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

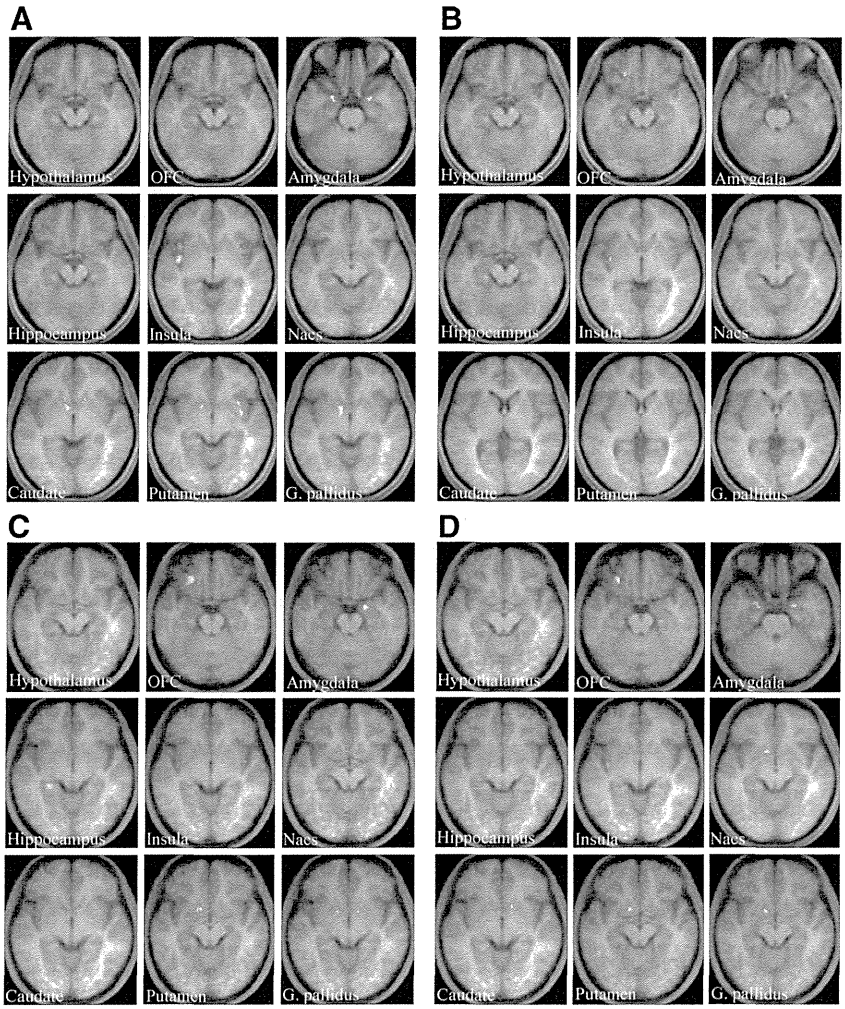

FIG. 1. Neural response to food-specific stimuli in healthy controls and leptin-off patients. Food-specific activations in ROI in the brains of controls (A and B) and patients (C and D) under fasting (A and C) and postprandial (B and D) conditions. Activation is overlaid onto the group average T1-weighted 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.

