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

Contrast	ROI area	Fasting Coordinate				Postprandial Coordinate			
		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.

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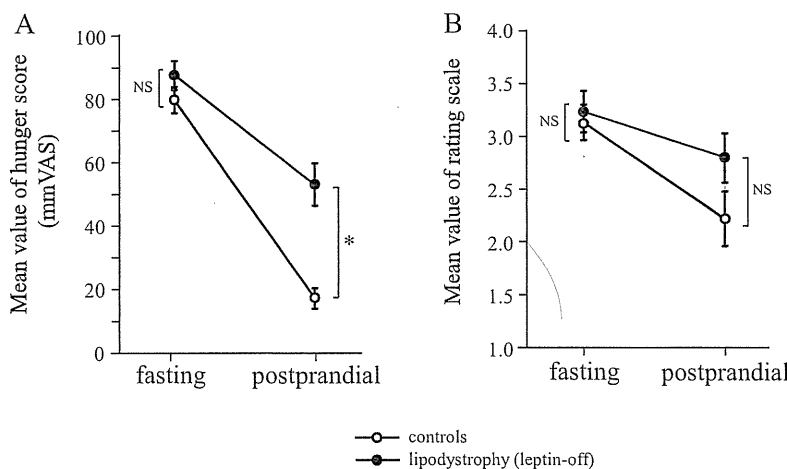
FIG. 2. Subjective feelings of appetite under fasting and postprandial conditions in healthy controls and leptin-off patients. A, Hunger scores on the 100-mm VAS before fMRI scan. B, Mean value of rating scores for food pictures during the fMRI scan. Data are means ± SEM (n = 10 in each group). *, $P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

A   **B**
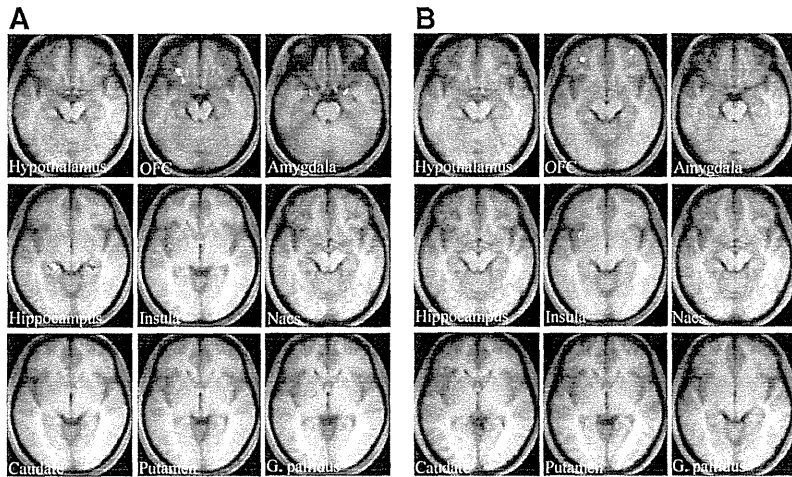
FIG. 3. Neural response to food-specific stimuli in leptin-on patients. Food-specific activations in ROI in the brain under fasting (A) and postprandial (B) conditions. Activation is overlaid onto the group average T1-weighted anatomical axial images (*right* is right side of the brain). The *brighter 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

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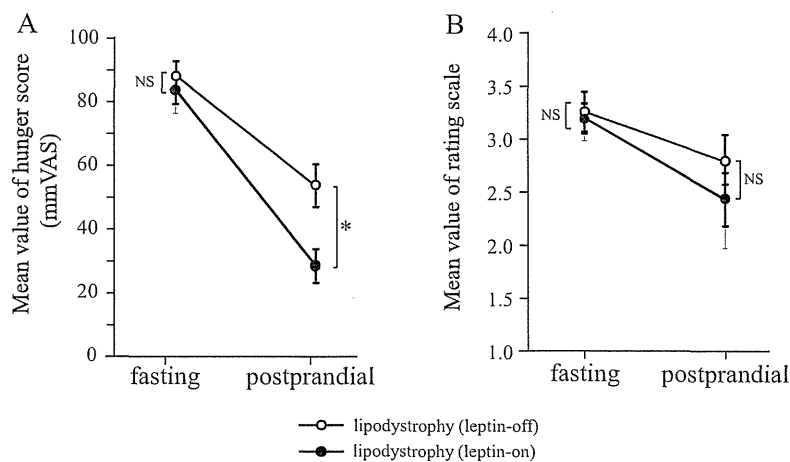
FIG. 4. Subjective feelings of appetite under fasting and postprandial conditions in patients with leptin-on and leptin-off. A, Hunger scores on the 100-mm VAS before the fMRI scan. B, Mean value of rating scores for food pictures during the fMRI scan. Data are means ± SEM (n = 10 in each group). *,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

Acknowledgments

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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-

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.

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 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.

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

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

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

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

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. 1C). In addition, L/A coadministration significantly decreased respiratory exchange ratio in the dark phase, indicating increased utilization of fat as the fuel source (Fig. 1D).

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. 2A). 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. 2B). 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. 2C).

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. 2D). Consistent with these findings, the glucose AUC after insulin injection was decreased only in L/A-treated mice (Fig. 2E).

