

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

(グレリン *O*-アシル基転移酵素ノックアウトマウスは高スクロース飼料給餌条件下において抗肥満性を示す)

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TITLE: 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 wild-type (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.

1. 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). Discoveries that administration of exogenous acylated-ghrelin stimulates food intake in humans (Neary *et al.* 2004, Wren *et al.* 2001) and rodents (Nakazato *et al.* 2001, Tschöp *et al.* 2000), 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 heightened expectations that inhibiting GOAT activity would offer a therapeutic option to treat obesity and other metabolic disorders as well as

GHSR1a 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 wild type (WT) mice given high-fat (HF) diet or standard chow (Sato *et al.* 2008, Sun *et al.* 2003, Wortley *et al.* 2004, 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 ghrelin acylation, 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) (Abizaid *et al.* 2006, Naleid *et al.* 2005). A recent investigation has shown that peripherally and centrally administered 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 (high-fat, MCT, and high-sucrose). We also performed glucose and insulin tolerance test to investigate whether insulin resistance elicited by high-calorie diet load was ameliorated in GOAT KO mice.

2. Materials and methods

Animals and housing

All the animals were maintained on 12:12 artificial light-dark 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) by electroporation using Nucleofector kits for mouse embryonic stem cells (Lonza) as directed program. Mouse ES cells were maintained on neomycin-resistant 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 x MEM non-essential amino acids solution, 55 nM 2-mercaptoethanol (these were from Life Technologies), 1.7 x 10³ unit/mL mouse leukemia inhibitory factor, 1 x 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'-acaccaaggatagtggtgcttc-3', reverse 5'-agagctccctggcgaaatcggtac-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 8-cell stage embryo from ICR mice (CLEA, 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 PrimeScript PLUS RT-PCR Kit (Takara Bio; Fig. 1C) as directed. Primers used were as follows: *Mboat4* forward 5'-gacagtgggcctcacattcag-3' and reverse 5'-gtggaaaaggtgtccaagatgc-3', glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) forward 5'-aaatggtgaaggctcggtgtg-3' and reverse 5'-tgaagggtcgttgatgg-3'.

Two-bottle-drinking test

After habituation of free access to two bottles of 5% (w/v) sucrose (Wako, Japan) and water for 1 week, twenty one C57BL/6 mice were assigned to one of three groups (n = 7 per 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 (saline group), 2.2 ± 0.1 g (acylated-ghrelin), 2.1 ± 0.2 g (unacylated-ghrelin), body weight (g) was 23.4 ± 0.1 g (saline group), 23.0 ± 0.3 (acylated-ghrelin), 23.1 ± 0.2 g (unacylated-ghrelin). No significant differences in sucrose consumption, mean body weight, and food intake between the groups are confirmed by one-way analysis of variance (ANOVA). The mice received an intraperitoneal (i.p.) injection of 1 mg/kg of acylated-ghrelin, 1 mg/kg of unacylated-ghrelin (Peptide Institute. Inc., Japan) or saline control, and two hours later, bottles were weighted.

For voluntary saccharin and sucrose consumption test, WT mice and GOAT KO mice (n = 20 per group) were allowed to freely access to two bottles for 6 days, with one containing 0.1% (w/v) saccharin (Sigma-Aldrich) and the other containing only water. Water and saccharin intakes were measured, and the data were analyzed and expressed as mL/g/day. The sucrose consumption test was carried out in the same way. WT mice and GOAT KO mice (n = 20 per group) had free access to food, water and 5% (w/v) sucrose solution for 6 days.

Body weight and food intake

Both WT and GOAT KO mice were allowed free access to water and one of the diet listed in Table 1 (n = 10 per group). Seven-week-old mice were fed high-fat diet (D12492, HF), MCT diet (D12331, MCT), high-fat and high-sucrose diet (D12445, HF + sucrose), or MCT and high-sucrose diet (D12327, MCT + sucrose). The diets were purchased from Research Diets, New Brunswick, NJ, USA. Body weight and food intake were measured every week. Food consumption was calculated by summarizing weekly amount of food intake. When food intake was measured, food spillage in the cage was collected and weighted to correct for overestimation of food intake. After body weight and food intake were monitored for 12 weeks, oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were conducted at 13 and 14 week, respectively. At the end of the study, 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 one week, and habituated to metabolic chambers for 5 days. Then, oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured over 40-minute intervals by mass spectrometry (ARCO-1000A, Arco System, Kashiwa, Japan) for 24 hours beginning from 10:00 AM. Respiratory quotient ($\text{RQ} = \text{VCO}_2/\text{VO}_2$) and energy expenditure ($3.815 + 1.232 * \text{RQ}$) 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.

Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

For, OGTT, mice (n = 10 per 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 15,000 rpm to obtain plasma samples. Plasma glucose level was measured using an automatic analysis instrument (Hitachi, Japan) and insulin concentration was measured by using ELISA kit (mouse insulin ELISA kit, Shionogi, Japan) (Imai *et al.* 2015).

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

Statistical analysis

All experimental results were expressed as mean $\pm$ 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.

3. Results

Effects of acylated- and unacylated-ghrelin on sucrose consumption

Two-bottle-drinking test showed acylated-ghrelin administrated 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, $p = 0.19$). Fig. 3D and E show 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 energy expenditure compared to WT mice in both the light and dark phases. In addition, respiratory quotient (RQ) was decreased in GOAT KO mice in both phases (Fig. 4C and D). There was not a significant difference in locomotor activity during the observation period (Fig. 4E).

Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

To investigate whether GOAT KO mice show improvement in glucose homeostasis accompanied by reduction of body weight, the oral glucose tolerance test (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.

4. Discussion

The study presented herein demonstrated that GOAT KO mice were protected from obesity on a high-fat, high-sucrose diet, implying that sucrose content in diet may have a significant impact on food intake behavior 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 high-fat diet-induced obesity model (Wortley *et al.* 2004, Zhao *et al.* 2010). Alternatively, Kirchner *et al.* showed that GOAT KO mice with MCT diet exhibited a slight resistance to obesity (Kirchner *et al.* 2009). Similarly, the present results showed no significant weight loss in GOAT KO mice on high-fat 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 high-fat 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 overconsumption of sugar-sweetened high-calorie foods. The exact reason why GOAT KO led to weight loss in mice on a high-fat, high-sucrose diet, but not on a high-fat diet remains elusive. As a possible explanation, it is tempting to assume that adding sucrose to high-fat diet may evoke palatability-driven food consumption much more than high-fat 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 high fat, because our previous 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 behavior 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.* in which GHSR1a-deficient mice did not exhibit any significant reduction in saccharin intake compared to WT mice in the absence of exogenous acylated-ghrelin injection (Disse *et al.* 2010). 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 caloric 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 GHSR1a-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 (Bermudez and Gao 2010, Bleich *et al.* 2009). In the US, 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 and 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 finding 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 no conflicts of interest.

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Figure legends

Figure 1 Generation of GOAT-deficient mice. (A) A schematic diagram of targeting strategy. Exon 1 of *Mboat4* gene was replaced with a PGK-Neo cassette. White boxes indicate exons of *Mboat4* gene; exon number is indicated below the boxes. Black arrows indicate the priming sites of PCR to pick out ES cell clones. Black bars indicate the probes for Southern hybridization. Restriction sites are abbreviated as follows: Bm = BamHI; RV = EcoRV; Kp = KpnI; RI = EcoRI. (B) Southern hybridization with BamHI-digested genomic DNA from the ES cells after homologous recombination. The wild-type (WT) signal at 9.0 kb and targeted alleles (KO) signal at 12.0 kb by the 3' probe and targeted allele (KO) signal at 4.5 kb by the Neo probe are respectively indicated. (C) Confirmation of disappearance of *Mboat4* gene expression in knockout mice by quantitative RT-PCR. Relative expression levels of *Mboat4* mRNA in the stomach and small intestine were determined using those of *Gapdh* mRNA as the internal control. The expression in males (M) and females (F) of homozygous (-/-) and heterozygous (-/+) knockout mice was compared to wild-type (+/+) male mice in each tissue arbitrarily set to 1.0.

