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Table 1. Metabolic parameters in CD and DIO mice.

	CD	DIO
Body weight (g)	34.2 ± 0.8	54.1 ± 1.0 **
Glucose (mg/dl)	117 ± 7	190 ± 7 **
Insulin (μ U/ml)	18.9 ± 3.2	126.0 ± 28.7 **
Leptin (ng/ml)	2.2 ± 0.6	42.1 ± 4.5 **

Results were presented by mean ± SEM (n = 14). Significantly different from CD mice in each group, ** $p < 0.01$.

Figure legends

Fig. 1. Fear-conditioning responses in CD and DIO mice.

Fear-conditioning responses in CD (closed circles) and DIO (open circles) mice. Freezing percentages of CD and DIO mice in the contextual conditioning test were measured every minute for 5 minutes. Freezing percentages of CD and DIO mice in the cued conditioning test were measured every minute for 3 minutes. Data points represented mean ± SEM (n = 9 - 14). Significantly different from CD mice, * $p < 0.05$, ** $p < 0.01$.

Fig. 2. Contents of (A) BDNF and (B) NT-3 in the cerebral cortex, hippocampus, amygdala and hypothalamus in CD and DIO mice. Results are presented as mean ± SEM (n = 18 - 29). Significantly different from CD mice, * $p < 0.05$, ** $p < 0.01$.

Fig. 3. Expressions of full-length TrkB (A) and TrkC (B) in the cerebral cortex, hippocampus, amygdala and hypothalamus in CD and DIO mice. Results are presented as mean ± SEM (n = 3 - 7). Significantly different from CD mice, * $p < 0.05$.

Fig. 1.

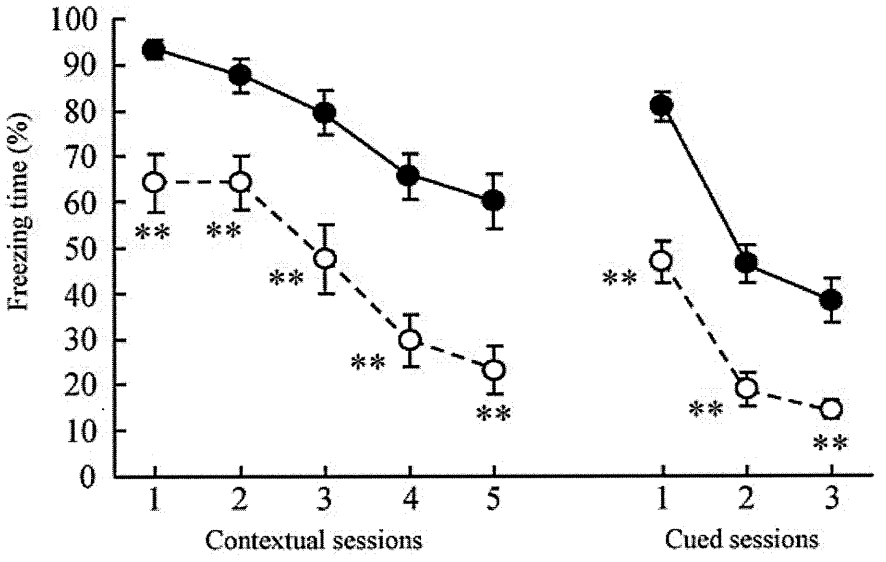
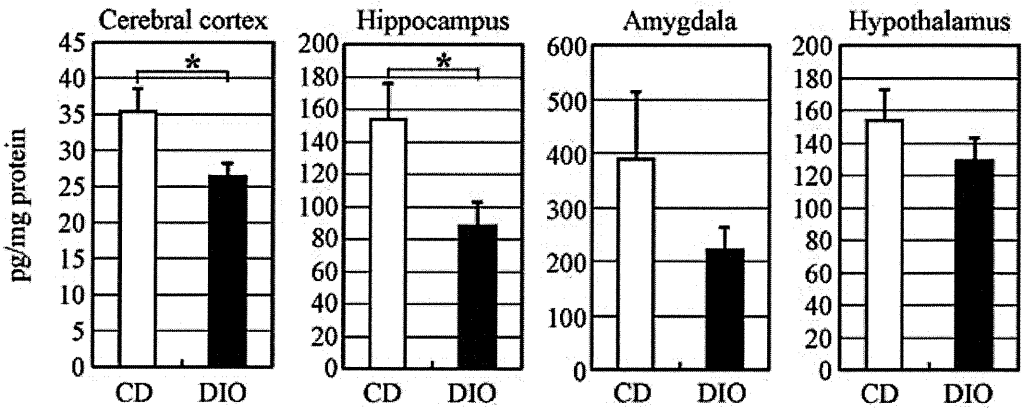


Fig. 2.

(A) BDNF



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(B) NT-3

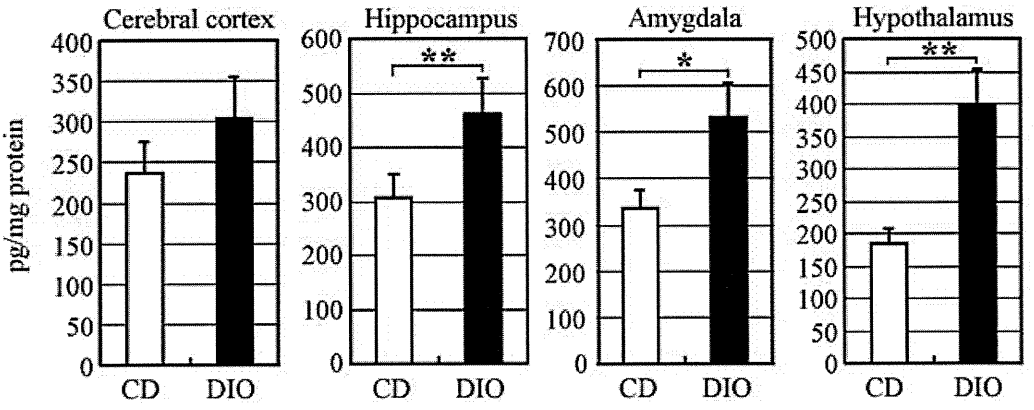

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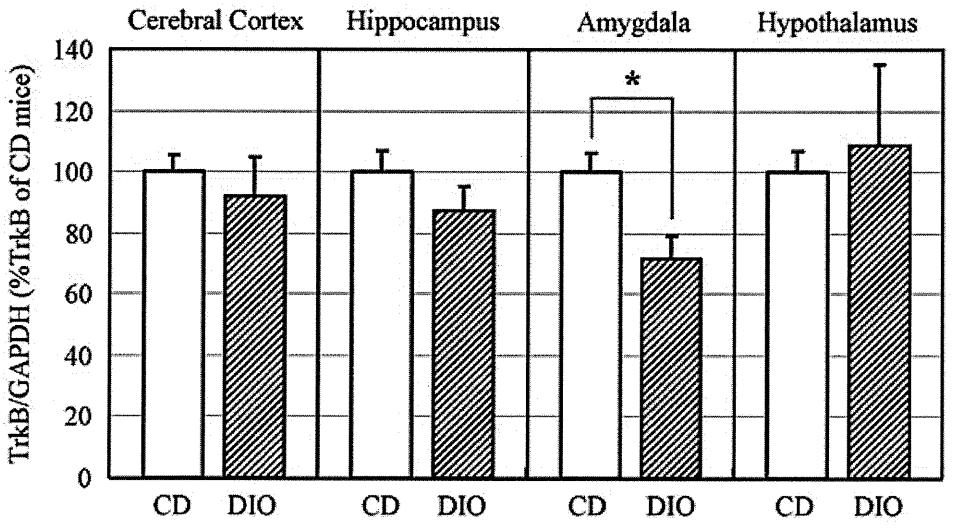
Fig. 3.

