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

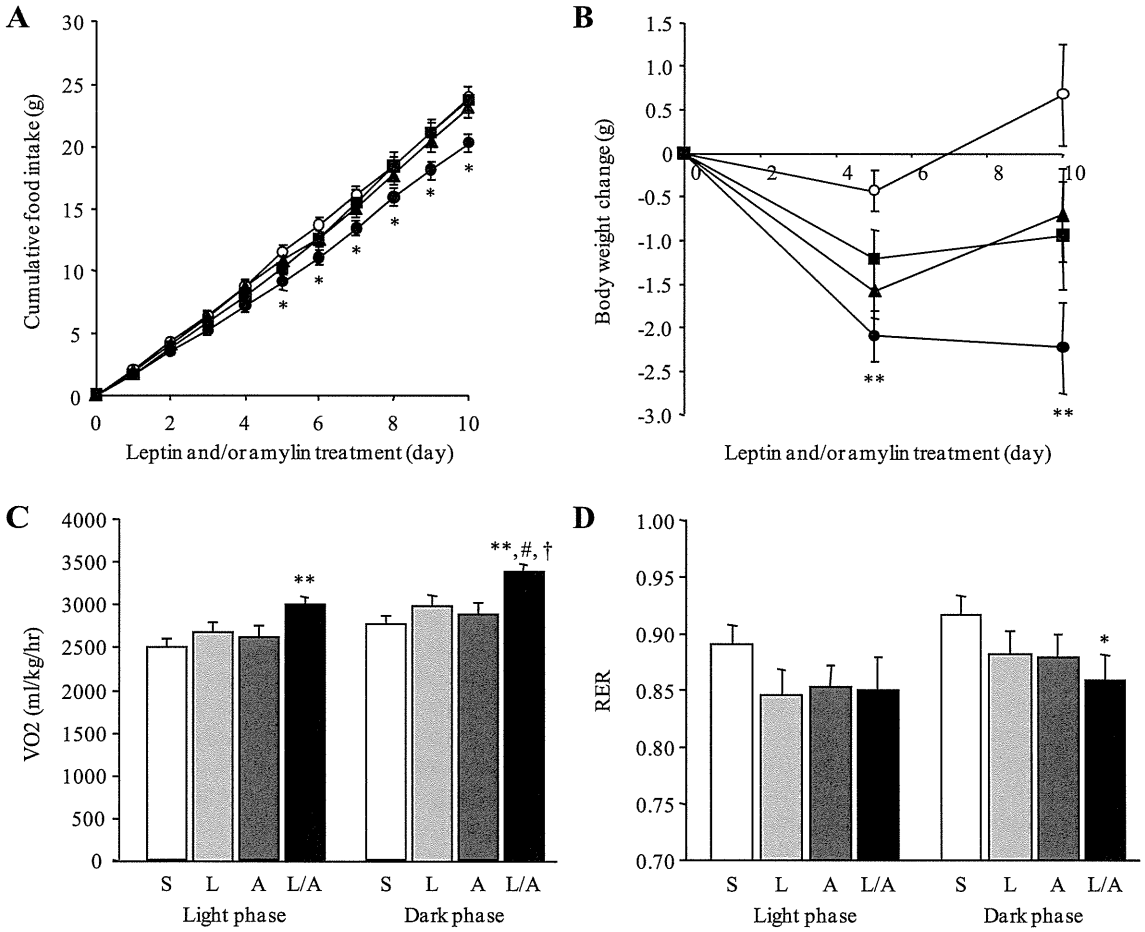

Fig. 1. Effect of leptin and/or amylin on food intake, body weight, energy expenditure, and respiratory exchange ratio (RER) in diet-induced obese (DIO) mice. Cumulative food intake (*A*) and change in body weight (*B*) during the treatment in saline- (S; ○), leptin- (L; ■), amylin- (A; ▲), and leptin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

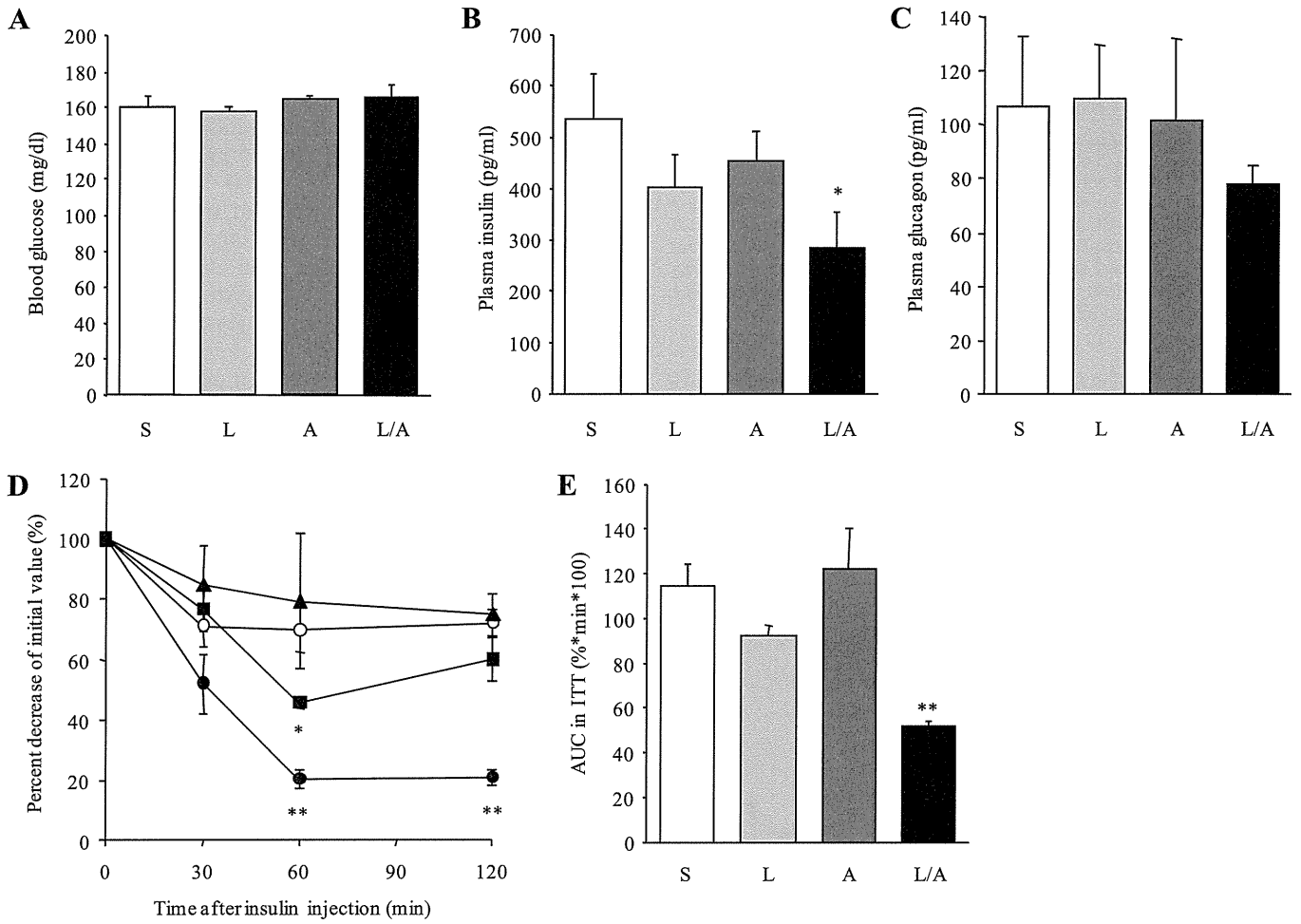

Fig. 2. Effect of L and/or A on glucose metabolism in DIO mice. Blood glucose (*A*), plasma insulin (*B*), and plasma glucagon levels (*C*) under ad libitum feeding on *day 14* in S, L, A, and L/A-treated mice. Values are means ± SE (n = 8–9/group). %Change of initial value of blood glucose levels (*D*) 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-