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
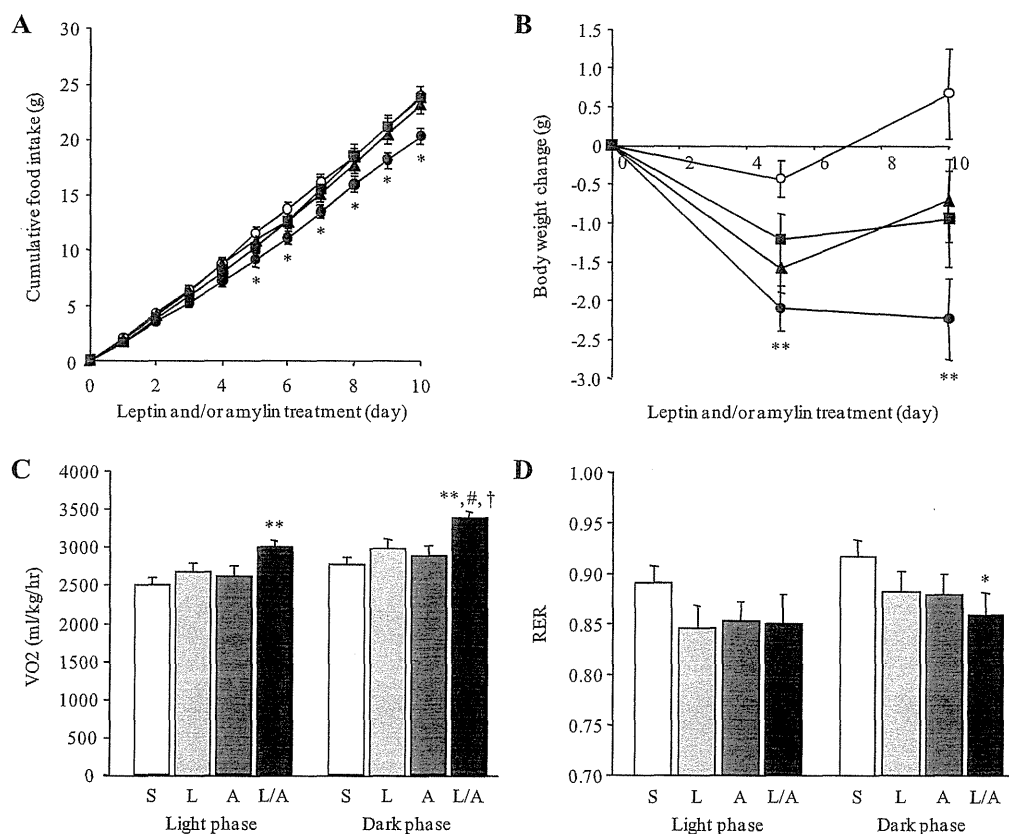