(A) Full-length TrkB



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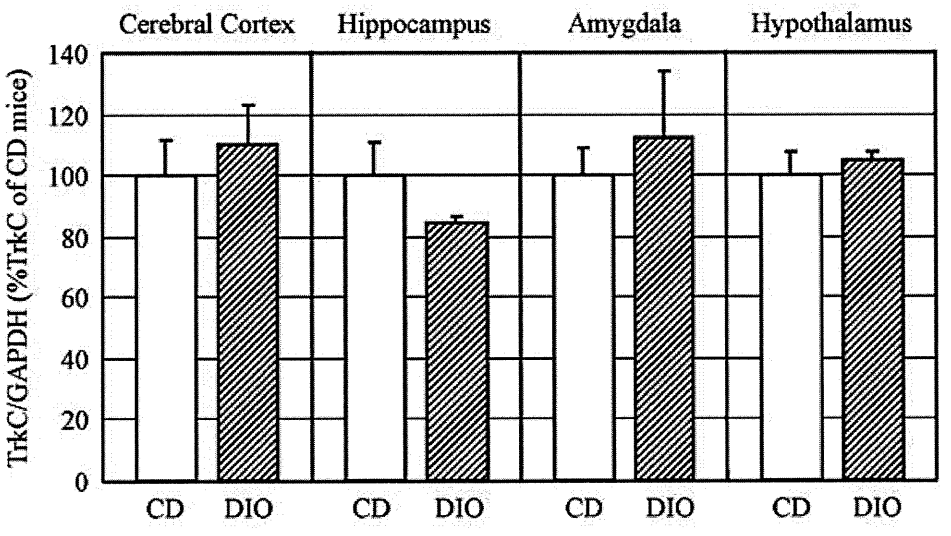
(B) Full-length TrkC



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Amylin improves the effect of leptin on insulin sensitivity in leptin-resistant diet-induced obese mice

Toru Kusakabe,[1] Ken Ebihara,[1,2] Takeru Sakai,[1] Licht Miyamoto,[1] Daisuke Aotani,[1] Yuji Yamamoto,[1] Sachiko Yamamoto-Kataoka,[1] Megumi Aizawa-Abe,[2] Junji Fujikura,[1] Kiminori Hosoda,[1,2] and Kazuwa Nakao[1]

[1]*Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine; and* [2]*Translational Research Center, Kyoto University Hospital, Kyoto, Japan*

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Kusakabe T, Ebihara K, Sakai T, Miyamoto L, Aotani D, Yamamoto Y, Yamamoto-Kataoka S, Aizawa-Abe M, Fujikura J, Hosoda K, Nakao K. Amylin improves the effect of leptin on insulin sensitivity in leptin-resistant diet-induced obese mice. *Am J Physiol Endocrinol Metab* 302: E924–E931, 2012. First published January 24, 2012; doi:10.1152/ajpendo.00198.2011.—Leptin enhances insulin sensitivity in addition to reducing food intake and body weight. Recently, amylin, a pancreatic β-cell-derived hormone, was shown to restore a weight-reducing effect of leptin in leptin-resistant diet-induced obesity. However, whether amylin improves the effect of leptin on insulin sensitivity in diet-induced obesity is unclear. Diet-induced obese (DIO) mice were infused with either saline (S), leptin (L; 500 $\mu g \cdot kg^{-1} \cdot day^{-1}$), amylin (A; 100 $\mu g \cdot kg^{-1} \cdot day^{-1}$), or leptin plus amylin (L/A) for 14 days using osmotic minipumps. Food intake, body weight, metabolic parameters, tissue triglyceride content, and AMP-activated protein kinase (AMPK) activity were examined. Pair-feeding and weight-matched calorie restriction experiments were performed to assess the influence of food intake and body weight reduction. Continuous L/A coadministration significantly reduced food intake, increased energy expenditure, and reduced body weight, whereas administration of L or A alone had no effects. L/A coadministration did not affect blood glucose levels during ad libitum feeding but decreased plasma insulin levels significantly (by 48%), suggesting the enhancement of insulin sensitivity. Insulin tolerance test actually showed the increased effect of insulin in L/A-treated mice. In addition, L/A coadministration significantly decreased tissue triglyceride content and increased AMPKα2 activity in skeletal muscle (by 67%). L/A coadministration enhanced insulin sensitivity more than pair-feeding and weight-matched calorie restriction. In conclusion, this study demonstrates the beneficial effect of L/A coadministration on glucose and lipid metabolism in DIO mice, indicating the possible clinical usefulness of L/A coadministration as a new antidiabetic treatment in obesity-associated diabetes.

obesity; diabetes; adenosine 5′-monophosphate-activated protein kinase

LEPTIN, AN ADIPOCYTE-DERIVED HORMONE, has a weight-reducing effect accompanied by reduction in food intake and increase in energy expenditure (11, 13). In general, in rodent models of diet-induced obesity and obese human, although leptin levels rise proportionally with adiposity (16, 23), the increased leptin fails to suppress the progression of obesity. Moreover, even high pharmacological doses of leptin have demonstrated only marginal, if any, effects on body weight in diet-induced obese (DIO) rodents and obese humans (8, 15). This leptin ineffectiveness is called leptin resistance.

Recently, it was shown that amylin, a pancreatic β-cell-derived hormone (4), restored a weight-reducing effect of leptin and that leptin/amylin coadministration effectively reduced body weight in DIO rats (34). Moreover, in overweight/obese humans, coadministration of the amylin analog pramlintide and the leptin analog metreleptin induced significantly greater weight loss than either pramlintide or metreleptin alone (32, 34).

Besides the weight-reducing effect, leptin has a wide range of effects, including an antidiabetic effect. We previously generated transgenic skinny mice (LepTg) overexpressing leptin under the control of the liver-specific human serum amyloid P component promoter, whose plasma leptin levels are elevated compared with those of obese human individuals (30). LepTg mice showed increased glucose metabolism. In LepTg mice, we have demonstrated that leptin increases insulin sensitivity with augmentation of liver and skeletal muscle insulin receptor signaling (30). In addition, LepTg mice had reduced tissue triglyceride contents along with increased energy expenditure through activation of AMP-activated protein kinase (AMPK) (37, 38), a key enzyme that mediates the effect of leptin on fatty acid β-oxidation in skeletal muscle (24).

Given the antidiabetic effect of leptin, we have demonstrated that leptin could be an antidiabetic drug for various types of diabetes, such as lipoatrophic, insulin-deficient, and type 2 diabetes, using animal models (7, 18, 25, 28, 29). In addition, we and others confirmed that leptin treatment effectively reduces food intake and improves insulin sensitivity, hyperglycemia, hypertriglyceridemia, and fatty liver in patients with lipoatrophic diabetes (2, 5, 6, 31). However, in DIO rodents and obese humans, the effect of leptin on insulin sensitivity is also attenuated because of leptin resistance (18).

Evidence indicating that leptin can stimulate insulin sensitivity independently of food intake and body weight reduction via central mechanisms has accumulated (9, 14, 17, 27). Amylin also activates multiple central nervous system regions to regulate both energy and glucose homeostasis (19, 21, 22). Therefore, it is possible that leptin and amylin interact with each other in the regulation of glucose metabolism. However, whether amylin improves the effect of leptin on insulin sensitivity in leptin-resistant obese subjects is unclear.

In this study, we demonstrated that leptin/amylin coadministration, unlike administration of leptin or amylin alone, enhances insulin sensitivity in leptin-resistant DIO mice in addition to reducing body weight accompanied by reduction in food intake and increase in energy expenditure, indicating the pos-

Address for reprint requests and other correspondence: K. Ebihara, Dept. of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan (e-mail: kebihara@kuhp.kyoto-u.ac.jp).

sible clinical usefulness of leptin/amylin coadministration as a new antidiabetic treatment in obesity-associated diabetes.

MATERIALS AND METHODS

Experimental animals. Eight-week-old male C57BL/6J mice were purchased from Japan SLC, Shizuoka, Japan. The mice were caged individually and kept under a 12:12-h light-dark cycle (lights on at 0900). The mice were fed a high-fat diet (D12451, 45% of energy as fat; Research Diets, New Brunswick, NJ) for 5 wk, with free access to water (termed DIO mice), before experiments. Body weight of DIO mice before experiments was significantly heavier than that of control mice fed a standard diet (NMF, 13% of energy as fat; Oriental Yeast, Tokyo, Japan) (32.6 ± 0.5 vs. 26.9 ± 0.4 g, $P < 0.01$). Metabolic characteristics of control and DIO mice are summarized in Table 1. The result of an insulin tolerance test (ITT) showed that DIO mice were insulin resistant compared with control mice. Animal care and all experiments were conducted in accordance with the Guidelines for Animal Experiments of Kyoto University and were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

Leptin and/or amylin infusion experiments. DIO mice were divided into four treatment groups [saline (S), leptin (L), amylin (A), and leptin plus amylin (L/A)] to be counterbalanced for starting body weight and blood glucose level. On *day 0*, all mice were implanted subcutaneously in the midscapular region with two osmotic minipumps (Alzet model 2002; Alza, Palo Alto, CA) containing either saline, leptin (500 $\mu g \cdot kg^{-1} \cdot day^{-1}$; Amgen, Thousand Oaks, CA), or amylin (100 $\mu g \cdot kg^{-1} \cdot day^{-1}$; Bachem, Torrance, CA). High-fat diet feeding was continued during the experiment.

