrelin signal plays an important role in the pathological process of obesity-related metabolic syndrome elicited by over-consumption of sugar-sweetened high-calorie foods. The exact reason why GOAT KO led to weight loss in mice on a HF, high-sucrose diet, but not on a HF diet remains elusive. As a possible explanation, it is tempting to assume that adding sucrose to HF diet may evoke palatability-driven food consumption much more than HF alone because ghrelin is implicated in hedonic as well as homeostatic food intake regulation, and because a diet rich in sucrose and fat is accepted as highly palatable and strongly preferred. In the present study, we did not utilize high sucrose diet without HF, because our previous

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**Figure 5**

Plasma glucose and insulin during OGTT. WT and GOAT KO mice fed high-fat (HF) diet, medium-chain triglyceride (MCT) diet, HF + sucrose diet, MCT + sucrose diet for 13 weeks were loaded with 0.5 g/kg glucose after overnight fasting. Plasma samples were collected before glucose load (0 min), and at 15, 30, 60, and 120 min after glucose load. A, C, E, and G show plasma glucose excursion during an OGTT and area under the curve (AUC), and B, D, F, and H show plasma insulin excursion and AUC under HF, MCT, HF + sucrose, MCT + sucrose diet respectively. Data are presented as mean ± S.E.M., *P < 0.05 and **P < 0.01 between WT and GOAT KO mice. NS, not significant.
studies have shown that addition of sucrose alone to the diet was not enough to evoke overweight in C57BL/6 strain. Anyway, this finding underscores the importance of macronutrient composition in diet in evaluating anti-obesity treatments using diet-induced obesity animal models.

The present study revealed that sucrose significantly affects feeding behaviour in GOAT KO mice. Previous reports have indicated that the ghrelin system is closely associated with sucrose consumption. Plasma acylated-ghrelin level was positively correlated with carbohydrate consumption, while inversely correlated with fat intake in rat (Beck et al. 2002). Peripheral and central acylated-ghrelin injection stimulated dopamine release in NAc, accompanied by increased sucrose consumption (Landgren et al. 2011, McCallum et al. 2011). In addition, a population-based genetic study in humans showed that pro-ghrelin gene (GHRL) haplotype was associated with sucrose preference (Landgren et al. 2011). These observations may have been interpreted to indicate that ghrelin signal may stimulate a reward system in the midbrain and prompt animals to seek foods with a sweet taste such as sucrose. On the other hand, as presented here in the two-bottle preference test, we showed that intake of saccharin, a non-nutritive sweetener, in GOAT KO mice was at the same level as that in WT mice, showing that preference for sweet taste was not reduced in GOAT KO mice. This is consistent with the study by Disse et al. (2010) in which GHS-R1a-deficient mice did not exhibit any significant reduction in saccharin intake compared to WT mice in the absence of exogenous acylated-ghrelin injection. There seems to be different physiological responses between sucrose and saccharin, as demonstrated by a study which has shown that saccharin cues evoke less phasic dopamine release in NAc than sucrose cues (McCutcheon et al. 2012), and by another study which has indicated that the calorific value of sucrose is enough to induce dopamine release in mice with aberrant taste (De Araujo et al. 2008). We did not analyze dopamine release in this study, and we consider this as a subject of future study. Taking these findings into account, it is speculated that nutritional value, rather than the sweet taste of sucrose may be significantly involved in regulating food preference in GOAT KO mice.

Consistent with the findings reported by other groups that showed increased energy expenditure in ghrelin- or GHS-R1a-deficient mice (Wortley et al. 2005, Lin et al. 2011), the current study indicated GOAT KO mice had higher energy expenditure under ad libitum feeding of sucrose-rich MCT diet, suggesting amelioration of obesity in GOAT KO mice is attributed to increased energy expenditure as well as suppression of overeating. In addition, GOAT KO mice exhibited a tendency of increased locomotor activity during the dark phase, which might contribute to increase in energy expenditure. However, these results should be interpreted with caution for two reasons. First, it is possible that higher energy
expenditure may be affected by lower body weight of GOAT KO mice, even though the current experiment was conducted at early stage of obesity to minimize the influence of difference in body weight and food intake (body weight: WT, 29.3±0.3 g, KO, 26.6±0.1 g; food intake/day: WT, 3.4±0.1 g, KO, 3.1±0.1 g). Second, no significant difference in body weight has been observed when food intake was similar between WT and GOAT KO mice fed on HF diet, raising a question about the contribution of energy expenditure in KO mice to lower body weight. In the future study, it would be necessary to compare the energy expenditure under the pair-fed feeding condition.

In conclusion, this report provides evidence that GOAT KO mice show marked resistance to diet-induced obesity when fed a high-sucrose diet, which led to amelioration of insulin resistance and adiposity. There has been a concern about excessive sucrose intake as a public health problem in relation to obesity, diabetes and cardiovascular diseases (Bleich et al. 2009, Bermudez & Gao 2010). In the USA, men and women consumed an average of 335 and 239 kcal from added sugars, equivalent to 12.8 and 13.2% of their total calorie intake (Ervin & Ogden 2013). A newly updated guideline from the World Health Organization (WHO) strongly recommends reducing the intake of free sugars to below 10% of total energy intake in both adults and children (World Health Organization Guideline 2015), and the American Heart Association published a scientific statement which recommends even stricter control of added sugar consumption (Johnson et al. 2009). Taking into account social demands to cut back sugar intake, our present findings is noteworthy for shedding light on a physiological function of ghrelin, and also showing a potential treatment strategy for treating obesity due to overconsumption of sweetened foods and drinks.

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.

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