walls (150 mm high) and two open arms with a small raised lip (3 mm). The maze was elevated to a height of 400 mm above the ground. At least 1 hr before the test, mice were transferred to a standby room (20 lx) that was separated from the test room. Experiments were performed between 13:00 and 15:00. Each mouse was placed on the center platform facing an open arm to initiate the test session. Mice were allowed to freely explore the apparatus under overhead fluorescent lighting (20 lx) for 5 min. Increased exploration of the relatively open arms is indicative of reduced anxiety-like behavior in this paradigm. Open/closed arm entries and time spent in the open/closed arms were scored. Arm entries were scored upon entry of the two front paws into the arm.

Measurement of BDNF and NT-3 contents in the brain

BDNF and NT-3 contents in the brain of CD and DIO mice fed CD and HFD, respectively, for 16 weeks were measured according to our previous report (3) using commercially available measurement kits for BDNF (BDNF Emax® ImmunoAssay System: Promega Inc., Madison, WI) and for NT-3 (NT-3 Emax® ImmunoAssay System: Promega Inc., Madison, WI).

Western blot analysis of TrkB and TrkC

Western blotting of full- length TrkB and TrkC in the brain of CD and HFD mice was performed according to our previous report (3). Full-length TrkB and TrkC were detected using rabbit polyclonal anti-TrkB antibody (sc-8316, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and rabbit polyclonal anti-TrkC antibody (sc-14025, Santa Cruz Biotechnology, Inc.), respectively. Results represent the

densitometry data relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) detected in each sample.

Data analysis

All values are given as the mean ± SEM. Statistical analysis of the data was carried out by analysis of variance (ANOVA) followed by Dunnett's multiple-range test. Statistical significance was defined as $p < 0.05$.

Results

Metabolic parameters in CD and DIO mice

The metabolic parameters in CD and DIO mice are shown in Table 1. The body weight of DIO mice was 1.6 times greater than that of CD mice. Plasma levels of glucose, insulin and leptin in DIO mice were significantly high compared with those in CD mice.

Fear-conditioning response

CD mice exhibited 93% freezing due to fear in the 1st session in the contextual conditioning response, and the freezing percentage gradually decreased during the sessions to reach 60% in the 5th session (Fig. 1). In DIO mice, the freezing percentage of the contextual fear response was significantly lower than that in CD mice in each session (Fig. 1). DIO mice exhibited 64% freezing percentage in the 1st session of the contextual fear response, and the freezing percentage subsequently decreased during the sessions to 23% in the 5th session (Fig. 1). Similarly, the freezing percentage of the cued fear response in DIO mice was 47% in the first session which was much lower

than the 81% in CD mice, and a significant decrease in freezing percentage of DIO mice was observed over the course of three cued sessions compared with CD mice (Fig. 1).

Jumping-vocalization test, spontaneous locomotor activity and elevated plus-maze test

To compare the sensitivities to foot shock between CD and DIO mice, the jumping-vocalization test was used. No difference in scores of jumping-vocalization test was found between CD (score: 3.2 ± 0.3; n=14) and DIO (score: 2.6 ± 0.1; n=14) mice. To explore the involvement of motor activity and anxiety in impaired fear-conditioning responses in DIO mice, spontaneous locomotor activity for 30 min following placement of mice into new cages and behaviors in the elevated plus-maze test were examined. Spontaneous locomotor activity was not different between CD and DIO mice after 16-week feeding of each diet (data not shown). Moreover, both entry times and time spent in the dark and light arms in the elevated plus-maze test were not different between CD and DIO mice (data not shown).

BDNF and NT-3 contents in the brain areas

BDNF contents in the cerebral cortex and hippocampus of DIO mice had significantly decreased to approximately 70% and 60% of CD mice, respectively (Fig. 2A). BDNF contents in the amygdala and hypothalamus of DIO mice also tended to decrease compared with those of CD mice (Fig. 2A). In contrast to the changes of BDNF contents, NT-3 contents in the hippocampus, amygdala and hypothalamus of DIO mice significantly increased to 150%, 165% and 230% of those of CD mice, respectively (Fig. 2B). NT-3 contents in the cerebral cortex also tended to be higher than those of CD mice

(Fig. 2B).

