Mice With Hyperghrelinemia Are Hyperphagic and Glucose Intolerant and Have Reduced Leptin Sensitivity

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OBJECTIVE—Ghrelin is the only known peripheral hormone to increase ingestive behavior. However, its role in the physiological regulation of energy homeostasis is unclear because deletion of ghrelin or its receptor does not alter food intake or body weight in mice fed a normal chow diet. We hypothesized that overexpression of ghrelin in its physiological tissues would increase food intake and body weight.

RESEARCH DESIGN AND METHODS—We used bacterial artificial chromosome transgenesis to generate a mouse model with increased ghrelin expression and production in the stomach and brain. We investigated the effect of ghrelin overexpression on food intake and body weight. We also measured energy expenditure and determined glucose tolerance, glucose stimulated insulin release, and peripheral insulin sensitivity.

RESULTS—Ghrelin transgenic (Tg) mice exhibited increased circulating bioactive ghrelin, which was associated with hyperphagia, increased energy expenditure, glucose intolerance, decreased glucose stimulated insulin secretion, and reduced leptin sensitivity.

CONCLUSIONS—This is the first report of a Tg approach suggesting that ghrelin regulates appetite under normal feeding conditions and provides evidence that ghrelin plays a fundamental role in regulating β-cell function. ***Diabetes*** **58:840–846, 2009**

Ghrelin is a 28-aa peptide that is expressed at high levels in the stomach. It is the endogenous ligand for the growth hormone secretagogue receptor and increases growth hormone secretion from the pituitary (1). Ghrelin also increases food intake and adiposity, suggesting a role in the control of energy homeostasis (2). Consistent with this, plasma ghrelin levels have been shown to increase before a meal and during fasting (3). Ghrelin circulates in two forms: the biologically active octanoylated form and the des-octanoyl form, which is thought to be biologically inactive (4). Recent data show that ghrelin a-acyltransferase (GOAT), a membrane-bound enzyme, is responsible for octanoylation of the serine 3 residue of ghrelin and confers biological activity (5,6).

Despite unequivocal pharmacological data, the evidence for a physiological role for ghrelin in the control of appetite is much less clear. Mice with targeted deletion of either ghrelin or the growth hormone secretagogue receptor exhibit an essentially normal metabolic phenotype when fed a regular chow diet, suggesting that ghrelin may have a redundant role in the regulation of food intake (7,8). When fed a high-fat diet, these mice are resistant to diet-induced obesity, exhibiting reduced adiposity and increased energy expenditure (9,10). More recent data suggest that these knockout models are not resistant to diet-induced obesity when backcrossed to a pure C57BLK6 genetic background. Despite this, calorie restriction in the pure-bred mice resulted in lower blood glucose in both knockout models (11). The conflicting food intake and body weight data from Tg models has made defining a key role for endogenous ghrelin in the control of appetite difficult. However, the data do consistently suggest that ghrelin may be important in the control of glucose homeostasis.

Ghrelin gain-of-function models have not produced the expected hyperphagic and obese phenotype (12–14). These models did not, however, exhibit increases in plasma bioactive ghrelin. Reed et al. developed a model in which ghrelin was overexpressed in the brain but not the stomach (15). In one Tg line, circulating bioactive ghrelin was found to be increased, but this was not associated with hyperphagia. The lack of an obese phenotype in these mice was attributed to developmental compensation, alterations in peripheral versus central nervous system ghrelin concentrations, and/or alterations in diurnal patterns of ghrelin release.

The production of bioactive ghrelin critically depends on its octanoylation by GOAT. To physiologically overexpress bioactive ghrelin, the ghrelin transgene must be expressed in tissues that also produce GOAT: the stomach and small intestines. We used the ghrelin promoter to drive ghrelin overexpression and generated mice with increased circulating levels of bioactive ghrelin. We then investigated the phenotype of these mice.

RESEARCH DESIGN AND METHODS

Generation of ghrelin Tg mice. We identified a bacterial artificial chromosome (BAC) containing the ghrelin gene RP23-441K11 (Invitrogen, Huntsville, AL). Tg mice were created using standard pronuclear injection techniques. F0 mice were mated with CBA/C57Bl6 mice, and Tg lines were maintained separately. Mice were maintained in cages under controlled temperature (21–23°C) and light (11 h light/13 h dark) with ad libitum access to food (RM1 diet; SDS UK Ltd) and water. Animal procedures performed were approved under the British Home Office Animals Scientific Procedures Act 1986.

Body weight, food intake, indirect calorimetry, and body composition. Mice were singly housed from weaning, and food intake and body weight were measured. Body composition of 16-week-old mice was calculated using the method of Salmon and Flatt (16). Metabolic parameters were obtained using the open-circuit Oxymax comprehensive lab animal monitoring system (Columbus Instruments, Columbus, OH) as previously described (17).

Glucose tolerance test and insulin tolerance test. An intraperitoneal glucose tolerance test (IP-GTT) was performed in conscious 16-week-old