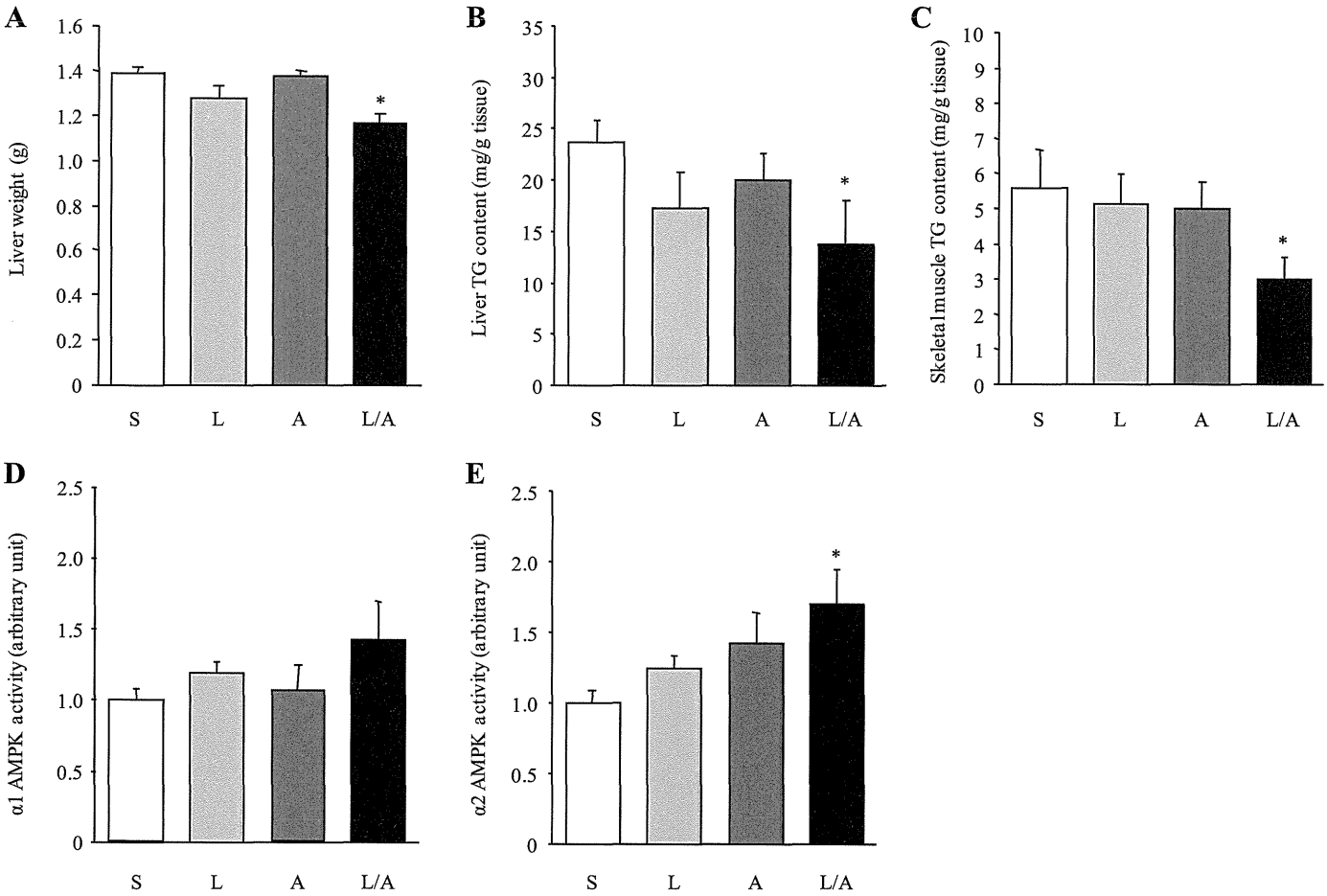

Fig. 3. Effect of L and/or A on tissue triglyceride (TG) content and skeletal muscle AMP-activated protein kinase (AMPK) activity in DIO mice. Liver size (*A*) and liver (*B*) and gastrocnemius muscle (*C*) TG contents on *day 14* in S, L, A, and L/A-treated mice. AMPKα1 (*D*) and AMPKα2 activity (*E*) 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

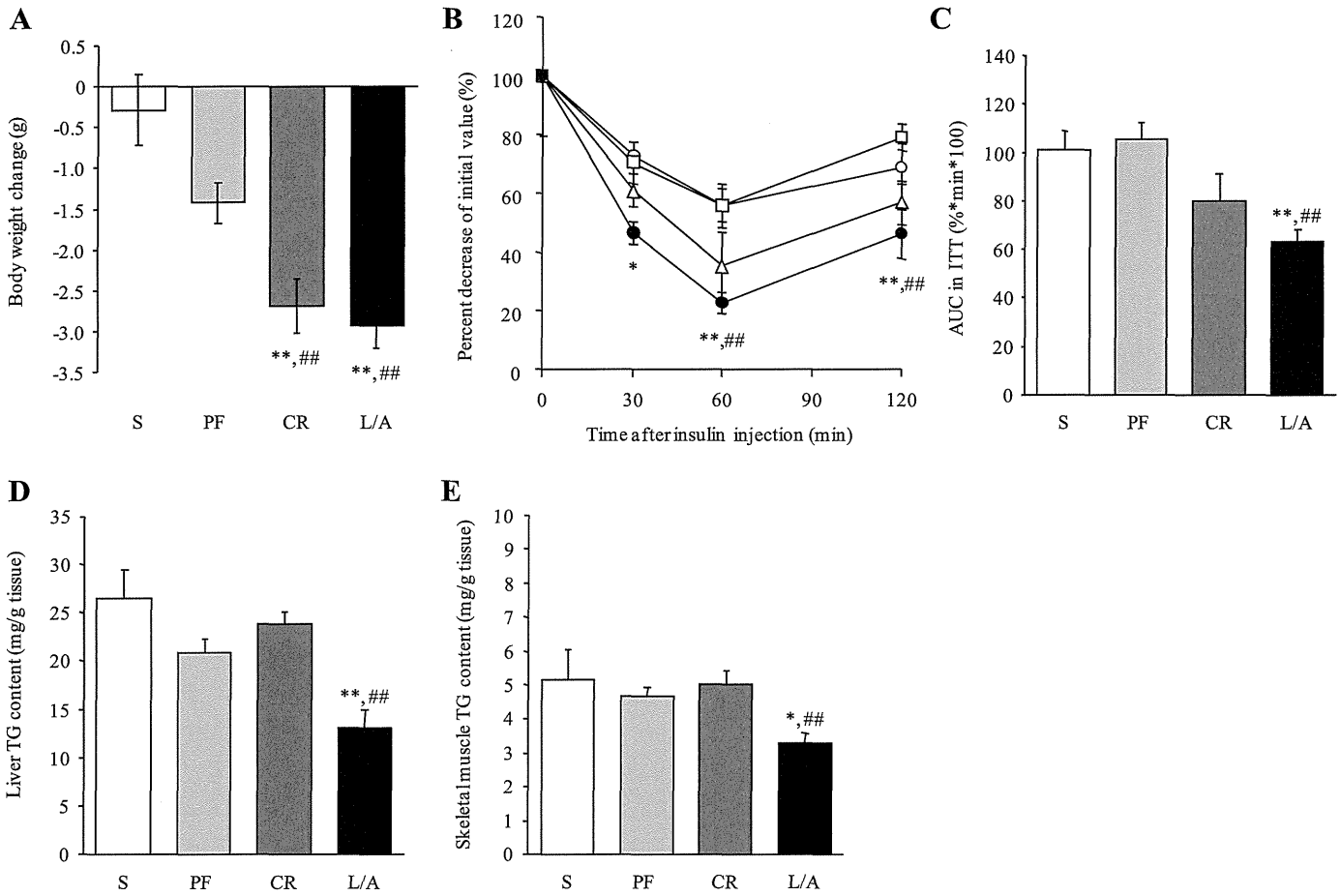

Fig. 4. Pair-feeding and weight-matched calorie restriction experiments. *A*: change in body weight on *day 10* in S, saline + pair-fed L/A-treated (PF), weight-matched DIO (CR), and L/A-treated mice. %Decrease of initial value of blood glucose levels (*B*) and AUC (*C*) during the ITT on *day 10* in 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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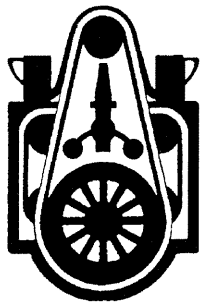

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ORIGINAL ARTICLE

Impairment of Fear-Conditioning Responses and Changes of Brain Neurotrophic Factors in Diet-Induced Obese Mice

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Recent epidemiological studies demonstrate that obesity is related to a high incidence of cognitive impairment. In the present study, cognitive behaviours in diet-induced obese (DIO) mice fed 60% high-fat diet for 16 weeks were compared with those in mice fed a control diet (CD) in fear-conditioning tests including both contextual and cued elements that preferentially depend on the hippocampus and amygdala, respectively. Furthermore, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) content in the brain areas was examined in both CD and DIO mice. In fear-conditioning tests, the freezing percentages of both contextual fear and cued fear responses in DIO mice were significantly lower than in CD mice. BDNF content in the cerebral cortex and hippocampus of DIO mice was significantly lower than that in CD mice. Its receptor, full-length TrkB, in the amygdala of DIO mice was significantly decreased compared to that in CD mice, although not in the cerebral cortex, hippocampus and hypothalamus. By contrast, NT-3 content in the hippocampus, amygdala and hypothalamus of DIO mice was significantly higher than that in CD mice. Its receptor, full-length TrkC, was not significantly different between CD and DIO mice. The present study demonstrates that DIO mice show impairment of both hippocampus-dependent contextual and amygdala-dependent cued responses in the fear-conditioning tests, as well as an imbalance in the interaction between the BDNF and NT-3 systems in the cerebral cortex, hippocampus and amygdala related to cognition and fear.