Figure 2 Two-bottle-drinking test. Mice were intraperitoneally administrated with 1 mg/kg of acylated-ghrelin, unacylated-ghrelin or saline and had a freely access to water and 5% sucrose solution for 2hr (A). Data were analyzed per g body weight. WT and GOAT KO mice were freely access to water and 5% sucrose solution (B), or 0.1% saccharin solution (C) for 6 days. Consumption of each solution was analyzed per g body weight per day (mL/g/day). Data are presented as mean $\pm$ SEM, ** $p < 0.01$ denotes the difference between water intake and 5% sucrose, 0.1% saccharin intake in the same genotype or group. # $p < 0.05$ denotes the difference between the saline

group and the acylated-ghrelin group. † $p < 0.05$ denotes the difference between the acylated-ghrelin group and the unacylated-ghrelin group. § $p < 0.05$ denotes the difference between WT and GOAT KO mice.

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 were measured every week, and the columns of food intake show total calorie consumption for 12 weeks (B, E, H, K). After WT and GOAT KO mice were fed diets for 15 weeks, mesenteric fat mass was dissected and weighted (C, F, I, L). Data are presented as mean $\pm$ SEM. * $p < 0.05$ ** $p < 0.01$ 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 hr 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 $\pm$ SEM. * $p < 0.05$ ** $p < 0.01$ between WT and GOAT KO mice. NS, not significant.

Figure 5 Plasma glucose and insulin during oral glucose tolerance test (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, 120 minutes after glucose load. A, C, E, G show plasma glucose excursion during an OGTT and area under the curve (AUC), and B, D, F, H show plasma insulin excursion and AUC under HF, MCT, HF + sucrose, MCT + sucrose diet, respectively. Data are presented as mean $\pm$ SEM, * $p < 0.05$ ** $p < 0.01$ between WT and GOAT KO mice. NS, not significant.

Figure 6 Plasma glucose during insulin tolerance test (ITT). WT and GOAT KO mice fed high-fat (HF) diet (A), medium-chain triglyceride (MCT) diet (B), HF + sucrose diet (C), MCT + sucrose diet (D) for 14 weeks were injected intraperitoneally with 0.5 U/kg of insulin. Plasma samples were collected before insulin injection (0 min), and at 30, 60, 90, 120 minutes after insulin load. Data are presented as mean $\pm$

SEM. * $p < 0.05$, ** $p < 0.01$ between WT and GOAT KO mice. NS, not significant.

Table 1 Dietary composition. HF, high fat: MCT, medium-chain triglycerides.

		Formula (kcal %)			
		HF	MCT	HF + sucrose	MCT + sucrose
		(D12492)	(D12331)	(D12445)	(D12327)
Protein		20	16	20	20
Carbohydrate	Sucrose	7	13	40	40
	Maltodextrin 10	13	13	0	0
Fat	Lard	60	0	40	0
	Coconut oil	0	58	0	40