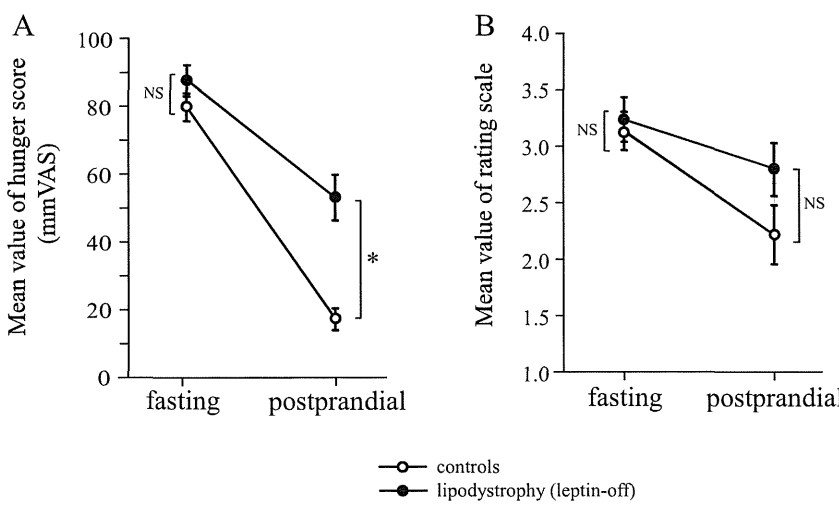

FIG. 2. Subjective feelings of appetite under fasting and postprandial conditions in healthy controls and leptin-off patients. A, Hunger scores on the 100-mm VAS before fMRI scan. B, Mean value of rating scores for food pictures during the fMRI scan. Data are means ± SEM (n = 10 in each group). *, $P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

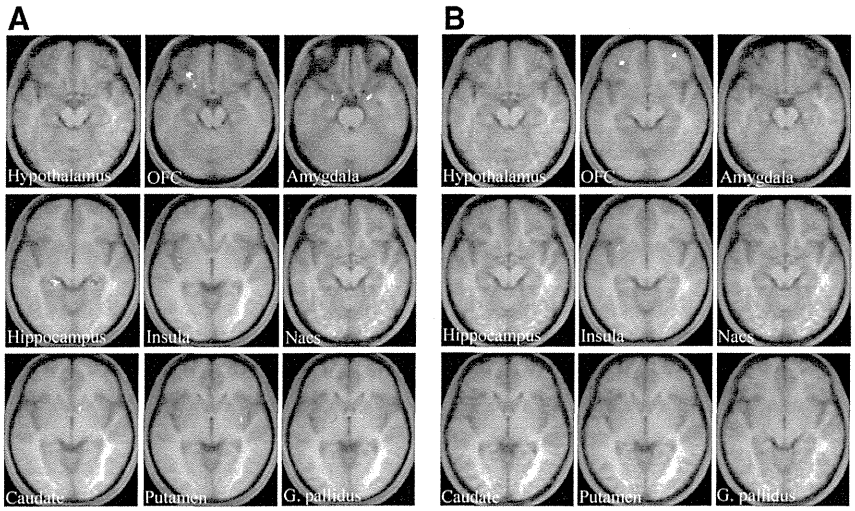

FIG. 3. Neural response to food-specific stimuli in leptin-on patients. Food-specific activations in ROI in the brain under fasting (A) and postprandial (B) conditions. Activation is overlaid onto the group average T1-weighted anatomical axial images (*right* is right side of the brain). The *brighter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

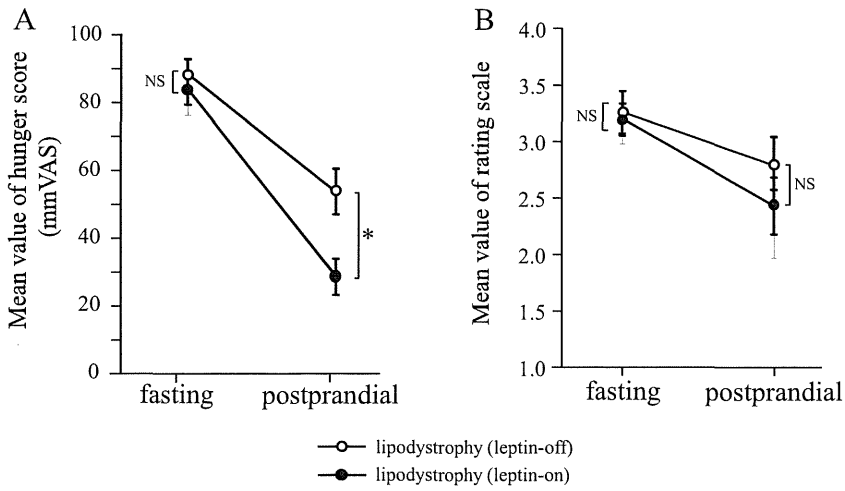

FIG. 4. Subjective feelings of appetite under fasting and postprandial conditions in patients with leptin-on and leptin-off. A, Hunger scores on the 100-mm VAS before the fMRI scan. B, Mean value of rating scores for food pictures during the fMRI scan. Data are means ± SEM (n = 10 in each group). *,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