Fig. 1. Effect of leptin and/or amylin on food intake, body weight, energy expenditure, and respiratory exchange ratio (RER) in diet-induced obese (DIO) mice. Cumulative food intake (A) and change in body weight (B) during the treatment in saline- (S; ○), leptin- (L; ■), amylin- (A; ▲), and leptin + 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.
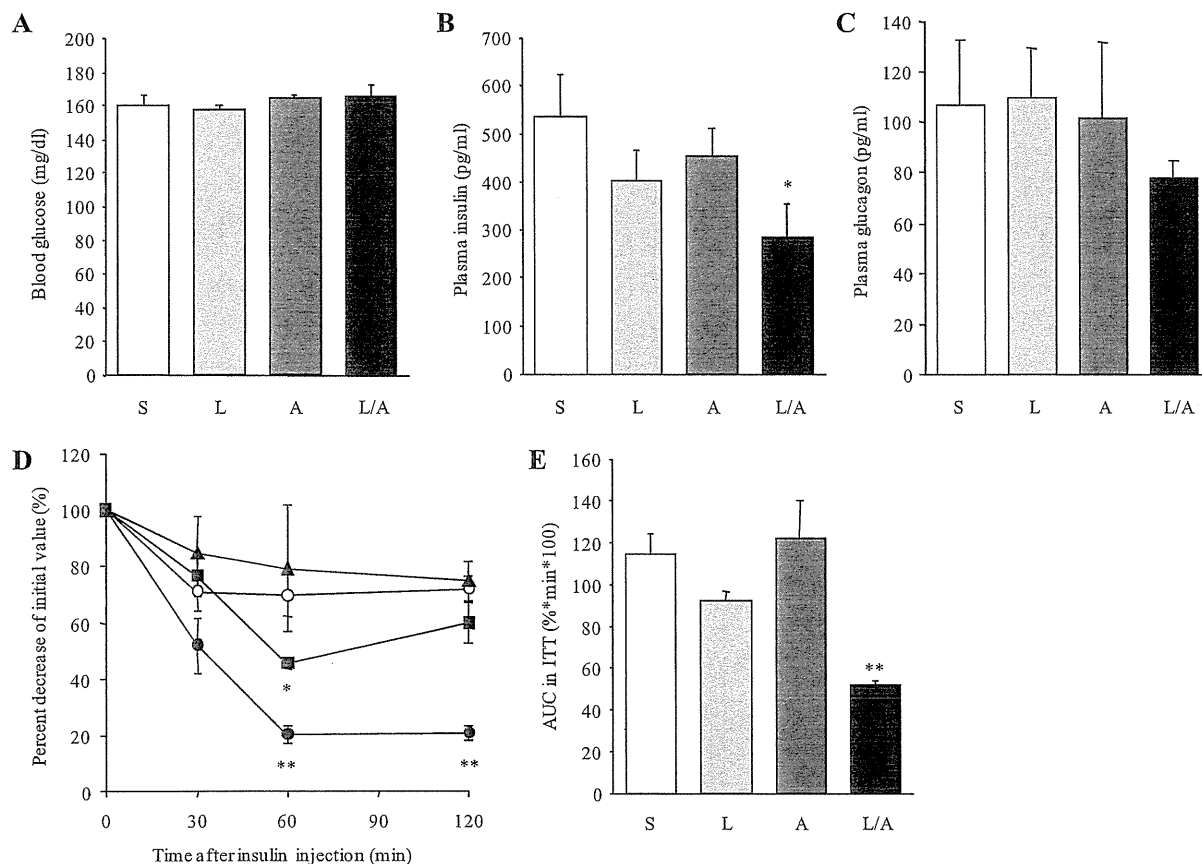
Fig. 2. Effect of L and/or A on glucose metabolism in DIO mice. Blood glucose (A), plasma insulin (B), and plasma glucagon levels (C) under ad libitum feeding on *day 14* in S, L, A, and L/A-treated mice. Values are means ± SE ($n = 8-9$/group). %Change of initial value of blood glucose levels (D) and 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. 3A). 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. 3D). 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. 3E), 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. 4A). 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. 4A). 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-
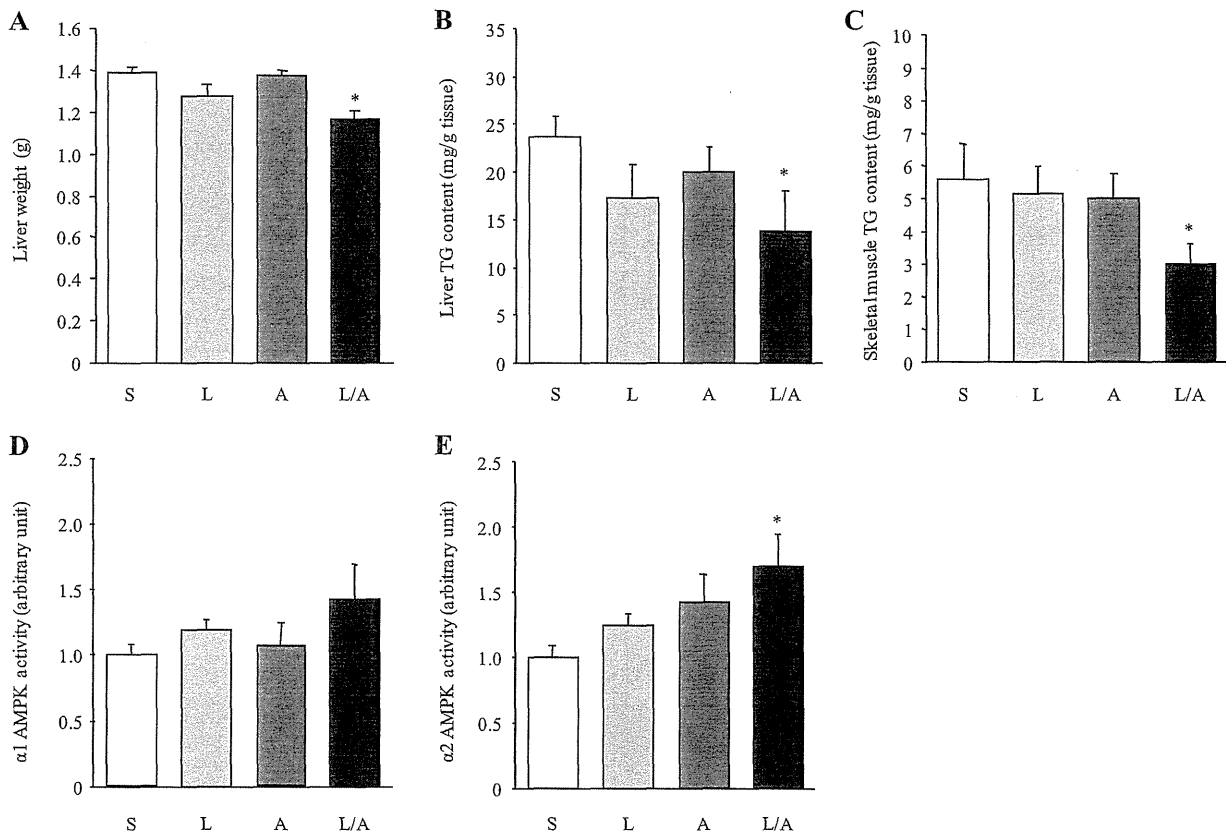
Fig. 3. Effect of L and/or A on tissue triglyceride (TG) content and skeletal muscle AMP-activated protein kinase (AMPK) activity in DIO mice. Liver size (A) and liver (B) and gastrocnemius muscle (C) TG contents on *day 14* in S, L, A, and L/A-treated mice. AMPKα1 (D) and AMPKα2 activity (E) on *day 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 μg·kg^{-1}·day^{-1}) and A (100 μg·kg^{-1}·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 μg·kg^{-1}·day^{-1}) and A (0–50 μg·kg^{-1}·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 μg·kg^{-1}·day^{-1}) and A (100 μg·kg^{-1}·day^{-1}) in the present study according to the first report (34). Administration of L (500 μg·g^{-1}·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 μg·kg^{-1}·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
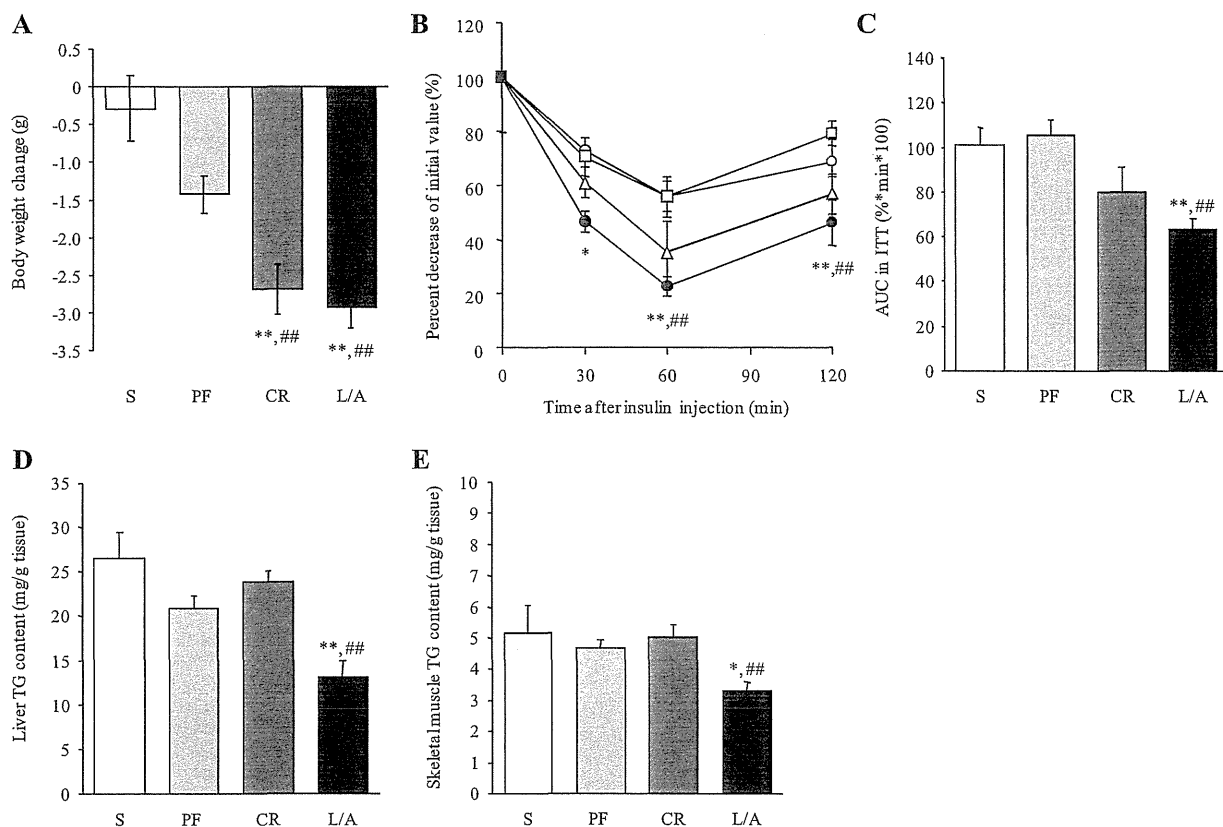
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Fig. 4. Pair-feeding and weight-matched calorie restriction experiments. *A*: change in body weight on *day 10* in S, saline + pair-fed L/A-treated (PF), weight-matched DIO (CR), and L/A-treated mice. %Decrease of initial value of blood glucose levels (*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 re-