Key words: high-fat diet, obese mouse, fear-conditioning test, cognition, brain neurotrophic factors.

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Obesity is defined as increased adipose mass resulting from chronic excess of energy intake over energy expenditure. Obesity is becoming a worldwide problem because it is associated with serious comorbidities, including a high incidence of type II diabetes and cardiovascular disease, and an increased risk of many forms of cancer. In addition, epidemiological studies have demonstrated that the incidence of depression and cognitive impairment is higher in obese subjects than in normal body weight subjects (1,2). We recently demonstrated that impaired leptin action in the hippocampus is involved in depression associated with diet-induced obesity in mice (3).

Energy homeostasis including food intake and energy consumption has been demonstrated to be regulated predominantly by orexigenic and anorexigenic systems in the hypothalamus. Recently, several lines of evidence have indicated that energy regulations are also modulated by extra-hypothalamic brain areas originally related to regulation of emotion and cognition, such as the nucleus accumbens, amygdala, hippocampus and cerebral cortex (4). These findings suggest that maintaining energy homeostasis and regulating emotion and cognition share common brain regions, as well as bidirectional interaction between energy regulation and emotional/cognitive functions. In this regard, obese rats fed saturated fat and refined sugar show an impaired acquisition and retention of spatial memory in the water maze test that is dependent on the hippocampus (5). Electrophysiological studies in genetically obese Zucker rats with leptin-receptor deficiency demonstrated that long-term potentiation (LTP) of the hippocampal CA1 region, which is closely related to memory formation and is predominately regulated by the glutamatergic system, especially NMDA receptors and AMPA receptors (6), is markedly impaired in comparison with lean rats (7). These findings suggest dysfunction of the hippocampus in obese animals. The amygdala, as well as the hippocampus, which has

been established as playing a pivotal role in regulation of fear, emotion and cognition (8,9), has been suggested to be involved in energy regulation because lesion of the amygdala has been reported to induce hyperphagia, 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).

Memory formation involves long-term structural alterations of synapses, so-called neuronal plasticity involving cellular and molecular mechanisms of synapse formation, neurite outgrowth, and behavioural adaptation (13). Cellular and molecular events involved with neuronal plasticity are under the range of action of neurotrophic factors, including brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) (14,15). BDNF and NT-3 act via high-affinity tyrosine kinase receptors, TrkB and TrkC, respectively (16,17). The BDNF system in the brain is demonstrated to have anti-obesity and anti-diabetic effects, as well as to regulate neural modelling and cognitive processes (18–21). Although the actions of NT-3 in the brain on energy regulation are not yet known, BDNF and NT-3 act in opposite directions in neurite outgrowth and neural activities (22,23). Moreover, glucocorticoid is reported to show an opposite effect in the regulation of BDNF and NT-3 expression in the brain (24).

To explore cognition in diet-induced obese (DIO) mice, in the present study, we examined the cognitive behaviour of DIO mice fed high-fat diet (HFD) using fear-conditioning tests involving regulation mainly by the hippocampus and amygdala (25), and also investigated BDNF and NT-3 content and the expression of their receptors, TrkB and TrkC, in the cerebral cortex, hippocampus, amygdala and hypothalamus of DIO mice compared to control mice.

Materials and methods

Animals and diets

Male C57BL/6J mice (6 weeks old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). They were housed under a 12 : 12 h light/dark cycle (lights on 07.00 h) at room temperature (23 ± 1 °C). The animals had *ad lib.* access to water and food. They were randomly divided into two groups: mice fed HFD (DIO: Research Diets, Inc., New Brunswick, NJ, USA; No. D12492: 524 kcal per 100 g) and mice fed control diet (CD: CE-2, CLEA Japan, Inc., Tokyo, Japan: 346.8 kcal per 100 g). Both groups were fed for 16 weeks. Experiments were performed between 13.00 and 15.00 h. All experiments were performed in accordance with the guidelines established by the Institutional Animal Investigation Committee at Kyoto University and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to optimise the comfort and minimise the use of animals.

Blood sampling and analysis of metabolic parameters

Blood samples were taken from the thoracic aorta using a syringe containing heparin sodium and aprotinin. The blood samples were centrifuged at 15,000×g for 2 min, and plasma was separated and stored at −20 °C until assayed. Plasma metabolic parameters were analysed in accordance with a previous study (3).

Fear-conditioning test

The fear-conditioning test was performed as described in a previous study (26). Briefly, training sessions consisted of pairing a neutral stimulus (conditioned stimulus; CS) of a tone and an aversive stimulus (unconditioned stimulus; US) of an electric foot shock. The conditioning chamber was surrounded by a sound-attenuated chest with an observation window. The foot shock was delivered via the grid floor composed of stainless steel rods. The tone was provided by a ventilation fan making a noise of 65 dB. On the first day, each mouse was trained ten times to associate foot shock with the tone, which was presented for 30 s as a conditioned stimulus and a 0.5-mA foot shock for 2 s as an unconditioned stimulus. Mice were then returned to their home cages. Twenty-four hours later, the contextual response and the cued response were observed. To examine the contextual conditioning response, each mouse was placed in the conditioning chamber without the tone for 5 min and freezing behaviour was measured every 1 min. Freezing was defined as the absence of all movement except for respiration. Freezing was monitored continuously by an observer and was recorded on a chart via a switch. Freezing time was summed, and the freezing percentage was calculated per minute. This response mainly depends on the hippocampus. Three hours after termination of the contextual conditioning response, the cued conditioning response was examined by placing each mouse in a new clear plastic cage with the tone for 3 min. Freezing behaviour was measured every 1 min. This response mainly depends on the amygdala.

Jumping–vocalisation response

To compare the responses to foot shock of DIO mice with those of CD mice, the test was performed as described in a previous study (26) with the foot shock box used in the experiment on contextual fear conditioning of CD and DIO mice. Each mouse was placed individually in the box. After a 3-min period of habituation to the test box, shock titrations were continued upwards and downwards in a stepwise manner (0.5 mA for 2 s). Jumping responses to the foot shock were scored as 0–3 and vocalisation responses to the foot shock were scored as 0–3. Response scores 0, 1, 2 and 3 indicate no response, a slight response, a moderate response and a marked response, respectively. Data are presented as the total score of these two responses.

Spontaneous locomotor activity

As described in our previous study (3), spontaneous locomotor activity was measured for 30 min immediately after CD, and DIO mice fed CD and HFD, respectively, for 16 weeks were placed in a new cage.

Elevated plus maze test

This test was performed in accordance with our previous study (27). The elevated plus maze (Muromachi Kikai Co., Ltd., Tokyo, Japan) was constructed of gray Plexiglas and consisted of four arms (length 300 mm, width 60 mm): two closed arms with high gray 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 h before the test, mice were transferred to a standby room (20 lux) that was separated from the test room. Experiments were performed between 13.00 and 15.00 h. 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 lux) for 5 min. Increased exploration of the relatively open arms is indicative of reduced anxiety-like behaviour 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.

Table 1. Metabolic Parameters in Control Diet (CD) and Diet-Induced Obese (DIO) Mice.

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

Results are presented as the mean ± SEM (n = 14). Significantly different from CD mice in each group: **P < 0.01.

Measurement of BDNF and NT-3 content in the brain

BDNF and NT-3 content in the brain of CD and DIO mice fed CD and HFD, respectively, for 16 weeks was measured in accordance with our previous study (3) using commercially available measurement kits for BDNF (BDNF Emax® ImmunoAssay System: Promega Inc., Madison, WI, USA) 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 in accordance with our previous study (3). Full-length TrkB and TrkC were detected using rabbit polyclonal anti-TrkB antibody (sc-8316; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and rabbit polyclonal anti-TrkC antibody (sc-14025; Santa Cruz Biotechnology, Inc.), respectively. Results represent the densitometry data relative to glyceraldehyde 3-phosphate dehydrogenase detected in each sample.

Statistical analysis

All values are provided as the mean ± SEM. Statistical analysis of the data was carried out by ANOVA followed by Dunnett's multiple-range test. $P < 0.05$ was considered statistically significant.