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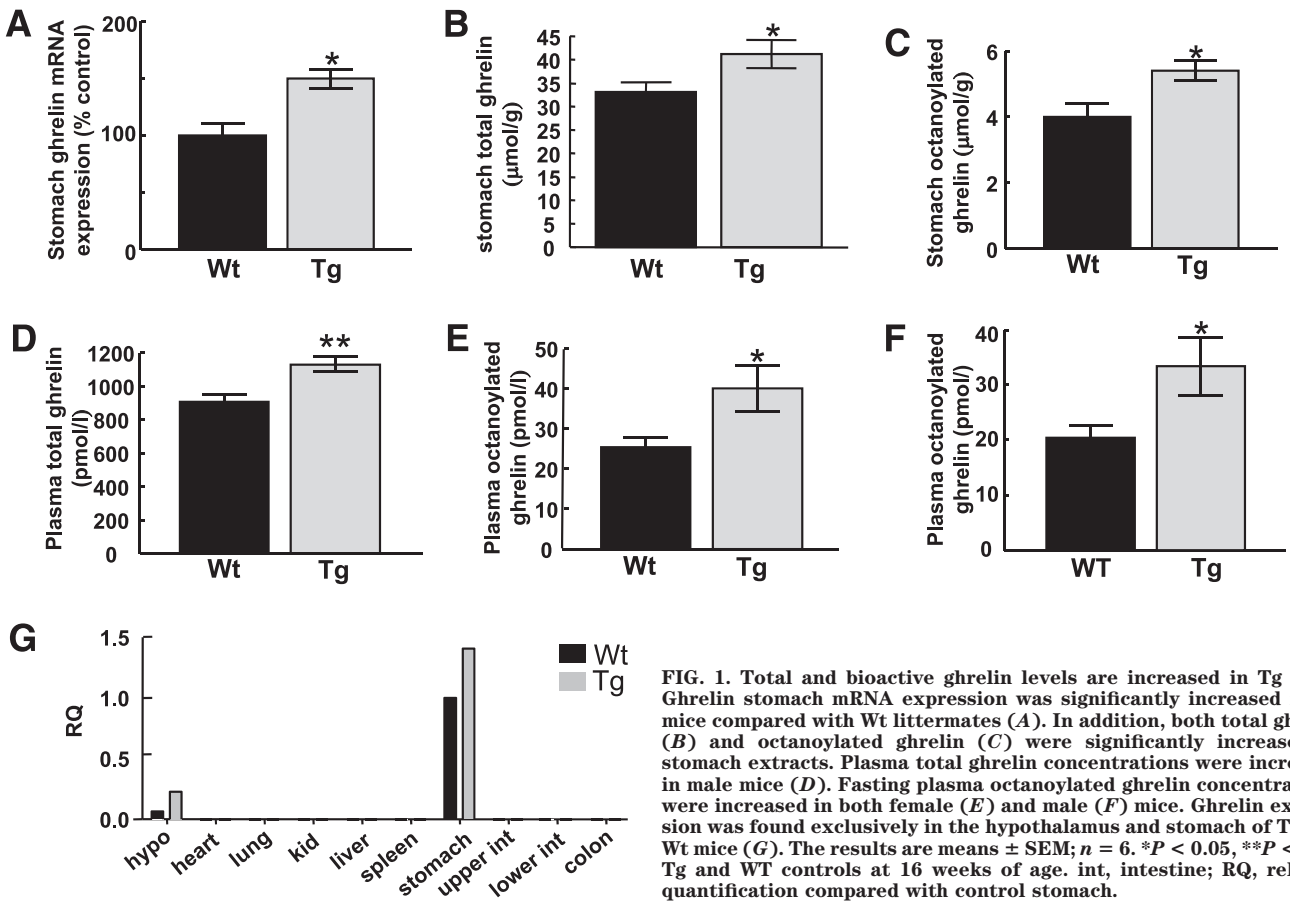

FIG. 1. Total and bioactive ghrelin levels are increased in Tg mice. Ghrelin stomach mRNA expression was significantly increased in Tg mice compared with Wt littermates (*A*). In addition, both total ghrelin (*B*) and octanoylated ghrelin (*C*) were significantly increased in stomach extracts. Plasma total ghrelin concentrations were increased in male mice (*D*). Fasting plasma octanoylated ghrelin concentrations were increased in both female (*E*) and male (*F*) mice. Ghrelin expression was found exclusively in the hypothalamus and stomach of Tg and Wt mice (*G*). The results are means ± SEM; $n = 6$. *$P < 0.05$, **$P < 0.01$ Tg and WT controls at 16 weeks of age. int, intestine; RQ, relative quantification compared with control stomach.

mice. After an 18-h fast, D-glucose (2 g/kg) was administered intraperitoneally and blood glucose was measured by tail bleeds at 0, 15, 30, 60, 120, and 150 min postglucose administration. For glucose-stimulated insulin release, blood glucose and insulin levels were measured at 0, 15, 30 and 60 min postglucose. The insulin tolerance test was performed similarly except that mice were fasted for 4 h before intraperitoneal administration of Humulin (1.5 units/kg) (Eli Lillly, Basingstoke, U.K.). Plasma glucose was measured using the Acensia Contour blood glucose monitoring system (Bayer HealthCare, Newbury, U.K.).

Measurement of circulating hormones. Whole blood was collected by cardiac puncture from fasted 16-week-old mice. Mouse plasma insulin and leptin concentrations were determined using reagents and methods from Crystal Chem (Downers Grove, IL). Plasma corticosterone was measured using a radioimmunoassay kit from MP Biomedicals (Orangeburg, NY). Plasma IGF-I levels were measured using reagents and methods from Immunodiagnostic Systems Ltd (Bolden, UK). Octanoylated ghrelin concentrations were analyzed using an ELISA kit from LINCO Research (St. Charles, MO).

Northern blot analysis and quantitative PCR. Tissues from 16-week-old mice were snap-frozen and RNA was extracted using TRI reagent. Northern blot analysis was used to determine uncoupling protein (UCP)-1 mRNA expression in brown adipose tissue and ghrelin mRNA expression in stomach. Real-time quantitative PCR analysis was performed using TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) using the ABI Prism 7900 Sequence Detection System according to the protocols provided by the manufacturer (Applied Biosystems, Melbourne, Australia). The relative mRNA transcript levels were calculated according to the $2^{-\Delta CT}$ method, with ΔCT being the difference in cycle threshold values between the target mRNA and the 18S internal control.