stored 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. 1C). 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.

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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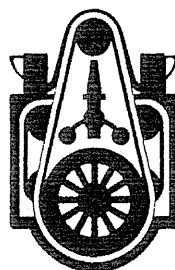
Intracerebroventricular Administration of C-Type Natriuretic Peptide Suppresses Food Intake via Activation of the Melanocortin System in Mice

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C-type natriuretic peptide (CNP) and its receptor are abundantly distributed in the brain, especially in the arcuate nucleus (ARC) of the hypothalamus associated with regulating energy homeostasis. To elucidate the possible involvement of CNP in energy regulation, we examined the effects of intracerebroventricular administration of CNP on food intake in mice. The intracerebroventricular administration of CNP-22 and CNP-53 significantly suppressed food intake on 4-h refeeding after 48-h fasting. Next, intracerebroventricular administration of CNP-22 and CNP-53 significantly decreased nocturnal food intake. The increment of food intake induced by neuropeptide Y and ghrelin was markedly suppressed by intracerebroventricular administration of CNP-22 and CNP-53. When SHU9119, an antagonist for melanocortin-3 and melanocortin-4 receptors, was coadministered with CNP-53, the suppressive effect of CNP-53 on refeeding after 48-h fasting was significantly attenuated by SHU9119. Immunohistochemical analysis revealed that intracerebroventricular administration of CNP-53 markedly increased the number of c-Fos–positive cells in the ARC, paraventricular nucleus, dorsomedial hypothalamus, ventromedial hypothalamic nucleus, and lateral hypothalamus. In particular, c-Fos–positive cells in the ARC after intracerebroventricular administration of CNP-53 were coexpressed with α-melanocyte-stimulating hormone immunoreactivity. These results indicated that intracerebroventricular administration of CNP induces an anorexigenic action, in part, via activation of the melanocortin system. *Diabetes* **62**:1500–1504, 2013

C -type natriuretic peptide (CNP) is a member of the natriuretic peptide family and has been demonstrated to be abundantly present in the brain, interestingly in discrete hypothalamic areas, such as the arcuate nucleus (ARC) of the hypothalamus, that play pivotal roles in energy regulation (1–3). Two predominant molecular forms of CNP in the porcine brain were reported to be a 22-residue peptide (CNP-22) and its N-terminally elongated 53-residue peptide (CNP-53) (1). Moreover, natriuretic peptide receptor-B (NPR-B), a CNP receptor, is also widely distributed in the brain and is reported to be abundantly expressed in the ARC of the

hypothalamus (4,5). These findings indicate the possibility that the brain CNP/NPR-B system may regulate energy homeostasis.

In the current study, we examined the effects of intracerebroventricular administration of CNP on food intake induced by refeeding after fasting and by orexigenic peptides, such as neuropeptide Y (NPY) and ghrelin. Also, we examined the involvement of the melanocortin system in the CNP actions.