Body weight and food intake. Body weight was measured on *days 0*, *5*, and *10*. Daily food intake was measured before and during the leptin and/or amylin infusion experiment.

Indirect calorimetry. Measurement of oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) was performed over a period of 48 h, after >72 h of acclimation, using an Oxymax indirect calorimeter (Columbus Instruments, Columbus, OH) on *days 4* and *5* (n = 4/group) for S, L, A, and L/A-treated mice. Respiratory exchange ratio [ratio of CO_2 production to O_2 ($\dot{V}CO_2/O_2$)], which indicates the relative contribution of fat and carbohydrate oxidation to overall metabolism, was calculated and averaged across the 48-h measurement session.

Metabolic variables. Blood was obtained from nonfasted mice between 1500 and 1700 at the end of the experiment. Blood glucose levels were measured by the glucose oxidase method using a reflectance glucometer (MS-GR102; Terumo, Tokyo, Japan). Plasma insulin levels were measured by enzyme immunoassay with an Insulin-EIA kit (Morinaga, Tokyo, Japan). Plasma glucagon levels were measured by enzyme immunoassay with a Glucagon-EIA kit (Yanaihara, Shizuoka, Japan). Plasma leptin levels were measured by an ELISA kit for mouse leptin (Millipore, Billerica, MA). Plasma amylin levels were measured by enzyme immunoassay using a mouse Amylin-EIA kit (Phoenix Pharmaceuticals, Burlingame, CA).

ITT. An ITT was performed on *day 10*. For the ITT, after a 4-h fast, mice were injected with 0.8 mU/g ip human regular insulin (Humulin R; Eli Lilly Japan, Kobe, Japan). Blood was sampled from the tail vein before and 30, 60, and 120 min after the insulin injection. Blood glucose levels were determined as described above. The area under the curve (AUC) during the ITT was calculated in each mouse.

Liver weight and tissue triglyceride content. Liver weight was measured at the end of the experiment. Liver and skeletal muscle triglyceride content were measured as described previously (18). Liver and gastrocnemius muscle were isolated at the end of the experiment and immediately frozen in liquid nitrogen, and lipids were extracted with isopropyl alcohol-heptane (1:1, vol/vol). After the solvent was evaporated, the lipids were resuspended in 99.5% (vol/vol) ethanol, and the triglyceride content was measured using the Triglyceride E-test Wako kit (Wako Pure Chemicals, Osaka, Japan).

Isoform-specific AMPK activity. AMPK activity was determined as described previously (18). Soleus muscles were isolated at the end of the experiment and immediately frozen in liquid nitrogen. To measure isoform-specific AMPKα1 and -α2 activity in soleus muscle, AMPK was immunoprecipitated from muscle lysates (200 μg of protein) with specific antibodies against the α_1- and α_2-subunits (Upstate Cell Signaling Solutions, Lake Placid, NY) bound to Protein A-Sepharose beads, and the kinase activity of the immunoprecipitates was measured using "SAMS" peptide and [γ-^{32}P]ATP.

Pair-feeding and weight-matched calorie restriction experiments. Pair-feeding experiments were performed to assess the influence of food intake reduction. In this experiment, DIO mice (mean body weight 31.2 ± 0.4 g) were divided into three treatment groups [S, saline + pair-fed L/A-treated mice (PF), and L/A] to be counterbalanced for starting body weight and blood glucose level. Saline, leptin, and amylin were infused using two osmotic minipumps, as described above. Pair-fed mice were fed the same amount of food consumed by L/A-treated mice on the previous day at the end of light phase once for 14 days. Body weight was measured on *days 0* and *10*. Weight-matched calorie restriction experiments were performed to assess the influence of body weight reduction. In this experiment, the food consumption of DIO mice (mean body weight 31.7 ± 0.5 g) was restricted to match their body weight to those of L/A-treated mice (weight-matched DIO mice, termed CR mice). CR mice were fed the ~70% amount of food consumed by S-treated mice on the previous day at the end of light phase at once for 14 days. An ITT was performed on *day 10* of these experiments. Liver and gastrocnemius muscle were obtained for triglyceride content measurements at the end of these experiments.

Statistical analyses. Data are expressed as means ± SE. Comparison between or among groups was by Student's t-test or ANOVA with Fisher's protected least significant difference test. $P < 0.05$ was considered statistically significant.

Table 1. *Metabolic characteristics of control and DIO mice*

Variable	Control (n = 6)	DIO (n = 9)
Blood glucose, mg/dl	142.4 ± 5.4	160.4 ± 6.6
Plasma insulin, pg/ml	466.9 ± 99.1	535.0 ± 87.6
AUC in ITT, %/min × 100	77.3 ± 10.5	102.5 ± 5.5*
Liver TG content, mg/g tissue	9.8 ± 0.8	23.6 ± 2.4**
Skeletal muscle TG content, mg/g tissue	5.2 ± 0.7	5.6 ± 1.1

Values are means ± SE. DIO, diet-induced obese; AUC, area under the curve; ITT, insulin tolerance test; TG, triglyceride. Blood glucose, plasma insulin, liver TG content, and skeletal muscle TG content were measured in saline-treated control and DIO mice at the end of the experiment. Blood samples were obtained during ad libitum feeding. AUC in ITT was measured on *day 10*. *$P < 0.05$ and **$P < 0.01$ vs. control mice.

RESULTS

Effect of leptin and/or amylin on food intake, body weight, and energy expenditure in DIO mice. Leptin and amylin were administered for 14 days in DIO mice, using osmotic minipumps. Plasma leptin and amylin levels at the end of the experiment were shown in Table 2. Administration of leptin (500 $\mu g \cdot g^{-1} \cdot day^{-1}$) was adequately effective in control mice fed a standard diet, as shown in our previous report (18), but it had no significant effect on food intake or body weight in DIO mice (Fig. 1, *A* and *B*), indicating that these DIO mice were in the leptin-resistant state. Administration of amylin (100 $\mu g \cdot g^{-1} \cdot day^{-1}$) had no effect on food intake or body weight in mice fed a standard diet (data not shown) or DIO mice (Fig. 1, *A* and *B*). However, L/A coadminis-

Table 2. *Plasma leptin and amylin levels in mice administered leptin and/or amylin*

Variable, ng/ml	Mouse Group			
	S	L	A	L/A
L	28.5 ± 5.6	53.0 ± 5.3*	19.7 ± 4.8	45.1 ± 6.6*†
A	1.7 ± 0.1	1.8 ± 0.2	2.7 ± 0.2**	2.9 ± 0.2**,##

Values are means ± SE for 8–9 mice in each group. S, saline; L, leptin; A, amylin; L/A, leptin + amylin. Plasma L and A levels were measured at the end of the experiment. Blood samples were obtained during ad libitum feeding. *$P < 0.05$ and **$P < 0.01$ vs. S-treated mice; ##$P < 0.01$ vs. L-treated mice; †$P < 0.05$ vs. A-treated mice in L/A-treated mice.

tration significantly reduced cumulative food intake for 10 days by 15.3% in DIO mice compared with saline administration (Fig. 1*A*). Body weight was decreased by 9.2% for 10 days of L/A coadministration (Fig. 1*B*).

To assess the effect of leptin and/or amylin on energy expenditure, indirect calorimetry was performed. L/A coadministration significantly increased $\dot{V}O_2$, a marker of energy expenditure, in both the light and dark phases (Fig. 1*C*). In addition, L/A coadministration significantly decreased respiratory exchange ratio in the dark phase, indicating increased utilization of fat as the fuel source (Fig. 1*D*).