Peripheral administration of ghrelin and PYY 3–36. At 16 weeks of age, ad libitum–fed mice were injected intraperitoneally with either saline or ghrelin (0.3 nmol/g) in a randomized blinded crossover design. A recovery period of 2 days was allowed between each study day. Mice were injected intraperitoneally in the early light phase at 9:00 A.M. Food intake was measured 1 h postinjection. At 16 weeks of age, in a randomized blinded crossover design, mice were injected intraperitoneally with either saline or leptin (3 μg/g). Before each study day, mice were fasted for 24 h and leptin was administered at 9:00 A.M.; food intake was measured at 1 and 4 h postinjection.

Values are means ± SEM unless otherwise stated. Differences in cumulative food intake through time were compared across experimental groups using generalized estimating equation curve analysis (Stata 9.1; Statacorp, College Station, TX). For analysis of the effect of peripheral administration of ghrelin and leptin, a paired Student's t test with a Bonferroni correction was used. All other comparisons were made using an unpaired Student's t test. P values <0.05 were considered significant.

RESULTS

Circulating bioactive ghrelin levels are increased in Tg mice. We identified a BAC, which contained the ghrelin gene and its promoter. Using this BAC, we generated Tg mice by standard pronuclear injection. Mice heterozygous for the ghrelin transgene had significantly increased ghrelin expression. Line L91 was chosen for further investigation. Ghrelin mRNA levels in the stomach were increased by ~150% in Tg mice compared with wild-type (Wt) littermates (Wt 2.24 ± 0.23 AU versus Tg 3.37 ± 0.19 AU, $n = 6$, $P < 0.005$; Fig. 1*A*). A similar increase was observed in stomach ghrelin content for both total and bioactive octanoylated ghrelin (total ghrelin: Wt 33.1 ± 01.9 μmol/g versus Tg 41.2 ± 3.0 μmol/g, $n = 12$, $P < 0.05$; octanoylated ghrelin: Wt 4.0 ± 0.4 μmol/g versus Tg 5.4 ± 0.3 μmol/g, $n = 5$, $P < 0.05$; Fig. 1*B* and *C*). This suggested that the transgene was increasing ghrelin expression and that transgene-derived ghrelin was subsequently octanoylated. The increased levels of stomach octanoylated ghrelin were reflected by similar increases in fasting plasma total ghrelin (Wt 907 ± 46 pmol/l versus Tg 1,130 ± 40 pmol/l, $n = 6$, $P < 0.01$; Fig. 1*D*) and octanoylated ghrelin in both males (Wt 25.39 ± 2.38 pmol/l versus Tg 40.07 ± 5.70 pmol/l, $n = 12$, $P < 0.05$; Fig. 1*E*) and females (Wt 20.38 ± 2.21 pmol/l versus Tg 33.38 ± 5.20 pmol/l, $n = 12$, $P < 0.05$, Fig. 1*F*). This suggests that the increased

bioactive ghrelin in the stomach was released into the circulation. To determine if the transgene expression matched the endogenous expression pattern for ghrelin, we measured ghrelin mRNA levels in various tissues. Ghrelin mRNA was found exclusively in the hypothalamus and stomach of both Wt and Tg mice (Fig. 1*G*). The increased stomach ghrelin expression was associated with a nonsignificant increase in stomach GOAT expression (Wt 1.16 ± 0.26 versus Tg 1.42 ± 0.7, $n = 6$). Two additional independent lines were produced that had smaller increases in circulating ghrelin levels and exhibited a phenotype similar to that of L91 (data not shown).

Phenotypic characterization of ghrelin overexpressing mice. Overexpression of ghrelin does not affect the growth hormone axis but inhibits the hypothalmic-adrenal-pituitary (HPA) axis. Administration of a single dose of ghrelin is known to stimulate growth hormone release (18). To study the possible effects of ghrelin overexpression on the growth hormone axis, we measured IGF-1 as a surrogate marker for growth hormone and longitudinal growth. At 16 weeks of age, there were no differences in circulating IGF-1 levels between Tg and Wt littermates (Wt 774 ± 49 ng/ml versus Tg 799 ± 80 ng/ml). There was also no difference in nose-to-anus length between genotypes (Wt 7.52 ± 0.09 cm versus Tg 7.57 ± 0.10 cm, $n = 10$). These data suggest that overexpression of bioactive ghrelin did not affect the growth hormone axis. Ghrelin is known to acutely affect the HPA axis; central administration of ghrelin increases corticosterone release, whereas intravenous administration inhibits corticosterone release in rats with high basal corticosterone levels (19,20). We observed a significant reduction in corticosterone levels in Tg mice (Wt 77.06 ± 8.05 ng/ml versus Tg 47.3 ± 6.34 ng/ml, $n = 12$–15, $P < 0.05$).