Figure 1

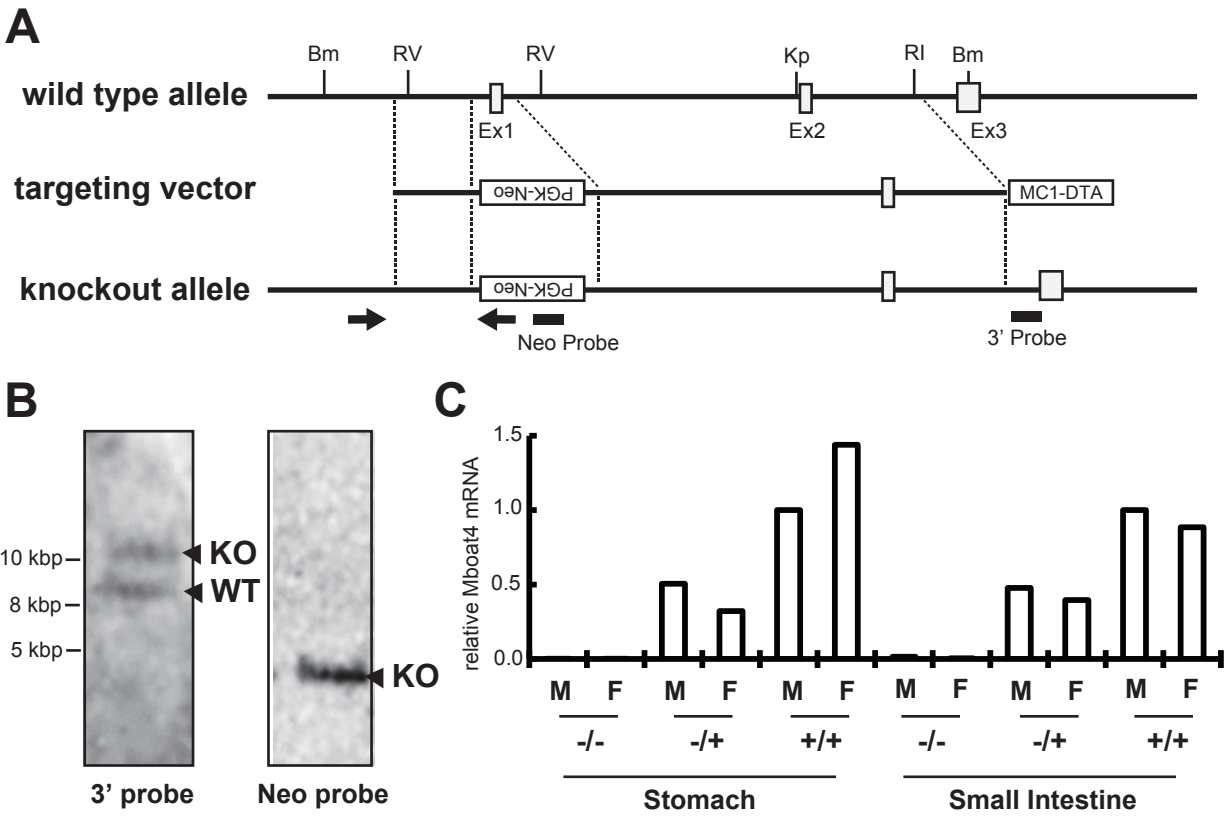


Figure 2