Effect of leptin and/or amylin on glucose metabolism in DIO mice. On *day 14*, there was no difference in blood glucose levels under ad libitum feeding among groups (Fig. 2*A*). On the other hand, L/A coadministration decreased plasma insulin levels significantly, whereas administration of L or A alone did not change plasma insulin levels, compared with saline administration (282.8 ± 69.6 vs. 535.0 ± 87.6 pg/ml, $P < 0.01$), indicating the improvement of insulin sensitivity in L/A-treated mice (Fig. 2*B*). Plasma glucagon levels of DIO mice were significantly higher than that of control mice (106.9 ± 26.0 vs. 45.0 ± 8.0 pg/ml, $P < 0.01$). L/A coadministration tended to suppress plasma glucagon levels, but not significantly (Fig. 2*C*).

To evaluate insulin sensitivity, we performed ITTs. The ITT actually showed greater decrease in glucose levels after insulin injection in L/A-treated mice than in L- or A-treated mice (Fig. 2*D*). Consistent with these findings, the glucose AUC after insulin injection was decreased only in L/A-treated mice (Fig. 2*E*).

Effect of leptin and/or amylin on liver weight, tissue triglyceride content, and AMPK activity in skeletal muscle in DIO mice. Because fat accumulation in insulin target tissues is considered to be one of the reasons for insulin resistance (36, 41), we examined liver and gastrocnemius muscle triglyceride

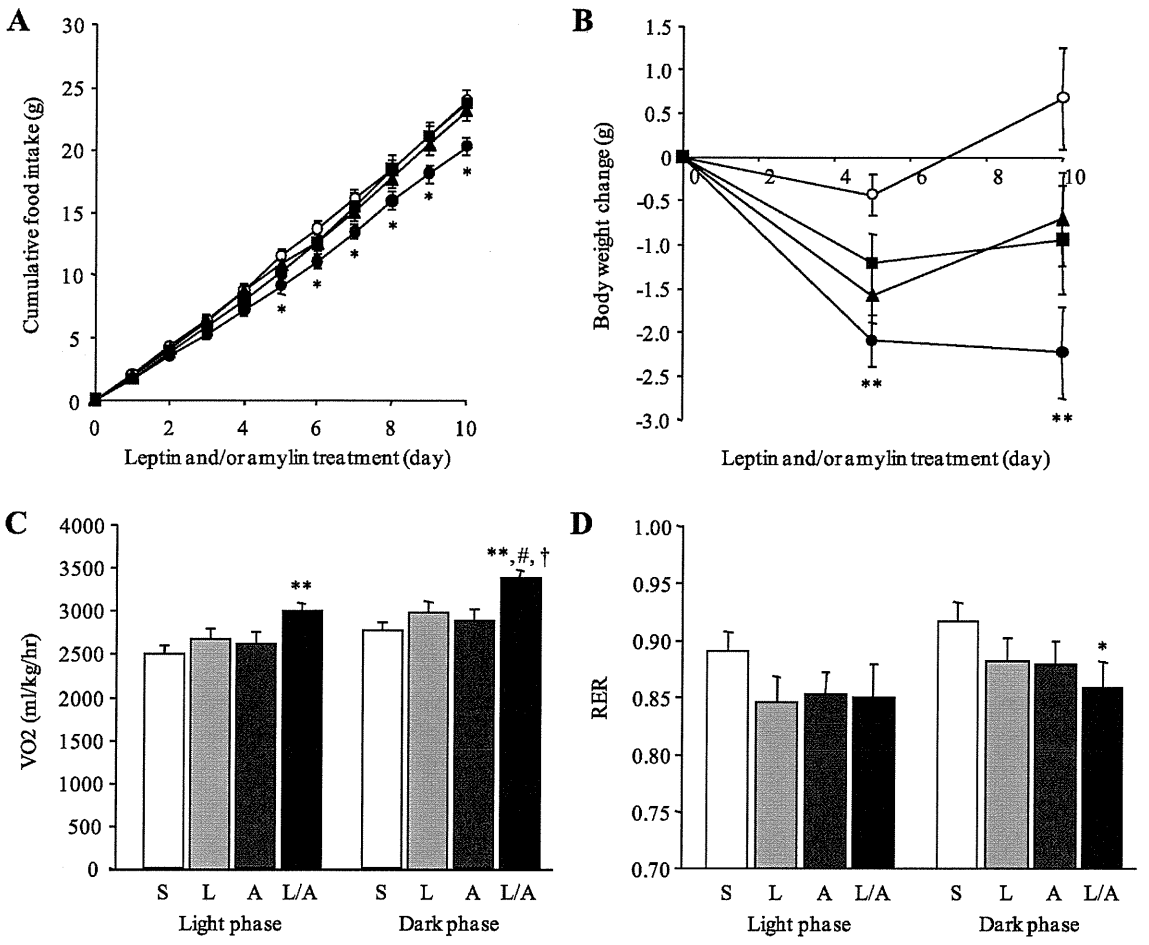

Fig. 1. Effect of leptin and/or amylin on food intake, body weight, energy expenditure, and respiratory exchange ratio (RER) in diet-induced obese (DIO) mice. Cumulative food intake (*A*) and change in body weight (*B*) during the treatment in saline- (S; ○), leptin- (L; ■), amylin- (A; ▲), and leptin + amylin (L/A)-treated mice (●). Values are means ± SE (n = 8–9/group). Oxygen consumption ($\dot{V}O_2$; *C*) and RER (*D*) during the treatment in S-, L-, A-, and L/A-treated mice. Values are means ± SE (n = 4/group). *$P < 0.05$ and **$P < 0.01$ vs. S-treated mice; #$P < 0.05$ vs. L-treated mice; †$P < 0.05$ vs. A-treated mice.