Expression of full-length TrkB and TrkC receptors in the brain areas

The expression of full-length TrkB in the amygdala of DIO mice significantly decreased to approximately 70% of CD mice, but not in the cerebral cortex, hippocampus and hypothalamus (Fig. 3A). The full-length TrkC expressions in four brain areas were not significantly different between CD and DIO mice (Fig. 3B).

Discussion

The present study demonstrated that DIO mice showed significant reduction of both hippocampus-dependent contextual and amygdala-dependent cued fear responses of fear-conditioning test. However, the response to electric foot shock, locomotor activity and anxiety-like behavior of DIO mice were the same as those of CD mice. Interestingly, BDNF contents in the cerebral cortex and hippocampus of DIO mice were significantly lower than those of CD mice, while NT-3 contents in the hippocampus, amygdala and hypothalamus of DIO mice were significantly higher than those of CD mice. The expression of full-length TrkB for BDNF in the amygdala of DIO mice significantly decreased compared that of CD mice, while the expressions of full-length TrkC for NT-3 in the brain regions were not different between CD and DIO mice. These findings demonstrated that DIO mice display impaired cognition in the fear-conditioning test with imbalanced interaction between BDNF and NT-3 systems in the cerebral cortex, hippocampus and amygdala related to cognition and fear.

Chronic dietary fat intake, especially saturated fatty acid intake, is reported to

contribute to deficits of hippocampus-dependent spatial cognition in the water maze test of rats (5, 6, 28). The adverse effects of high-dense diets on learning and memory have been associated with impaired hippocampal synaptic plasticity and suppressed neurogenesis (29, 30, 31).

Long-term structural alterations of synapses, so-called neuronal plasticity, are regulated by several synaptic molecules including neurotrophic factors, such as BDNF (15), and have been demonstrated to be essential for spatial learning performance which is dependent primarily on hippocampal functions (32). Animals lacking BDNF show deficits in LTP related to processes of learning and memory, and in hippocampus-dependent spatial learning, which can be amended by exogenous BDNF (32). Although the mechanisms by which a high-fat diet can affect BDNF expression are largely unknown, in the present study the feeding of high-fat diet or obesity led to reduction of BDNF contents in the hippocampus and cerebral cortex to the extent that cognitive performance was compromised. In contrast to the decrease in BDNF contents, the present study demonstrated that NT-3 contents were significantly increased in the hippocampus, amygdala and hypothalamus of DIO mice compared with those of CD mice. BDNF and NT-3 oppose one another in regulating the dendritic growth of pyramidal neurons in the hippocampus and neural activity (22, 23). NT-3 was reported to inhibit the dendritic growth stimulated by BDNF (22). The amygdala, which is well established to play a pivotal role in regulation of fear, emotion and cognition (8, 9), is suggested to be involved in energy regulation because lesion of the amygdala has been reported to induce hyperphasia, resulting in marked obesity (10, 11). Moreover, the amygdala has recently been demonstrated to be one of the brain regions regulating appetite via activation of the melanocortin system (12). Taken together, these findings

suggest that the impaired fear-conditioning response in DIO mice is attributed to the decrease of BDNF which facilitates memory processes and the antagonistic actions of NT-3 against BDNF in the hippocampus and amygdala, while the present study did not address the mechanisms of the changes in BDNF and NT-3 contents in the brain of DIO mice.

Several lines of electrophysiological and behavioral evidence demonstrate that leptin and insulin enhance hippocampal synaptic plasticity and improve learning and memory (31, 33). Electrophysiological studies in genetically obese Zucker rats with leptin-receptor deficiency demonstrated that LTP of the hippocampal CA1 region, which is closely related to learning and the formation of memory and is regulated by NMDA and AMPA receptors (6), is markedly impaired in comparison with lean rats (7). Streptozotocin-treated insulin-deficient rats are reported to exhibit impaired cognition in the water maze test which is dependent on the hippocampus (34). Therefore, it seems likely that impairment of actions of leptin or insulin might be attributable to cognitive deficits in obesity and diabetes mellitus (35, 36). Although there is no direct evidence for the impairment of cognition in DIO mice, the impaired cognitive behaviors of fear-conditioning tests observed in this study may be, in part, mediated by decreased inherent functions of leptin and insulin in the brain in spite of high plasma levels of leptin and insulin, so-called leptin resistance or insulin resistance associated with obesity.