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

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

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

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

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

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

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

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

RESULTS

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

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

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

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

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

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

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

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

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

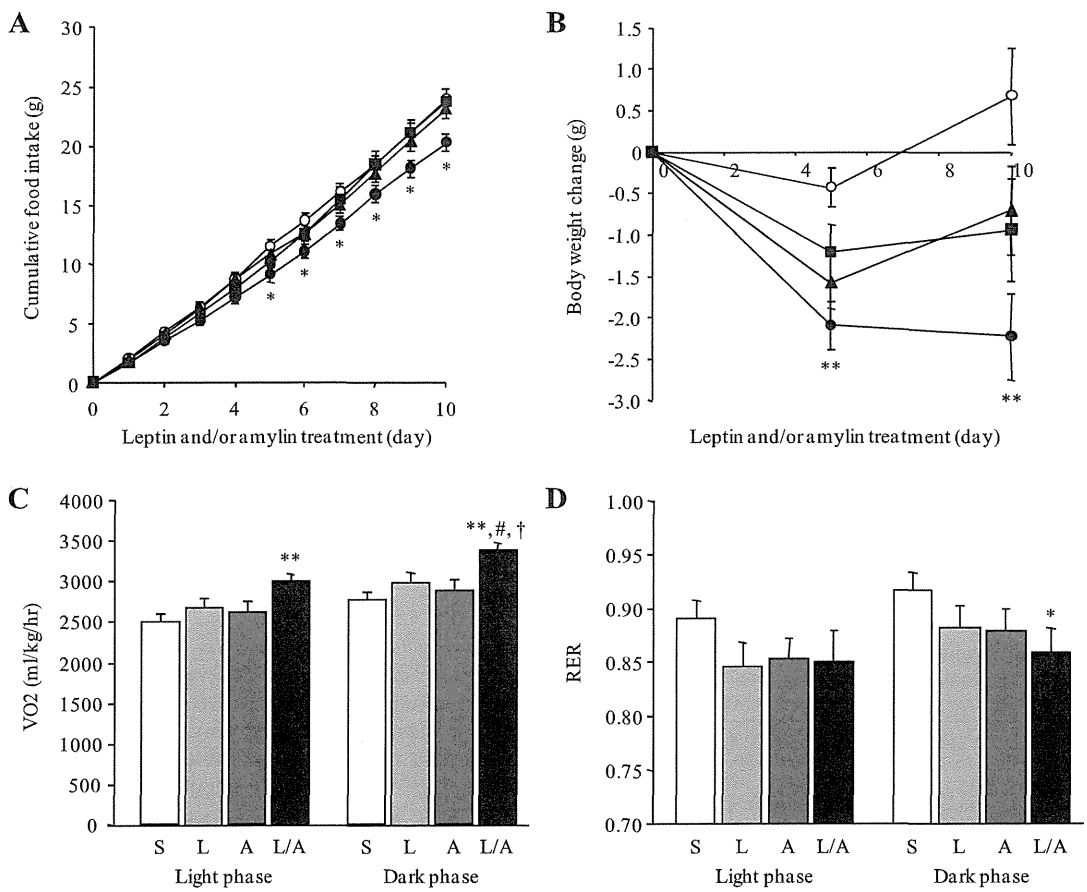

Fig. 1. Effect of leptin and/or amylin on food intake, body weight, energy expenditure, and respiratory exchange ratio (RER) in diet-induced obese (DIO) mice. Cumulative food intake (*A*) and change in body weight (*B*) during the treatment in saline- (S; ○), leptin- (L; ■), amylin- (A; ▲), and leptin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