Bioactive ghrelin overexpression increases both food intake and energy expenditure. Chronic pharmacological administration of ghrelin significantly increases food intake and body weight. However, previous studies of genetic overexpression of ghrelin have not produced the expected hyperphagic and obese phenotype. To determine the effects of overexpression of bioactive ghrelin on energy homeostasis, we monitored food intake and body weight of mice fed regular chow. Tg mice exhibited hyperphagia compared with Wt controls. Daily food intake was increased in both male and female mice from 6 weeks of age (males: Wt 98.8 ± 2.2 kj/day versus Tg 107.0 ± 3.3 kj/day, $n = 6$, $P < 0.05$; females: Wt 51.25 ± 1.25 kj versus Tg 56.13 ± 1.9 kj, $n = 6$, $P < 0.05$) until the end of the study at 16 weeks of age (males: Wt 99.5 ± 1.2 kj/day versus Tg 114.3 ± 5.0 kj/day, $n = 6$, $P < 0.05$; females: 54.25 ± 1.75 kj/day versus 61.63 ± 2.13 kj/day, $n = 6$, $P < 0.05$; Fig. 2*A*). In accordance with increased daily food intake, cumulative food intake was also increased by 13% between 5 and 16 weeks of age in Tg mice (males: Wt 7,440 ± 68 kj versus Tg 8,480 ± 379 kj; females: Wt 4,087 ± 115 kj versus Tg 4,544 ± 148 kj, $n = 6$, $P < 0.05$; Fig. 2*C*). This increase in food intake would be expected to significantly increase both weight gain and adiposity. However, the increased energy intake did not result in increased weight gain in either male or female Tg mice (Fig. 2*B* and *D*). There was also no change in adiposity or lean mass between the two groups (Fig. 2*E*). Consistent with this finding, circulating leptin levels were unaltered between Tg and Wt mice (Wt 2.08 ± 0.68 ng/ml versus Tg 2.66 ± 0.63 ng/ml; Fig. 2*F*). The hyperphagia without increased body weight was indicative of increased energy expenditure. To determine the mechanism by which Tg mice remained lean in the face of increased food intake, we measured UCP-1 expression levels in brown adipose tissue as a surrogate marker of energy expenditure. UCP-1 expression levels were found to be significantly increased in Tg mice compared with Wt controls (Wt 4.42 ± 0.45 AU versus Tg 5.62 ± 0.5 AU; Fig. 3*A*), suggesting that energy expenditure was increased in Tg mice. This conclusion was supported by indirect calorimetry, which indicated that oxygen consumption was increased during both the light and dark cycles (Fig. 3*B*). Energy expenditure was found to be increased by 15% in Tg mice compared with Wt controls (hourly average V_{O_2} wt 5,458 ± 114 ml/h/kg versus Tg 6,207 ± 367 ml/h/kg, $n = 6$–8, females, $P < 0.01$; Fig. 3*C*). In addition, we measured locomotor activity and found no differences in either dark or light cycle locomotor activity between genotypes (Fig. 3*D*). This suggested that the normal body weight observed in the Tg mice was a consequence of increased basal metabolic rate rather than an increase in locomotor activity. Exogenous ghrelin administration has been shown to decrease fat use, whereas mice with targeted deletion of ghrelin have increased fat use when fed a high-fat diet. Our ghrelin overexpressing mice did not show an altered respiratory exchange ratio suggesting chronic exposure to ghrelin does not alter nutrient partitioning in our model (Fig. 3*E*).

Bioactive ghrelin overexpression decreases glucose-stimulated insulin release. Pharmacological administration of ghrelin inhibits glucose stimulated insulin release, whereas both knockout and overexpression models of the ghrelin system exhibit changes in glucose homeostasis (15,21,22). Our Tg mice had similar fasting plasma glucose concentrations as Wt littermates (Wt 5.1 ± 0.4 mmol/l versus Tg 5.5 ± 0.4 mmol/l, mean ± SEM, $n = 6$). Despite similar plasma glucose levels, Tg mice had elevated fasting plasma insulin levels, although this was not statistically significant (Wt 0.37 ± 0.1 ng/ml, $n = 9$; Tg 0.61 ± 0.17 ng/ml, $n = 5$; $P = 0.2$). We carried out IP-GTT to further explore the effects of ghrelin on glucose homeostasis. Tg mice were glucose intolerant with significantly increased plasma glucose concentrations at 30 and 60 min (Fig. 4*A*) after glucose (2 g/kg) injection compared with Wt controls (area under the curve [AUC]: Wt 1,253 ± 58 versus 1,769 ± 158 Tg, $n = 6$, $P < 0.01$; Fig. 4*B*). This was indicative of either increased insulin resistance or impaired glucose-stimulated insulin release. To determine the mechanism, we measured glucose-stimulated insulin release and performed insulin tolerance tests. After insulin administration, there were no differences in plasma glucose concentrations between genotypes (AUC: Wt 5,301 ± 388 versus Tg 5,740 ± 438, $n = 6$–10; Fig. 4*C* and *D*). Glucose-stimulated insulin release was significantly inhibited in Tg mice during an IP-GTT (glucose and insulin percent baseline, Fig. 4*E* and *F;* insulin AUC: Wt 25116 ± 6,182 versus Tg 11202 ± 2,136; Fig. 4*G*). These results suggest that ghrelin inhibits glucose-stimulated insulin release but has no effect on insulin sensitivity.

Bioactive ghrelin overexpressing mice are equally sensitive to exogenous ghrelin but have reduced leptin sensitivity. To determine if overexpression of ghrelin attenuated the response to exogenously administered ghrelin, we measured 1-h food intake after intraperitoneal injection of ghrelin (0.3 nmol/g). Ghrelin was equally potent at increasing 1-h food intake in both genotypes (Wt 0.02 ± 0.01 g versus 0.11 ± 0.03 g saline versus ghrelin, mean ± SEM, $n = 6$, $P < 0.01$; Tg 0.03 ± 0.01 g