RESEARCH DESIGN AND METHODS

Animals and diets. Male C57BL/6J mice (6 weeks old) obtained from Japan SLC (Shizuoka, Japan) were housed in plastic cages in a room kept at a room temperature of 23 $\pm$ 1°C and a 12:12-h light–dark cycle (lights turned on at 9:00 A.M.). The mice had ad libitum access to water and food (CE-2; CLEA Japan, Tokyo, Japan). All experiments were performed at 10 weeks of age 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 optimize comfort and to minimize the use of animals.

Peptides. CNP-22, CNP-53, ghrelin, and NPY were purchased from Peptide Institute (Osaka, Japan). SHU9119 was purchased from Bachem AG (Bubendorf, Switzerland).

Intracerebroventricular injection. Intracerebroventricular injection was performed according to our previous report (6).

Measurement of food intake

Fasting-refeeding. Mice were fasted for 48 h and then refed for 4 h. Water was available ad libitum during the experiments. The intracerebroventricular or intraperitoneal administration of CNP-22 or CNP-53 was performed just before refeeding. Food intake was measured for 4 h of refeeding. At the end of experiments, the hypothalamus was collected for examination of the expressions of mRNA for neuropeptides (7).

Nocturnal food intake. To assess the effect of intracerebroventricular administration of CNP-22 or CNP-53 on nocturnal food intake, peptides were injected intracerebroventricularly 1 h before the beginning of the dark phase. Food intake was measured for 15 h after intracerebroventricular injection. Water was available ad libitum during the experiments.

Food intake induced by NPY and ghrelin. The experiments were performed from 11:00 A.M. to 3:00 P.M. CNP-22 or CNP-53 was intracerebroventricularly administrated just before intracerebroventricular injection of NPY (5 nmol/mouse) or intraperitoneal injection of ghrelin (100 nmol/kg). Food intake was measured for 4 h after peptide injection. In these experiments, food and water were available ad libitum.

PCR. The extraction of mRNA and quantitative real-time RT-PCR were performed according to our previous report (8). Primers for preopiomelanocortin, cocaine and amphetamine-related peptide, *NPY*, agouti gene-related peptide (*AgRP*) and glyceraldehyde 3-phosphate dehydrogenase are shown in Supplementary Table 1.

Immunohistochemistry for c-Fos and α-MSH in the hypothalamus. The immunohistochemical methods and the stereotaxic coordinates for the hypothalamic nuclei were based on our previous report (6). Briefly, mice were anesthetized with pentobarbital at 1 h after intracerebroventricular injection of CNP-53 (1.5 nmol/mouse) and perfused with 50 mL 0.1 mol/L PBS, followed by 50 mL ice-cold 4% paraformaldehyde in 0.1 mol/L PBS. Sections of 30-μm thickness were cut with a cryostat. According to the mouse brain atlas (9), cross-sections were selected in correspondence to -1.70 mm [ARC, lateral hypothalamus (LH), dorsomedial hypothalamus (DMH), ventromedial hypothalamic

nucleus (VMH)] and to −0.82 mm [paraventricular nucleus (PVN)], relative to bregma. For c-Fos and α-melanocyte–stimulating hormone (α-MSH) protein staining, the sections were incubated with antic-Fos rabbit antibody (Ab-5; 1:5,000; Oncogene Science, Cambridge, MA) and antiα-MSH sheep antibody (AB5087; 1:10,000; EMD Millipore, Billerica, MA), respectively. The antibody was detected using the Vectastain ABC Elite kit (PK-6101; Vector Laboratories, Burlingame, CA) and a diaminobenzidine substrate kit (SK-4100; Vector Laboratories) was used for visualization. The second antibodies for fluorescence visualization used were goat anti-rabbit488 (A11008; 1:200; Life Technologies, Carlsbad, CA) for antic-Fos rabbit antibody and goat anti-sheep546 (A21098; 1:200; Life Technologies) for antiα-MSH sheep antibody.

Data analysis. All values are given as the mean ± SEM. Statistical analysis of the data were performed by ANOVA, followed by the Tukey-Kramer test. Statistical significance was defined as $P < 0.05$.
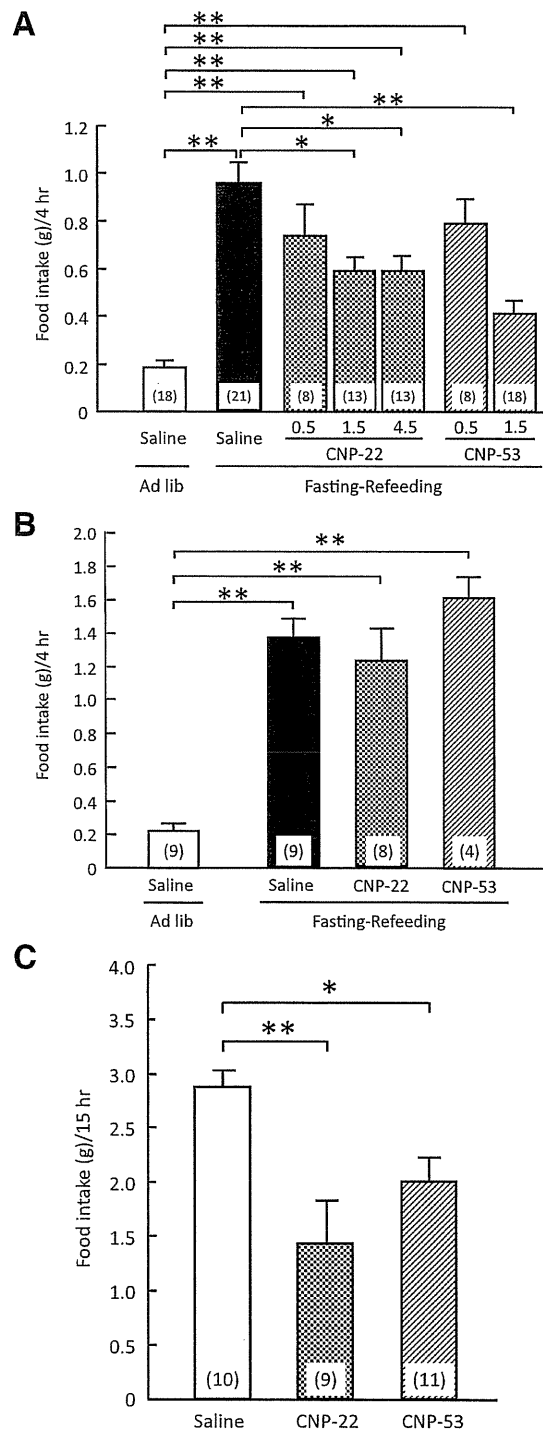
RESULTS