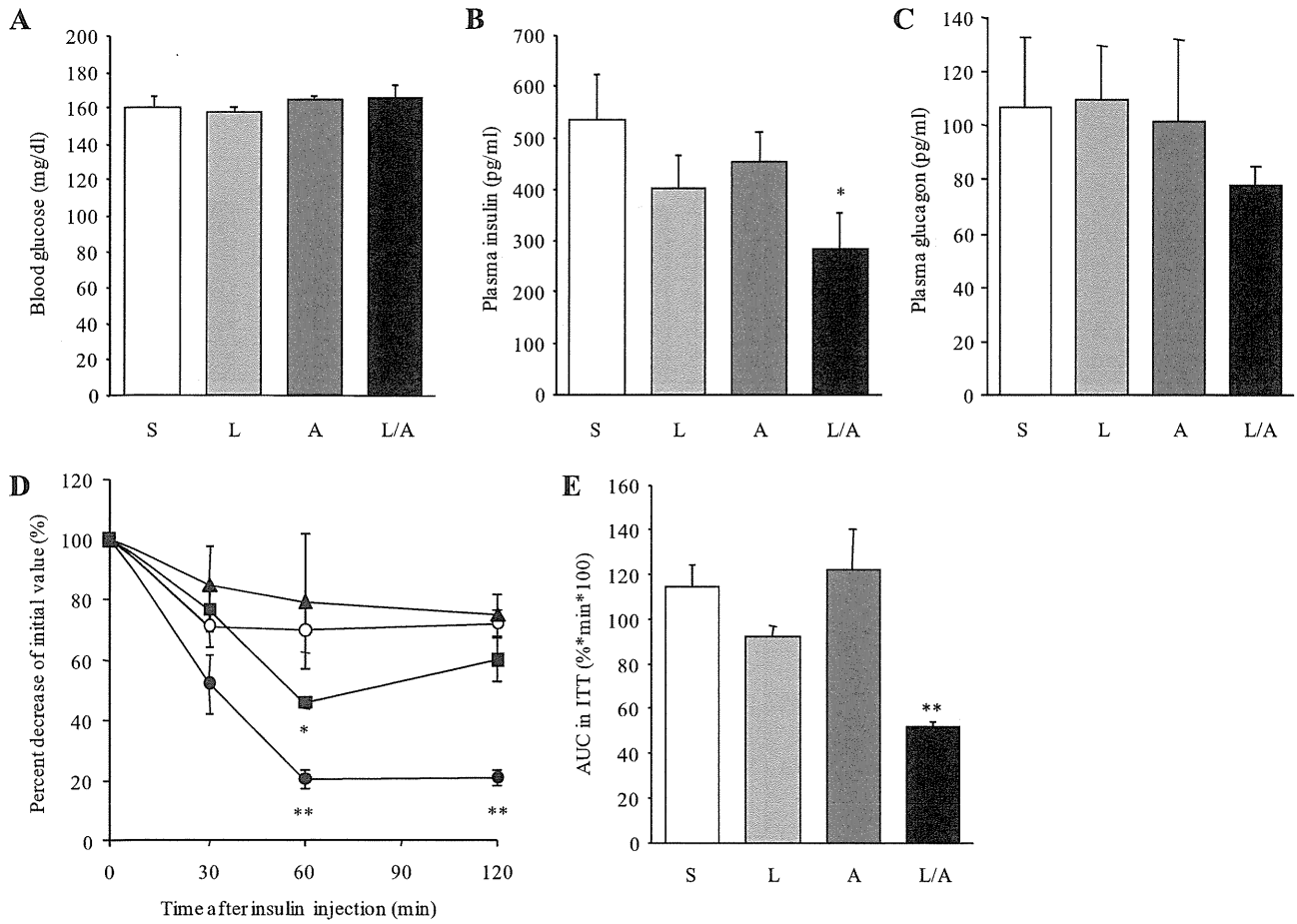

Fig. 2. Effect of L and/or A on glucose metabolism in DIO mice. Blood glucose (*A*), plasma insulin (*B*), and plasma glucagon levels (*C*) under ad libitum feeding on *day 14* in S, L, A, and L/A-treated mice. Values are means ± SE (n = 8–9/group). %Change of initial value of blood glucose levels (*D*)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