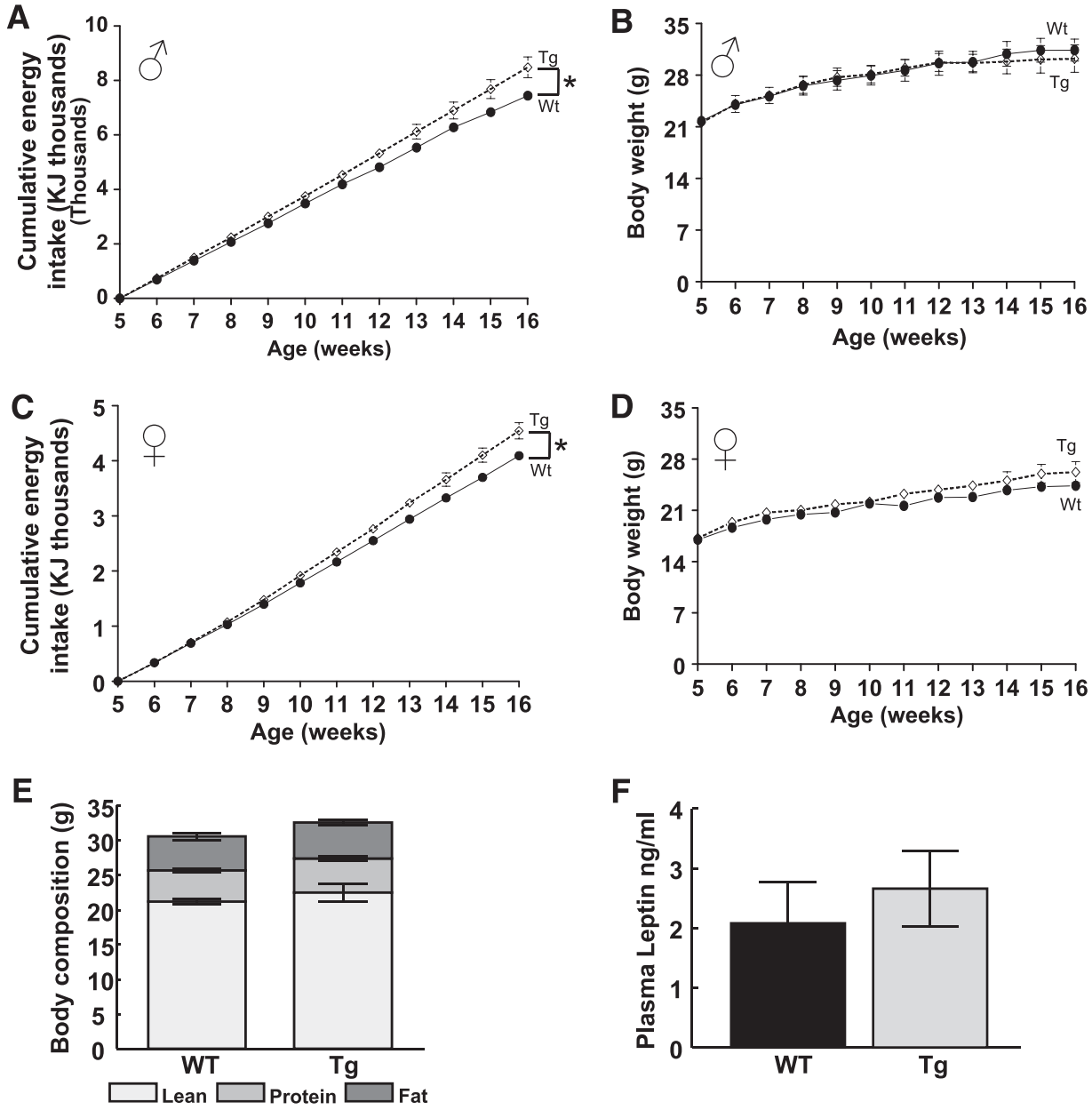

FIG. 2. Overexpression of bioactive ghrelin increases food intake. Cumulative food intake was measured from 5 to 16 weeks of age in both male (*A*) and female (*C*) mice fed on regular chow. Body growth curves from 5 to 16 weeks of age for male (*B*) and female (*D*) mice. Body composition (*E*; □, lean; ▨, protein; ■, fat) and plasma leptin concentrations (*F*) were measured in 16-week-old male mice. ● (solid lines), Wt; ◇ (broken lines), Tg. The results are means ± SEM; $n = 6–8$. *$P < 0.05$, **$P < 0.01$ Tg and Wt controls.

versus 0.12 ± 0.03 g saline versus ghrelin, mean ± SEM, $n = 6$, $P < 0.01$; Fig. 5*A*).

Within hypothalamic feeding centers, ghrelin and leptin have been shown to be functional antagonists (23). To determine if ghrelin overexpression altered leptin sensitivity, we measured the effect of peripherally administered leptin (3 μg/g) on food intake. Tg mice were less sensitive to the anorexigenic effect of leptin than Wt controls. Leptin significantly reduced food intake 0 to 1 h postadministration in Wt but not in Tg mice compared with saline (saline versus leptin: Wt 0.54 ± 0.12 g versus 0.22 ± 0.05 g, $P < 0.05$; Tg 0.69 ± 0.15 g versus 0.62 ± 0.12 g; P = nonsignificant, mean ± SEM, $n = 6$; Fig. 5*B*). Four hours postadministration, leptin significantly reduced food intake in both genotypes (saline versus leptin: Wt 1.08 ± 0.15 g versus 0.57 ± 0.12 g, Tg 1.33 ± 0.17 g versus 0.99 ± 0.12 g, mean ± SEM, $n = 6$, $P < 0.05$; Fig. 5*C*). The magnitude of the reduction in food intake, however, was less in Tg than Wt mice. Leptin reduced 4-h food intake by approximately half in Wt animals, but only by one-fourth in Tg animals. These results suggest that Tg mice are less sensitive to the effects of leptin but equally sensitive to ghrelin.