Effects of intracerebroventricular administration of CNP-22 and CNP-53 on food intake at refeeding after fasting. The intracerebroventricular administration of CNP-22 (1.5 and 4.5 nmol/mouse) and CNP-53 (1.5 nmol/mouse) significantly suppressed food intake during 4-h refeeding after 48-h fasting in comparison with data from saline-treated mice (Fig. 1A). In this experiment, CNP-53 (1.5 nmol), but not other treatments, induced significant reduction of body weight compared with saline treatment (Supplementary Table 2). The mRNA expressions of pre-opiomelanocortin and cocaine and amphetamine-related peptide significantly decreased, and the mRNA expressions of *NPY* and *AgRP* significantly increased after refeeding compared with control animals (Supplementary Fig. 1). The intracerebroventricular administration of CNP-53 did not influence the mRNA expressions of these neuropeptides in the hypothalamus (Supplementary Fig. 1). Next, the peripheral action of CNP on food intake was examined when a 10-fold greater dose than intracerebroventricular injection of each CNP was intraperitoneally administered. The intraperitoneal administrations of CNP-22 (1.5 μmol/kg) and CNP-53 (0.5 μmol/kg) did not change the food intake during 4-h refeeding after 48-h fasting (Fig. 1B), nor were there changes in body weight (Supplementary Table 3).
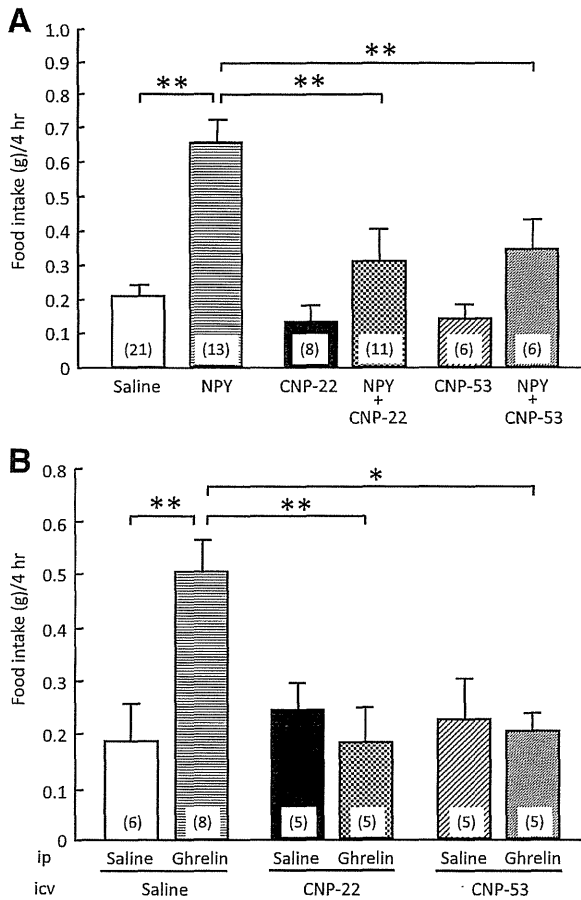
The intracerebroventricular administrations of CNP-22 (4.5 nmol/mouse) and CNP-53 (1.5 nmol/mouse) at 1 h before the start of the dark phase significantly suppressed nocturnal food intake compared with saline treatment (Fig. 1C).

Effect of intracerebroventricular administration of CNP-22 and CNP-53 on NPY-induced and ghrelin-induced food intake. When CNP-22 (4.5 nmol/mouse) and CNP-53 (1.5 nmol/mouse) were concomitantly administered intracerebroventricularly with NPY, they significantly suppressed the food intake induced by NPY compared with that of saline treatment (Fig. 2A). When CNP-22 (4.5 nmol/mouse) and CNP-53 (1.5 nmol/mouse) were administered intracerebroventricularly with ghrelin, they significantly suppressed the food intake induced by ghrelin compared with that of saline treatment (Fig. 2B).