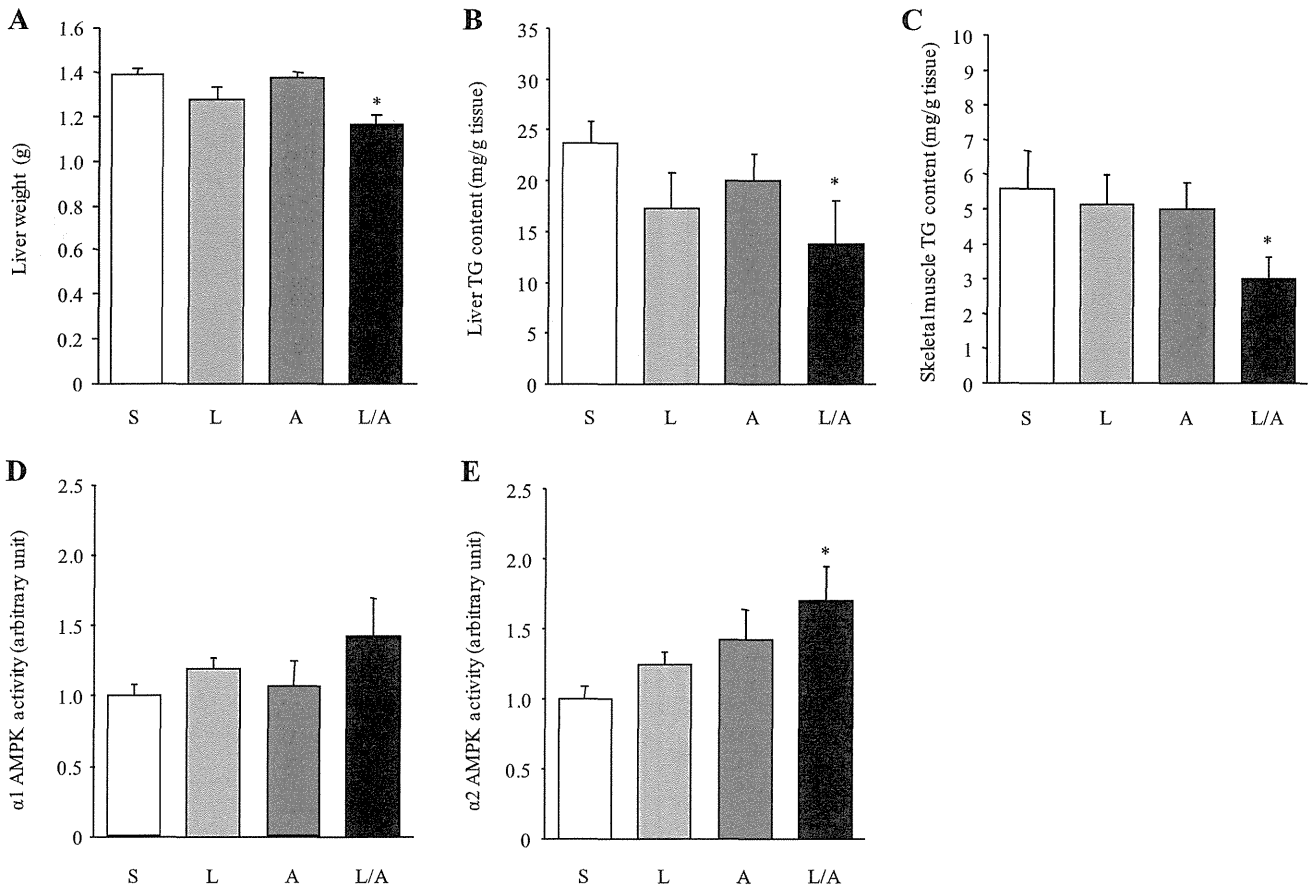

Fig. 3. Effect of L and/or A on tissue triglyceride (TG) content and skeletal muscle AMP-activated protein kinase (AMPK) activity in DIO mice. Liver size (*A*) and liver (*B*) and gastrocnemius muscle (*C*) TG contents on *day 14* in S, L, A, and L/A-treated mice. AMPKα1 (*D*) and AMPKα2 activity (*E*)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