DISCUSSION

Bioactivity of ghrelin is conferred by octanoylation of its serine 3 residue. This reaction is catalyzed by the enzyme GOAT. Bioactive ghrelin is only produced in GOAT-expressing tissues. In the mouse, GOAT is expressed exclusively in the gastrointestinal tract (5,6). To increase bioactive ghrelin concentrations, the ghrelin transgene should ideally be expressed in tissues that produce endog-

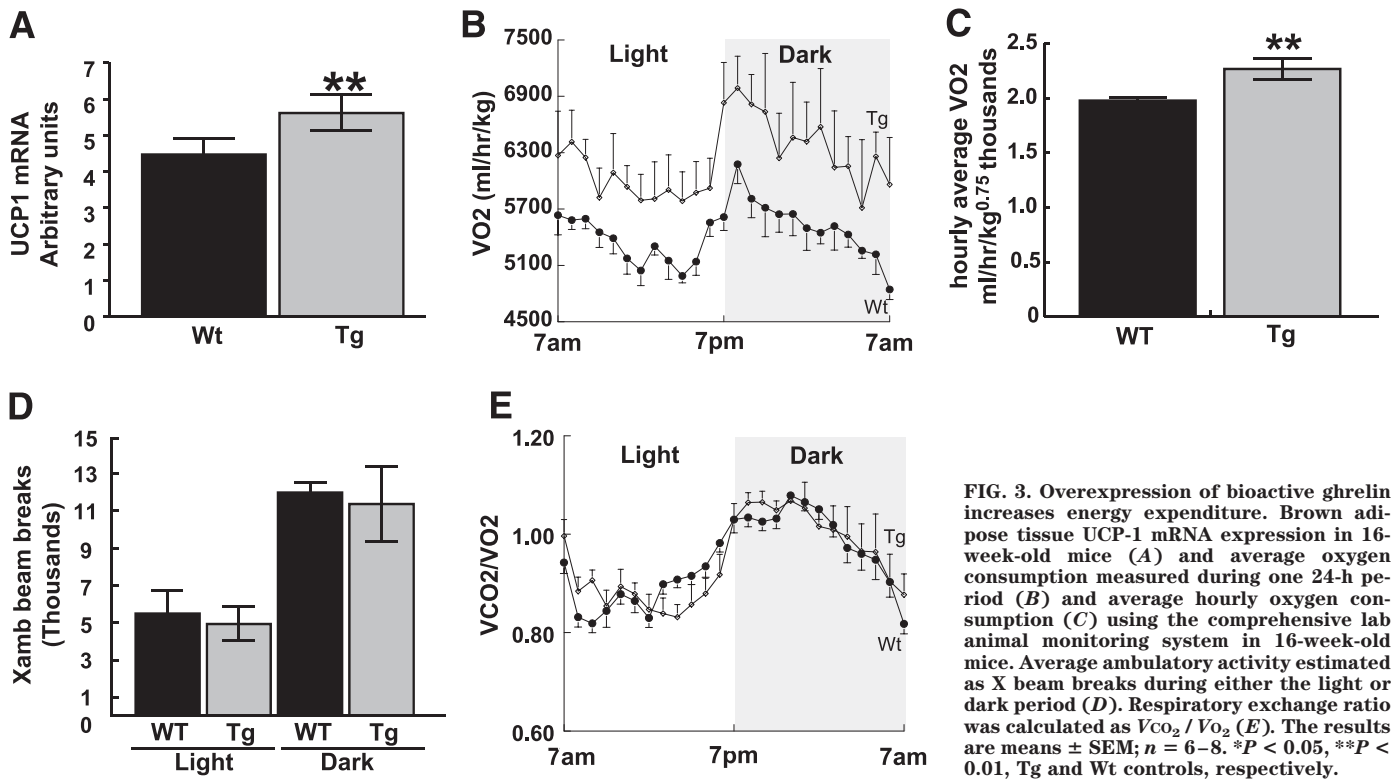

FIG. 3. Overexpression of bioactive ghrelin increases energy expenditure. Brown adipose tissue UCP-1 mRNA expression in 16-week-old mice (*A*) and average oxygen consumption measured during one 24-h period (*B*) and average hourly oxygen consumption (*C*) using the comprehensive lab animal monitoring system in 16-week-old mice. Average ambulatory activity estimated as X beam breaks during either the light or dark period (*D*). Respiratory exchange ratio was calculated as $V\mathrm{CO}_2 / V\mathrm{O}_2$ (*E*). The results are means ± SEM; $n = 6–8$. *$P < 0.05$, **$P < 0.01$, Tg and Wt controls, respectively.

enous ghrelin and GOAT. To do this, we chose to drive ghrelin transgene expression using its own promoter. The approach successfully increased both stomach and plasma concentrations of bioactive ghrelin. Our data constitute the first report of the targeted overexpression of bioactive ghrelin in its physiological sites of production: the stomach and hypothalamus.

Exogenously administered ghrelin has been shown to have powerful effects on food intake (2,24). Despite the overwhelming pharmacological evidence, data from Tg models have not supported the expected role for ghrelin in the control of appetite, leading to the suggestion that ghrelin is not a critical regulator of appetite. On the contrary, our data suggest it is an important regulator of food intake. Overexpression of bioactive ghrelin in our model causes hyperphagia, increased energy expenditure, and glucose intolerance. The increased food intake suggests ghrelin may physiologically regulate appetite under normal feeding conditions.

The hyperphagia observed in our Tg mice was in contrast to the phenotype described for mice with nonspecific neuronal overexpression of ghrelin (15). These mice are reported to exhibit an increase in plasma octanoylated ghrelin without an increase in feeding behavior. A likely explanation for this difference is the nonspecific nature of the central nervous system overexpression. Results from this model are likely to be pharmacological because physiologically only a few neurones in the hypothalamus produce ghrelin. The hyperphagic phenotype is also in contrast to mice with targeted deletion of the ghrelin gene ($ghr^{-/-}$) or its receptor. These mice have normal growth rates and appetites when fed on regular chow (7). A possible explanation for this is that knockout mice are susceptible to developmental compensation, which can mask the true role of the gene in question.