Effect of melanocortin receptor antagonist, SHU9119, on the anorectic effect of CNP. To examine its involvement in the anorectic effect of CNP, SHU9119 was administered intracerebroventricularly together with CNP-53 (1.5 nmol/mouse). SHU9119 (1 nmol/mouse) significantly attenuated the suppressive action of CNP-53 on the food intake during 4-h refeeding after 48-h fasting, whereas SHU9119 itself significantly enhanced the increase of food intake in comparison with mice administered saline treatment (Fig. 3).
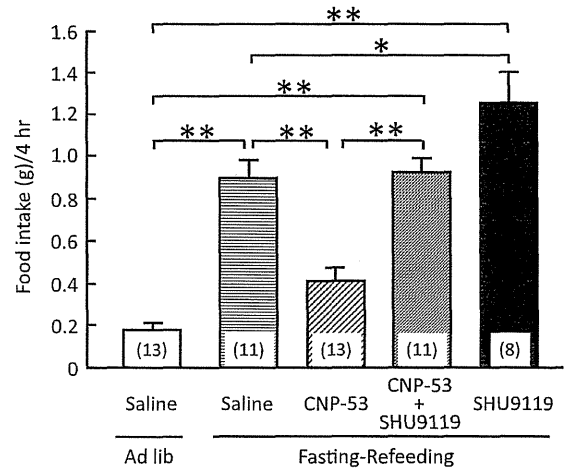

FIG. 1. Effects of CNP on refeeding after fasting. *A*: Effects of intracerebroventricular administration of CNP-22 (0.5, 1.5, and 4.5 nmol/mouse) and CNP-53 (0.5 and 1.5 nmol/mouse) on 4-h refeeding after 48-h fasting in mice. Food intake was observed for 4 h after refeeding. *B*: Effects of intraperitoneal administration of CNP-22 (1.5 μmol/kg) and CNP-53 (0.5 μmol/kg) on 4-h refeeding after 48-h fasting in mice. Food intake was observed for 4 h after refeeding. *C*: Effects of intracerebroventricular administration of CNP-22 (4.5 nmol/mouse) and CNP-53 (1.5 nmol/mouse) on nocturnal food intake in mice. Food intake was observed for 15 h after intracerebroventricular injection. Data represent mean ± SEM. The number of mice is given in parentheses. Significant differences: *P < 0.05, **P < 0.01.

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A

B

FIG. 2. Effects of CNP-22 and CNP-53 on food intake induced by NPY and ghrelin. *A*: Effects of intracerebroventricular administration of CNP-22 (4.5 nmol/mouse) and CNP-53 (1.5 nmol/mouse) on NPY-induced (5 nmol/mouse, intracerebroventricular) food intake in mice. Food intake was observed for 4 h after coadministration of NPY and CNP. *B*: Effects of intracerebroventricular administration of CNP-22 (4.5 nmol/mouse) and CNP-53 (1.5 nmol/mouse) on ghrelin-induced (100 nmol/kg, intraperitoneal) food intake in mice. Food intake was observed for 4 h after coadministration of ghrelin and CNP. Data represent mean ± SEM. The number of mice is given in parentheses. Significant differences: *$P < 0.05$, **$P < 0.01$.

c-Fos–immunoreactive cells in the hypothalamus after intracerebroventricular administration of CNP. To understand the neuronal pathway involved in the anorectic actions of CNP, the expression of c-Fos, one of the markers of neuronal activation, was monitored by immunohistochemical examination at 1 h after intracerebroventricular injection of CNP-53 (1.5 nmol/mouse). The numbers of c-Fos–immunoreactive cells in the ARC, PVN, and DMH were predominantly increased after intracerebroventricular injection of CNP-53 in comparison with saline treatment (Fig. 4*A*). The c-Fos–positive cells were also moderately increased in the VMH and LH (Fig. 4*A*). Next, we examined whether c-Fos immunoreactivity coexisted with α-MSH–containing cells. In the ARC of saline-treated mice, only a few α-MSH–immunoreactive cells showed weak c-Fos immunoreactivity (Fig. 4*B*). However, c-Fos–immunoreactive cells that increased with intracerebroventricular administration of CNP-53 in the ARC expressed a large amount of α-MSH immunoreactivity (Fig. 4*B*).
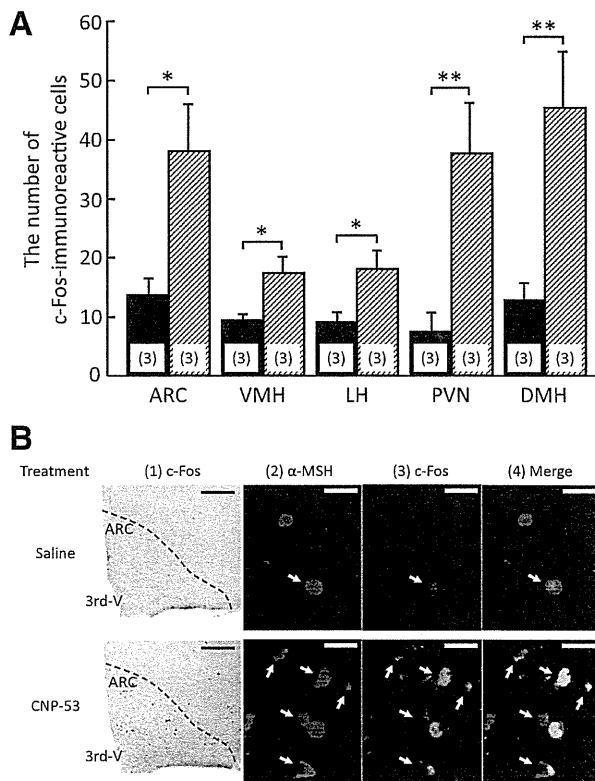

FIG. 3. Effects of intracerebroventricular administration of CNP-53 (1.5 nmol/mouse) and SHU9119 (1 nmol/mouse) on refeeding after 48-h fasting in mice. Food intake was observed for 4 h after refeeding. Data represent mean ± SEM. The number of mice is given in parentheses. Significant differences: *$P < 0.05$, **$P < 0.01$.