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 in CD mice. Plasma levels of glucose, insulin and leptin in DIO mice were significantly high compared to those in CD mice.

Fear-conditioning response

CD mice exhibited 93% freezing as a result of fear in the first session in the contextual conditioning response, and the freezing percentage gradually decreased during the sessions to reach 60% in the fifth 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 first session of the contextual fear response, and the freezing percentage subsequently decreased during the sessions to 23% in the fifth 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 to CD mice (Fig. 1).

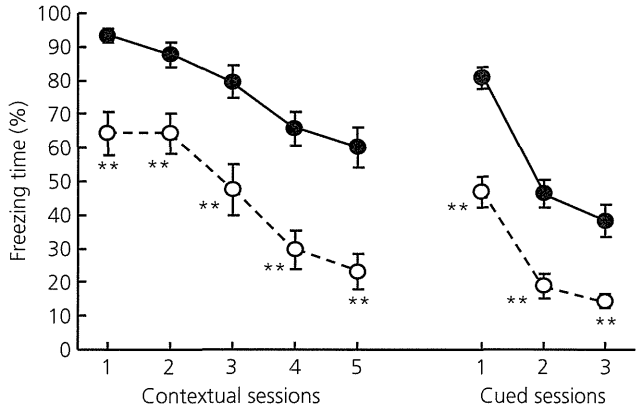

Fig. 1. Fear-conditioning responses in control diet (CD) and diet-induced obese (DIO) mice. Fear-conditioning responses in CD (closed circles) and DIO (open circles) mice. Freezing percentages of CD and DIO mice in the contextual conditioning test were measured every minute for 5 min. Freezing percentages of CD and DIO mice in the cued conditioning test were measured every minute for 3 min. Data points represent the mean ± SEM (n = 9–14). Significantly different from CD mice: *P < 0.05, **P < 0.01.

Jumping–vocalisation test, spontaneous locomotor activity and elevated plus maze test

To compare the sensitivities to foot shock between CD and DIO mice, the jumping–vocalisation test was used. No difference in scores of jumping–vocalisation 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 after placement of mice into new cages and behaviours in the elevated plus maze test were examined. Spontaneous locomotor activity was not different between CD and DIO mice after 16 weeks of feeding 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 content in the brain areas

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

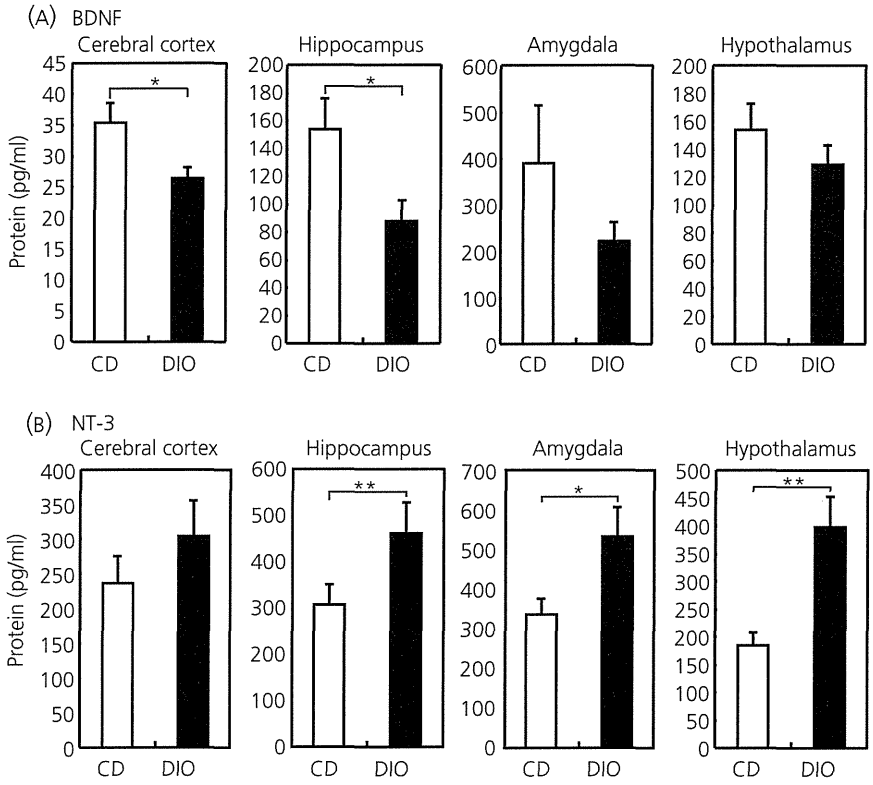

Fig. 2. Content of (A) brain-derived neurotrophic factor (BDNF) and (B) neurotrophin-3 (NT-3) in the cerebral cortex, hippocampus, amygdala and hypothalamus in control diet (CD) and diet-induced obese (DIO) mice. Results are presented as the mean ± SEM (n = 18–29). Significantly different from CD mice: *P < 0.05, **P < 0.01.

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, although not in the cerebral cortex, hippocampus and hypothalamus (Fig. 3A). Full-length TrkC expression in the four brain areas was not significantly different between CD and DIO mice (Fig. 3B).

Discussion

The present study demonstrated that DIO mice showed a significant reduction of both hippocampus-dependent contextual and amygdala-dependent cued fear responses of fear-conditioning test. However, the responses to electric foot shock, locomotor activity and anxiety-like behaviour of DIO mice were the same as those of CD mice. Interestingly, BDNF content in the cerebral cortex and hippocampus of DIO mice was significantly lower than that in CD mice, whereas NT-3 content in the hippocampus, amygdala and hypothalamus of DIO mice was significantly higher than that in CD mice. The expression of full-length TrkB for BDNF in the amygdala of DIO mice significantly decreased compared to that in CD mice, whereas the expression of full-length TrkC for NT-3 in the brain regions was not different between CD and DIO mice. These findings demonstrate that DIO mice display impaired cognition in the fear-conditioning test with an 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 (29–31).

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 (15). 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 (15). Although the mechanisms by which a HFD can affect BDNF expression are largely unknown, in the present study, the feeding of HFD or obesity led to a reduction of BDNF content in the hippocampus and cerebral cortex to the extent that cognitive performance was compromised. By contrast to the decrease in BDNF content, the present study demonstrated that NT-3 content was significantly increased in the hippocampus, amygdala and hypothalamus of DIO mice compared to that in CD mice. BDNF and NT-3 oppose one another in regulating

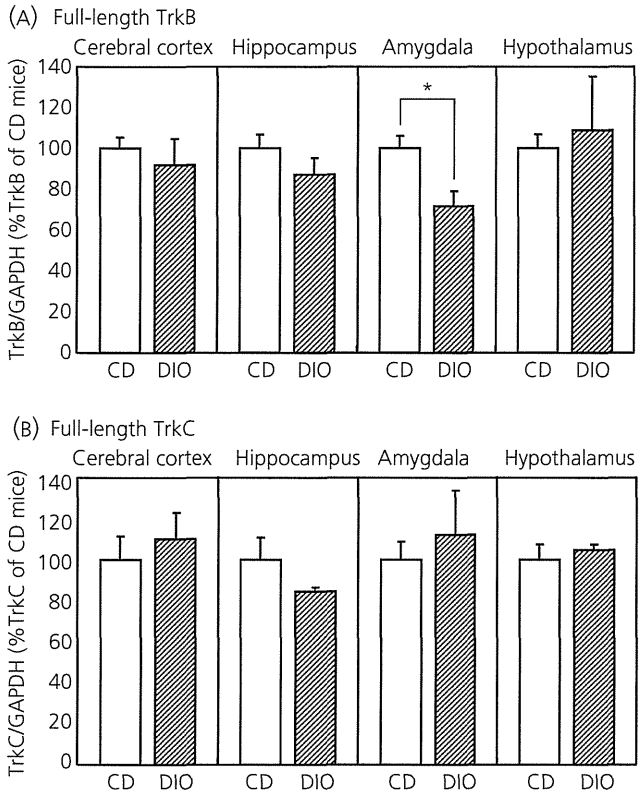

Fig. 3. Expression of full-length TrkB (A) and TrkC (B) in the cerebral cortex, hippocampus, amygdala and hypothalamus in control diet (CD) and diet-induced obese (DIO) mice. Results are presented as the mean ± SEM (n = 3–7). Significantly different from CD mice: *P < 0.05. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

the dendritic growth of pyramidal neurones 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 as playing 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, although the present study did not address the mechanisms responsible for changes in BDNF and NT-3 content in the brain of DIO mice.