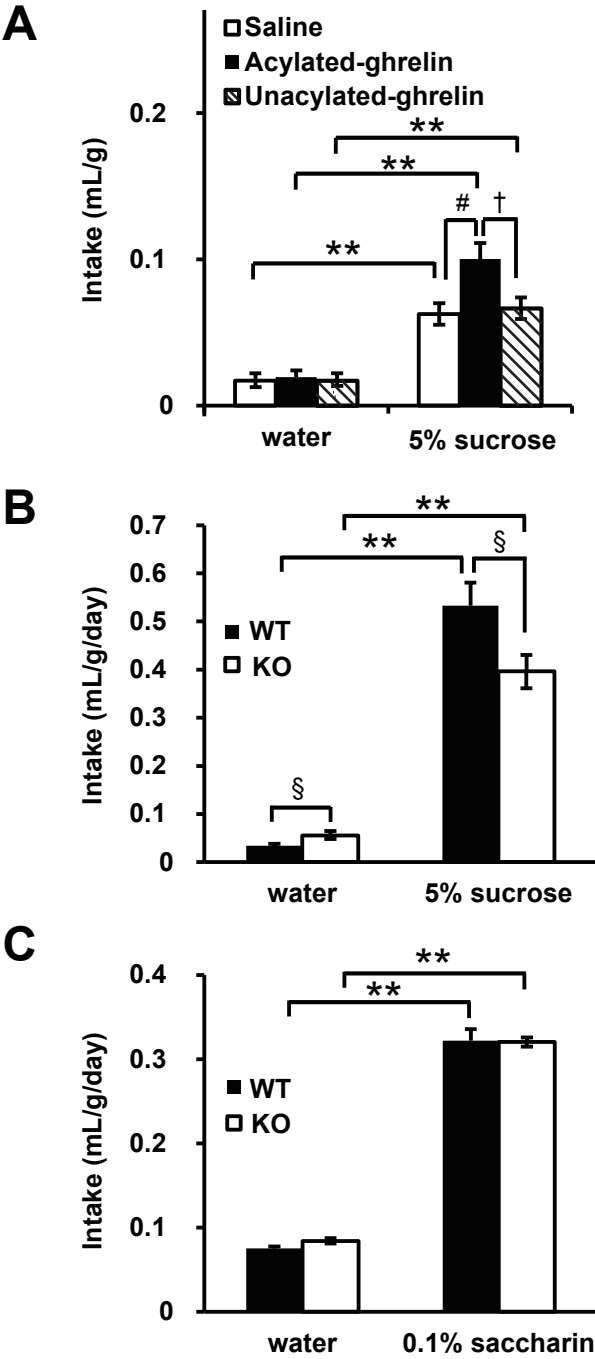


Figure 3

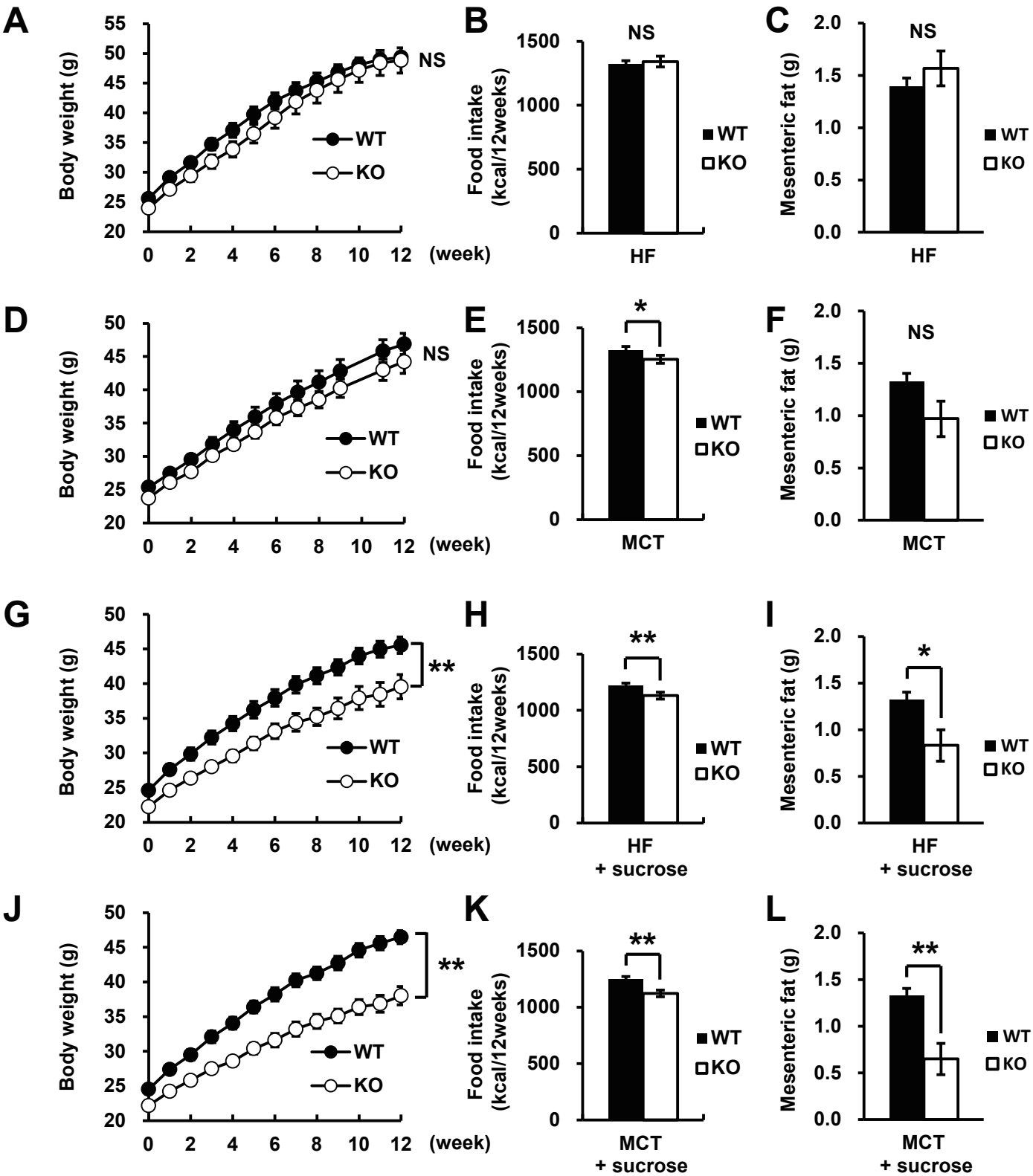