DISCUSSION

The current study demonstrated that intracerebroventricular administration of CNP-22 and CNP-53, but not intraperitoneal injection, led to significant reduction of food intake induced by fasting–refeeding. This reduction was inhibited by the melanocortin-3 receptor (MC3R)/melanocortin-4 receptor (MC4R) antagonist SHU9119. In addition, CNP significantly suppressed nocturnal food intake and orexigenic actions induced by NPY and ghrelin. The immunohistochemical study revealed that intracerebroventricular administration of CNP-53 increased the number of c-Fos–expressing cells containing α-MSH in the hypothalamus. These findings indicated that the intracerebroventricular administration of CNP exhibits anorexigenic actions partially via activation of the melanocortin system, although the doses of CNP used in the current study could be pharmacological doses.

The hypothalamus is considered to be an important region in regulating energy homeostasis. In particular, the ARC in the hypothalamus contains both an orexigenic peptide, NPY, and an anorexigenic peptide, α-MSH, and is postulated to be involved in the first-order regulation of food intake. Synthetic MC3R/MC4R agonists, melanotan II, and [Nle⁴-D-Phe⁷]–α-MSH completely blocked food deprivation–induced increase in food intake as well as the food intake stimulated by intracerebroventricular administration of NPY (10,11). Regarding the reciprocal interactions of α-MSH and NPY, melanocortin neurons in the ARC project to the PVN (12). In the current study, intracerebroventricular administration of CNP significantly suppressed food intake after fasting, which was antagonized by SHU9119. Our results also showed that CNP suppressed NPY-induced food intake. Taken together, these findings indicate that CNP exhibits anorexigenic actions via activation of MC3R/MC4R downstream signaling. However, mRNA expressions of preopiomelanocortin, cocaine and amphetamine-related peptide, *NPY*, and *AgRP* in the hypothalamus after the intracerebroventricular injection of CNP-53 in fasting–refeeding experiment did not change compared with those after saline. The reason for this

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FIG. 4. The c-Fos–immunoreactive cells in the hypothalamus after intracerebroventricular administration of CNP-53 (1.5 nmol/mouse). *A:* Number of c-Fos–immunoreactive cells after saline and CNP-53 treatments. Data represent mean ± SEM. The number of mice is given in parentheses. Significant differences: *P < 0.05, **P < 0.01. *B:* c-Fos–immunoreactive cells induced by intracerebroventricular administration of saline and CNP-53 (1). 3rd-V, the third ventricular. Scale bars, 100 μm. Coexistence of α-MSH (red) and c-Fos (green) immunoreactivity in the ARC (2–4) after saline (*upper*) and CNP-53 (1.5 nmol/mouse; *lower*) treatments. White arrows indicate cells expressing both α-MSH and c-Fos immunoreactivity. 3rd-V, the third ventricular. Scale bars, 20 μm.

discrepancy may lie in the experimental condition, time course, and regional specificity. To clarify this discrepancy, further examinations will be required.

This study demonstrated that the intracerebroventricular administration of CNP significantly suppressed the nocturnal food intake. Robust feeding during the nocturnal phase of the daily light–dark cycle was demonstrated to be attributed to the upregulation of NPY and its receptors (13). These findings indicate that CNP may decrease food intake in the nocturnal phase via suppression of NPY action.

In the current study, CNP significantly suppressed the increase in food intake induced by ghrelin, an orexigenic hormone secreted by the stomach (14). NPR-B, a CNP receptor, has been identified in appetite-regulating regions, such as the ARC, VMH, PVN, DMH, and LH (15). The systemic administration of ghrelin significantly increased NPY and AgRP expression in the ARC of the hypothalamus in fed and fasted rats (15), resulting in hyperphagia. The intracerebroventricular injection of melanotan II caused a significant decrease in ghrelin-induced food intake (16). These findings suggest that the actions of ghrelin are modulated by α-MSH and NPY systems. Furthermore, plasma ghrelin and hypothalamic ghrelin receptor mRNA

expression are reported to be increased after fasting (17,18). These findings suggest the possibility that intracerebroventricular administration of CNP activates the melanocortin system, which subsequently inhibits the action of NPY, resulting in a reduced increase of food intake induced by ghrelin.

To assess which hypothalamic nucleus is involved in the anorexigenic action of CNP, a marker for neuronal activity, c-Fos expression in the hypothalamus was examined after intracerebroventricular administration of CNP-53. The intracerebroventricular administration of CNP-53 significantly increased the number of c-Fos–expressing cells in several hypothalamic nuclei, such as ARC, PVN, DMH, VMH, and LH, indicating that CNP-53 directly or indirectly stimulates neurons in these hypothalamic nuclei. Especially in the ARC, the result was an increased number of c-Fos–immunoreactive cells containing α-MSH immunoreactivity, indicating that CNP stimulates α-MSH–containing neurons. This possibility is supported by the finding that the suppressive action of CNP-53 on food intake was blocked by concomitant administration of SHU9119, an MC3R/MC4R antagonist.

The current study has demonstrated the anorexigenic action of intracerebroventricular administration of CNP via activation of the melanocortin system. To define the precise effect of CNP in the brain on food intake, further investigation using mice with inducible brain-specific deletion of CNP or NPR-B/NPR-C will be required.

From the present findings, we postulate the possible mechanism for anorexigenic action of exogenous CNP to be as follows: CNP directly or indirectly acts on α-MSH–containing neurons and subsequently stimulates α-MSH release, resulting in suppression of food intake induced by NPY and ghrelin. This possible mechanism may apply to the suppressive effects of CNP on food intake after fasting and in the nocturnal phase. Further work is needed to define the pathophysiological significance of brain CNP in regulation of food intake.

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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.

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.