Several lines of electrophysiological and behavioural evidence demonstrate that leptin and insulin enhance hippocampal synaptic plasticity and improve learning and memory (31,32). 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 compared to 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 (33). Therefore, it is likely that impairment of actions of leptin or insulin might be attributable to cognitive deficits in obesity and diabetes mellitus (34,35). Although there is no direct evidence for the impairment of cognition in DIO mice, the impaired cognitive behaviours of fear-conditioning tests observed in the present study may be partly mediated by decreased inherent functions of leptin and insulin in the brain, despite high plasma levels of leptin and insulin, giving rise to the 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 content decreases in the hippocampus and cerebral cortex of DIO mice, whereas NT-3 content increases in the hippocampus, amygdala and hypothalamus of DIO mice, compared to CD mice. The expression of TrkB in the amygdala of DIO mice decreases compared to CD mice. These findings suggest that consumption of a HFD may contribute to aspects of dysfunction in the central nervous system.

Acknowledgements

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Functional Magnetic Resonance Imaging Analysis of Food-Related Brain Activity in Patients with Lipodystrophy Undergoing Leptin Replacement Therapy

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Context: Lipodystrophy is a disease characterized by a paucity of adipose tissue and low circulating concentrations of adipocyte-derived leptin. Leptin-replacement therapy improves eating and metabolic disorders in patients with lipodystrophy.

Objective: The aim of the study was to clarify the pathogenic mechanism of eating disorders in lipodystrophic patients and the action mechanism of leptin on appetite regulation.

Subjects and Interventions: We investigated food-related neural activity using functional magnetic resonance imaging in lipodystrophic patients with or without leptin replacement therapy and in healthy controls. We also measured the subjective feelings of appetite.

Results: Although there was little difference in the enhancement of neural activity by food stimuli between patients and controls under fasting, postprandial suppression of neural activity was insufficient in many regions of interest including amygdala, insula, nucleus accumbens, caudate, putamen, and globus pallidus in patients when compared with controls. Leptin treatment effectively suppressed postprandial neural activity in many of these regions of interest, whereas it showed little effect under fasting in patients. Consistent with these results, postprandial formation of satiety feeling was insufficient in patients when compared with controls, which was effectively reinforced by leptin treatment.

Conclusions: This study demonstrated the insufficiency of postprandial suppression of food-related neural activity and formation of satiety feeling in lipodystrophic patients, which was effectively restored by leptin. The findings in this study emphasize the important pathological role of leptin in eating disorders in lipodystrophy and provide a clue to understanding the action mechanism of leptin in human, which may lead to development of novel strategies for prevention and treatment of obesity. ***(J Clin Endocrinol Metab* 97: 3663–3671, 2012)**

Lipodystrophy is a disease characterized by a paucity of adipose tissue due to genetic or acquired conditions that alter the ability to store triglyceride in adipose tissue (1–4). Patients with lipodystrophy have abnormally low circulating concentrations of adipocyte-derived leptin and frequently develop a wide range of met-

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Abbreviations: BMI, Body mass index; CGL, congenital generalized lipodystrophy; FDR, false discovery rate; fMRI, functional magnetic resonance imaging; ROI, region of interest; VAS, visual analog scale.

abolic disorders including insulin-resistant diabetes, hypertriglyceridemia, and fatty liver (1, 5, 6). Lipodystropic patients also exhibit eating disorders, which makes diet therapy difficult (7).

We and others have demonstrated that leptin-replacement therapy effectively improves metabolic disorders in patients with lipodystrophy (1, 8, 9). In this context, leptin was also shown to suppress appetite in lipodystrophic patients (7, 10). Leptin treatment decreased satiation time, *i.e.* the time to voluntary cessation of eating, and increased satiety time, *i.e.* the time to hunger sufficient to consume a full meal. However, there is no report on the comparison of eating behaviors between healthy subjects and patients with lipodystrophy. Therefore, the pathophysiological role of leptin in eating disorders in patients with lipodystrophy remains unclear.

Leptin is a hormone secreted by the adipocytes, which serves to communicate the status of body energy store to the central nervous system and controls eating behavior and energy expenditure (11–16). From experimental studies in human and animals, it has long been established that leptin suppresses energy intake mainly by acting on the hypothalamus (7, 17, 18). However, there is little information about how the neural networks including the hypothalamus are influenced by leptin signals. Recently the advent of functional neuroimaging techniques such as functional magnetic resonance imaging (fMRI) has been providing novel insights into homeostatic and hedonic aspects of human eating behavior. fMRI measurements of food-related neural activity in congenital leptin-deficient patients were reported (19–21). These studies revealed that leptin treatment modulates neural activity in reward- and food-related areas such as the ventral striatum and orbitofrontal cortex.

In the present study, to reveal the pathogenic mechanism of eating disorders in lipodystrophic patients, we measured food-related neural activity by fMRI scans and investigated subjective feelings of appetite under both fasting and postprandial conditions in patients and age- and sex-matched healthy subjects. In addition, we performed the same sequential analyses in the same patients with leptin-replacement therapy. Data from these experiments might provide useful notions to understand the pathological role of leptin in eating disorders associated with lipodystrophy and action mechanism of leptin on appetite regulation.

Materials and Methods

Subjects

Ten patients with lipodystrophy and 10 healthy subjects participated in the study. Among the 10 patients, six had congenital generalized lipodystrophy (CGL), two had acquired generalized lipodystrophy and the remaining two had Dunnigan-type partial lipodystrophy. Five of the six CGL patients were homozygous or compound heterozygous for mutations in the *seipin* gene (2). The etiology of the remaining CGL patient was unknown. One of the two patients with Dunnigan-type partial lipodystrophy was heterozygous for a mutation in the *LMNA* gene, whereas the other patient had an unknown etiology. For controls, age- and sex-matched healthy subjects with normal weight [body mass index (BMI) between 18.5 and 25.0 kg/m^2] were recruited. None of the control subjects had a past or present history of psychiatric, neurological, endocrine, metabolic, gastrointestinal, or eating disorders, and none was taking medications at the time of study. For both patients and controls, individuals with contraindications for magnetic resonance imaging scanning including claustrophobia and the presence of a cardiac pacemaker or other metallic fragments in the body were excluded. All the subjects had been stable at their body weight for at least 3 months before recruitment. Characteristics of all the subjects are summarized in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org. All the subjects were right-hand dominant according to the Edinburgh Handedness Inventory (22). The means of BMI and basal plasma leptin concentrations in patients were apparently lower than those in controls. All the patients had received leptin-replacement therapy as described below for more than 2 months. For patients, the entire study was conducted during their hospitalization period at Kyoto University Hospital. Study protocols were approved by the Ethical Committee of Kyoto University Graduate School of Medicine. After detailed explanation of the study design and any potential risks, written informed consent was obtained from all subjects before study initiation.

Leptin- replacement therapy

Recombinant methionyl human leptin (meterleptin) was provided by Amylin Pharmaceuticals, Inc. (San Diego, CA). Meterleptin was administered sc once a day at the physiological replacement dose on the basis of information provided by Amylin (1).

Study design

All the fMRI scans were performed at Kyoto University Hospital between 1300 and 1400 h under fasting and postprandial conditions on separate days (Supplemental Fig. 1A). For the fasting condition, subjects were prohibited from eating for 18 h from the night before the examination. For the postprandial condition, subjects ate a meal 1 h before the examination. In addition, fMRI scans were performed for patients with and without leptin treatment (leptin-on and leptin-off, respectively) under both fasting and postprandial conditions. For the leptin-off condition, leptin-replacement therapy was discontinued for more than 4 d. All the subjects were given practice trials outside the scanner and were familiarized with scanning procedures and safety regulations.

fMRI procedures

Blood oxygen level-dependent (BOLD) response to stimuli was measured by fMRI on a 3-Tesla Trio MRI scanner (Siemens, Erlangen, Germany). Whole-brain images were acquired in axial

orientation using the following parameters: repetition time, 3000 msec; echo time, 30 msec; flip angle, 90°; voxel size, 3 × 3 × 3 mm; field of view, 192 × 192 mm; matrix size, 64 × 64; and number of slices, 48. The experiment was conducted in three separate sessions of 18 min, 42 sec each. In each session, 45 food and 30 nonfood pictures were presented randomly in an event-related design (Supplemental Fig. 1B). Food pictures were chosen to suit each subject's taste based on preliminary hearing investigations and included various kinds of food, such as warm meals, desserts, fruits, and vegetables (Supplemental Fig. 1C). Nonfood pictures contained scenery comprising naturally occurring objects, such as trees, bushes, grass, rocks, water, and flowers (Supplemental Fig. 1B). Each picture was presented for 5 sec, followed by 3 sec for the rating image (Supplemental Fig. 1C). Although subjects were presented with rating image, they were asked to rate how much they liked to eat each food or how much they liked each nonfood picture on a scale of 1 (not so appealing) to 4 (highly appealing) by pressing a button with their dominant hand. Next, a mosaic picture was presented for 7 sec as a resting baseline. All pictures were projected onto a screen in the scanner room using Presentation version 9.6 software (Neurobehavioral Systems, Albany, CA) and viewed through a mirror mounted on the head coil. Subjects were instructed to focus all their attention on the pictures.