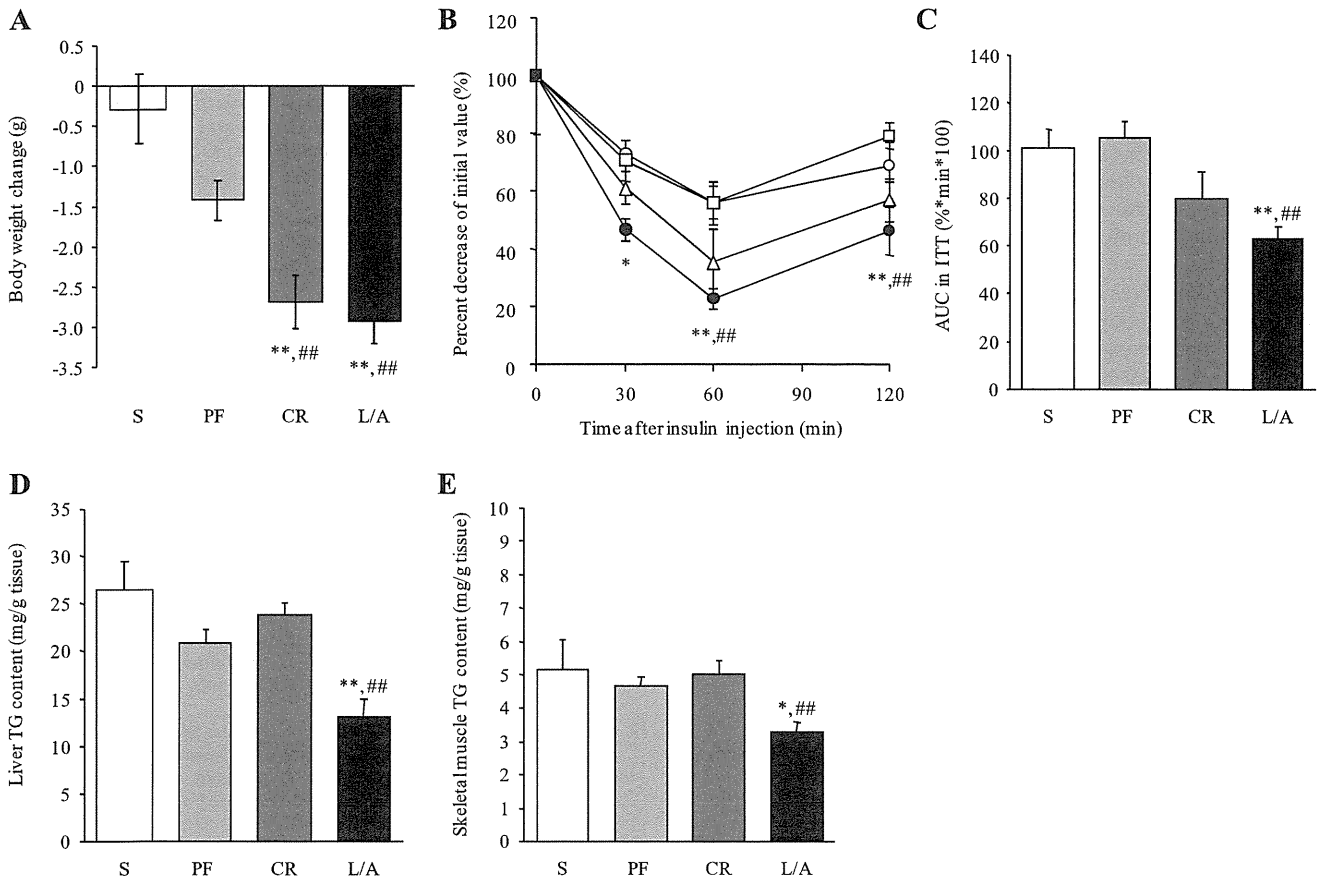

Fig. 4. Pair-feeding and weight-matched calorie restriction experiments. *A*: change in body weight on *day 10* in S, saline + pair-fed L/A-treated (PF), weight-matched DIO (CR), and L/A-treated mice. %Decrease of initial value of blood glucose levels (*B*) and AUC (*C*) during the ITT on *day 10* in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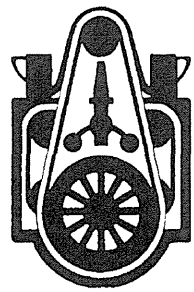