To fully interpret Tg studies, it is important to consider the genetic background of the model. This is particularly true for mice with targeted deletion of genes in which it has been suggested that c57blk/6j genetic traits cosegregate in the null mice, whereas 129Sv traits are more influential in the Wt controls (7). This can lead to misinterpretation of results because c57bl/6j mice are more obesity prone than 129Sv mice. In classical Tg mice, however, there is no evidence that the transgene cosegregates with a particular background and, therefore, the Tg and Wt littermates are likely to be of similar genetic background. Backcrossing our model to a pure C57bl/6j background may indeed increase the magnitude of the observed phenotype.

Our Tg mice were hyperphagic but did not have an increase in body weight, which suggested they had increased energy expenditure. Tg mice were found to have increased oxygen consumption and brown adipose tissue UCP-1 expression, both of which are surrogate markers of energy expenditure. This is in contrast to the pharmacological administration of ghrelin, which decreases energy expenditure (25). It is possible that the increased metabolic rate in Tg mice is the result of an indirect effect of ghrelin, which manifests itself after chronic exposure. Circulating corticosterone levels, for example, were suppressed in our Tg mice. Because corticosterone is known to suppress energy expenditure, the increased metabolic rate observed in our Tg mice may be attributed to an indirect effect of their attenuated corticosterone levels (26).

It is generally accepted that ghrelin activates the HPA axis, and this is thought to occur at the level of the hypothalamus (19). In contrast, corticosterone levels were reduced in our Tg mice. When given intravenously, however, growth hormone secretagogues (GHSs) reduce corticosterone in rats with high basal corticosterone levels (20). It has therefore been suggested that high circulating levels of glucocorticoids feed back to reduce the ACTH response to GHS. A similar mechanism could account for

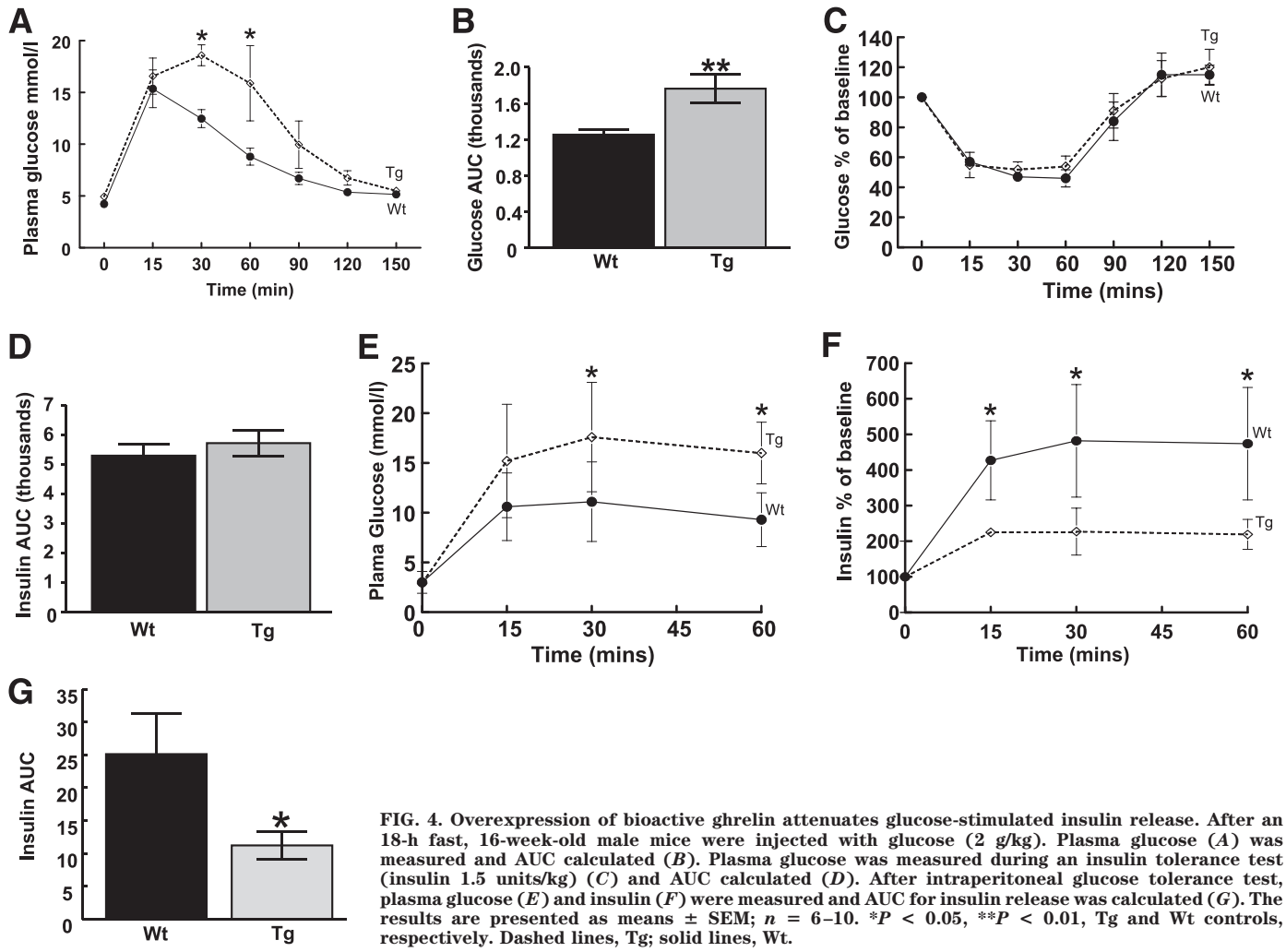

FIG. 4. Overexpression of bioactive ghrelin attenuates glucose-stimulated insulin release. After an 18-h fast, 16-week-old male mice were injected with glucose (2 g/kg). Plasma glucose (*A*) was measured and AUC calculated (*B*). Plasma glucose was measured during an insulin tolerance test (insulin 1.5 units/kg) (*C*) and AUC calculated (*D*). After intraperitoneal glucose tolerance test, plasma glucose (*E*) and insulin (*F*) were measured and AUC for insulin release was calculated (*G*). The results are presented as means ± SEM; $n = 6–10$. *$P < 0.05$, **$P < 0.01$, Tg and Wt controls, respectively. Dashed lines, Tg; solid lines, Wt.