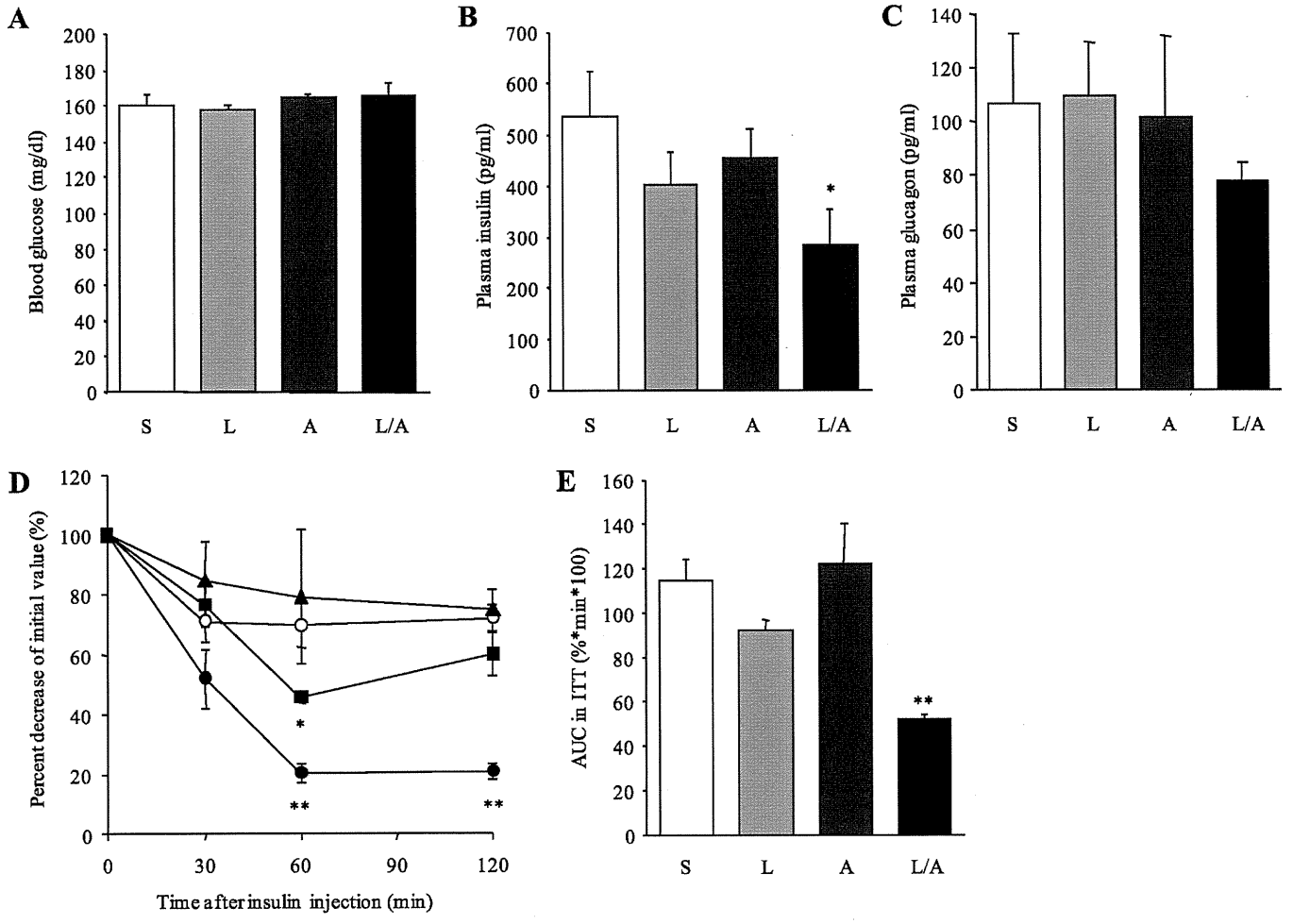

Fig. 2. Effect of L and/or A on glucose metabolism in DIO mice. Blood glucose (*A*), plasma insulin (*B*), and plasma glucagon levels (*C*) under ad libitum feeding on *day 14* in S, L, A, and L/A-treated mice. Values are means ± SE (n = 8–9/group). %Change of initial value of blood glucose levels (*D*) and area under the curve (AUC; *E*) during the insulin tolerance test (ITT) on *day 10* in S (○), L (■), A (▲), and L/A-treated mice (●). Values are means ± SE (n = 4/group). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. S-treated mice.

contents. Liver weight was significantly decreased (by 16%) in L/A-treated mice compared with that in S-treated mice (Fig. 3*A*). In addition, L/A coadministration significantly decreased triglyceride contents in liver (by 42%) and skeletal muscle (by 46%), whereas administration of L or A alone did not decrease tissue triglyceride contents compared with saline administration (Fig. 3, *B* and *C*).

Leptin has been shown to decrease skeletal muscle triglyceride content in part by increasing fatty acid β-oxidation through AMPKα2 activation in skeletal muscle (24). Therefore, we measured AMPK activity in soleus muscle, where the effect of leptin on AMPK activation was pronounced (24). AMPKα1 activity in soleus muscle was not changed significantly in any group of mice compared with S-treated mice (Fig. 3*D*). On the other hand, AMPKα2 activity in soleus muscle was increased significantly only in L/A-treated mice (by 71%) compared with those in S-treated mice (Fig. 3*E*), consistent with the results of tissue triglyceride contents.

Pair-feeding and weight-matched calorie restriction experiments. We performed pair-feeding experiments to assess whether the body weight reduction and the enhancement of insulin sensitivity by L/A coadministration was associated with food intake reduction. Pair-feeding to L/A-treated mice reduced body weight in DIO mice significantly, but the change was apparently smaller than in L/A-treated mice (Fig. 4*A*). In addition, PF mice showed neither the improvement in insulin sensitivity (Fig. 4, *B* and *C*) nor the decrease in triglyceride contents of liver and skeletal muscle (Fig. 4, *D* and *E*), in contrast to L/A-treated mice.

Then, we performed weight-matched calorie restriction experiments to assess whether the enhancement of insulin sensitivity by L/A coadministration was associated with body weight reduction. To match the body weight to L/A-treated mice, the food intake was restricted to 70% of S-treated mice in CR mice (Fig. 4*A*). In this condition, CR mice showed neither the improvement of insulin sensitivity (Fig. 4, *B* and *C*) nor the decrease in triglyceride contents of liver and skeletal muscle (Fig. 4, *D* and *E*), in contrast to L/A-treated mice.

DISCUSSION

Leptin could be an ideal drug for obesity-associated diabetes because it has both a weight-reducing effect and an antidiabetic effect. However, even high pharmacological doses of leptin elicit only marginal weight loss in non-leptin-deficient DIO rodents and humans (8, 15), whereas leptin replacement ther-

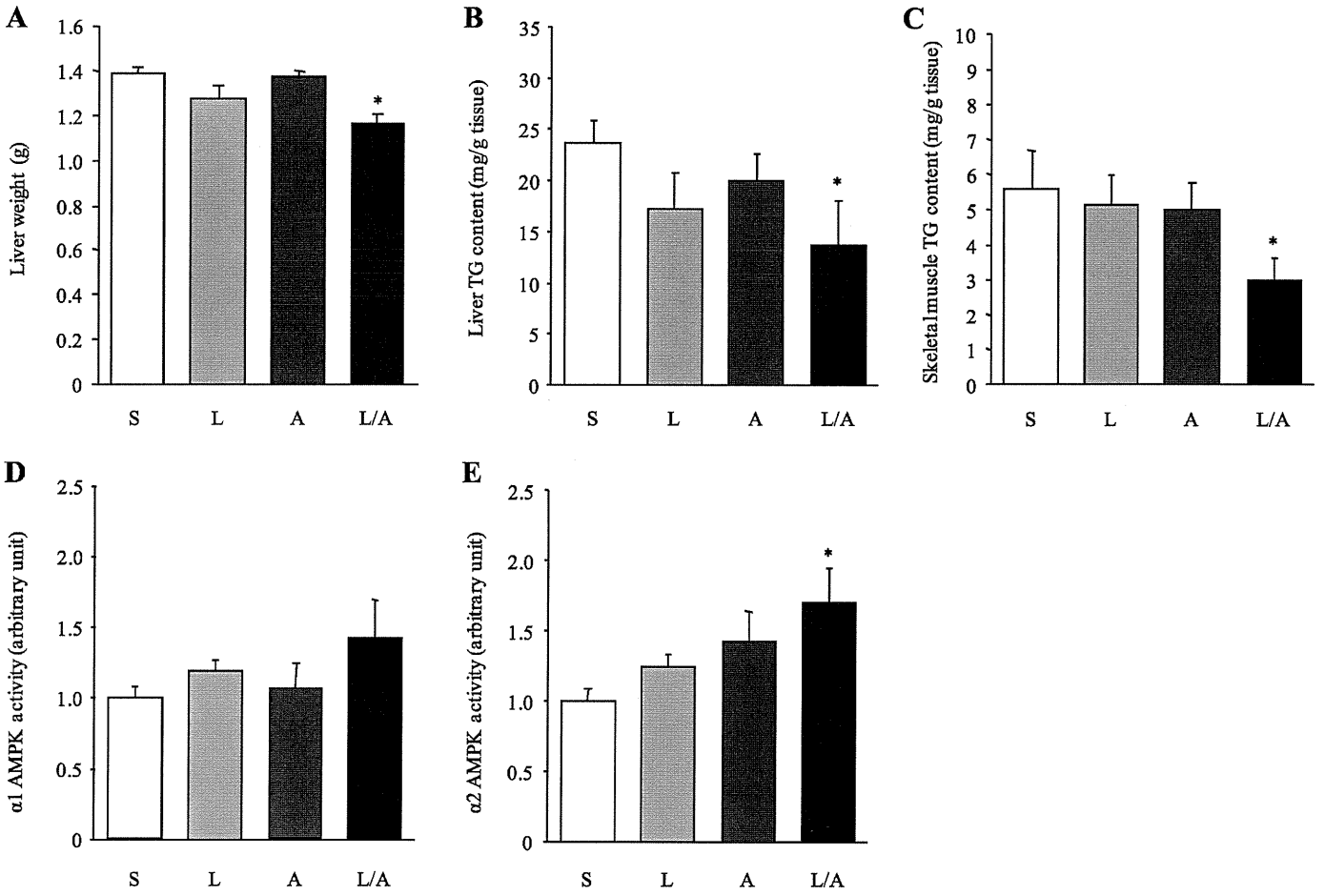

Fig. 3. Effect of L and/or A on tissue triglyceride (TG) content and skeletal muscle AMP-activated protein kinase (AMPK) activity in DIO mice. Liver size (*A*) and liver (*B*) and gastrocnemius muscle (*C*) TG contents on *day 14* in S, L, A, and L/A-treated mice. AMPKα1 (*D*) and AMPKα2 activity (*E*) on *day 14* in soleus muscle of S, L, A, and L/A-treated mice. Values are means ± SE (n = 8–9/group). *$P < 0.05$ vs. S-treated mice.

apy induces profound weight loss in leptin-deficient mice and humans (10, 13). The obese state is thus thought to be associated with leptin resistance, wherein overweight/obese individuals become insensitive to high circulating leptin levels. Sensitizing agents of leptin's effects are expected to treat obesity-associated diabetes comprehensively. In this study, we demonstrated that L/A coadministration not only reduced food intake and body weight but also enhanced insulin sensitivity accompanied by an increase of AMPKα2 activity in skeletal muscle and decrease of tissue triglyceride contents in leptin-resistant DIO mice. Our results indicate the possible clinical usefulness of L/A coadministration as a new antidiabetic treatment in obesity-associated diabetes.