The present study has shown that DIO mice exhibit impairment of both hippocampus-dependent contextual and amygdala-dependent cued responses of the fear-conditioning test. Moreover, BDNF contents in the hippocampus and cerebral cortex and of DIO mice, while NT-3 contents increase in the hippocampus, amygdala

and hypothalamus of DIO mice, in comparison to CD mice. The expression of TrkB in the amygdala of DIO mice decreases compared with CD mice. These findings suggest that high-fat diet consumption may contribute to aspects of dysfunction in the central nervous system.

Acknowledgments

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Conflict of interest

The authors declared no conflict of interest.

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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

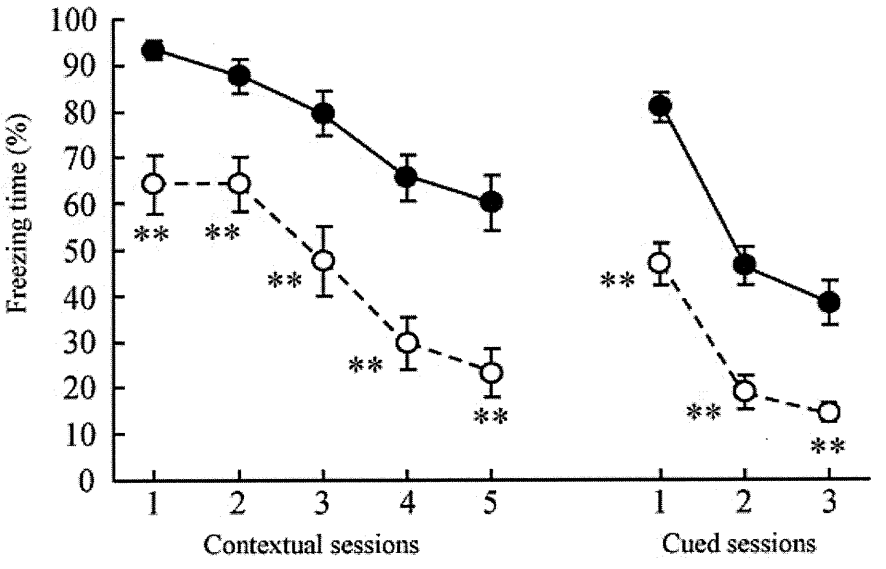
Fig. 1. Fear-conditioning responses in CD and DIO mice.

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, * $p < 0.05$.

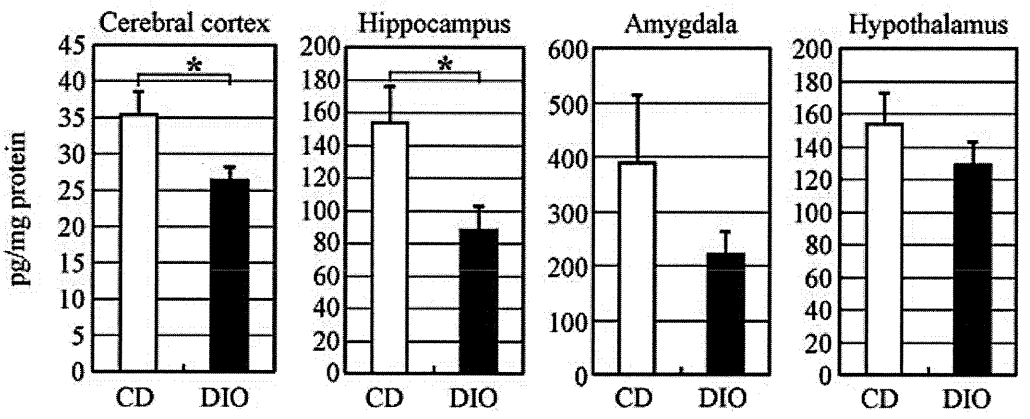
Fig. 1.



1

Fig. 2.