Figure 4

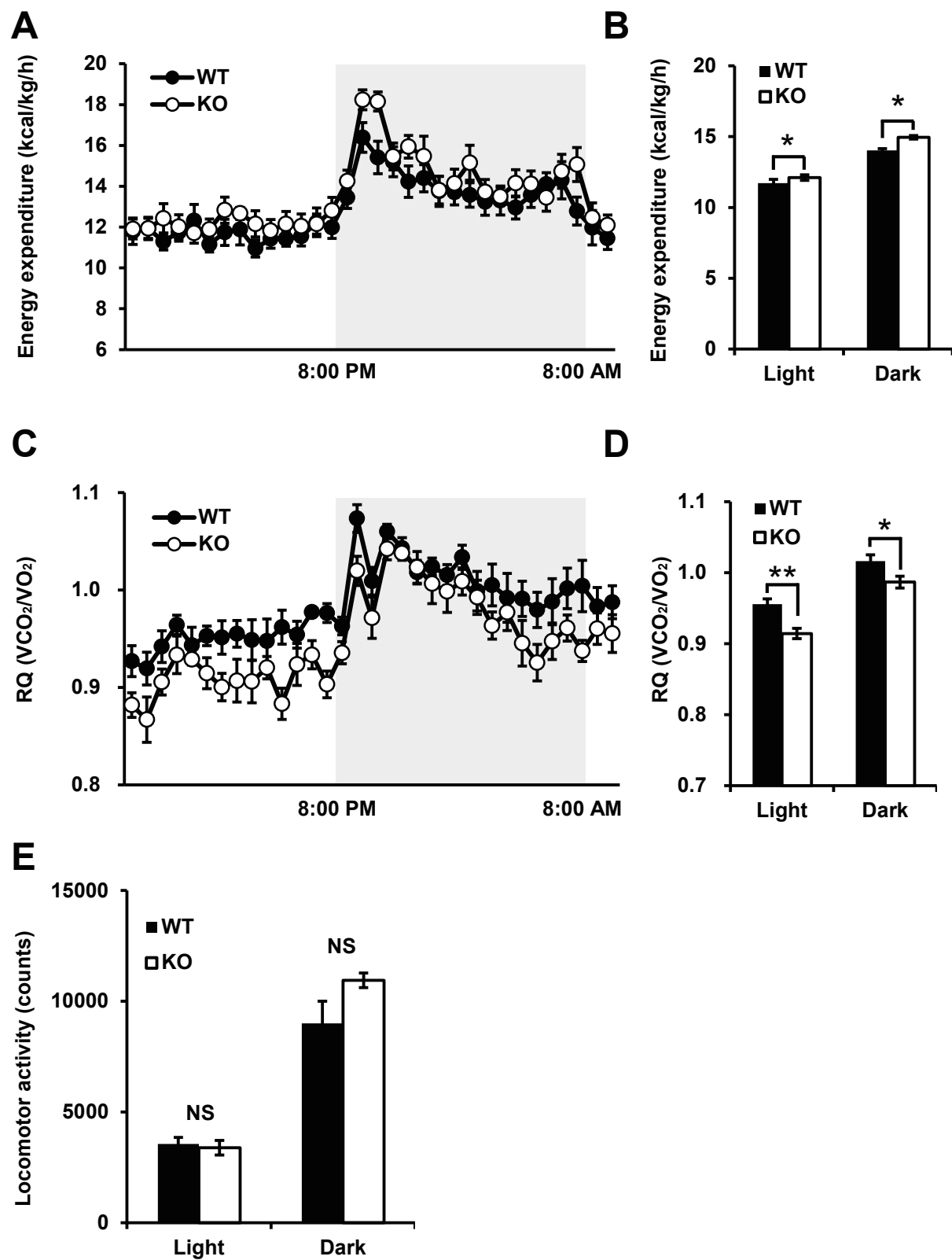