Recently, coadministration of L (500 $\mu g \cdot kg^{-1} \cdot day^{-1}$) and A (100 $\mu g \cdot kg^{-1} \cdot day^{-1}$) was shown to result in a synergistic fat-specific body weight reduction in DIO rats (34). The synergistic antiobesity effect of leptin and amylin was established by the response surface methodology analysis using lower dose ranges of L (0–125 $\mu g \cdot kg^{-1} \cdot day^{-1}$) and A (0–50 $\mu g \cdot kg^{-1} \cdot day^{-1}$) in DIO rats (39). However, because the study of L/A coadministration was not fully examined in mice, the adequate doses of L and A were unclear in DIO mice. Therefore, we chose L (500 $\mu g \cdot kg^{-1} \cdot day^{-1}$) and A (100 $\mu g \cdot kg^{-1} \cdot day^{-1}$) in the present study according to the first report (34). Administration of L (500 $\mu g \cdot g^{-1} \cdot day^{-1}$) had no significant effect on food intake or body weight in DIO mice (Fig. 1, *A* and *B*). Although amylin itself has been shown to dose-dependently reduce food intake and body weight (20, 26), administration of A (100 $\mu g \cdot kg^{-1} \cdot day^{-1}$) was not effective in our DIO mice (Fig. 1, *A* and *B*). Under these conditions, L/A coadministration reduced food intake and body weight in DIO mice in a greater than mathematically additive manner (Fig. 1, *A* and *B*). Our data support that L/A coadministration is a useful treatment for obesity beyond species difference. With the dose of leptin used in the present study, the plasma leptin level in DIO mice increased to 45.1–53.0 ng/ml (Table 2), which can be seen in human obese subjects. In addition, higher leptin levels were obtained in the obese human clinical trial without any clinically significant adverse effects on major organ systems (15). Therefore, the leptin level achieved with the dose used in the present study could be clinically applied in humans.

In general, amylin is considered not to affect insulin secretion and insulin sensitivity but rather to complement the effects of insulin on circulating glucose levels through two main mechanisms (43). First, amylin suppresses postprandial glucagon secretion, thereby decreasing glucagon-stimulated hepatic glucose output following nutrient ingestion (12). Second, amylin also slows the rate of gastric emptying and thus the rate at which nutrients are delivered from the stomach to the small intestine for absorption (44, 45). On the other hand, leptin is

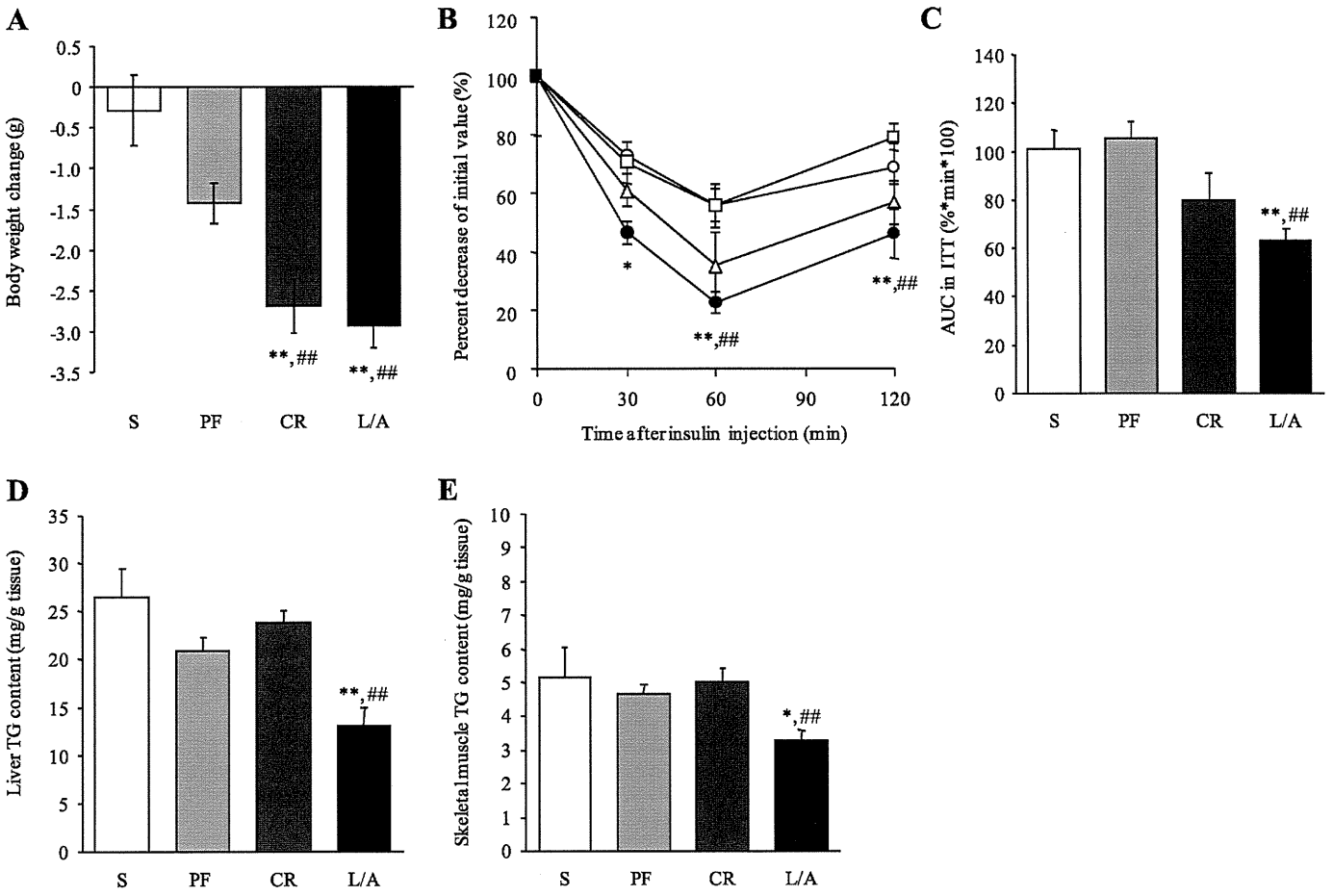

Fig. 4. Pair-feeding and weight-matched calorie restriction experiments. *A*: change in body weight on *day 10* in S, saline + pair-fed L/A-treated (PF), weight-matched DIO (CR), and L/A-treated mice. %Decrease of initial value of blood glucose levels (*B*) and AUC (*C*) during the ITT on *day 10* in S (○), PF (□), CR (△), and L/A-treated mice (●). Liver (*D*) and gastrocnemius muscle (*E*) TG contents on *day 14* in S, PF, CR, and L/A-treated mice. Values are means ± SE (n = 7–12/group). *$P < 0.05$ and **$P < 0.01$ vs. S-treated mice; ##$P < 0.01$ vs. PF mice.

considered to increase insulin sensitivity with augmentation of insulin receptor signaling in insulin target organs such as the liver and skeletal muscle (30) and suppress secretion of glucagon (28, 42). In this study, the tendency toward a decrease, but not a significant one, in plasma glucagon levels was observed in L/A-treated mice (Fig. 2*C*). Further studies are needed to evaluate the effect of leptin on plasma glucagon in DIO mice. Administration of L or A alone did not affect insulin sensitivity in DIO mice (Fig. 2, *A–D*). However, L/A coadministration effectively enhanced insulin sensitivity in DIO mice (Fig. 2, *A–D*). Taken together, our results indicate that amylin improved the insulin-sensitizing action of leptin in DIO mice.