(A) BDNF



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(B) NT-3

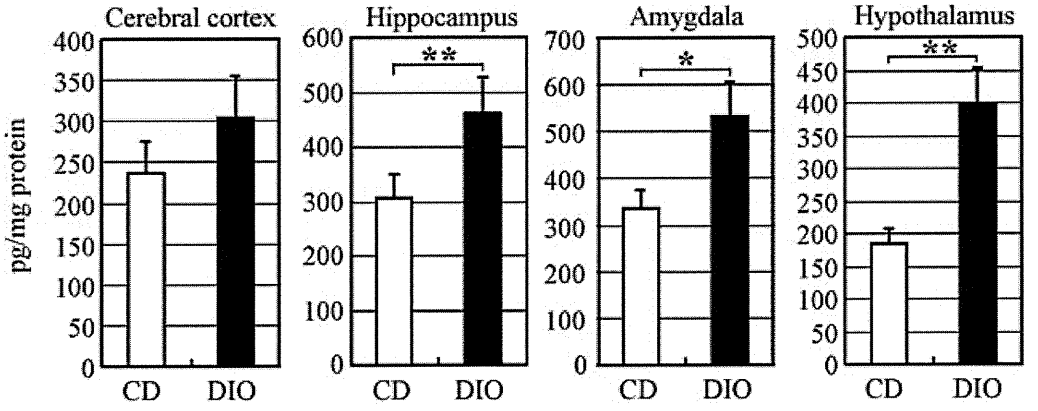

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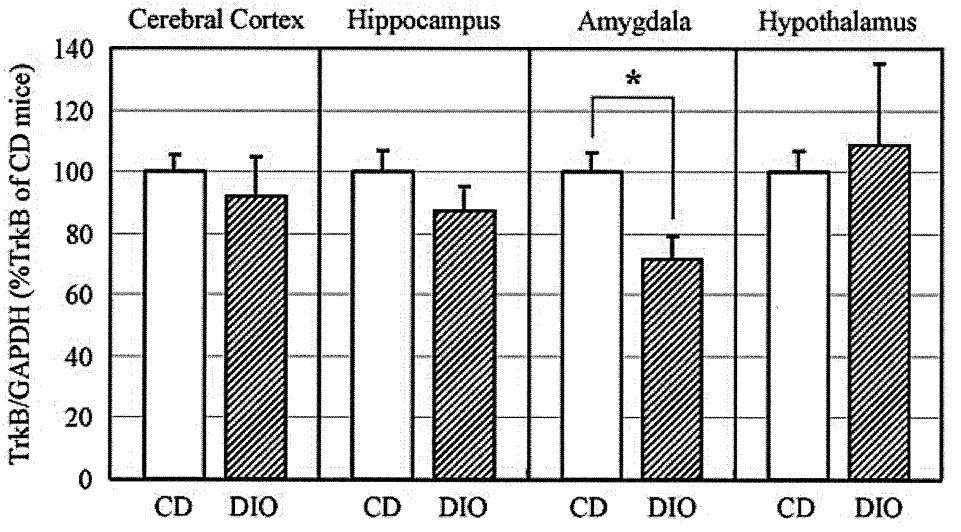
Fig. 3.

(A) Full-length TrkB



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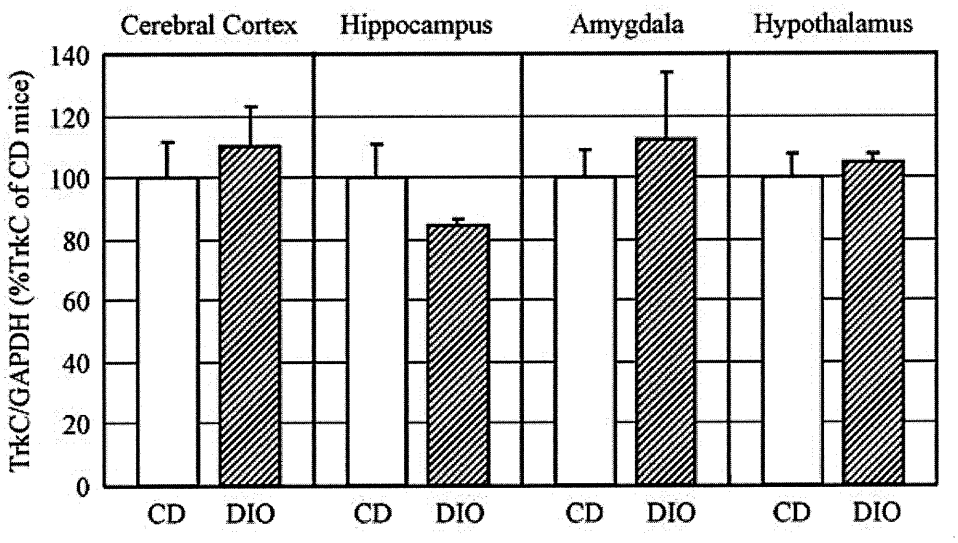
(B) Full-length TrkC