Image processing and statistical analysis of fMRI data

The fMRI data were preprocessed and statistically analyzed using SPM2 (Wellcome Department of Cognitive Neuroscience, University College London, London, UK) and MATLAB 6.5 (The Mathworks Inc., Natick, MA). Functional images were realigned to the first image and normalized into the Montreal Neurological Institute coordinate by an echo planar imaging template. Normalized images were then smoothed with a 6-mm full-width-at-half-maximum isotropic Gaussian kernel. The functional data were temporally filtered using an autoregressive model and a high pass filter with a cutoff of 128 sec. Five experimental conditions (food picture, nonfood picture, rating for food picture, rating for nonfood picture, and pressing button) were modeled by a function convolved with a hemodynamic response function in the general linear model, and an activation parameter was estimated at each voxel for each stimulus type. Significant signal changes were identified with a voxel-by-voxel analysis on the basis of a comparison of the mean signal amplitude during the periods of stimulation and those of resting baselines, as determined by *t* test comparisons. At the first level, a statistical parametric map for comparing brain activation to food greater than nonfood was generated for each subject and each condition. These contrast images were then entered into a second level random effect analysis. In the random effects analysis, one-sample *t* test resulted in images for within-group analysis. For between-group analysis, two-sample *t* tests created images for control *vs.* patient comparison, and paired *t* tests created images for leptin-on *vs.* leptin-off comparison. Finally, we transformed the *t* statistics into Z-scores and generated a Z-score map image. The Z-score maps were then superimposed onto the magnetic resonance images to allow visual inspection of the composite images. We set the significance threshold at $P < 0.05$, false discovery rate (FDR) corrected, for whole-brain analysis, and $P < 0.005$, uncorrected, for region of interest (ROI) analysis with a spatial extent of 10 contiguous voxels. For ROI analysis, brain regions known to be involved in energy homeostasis and appetite regulation were chosen on the basis of previous comparable fMRI studies (23–29). These regions included the hypothalamus, orbitofrontal cortex, amygdala, hippocampus, insula, nucleus accumbens, caudate, putamen, and globus pallidus. ROI were defined using the Wake Forest University Pickatlas (30) and the AAL Talairach Daemon atlas (Research Imaging Center, University of Texas Health Science Center, San Antonio, TX) (31). Regions that were unavailable in these libraries (*e.g.* nucleus accumbens) were drawn within the Wake Forest University Pickatlas using three-dimensional spheres centered at a voxel location determined based on a relevant fMRI study (23).

Measurement of subjective feelings

The participants were asked to provide subjective hunger ratings on a 100-mm visual analog scale (VAS) immediately before every scanning to assess their hunger feelings (32, 33). Higher scores indicated stronger hunger. In addition, appetite was also measured using the mean value of the rating scale for 135 food pictures while viewing them in the scanner. Higher values indicated stronger desire to eat the food in each picture.

Biochemical analyses

Blood samples were obtained in the fasting state. Plasma glucose concentrations were determined by a glucose oxidase method (Arkrey Marketing Inc., Tokyo, Japan), and plasma insulin concentrations were determined by use of an enzyme immunoassay method (TOSOH, Corp., Tokyo, Japan). Plasma leptin concentrations were determined by a competitive RIA method (Millipore Inc., Billerica, MA).

Statistical analysis

Differences between patients and controls in age, BMI, plasma leptin concentration, plasma glucose, and plasma insulin were determined using unpaired *t* tests. Differences between biochemical values under leptin-on and leptin-off conditions were determined by paired two-tailed *t* tests. Differences between patients and controls regarding VAS hunger scores and rating scores for food pictures were calculated using repeated measure ANOVA. $P < 0.05$ was considered statistically significant.

Results

Comparison of neural response to food-specific stimuli between healthy controls and patients with lipodystrophy

A within-group analysis of controls and patients for the contrast of food greater than nonfood revealed no significant activation in whole brain analysis at a significance level of $P < 0.05$ (FDR corrected). With a within-group ROI analysis for the contrast food greater than nonfood in healthy controls, significant activation was detected in the bilateral orbitofrontal cortex, amygdala, insula, caudate, putamen, and globus pallidus under the fasting conditions (Fig. 1A). However, significant activation was detected only in the bilateral orbitofrontal cortex and left insula under the postprandial conditions (Fig. 1B). On the other

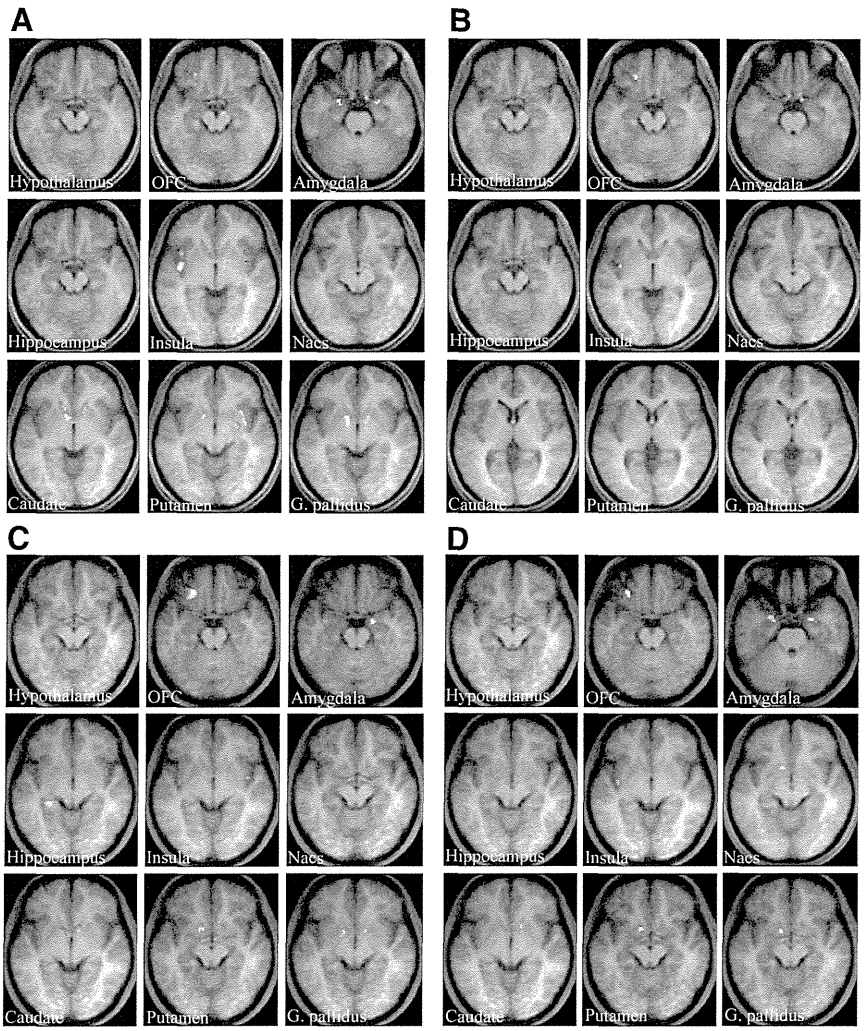

FIG. 1. Neural response to food-specific stimuli in healthy controls and leptin-off patients. Food-specific activations in ROI in the brains of controls (A and B) and patients (C and D) under fasting (A and C) and postprandial (B and D) conditions. Activation is overlaid onto the group average T1-weighted anatomical axial images (*right* is right side of the brain). The *brighter yellow color* represents the higher Z-score. ROI areas are the hypothalamus, orbitofrontal cortex (OFC), amygdala, hippocampus, insula, nucleus accumbens (Nacs), caudate, putamen, and globus pallidus (G. pallidus).

hand, in leptin-off patients, significant activation was detected in the left orbitofrontal cortex, right amygdala, left hippocampus, bilateral insula, bilateral caudate, left putamen, and bilateral globus pallidus under the fasting conditions (Fig. 1C). Significant activation was also detected in most of these areas under the postprandial conditions (Fig. 1D). Coordinates and maximum Z-scores in ROI areas under fasting and postprandial conditions in controls and patients are shown in Supplemental Table 2.