One of the mechanisms by which leptin enhances insulin sensitivity is the reduction of fat accumulation in insulin target organs by activation of the AMPKα2 in skeletal muscle (24, 37, 38). In this study, we demonstrated that only L/A coadministration significantly reduced liver and skeletal muscle triglyceride contents accompanied by AMPKα2 activation in the skeletal muscle (Fig. 3, *A–E*). Previously, we demonstrated that AMPK in skeletal muscle was activated and insulin sensitivity enhanced in LepTg mice. High-fat diet feeding diminished both the activation of AMPK and the enhancement of insulin sensitivity, and diet substitution to standard diet restored them in LepTg mice, indicating that AMPK activity in skeletal muscle closely parallels insulin sensitivity (37). Based on the results of LepTg mice, we proposed that the AMPK activity in peripheral tissues could be a novel biochemical marker of leptin sensitivity in vivo (37). Therefore, the increase of AMPK activity in L/A-treated mice suggests that amylin improved leptin sensitivity in leptin-resistant DIO mice.

For the treatment of obesity-associated diabetes, it is universally accepted that dietary management is used initially with specific emphasis on weight reduction, because weight reduction leads to improvement in deteriorated glucose metabolism (1, 3). Therefore, to assess the influence of food intake and body weight reduction, we compared insulin sensitivity and tissue triglyceride contents among PF, CR, and L/A-treated mice. In this study, PF mice did not show reduced body weight compared with L/A-treated mice (Fig. 4*A*). Because amylin-induced weight loss was attributable primarily to reduced food intake (20, 33, 35), weight loss in L/A-treated mice suggests additional mechanisms such as restoration of leptin's effect on energy expenditure. In previous analyses of calorie restriction effects on metabolism, calorie restriction was accompanied by an expected counterregulatory decline in energy expenditure in rodents (39). However, in this study, we showed that L/A coadministration increased energy expenditure significantly,

whereas it reduced food intake (Fig. 1*C*). In addition, CR mice, whose food consumption was restricted to match their body weight to those of the L/A-treated mice, showed neither the improvement of insulin sensitivity (Fig. 4, *B* and *C*) nor the decrease in liver and skeletal muscle triglyceride contents (Fig. 4, *D* and *E*). These results showed that the improvement of insulin sensitivity and the decrease in tissue triglyceride contents by L/A coadministration were achieved by other mechanisms besides calorie restriction.

In conclusion, we demonstrated that L/A coadministration effectively improves insulin sensitivity in addition to reducing food intake and body weight in DIO mice. Our data indicate that L/A coadministration could be a new antidiabetic treatment in obesity-associated diabetes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.K., K.E., and K.N. did the conception and design of the research; T.K., T.S., and L.M. performed the experiments; T.K., T.S., and L.M. analyzed the data; T.K., K.E., T.S., L.M., D.A., Y.Y., S.Y.-K., M.A.-A., J.F., K.H., and K.N. interpreted the results of the experiments; T.K. prepared the figures; T.K. drafted the manuscript; T.K. and K.E. edited and revised the manuscript; T.K., K.E., and K.N. approved the final version of the manuscript.

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Transgenic overexpression of intraislet ghrelin does not affect insulin secretion or glucose metabolism in vivo

Mika Bando,[1,3] Hiroshi Iwakura,[1] Hiroyuki Ariyasu,[1] Hiroshi Hosoda,[4] Go Yamada,[2] Kiminori Hosoda,[2,3] Souichi Adachi,[3] Kazuwa Nakao,[2] Kenji Kangawa,[4] and Takashi Akamizu[1,5]

[1]*Ghrelin Research Project, Translational Research Center,* [2]*Department of Medicine and Clinical Science, Endocrinology, and Metabolism, and* [3]*Department of Human Health Sciences, Kyoto University Hospital, Kyoto University Graduate School of Medicine, Kyoto;* [4]*National Cerebral and Cardiovascular Center Research Institute, Osaka; and* [5]*The First Department of Medicine, Wakayama Medical University, Wakayama, Japan*

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Bando M, Iwakura H, Ariyasu H, Hosoda H, Yamada G, Hosoda K, Adachi S, Nakao K, Kangawa K, Akamizu T. Transgenic overexpression of intraislet ghrelin does not affect insulin secretion or glucose metabolism in vivo. *Am J Physiol Endocrinol Metab* 302: E403–E408, 2012. First published November 22, 2011; doi:10.1152/ajpendo.00341.2011.—Whereas ghrelin is produced primarily in the stomach, a small amount of it is produced in pancreatic islets. Although exogenous administration of ghrelin suppresses insulin secretion in vitro or in vivo, the role of intraislet ghrelin in the regulation of insulin secretion in vivo remains unclear. To understand the physiological role of intraislet ghrelin in insulin secretion and glucose metabolism, we developed a transgenic (Tg) mouse model, rat insulin II promoter ghrelin-internal ribosomal entry site-ghrelin *O*-acyl transferase (RIP-GG) Tg mice, in which mouse ghrelin cDNA and ghrelin *O*-acyltransferase are overexpressed under the control of the rat insulin II promoter. Although pancreatic desacyl ghrelin levels were elevated in RIP-GG Tg mice, pancreatic ghrelin levels were not altered in animals on a standard diet. However, when Tg mice were fed a medium-chain triglyceride-rich diet (MCTD), pancreatic ghrelin levels were elevated to ~16 times that seen in control animals. It seems likely that the gastric ghrelin cells possess specific machinery to provide the octanoyl acid necessary for ghrelin acylation but that this machinery is absent from pancreatic β-cells. Despite the overexpression of ghrelin, plasma ghrelin levels in the portal veins of RIP-GG Tg mice were unchanged from control levels. Glucose tolerance, insulin secretion, and islet architecture in RIP-GG Tg mice were not significantly different even when the mice were fed a MCTD. These results indicate that intraislet ghrelin does not play a major role in the regulation of insulin secretion in vivo.

pancreas; ghrelin D-acyltransferase

GHRELIN IS A 28-AMINO ACID PEPTIDE HORMONE, with a unique modification of acylation at the third serine residue, first described by Kojima et al. (17) in 1999. The acyl modification of ghrelin is mediated by the recently discovered enzyme ghrelin *O*-acyl transferase (29), and the modification is essential for ghrelin binding to its cognate receptor (12). Ghrelin is produced primarily in the stomach, but small amounts of ghrelin are also produced in pancreatic islets (1, 5, 8, 10, 12, 26, 27). Controversy remains about which type of islet cell produces ghrelin (5, 20, 26, 27). Date et al. (5) reported that ghrelin is present in α-cells in humans and rats, whereas Volante et al. (26) reported that ghrelin is produced by β-cells in humans. In contrast, Wierup and colleagues (27, 28) and Prado et al. (20) reported that ghrelin-expressing cells comprise a new islet cell type distinct from α-, β-, and δ-cells and PP cells in human, rat, and mouse islets.

Exogenous ghrelin suppresses insulin secretion from pancreatic β-cells in vitro (4, 9, 22) or in vivo (3, 22, 25). Although several studies have demonstrated contradictory results (1, 5, 11, 18, 24), data from genetically engineered mice are consistent with this concept. Chronic elevation of plasma ghrelin levels suppresses insulin secretion, inducing glucose intolerance in transgenic mice (2, 13, 21), whereas ablation of ghrelin improves glucose tolerance by enhancing insulin secretion in diet-induced obesity (7) or *ob/ob* mouse models (23). Although in vitro studies demonstrate that intraislet ghrelin can suppress insulin secretion from isolated islets (6), the physiological role of intraislet ghrelin on the regulation of insulin secretion in vivo is unclear. Because only minimal amounts of ghrelin are produced by the pancreas compared with that made by the stomach (15), the effect of stomach-derived ghrelin may overpower the effects of intraislet ghrelin in vivo.

In this study, we developed a transgenic mouse model in which the ghrelin and ghrelin *O*-acyltransferase (GOAT) genes are overexpressed by pancreatic β-cells under the control of the rat insulin II promoter (RIP) to ascertain the physiological role of intraislet ghrelin on insulin secretion and glucose metabolism in vivo.