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

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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-

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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*

	Mouse Group			
Variable, ng/ml	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

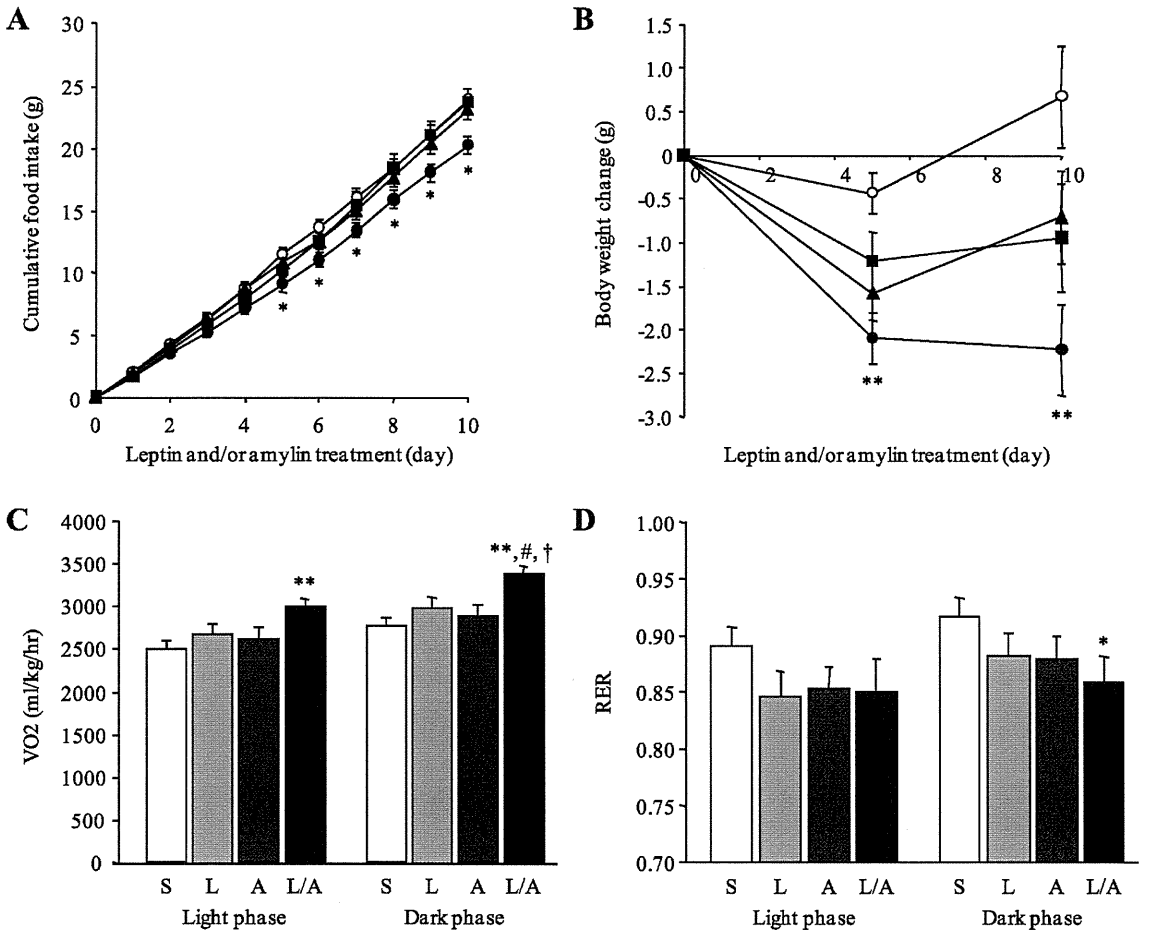

Fig. 1. Effect of leptin and/or amylin on food intake, body weight, energy expenditure, and respiratory exchange ratio (RER) in diet-induced obese (DIO) mice. Cumulative food intake (*A*) and change in body weight (*B*) during the treatment in saline- (S; ○), leptin- (L; ■), amylin- (A; ▲), and leptin + 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.