Next, we directly compared the contrast food greater than nonfood between controls and patients by a between-group ROI analysis (Table 1). Under the fasting conditions, a significant difference in activity was detected between controls and patients only in the left insula and left caudate. Activity was down-regulated in the left insula and up-regulated in the left caudate in patients compared with controls. On the other hand, under the postprandial conditions, a significant difference in activity was detected in many areas, including the right orbitofrontal cortex, right amygdala, left insula, left nucleus accumbens, bilateral caudate, left putamen, and left globus pallidus between controls and patients. Activity was up-regulated in all these areas except the right orbitofrontal cortex in patients.

These results indicate that the suppression of neuronal response to food-specific stimuli after a meal is attenuated in patients with lipodystrophy compared with healthy subjects.

Comparison of subjective feelings of appetite between healthy controls and patients with lipodystrophy

Subjective feelings of appetite were evaluated in healthy controls and leptin-off patients. Mean values of the self-reported hunger score on a 100-mm VAS were not significantly different between controls and patients under the fasting conditions (controls: 79.90 ± 4.11; patients: 87.50 ± 4.55) (Fig. 2A). In contrast, under the postprandial conditions, the score was significantly higher in patients than in controls (controls: 17.00 ± 3.09; patients: 53.0 ± 6.76). Consistent with the VAS results, mean values of rating scores for the 135 food pictures were also not different between controls and patients under the fasting conditions (controls: 3.11 ± 0.13; patients: 3.21 ± 0.20), but they tended to be higher in patients than in controls under the postprandial conditions (controls: 2.20 ± 0.24; patients: 2.78 ± 0.23) (Fig. 2B).

These results indicate that the formation of a satiety feeling after a meal is attenuated in patients with lipodystrophy compared with healthy subjects.

Effects of the leptin-replacement therapy on neural response to food-specific stimuli in patients with lipodystrophy

A within-group analysis of leptin-on patients for the contrast food greater than nonfood revealed no significant activation in whole brain analysis at a significance level of $P < 0.05$ (FDR corrected). With a within-group ROI anal-

TABLE 1. Between-group (controls *vs.* leptin-off patients) comparison of brain activations for the contrast food greater than nonfood

		Fasting				Postprandial			
		Coordinate				Coordinate			
Contrast	ROI area	x	y	z	Z-score	x	y	z	Z-score
Controls greater than patients (leptin-off)	Hypothalamus								
	Orbitofrontal cortex					36	44	−12	3.36
	Amygdala								
	Hippocampus								
	Insula	−42	−6	0	3.35				
	Nucleus accumbens								
	Caudate								
	Putamen								
	Globus pallidus								
Patients (leptin-off) greater than controls	Hypothalamus								
	Orbitofrontal cortex								
	Amygdala					22	−4	−22	2.92
	Hippocampus								
	Insula					−46	−12	12	3.10
	Nucleus accumbens					−8	10	−6	3.12
	Caudate					14	2	14	3.21
		−6	10	14	3.46	−8	8	−6	3.50
	Putamen					−10	8	−6	3.48
	Globus pallidus					−10	8	−4	3.41

Coordinate indicates the highest activity voxel of the cluster by Montreal Neurological Institute systems. Negative x-axis coordinates indicate left hemisphere. Z-score represents level of significance.

ysis for the contrast food greater than nonfood under the fasting conditions, significant activation was detected in many brain areas, such as the bilateral orbitofrontal cortex, bilateral amygdala, bilateral hippocampus, bilateral insula, right caudate, right putamen, and bilateral globus pallidus, in leptin-on patients (Fig. 3A). In contrast, neural activity under the postprandial conditions was effectively reduced and significant activation was detected only in the bilateral orbitofrontal cortex and left insula in leptin-on patients (Fig. 3B). Coordinates and maximum Z-scores in ROI areas under the fasting and postprandial conditions in leptin-on patients are shown in Supplemental Table 3.

Next, we directly compared the contrast food greater than nonfood between leptin-on and leptin-off patients by a between-group ROI analysis (Table 2). Under the fasting conditions, a significant difference in neural activity was detected between leptin-on and leptin-off patients only in the left caudate, in which the activity was down-regulated by leptin-replacement therapy in the patients. In contrast, a significant difference in activity was detected in many areas, including the right orbitofrontal cortex, left amygdala, left hippocampus, left insula, bilateral caudate, and left putamen, under the postprandial conditions. The activity was down-regulated in all these areas except the right orbitofrontal cortex by leptin-replacement therapy.

These results indicate that leptin-replacement therapy enhances the suppression of neural response to food-specific stimuli after meal in patients with lipodystrophy.

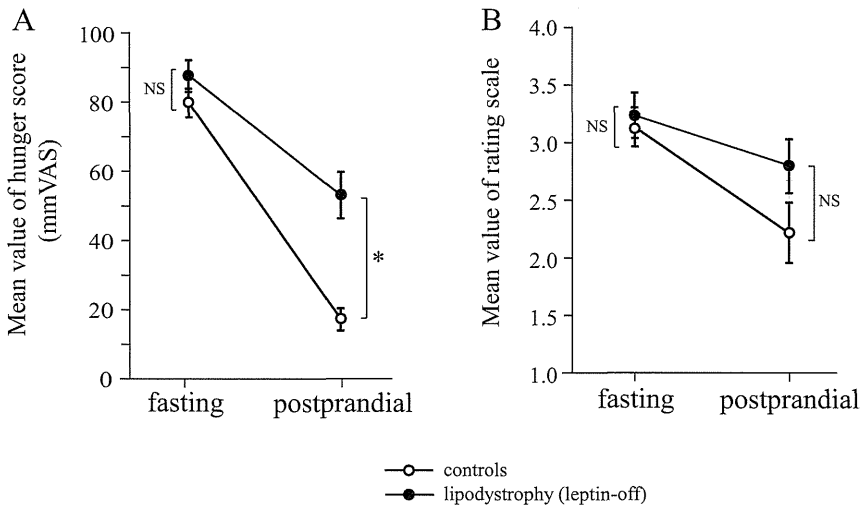

FIG. 2. Subjective feelings of appetite under fasting and postprandial conditions in healthy controls and leptin-off patients. A, Hunger scores on the 100-mm VAS before fMRI scan. B, Mean value of rating scores for food pictures during the fMRI scan. Data are means ± SEM (n = 10 in each group). *, $P < 0.01$ (repeated measure ANOVA).

Effects of the leptin-replacement therapy on subjective feelings of appetite in patients with lipodystophy

We compared subjective feelings of appetite between leptin-on and leptin-off

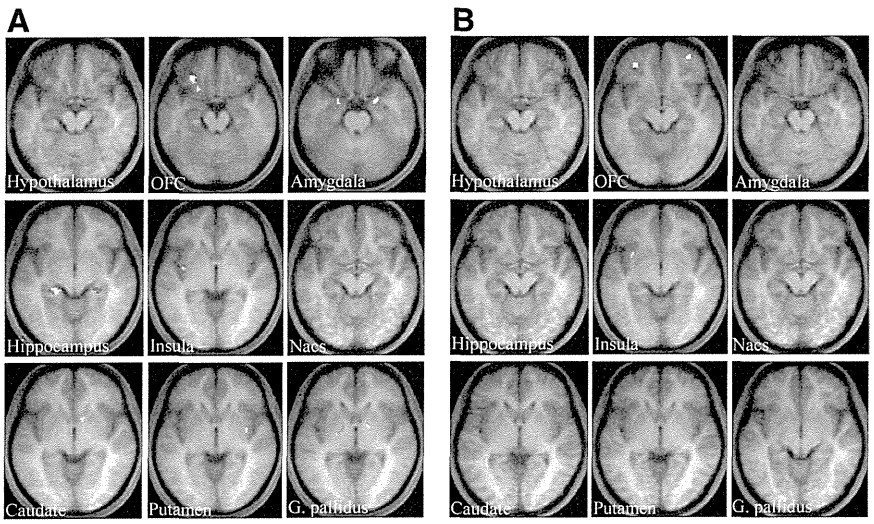

FIG. 3. Neural response to food-specific stimuli in leptin-on patients. Food-specific activations in ROI in the brain under fasting (A) and postprandial (B) conditions. Activation is overlaid onto the group average T1-weighted anatomical axial images (*right* is right side of the brain). The *brighter yellow color* represents the higher Z-score. ROI areas are the same as described in Fig. 1.

patients. Although plasma leptin levels were significantly higher in leptin-on than in leptin-off patients, plasma glucose and insulin levels were not affected by the discontinuation of leptin-replacement therapy for approximately 4 d (Supplemental Table 4). Mean values of self-reported hunger score on a 100-mm VAS were not significantly different between leptin-on and leptin-off patients under the fasting conditions (leptin-on: 83.10 ± 4.40; leptin-off: 87.50 ± 4.55) (Fig. 4A). In contrast, the score was significantly higher in leptin-off than in leptin-on patients (leptin-on: 27.70 ± 5.39; leptin-off: 53.0 ± 6.76) under the postprandial conditions. Consistent with the VAS results, mean values of rating scores for the 135 food pictures were also not different between leptin-on and leptin-off patients under the fasting conditions (leptin-on: 3.17 ± 0.17; leptin-off: 3.21 ± 0.20), but they tended to be higher in the leptin-off than in the leptin-on patients under the postprandial conditions (leptin-on: 2.40 ± 0.26; leptin-off: 2.78 ± 0.23) (Fig. 4B).