MATERIALS AND METHODS

Generation of RIP-ghrelin-GOAT transgenic mice. We designed a fusion gene comprised of RIP, mouse ghrelin cDNA, internal ribosomal entry site (IRES), and mouse GOAT cDNA coding sequences. The purified fragment (10 μg/ml) was microinjected into the pronuclei of fertilized C57/B6J mouse eggs (SLC, Shizuoka, Japan). Viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (SLC) by using standard techniques. Transgenic founder mice were identified by Southern blot analyses of tail DNA, using a mouse ghrelin cDNA fragment as a probe. For experimentation, we utilized heterozygous transgenic mice. Animals were maintained on a 12:12-h light-dark cycle and fed a standard diet (SD; CE-2, 352 kcal/100 g; Japan CLEA, Tokyo, Japan) or a MCTD containing 45% Dermol M5 (C8:60%, C10:40%; Research Diets, New Brunswick, NJ) as indicated. All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

Address for reprint requests and other correspondence: H. Iwakura, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan (e-mail: hiwaku@kuhp.kyoto-u.ac.jp).

Measurement of plasma and tissue ghrelin concentrations. Blood was drawn from the proximal end of the portal vein under ether anesthesia, transferred immediately to chilled siliconized glass tubes containing Na_2-EDTA (1 mg/ml) and aprotinin (1,000 KIU/ml), and centrifuged at 4°C. Hydrogen chloride was added to the samples at a final concentration of 0.1 N immediately after separation of plasma. Plasma was immediately frozen and stored at −80°C until assay. Plasma ghrelin concentration was determined by AIA-600 II (Tosoh, Tokyo, Japan).

To measure tissue ghrelin concentrations, pancreata or stomachs were isolated from mice and then boiled for 5 min in the 10-fold vol/wt of water. Acetic acid was added to each solution to adjust the final concentration to 1 M before tissue homogenization. We determine the tissue ghrelin concentration in supernatants obtained after centrifugation by radioimmunoassay (RIA) using anti-ghrelin [13–28] (C-RIA) and anti-ghrelin [1–11] (N-RIA) antisera, as described previously (12, 15).

Real-time quantitative RT-PCR. Total RNA was extracted from pancreata using an RNeasy Protect mini kit (Qiagen, Hilden, Germany). Reverse transcription (RT) was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems), using the following primers and TaqMan probes: mouse ghrelin (sense, 5′-GCATGCTCGGATGGACATG-3′; antisense, 5′-TGGTGGCTTCTTGGATTCCT-3′; TaqMan probe, 5′-AGCCCAGAGCACCAGAAAGCCCA-3′); mouse insulin (sense, 5′-CAGCTATAATCAGAGACCATCAGCAA-3′; antisense, 5′-GGGTAGGAAGTGCACCAACAG-3′; TaqMan probe, 5′-CAGGTCATTGTTTCAAC-3′); GOAT (sense, 5′-AGGGACTCTAGGAAGGACAG-3′; antisense, 5′-CCCATCTGAAAGAAGAAGGT-3′, with Power SybrGreen). Data were normalized to the content of 18S rRNA in each sample.

Glucose tolerance tests. For glucose tolerance testing, the ad libitum-fed mice were injected intraperitoneally with 1.5 g/kg glucose. Blood was sampled from the tail veins before and 30, 60, 90, and 120 min after the injection. Blood glucose levels were determined by the glucose oxidase method using a Glutest sensor (Sanwa Kagaku, Kyoto, Japan).

Insulin release. Ad libitum-fed mice were injected with 3.0 g/kg glucose intravenously. Plasma was sampled from a retroorbital vein before and 2 or 30 min after injections into heparin-coated tubes. Insulin concentrations were measured by a high-range speedy mouse insulin kit (Morinaga, Yokohama, Japan).

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were immunostained using the avidin-biotin peroxidase complex method (Vectastain "ABC" Elite Kit; Vector Laboratories, Burlingame, CA), as described previously (14). Serial sections of 5-μm thickness were incubated with anti-COOH-terminal ghrelin (1:1,000) (17) and anti-NH_2-terminal ghrelin (1:2,000) (17), anti-glucagon (1:500), anti-insulin (1:500), anti-somatostatin (1:500), and anti-pancreatic polypeptide (1:500; DAKO, Glostrup, Denmark) antisera.

Statistical analysis. All values are expressed as means ± SE. The statistical significance of the differences in mean values was assessed by ANOVA with a post hoc test (Tukey's test) or Student's *t*-test as appropriate. Differences with $P < 0.05$ were considered significant. Statistical analyses were performed using Statcel2 (OMS, Saitama, Japan).

RESULTS

Generation of RIP-ghrelin-IRES-GOAT transgenic mice. After the RIP-ghrelin-IRES-GOAT transgene was injected into 286 eggs, we obtained three lines (3–4, 9–3, and 11–5) confirmed to be rat insulin II promoter-ghrelin-IRES-GOAT transgenic (RIP-GG Tg) mice. For further analyses, we selected the 9–3 line, which had the highest expression of ghrelin and GOAT mRNA in the pancreas (data not shown). The expression levels of pancreatic ghrelin mRNA in the 9–3 line of RIP-GG Tg mice were ~20-fold higher than those seen in controls (Fig. 1*B*), whereas GOAT mRNA levels were ~80-fold higher than those in controls (Fig. 1*C*). There was also an increment in ghrelin and GOAT mRNA levels in the hypothalamus of RIP-GG Tg mice (non-Tg vs. Tg: ghrelin, 1.0 ± 0.28 vs. 25.6 ± 5.6; GOAT, 1.0 ± 0.26 vs. 5,735.5 ± 1,189.1, arbitrary unit; $n = 8$, $P < 0.01$).

Pancreatic and plasma ghrelin levels in RIP-GG Tg mice. Total ghrelin levels measured by C-RIA were significantly elevated in the pancreata of RIP-GG Tg mice on a SD or MCTD (Fig. 2*A*). However, the ghrelin levels measured by N-RIA were elevated only when RIP-GG Tg mice were fed a MCTD (Fig. 2*B*). Although ghrelin levels 16-fold higher than those seen in control littermates were observed in the pancreata of RIP-GG Tg mice fed a MCTD, these absolute levels were low compared with those isolated from stomach (Fig. 2, *D* and *E*). Furthermore, the ratio of ghrelin to total ghrelin in the pancreas of RIP-GG Tg mice was significantly low on SD, which was elevated on a MCTD (Fig. 2*C*). Still, the level was significantly lower compared with that of the stomach (Fig. 1*F*).

Immunohistochemistry showed that the ghrelin-like immunoreactivities were increased in the core of the islets of RIP-GG Tg mice on a MCTD (Fig. 3), indicating that increased tissue levels of pancreatic ghrelin were originated from β-cells.

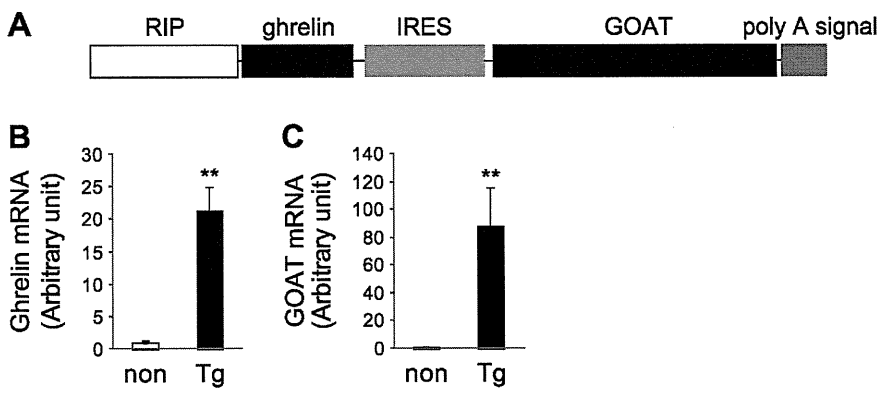

Fig. 1. Constructs of rat insulin II promoter (RIP)-ghrelin-internal ribosomal entry site (IRES)-ghrelin *O*-acyltransferase (GOAT) transgenic (RIP-GG Tg) mice and the expression levels of ghrelin and GOAT mRNA in the pancreas. *A*: we designed a fusion gene containing RIP, mouse ghrelin cDNA, IRES, and mouse GOAT cDNA. *B* and *C*: the resultant expression levels of ghrelin (*B*) and GOAT mRNA (*C*) in the pancreata of RIP-GG Tg mice. Non, nontransgenic littermate; Tg, RIP-GG Tg mice; $n = 7–11$, $^{**}P < 0.01$ compared with non.