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Clinical Study

Past Obesity as well as Present Body Weight Status Is a Risk Factor for Diabetic Nephropathy

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Aims. We analyzed the prevalence of nephropathy according to past body weight status in Japanese subjects with type 2 diabetes because the influence of past obesity on diabetic complications is not certain. *Methods.* We examined the prevalence of nephropathy in 2927 subjects with type 2 diabetes mellitus according to current BMI and maximum BMI in the past. We defined "current obesity" as BMI on hospitalization of 25 or more, "previous obesity" as BMI on hospitalization of less than 25 and self-reported maximum BMI in the past of 25 or more, and "continuously lean" as maximum BMI of less than 25. *Results.* The prevalence of nephropathy was significantly higher in subjects with current obesity (40.6%) or previous obesity (35.6%) than in those who were continuously lean (24.3%) ($P < 0.017$). In logistic regression analysis, previous obesity, as well as current obesity, was a significant risk factor for nephropathy, independent of sex, age, disease duration, hypertension, dyslipidemia, HbA1c, and diabetic retinopathy. *Conclusions.* Obesity in the past, as well as the present body weight status, was a risk factor for diabetic nephropathy.

1. Introduction

The majority of Japanese patients with type 2 diabetes mellitus are not obese, as reported by Kosaka and Ogata more than 50 years ago [1]. This is a well-known fact about Japanese type 2 diabetes mellitus [2]. Eastern Asian subjects might share common characteristics of the disease, comparable to those reported in Korean people [3]. It is, therefore, debatable whether we can apply epidemiological evidence obtained from studies of Caucasian subjects with type 2 diabetes and obesity to eastern Asian people, especially in the fields of diabetic complications, which are influenced not only by hyperglycemia but also by obesity. For example, type 2 diabetes is a relative risk factor for cardiovascular disease in Asians, as in Western societies, but the absolute risk differs greatly between these populations [4–7].

Recently, the "legacy effect" of intensive glycemic control early after the diagnosis of diabetes was advocated based on the UKPDS follow-up study [8]. However, there is no report about the legacy effect of past obesity over a lifetime on diabetic complications. Although there have been inconsistent results as to whether obesity is a risk for diabetic nephropathy [9–12], it was reported that current obesity and maximum past body mass index (BMI) were significant risk factors for diabetic nephropathy in the Japanese [12]. Although Caucasian subjects with type 2 diabetes usually maintain their body weight status during their disease course, the majority of patients of eastern Asian ethnicity begin to lose body weight from around the time of diagnosis of diabetes [3, 13]. This was easily overlooked, even if the effect of obesity in the past persisted for a long time, because they were already nonobese at the start of clinical follow-up. To clarify the influence of

past obesity on diabetic complications is an important clinical concern in patients of eastern Asian ethnicity.

We therefore analyzed the difference in the prevalence of diabetic nephropathy in Japanese type 2 diabetics according to their history of body weight status to clarify the effect of past obesity on diabetic nephropathy.

2. Materials and Methods

We examined subjects with type 2 diabetes whose estimated glomerular filtration rate (eGFR) was 30 mL/min/1.73 m^2 or more and who were admitted to Saiseikai Central Hospital from January 1999 to December 2004 ($n = 1834$) or Keio University Hospital from April 1998 to September 2010 ($n = 1093$) for the management of metabolic control. The study protocol was reviewed and approved by the ethics committee of both hospitals. Those subjects in whom the etiology of renal disease was strongly suspected to be other than diabetic nephropathy were excluded. According to the history of body weight status and the Japanese criteria for obesity [14], we defined "current obesity" as BMI on hospitalization of 25 or more, "previous obesity" as BMI on hospitalization of less than 25 and self-reported maximum BMI in the past of 25 or more, and "continuously lean" as maximum BMI of less than 25.