the reduced corticosterone in our Tg mice. Chronic exposure to ghrelin may cause attenuation of the HPA axis. Pharmacologically, ghrelin is a powerful GHS acutely. We found the growth hormone axis of our Tg mice to be normal; IGF-1 levels and linear growth were unaltered between genotype. A potential explanation for this is that the growth hormone axis becomes less sensitive to ghrelin after chronic exposure. In agreement with this, others have found that chronic ghrelin or GHS treatment does not affect circulating levels of growth hormone, IGF-1, or linear growth in rodents (27,28). In addition, the finding that targeted deletion of the growth GHS receptor or ghrelin also failed to produce any alteration in the growth hormone axis (7,8).

Ghrelin has been shown to alter glucose homeostasis in humans and rats (21,29). It powerfully inhibits glucose-stimulated insulin release (30). In support of this, ghrelin deletion has been shown to improve glucose tolerance during an IP-GTT by amplifying glucose-stimulated insulin release (22). Mice with ghrelin neuronal overexpression

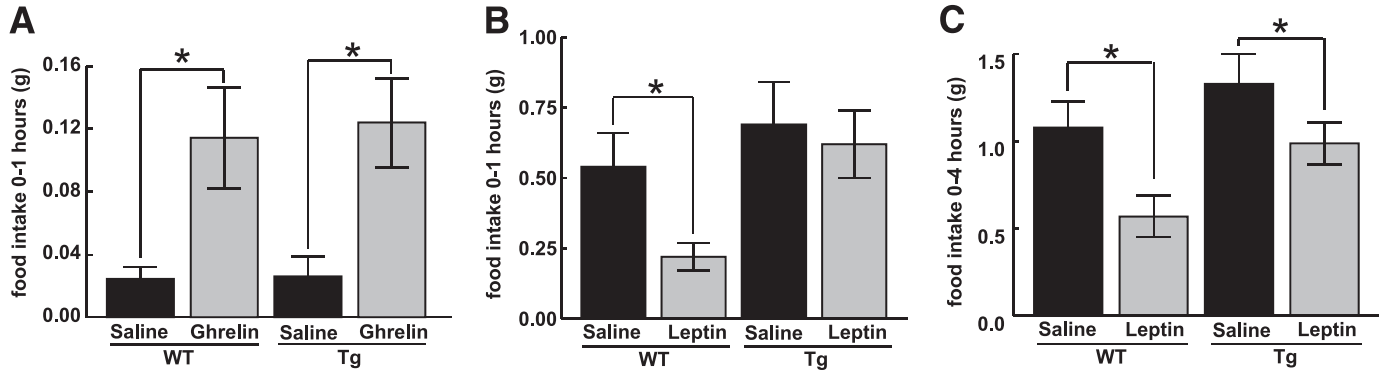

FIG. 5. Ghrelin overexpressing Tg mice are sensitive to ghrelin but have reduced leptin sensitivity. After intraperitoneal injection of ghrelin (0.3 nmol/g) or saline to fed 16-week-old male Tg and Wt mice, 0- to 1-h food intake was measured (*A*). After intraperitoneal administration of leptin (3 μg/g) or saline to fasted 16-week-old male Tg and Wt mice, 0- to 1-h food intake was measured (*B*) as well as 0- to 4-h food intake (*C*). The results are means ± SEM; $n = 9–10$. *$P < 0.05$ saline versus ghrelin or leptin treatment.

develop age-related glucose intolerance (15). Our Tg mice were glucose intolerant as a result of an inhibition of glucose-stimulated insulin release. Our data suggested that ghrelin overexpression did not alter insulin sensitivity. This was tested using a relatively high dose of insulin, however. It is therefore possible that there are subtle effects on insulin sensitivity, which would not be detectable at the doses of insulin used. Lower doses of insulin or the use of hyperinsulinemic/euglycemic clamp studies would provide firmer evidence of the effect of ghrelin overexpression on insulin sensitivity. These results suggest that ghrelin has an important role in regulating β-cell function and glucose homeostasis. Indeed, the weight of evidence supporting the role of ghrelin in the regulation of β-cell function could indicate a more physiologically important function in the control of glucose homeostasis than appetite regulation.

Ghrelin and leptin are known to have opposing effects on the orexigenic neurones expressing neuropeptide Y and agouti-related protein within the arcuate nucleus of the hypothalamus (31,32). It has been suggested that ghrelin and leptin may act as functional antagonists at this neuronal population to control energy homeostasis. Consistent with this hypothesis, Tg mice were as sensitive to exogenous ghrelin as Wt mice but less sensitive to the anorexigenic effects of leptin.

From our data, we conclude that ghrelin has important physiological roles in the control of energy homeostasis. Chronic overexpression of bioactive ghrelin increases food intake but does not alter long-term body weight gain because of a paradoxical increase in energy expenditure. We also found that ghrelin plays an important role in β-cell function by inhibiting glucose-stimulated insulin release. Given the genetic and pharmacological data in support of this finding, ghrelin's actions on glucose homeostasis could be viewed as more important than its effects on appetite. These data suggest that strategies designed to antagonize ghrelin function may reduce appetite and improve glucose homeostasis.

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No potential conflicts of interest relevant to this article were reported.

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