These results indicate that leptin-replacement therapy enhances the formation of satiety after meal in patients with lipodystrophy. These results were consistent with the results of fMRI analysis.

Discussion

This is the first report that demonstrates the difference in food-related neural activity between patients with lipodystrophy and healthy controls. A significant difference in food-related neural activity between patients and controls was detected in many brain areas under the postprandial

TABLE 2. Between-group (leptin-on *vs.* leptin-off patients) comparison of brain activations for the contrast food greater than nonfood

		Fasting				Postprandial			
		Coordinate				Coordinate			
Contrast	ROI area	x	y	z	Z-score	x	y	z	Z-score
Leptin-on greater than leptin-off	Hypothalamus								
	Orbitofrontal cortex					32	48	−10	2.98
	Amygdala								
	Hippocampus								
	Insula								
	Nucleus accumbens								
	Caudate								
	Putamen								
	Globus pallidus								
Leptin-off greater than leptin-on	Hypothalamus								
	Orbitofrontal cortex								
	Amygdala					−22	0	−20	2.98
	Hippocampus					−18	−8	−16	3.19
	Insula					−42	−16	10	4.26
	Nucleus accumbens								
	Caudate					6	8	−8	3.46
		−4	6	0	3.02	−4	6	−8	3.34
	Putamen					−8	8	−8	2.90
	Globus pallidus								

Coordinate indicates the highest activity voxel of the cluster by Montreal Neurological Institute systems. Negative x-axis coordinates indicate left hemisphere. Z-score represents level of significance.

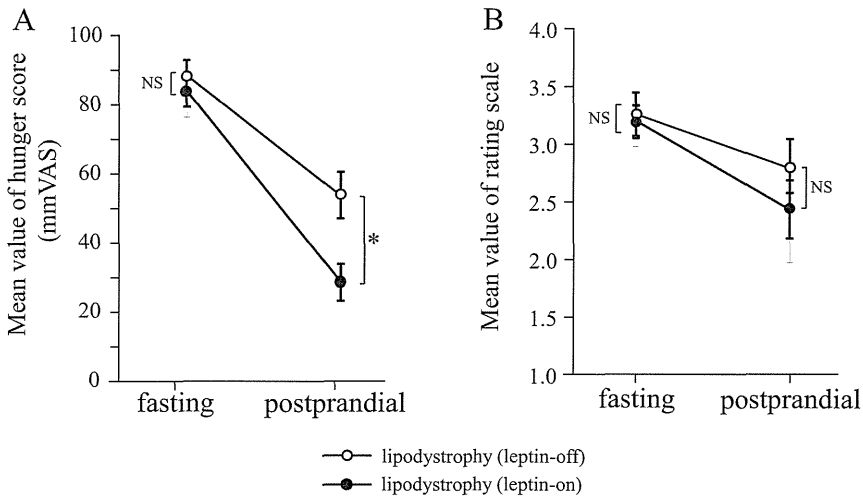

FIG. 4. Subjective feelings of appetite under fasting and postprandial conditions in patients with leptin-on and leptin-off. A, Hunger scores on the 100-mm VAS before the fMRI scan. B, Mean value of rating scores for food pictures during the fMRI scan. Data are means ± SEM (n = 10 in each group). *, $P < 0.01$ (repeated measure ANOVA).

conditions but in only a few brain areas under the fasting conditions (Table 1 and Supplemental Table 2 and Fig. 1). In addition, leptin-replacement therapy effectively restored neural activity in many brain areas under the postprandial conditions in patients with lipodystrophy (Table 2 and Supplemental Table 3 and Fig. 3).

The present study also indicates that leptin deficiency in patients accounts for a large part of the difference in postprandial neural activity in response to food stimuli between patients and controls. Indeed, in direct comparison between leptin-on patients and healthy controls (data not shown), a significant difference in food-related neural activity was detected only in the left globus pallidus, even under the postprandial condition. Alternatively, differences in neural activity in the globus pallidus may be due to factors other than leptin.

In the present study, we found that leptin treatment increased food-related neural activity in the orbitofrontal cortex, a region involved in satiety or the receipt of food reward (34–36), and suppressed activity in regions involved in hunger or the anticipation of food reward such as the amygdala, hippocampus, insula, caudate, and putamen (37–40) in patients under the postprandial conditions. In individuals with congenital leptin deficiency, leptin treatment also increased neural activity in the orbitofrontal cortex and reduced activity in the striatum, insula, amygdala, and substantia nigra/ventral tegmental area (19–21). Although results from the present study are not fully consistent with results from these previous reports on congenital leptin deficiency (19–21), they are consistent in that leptin enhances the neural activity in the regions involved in satiety and suppresses activity in regions involved in hunger (31). Furthermore, the present study demonstrates that leptin does not affect food-related neural activity in these regions under the fasting conditions.

This is also the first report that demonstrates the difference in appetite between patients with lipodystrophy and healthy controls. Consistent with neural activity, postprandial satiety was significantly reduced in patients compared with controls (Fig. 2), whereas there was no apparent difference in hunger under the fasting. Because leptin-replacement therapy effectively increased postprandial satiety and did not affect hunger under the fasting in patients (Fig. 4), leptin deficiency in patients accounts for a large part of the difference in postprandial satiety between patients and controls.

In the present study, to avoid the secondary effects of long-term leptin treatment such as changes in plasma glucose and insulin levels, fMRI scans and measurement of subjective feelings in leptin-off patients were performed within a short time after the discontinuation of leptin treatment. In patients who had been receiving leptin treatment for at least 2 months, no significant changes in glucose and insulin levels were observed after 4 d of discontinuation (Supplemental Table 4). Therefore, changes in food-related neural activity or feelings of appetite caused by leptin treatment were considered to be acute effects of leptin in this study.

The primary advantage of the present study lies in its imaging task methodology. First, the subjects were presented with 225 images during scanning, which was probably greater in numbers than those in any other previous studies. We also selected food pictures on the basis of an individual's food preference to maximize the saliency value of the food stimulus as a reinforcer for the subjects. Second, we used an event-related design in the imaging task to minimize habituation to each stimulus. Third, the subjects were instructed to press buttons to rate stimuli while viewing the rating images, not food or nonfood images. Thus, performance-related activation in the motor cortex (decision making, control mechanisms) was minimized during identification of neural activity elicited by the stimulus. Fourth, rating tasks were performed not only for food but also for nonfood stimuli. Therefore, the intensity of attention paid to stimuli was likely to have been comparable during food and nonfood picture presentation, which enabled us to disregard an effect arising from variance in attention while viewing, when we analyzed the contrast food greater than non-

food. We believe that these methodologies increased the reliability of obtained results.

Despite its many advantages, this study has some limitations. First, because of the relatively small sample size and genetic or phenotypic heterogeneity of the sample, statistical power was not sufficient. Second, we did not operate a diet and lifestyle standardization of the subjects sufficiently, which might affect their activity of reward systems. Our results need to be confirmed by further studies with a larger sample number and more homogeneous and standardized group of subjects. Furthermore, no significant blood oxygen level-dependent changes were observed in whole-brain analysis with a threshold of $P < 0.05$ (FDR corrected). Therefore, we used conservative analytic techniques and limited our investigation to ROI and possibly too liberal statistical thresholds. Besides our ROI, there must be many other brain regions, which are involved in feeding behaviors and are altered in patients with lipodystrophy. Additional whole-brain analysis with a larger sample number and more homogeneous and standardized group of subjects is required to accomplish this goal.

In conclusion, the present study using fMRI demonstrated the insufficiency of postprandial suppression of food-related neural activity and formation of satiety feeling in patients with lipodystrophy, which might be largely due to leptin deficiency. This study also demonstrated that leptin has little involvement in the regulation of neural activity and eating behavior under fasting, whereas leptin plays a significant role in these regulations under postprandial condition. The notion provided in the present study including information on ROI regulated by leptin might be useful for understanding the neural networks affected in obesity and eating disorder in leptin-deficient state and guiding the development of new pharmaceuticals for these conditions.

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