Figure 5

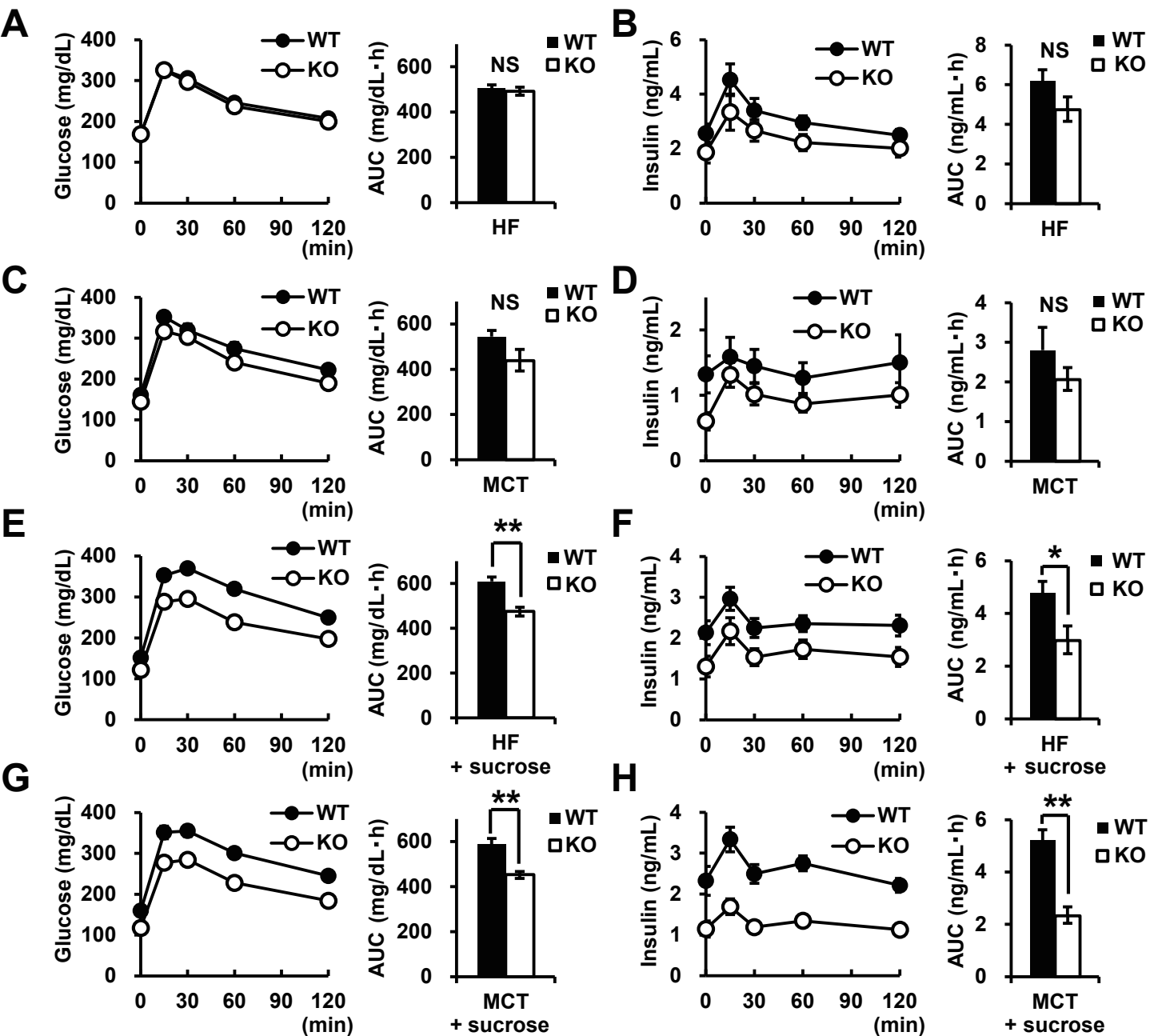


Figure 6