HbA1c level on admission was determined by high-performance liquid chromatography (HPLC: Arkray Inc., Kyoto, Japan) according to the recommended method by the Japan Diabetes Society (JDS) at that time and converted to the National Glycohemoglobin Standardization Program (NGSP) value [15]. eGFR (mL/min/1.73 m^2) was calculated as $194 \times Cr^{-1.094} \times Age^{-0.287}$ (with further multiplication by 0.739 for female subjects) using the equation provided by the Japanese Society of Nephrology [16].

Subjects with albumin excretion rate (AER) of 20 μg/min or more in 24-hour urine were considered to have diabetic nephropathy. All subjects underwent funduscopic examination by trained ophthalmologists during or just before admission. The diagnosis of diabetic retinopathy was made based on the Davis classification [17]. Hypertension was defined as systolic blood pressure >140 mmHg, diastolic blood pressure >90 mmHg, or the prescription of antihypertensive medication. Dyslipidemia was defined as LDL cholesterol >3.63 mmol/L, triglyceride >1.72 mmol/L, HDL cholesterol <1.04 mmol/L, or the prescription of lipid-lowering medication.

Continuous variables are expressed as mean ± SD. Differences in baseline characteristics among the obesity categories were analyzed by ANOVA and chi-squared test. Chi-squared test was also performed to evaluate differences in prevalence, with Bonferroni's correction for post hoc multiple comparisons. As a result, the probability equivalent to the usual $P = 0.05$ was $P = 0.017$. Logistic regression analysis with forced entry method was performed to detect significant independent predictors of diabetic nephropathy. We adopted as covariates factors such as sex, age, disease duration, hypertension, dyslipidemia, HbA1c, and obesity status (current obesity, past obesity, and continuously lean) in this study. Obesity status was converted to two dichotomous variables with dummy coding. $P < 0.05$ was considered statistically significant. All analyses were performed using IBM SPSS software ver. 18.0 (SPSS Inc., an IBM company, Japan).

3. Results

We analyzed a total of 2927 persons (males 2038, females 889, age 59.3 ± 10.6 years, BMI 24.0 ± 4.0, duration of diabetes 10.4 ± 28.0 years, HbA1c 9.3 ± 2.8%). The subjects' characteristics are shown in Table 1. The prevalence of current obesity in the total subjects was 33.6%, previous obesity 41.5%, and a continuous lean state 24.8%. The prevalence of nephropathy was significantly different among the categories, and both currently obese (40.6%) and previously obese (35.6%) patients had a significantly higher prevalence of diabetic nephropathy than that in continuously lean patients (24.3%) ($P < 0.017$).

When we divided the patients into quartiles according to current BMI, the prevalence of nephropathy significantly increased as current BMI increased (Figure 1, $P < 0.001$). When we similarly divided them into quartiles according to previous maximum BMI, the prevalence of nephropathy significantly increased as previous maximum BMI increased (Figure 1, $P < 0.001$). Current BMI and previous maximum BMI were highly correlated with each other ($r = 0.785$, $P < 0.001$).

In logistic regression analysis, both current obesity and previous obesity revealed a significant odds ratio for nephropathy, as well as diabetic retinopathy, independent of sex, age, disease duration, hypertension, dyslipidemia, and HbA1c (Table 2).

4. Discussion

We confirmed that previous obesity, as well as present obesity, was closely associated with nephropathy in type 2 diabetes. The notable finding of this study was that obesity is an independent risk factor, not only if it is present, but also if it was present in the past. This might indicate a legacy effect of obesity on nephropathy. The mechanism is the theme for investigation of how obesity in the past can influence diabetic complications over time.

Both current BMI and previous maximum BMI were associated with the nephropathy, as demonstrated in Figure 1. However, current BMI and previous maximum BMI were highly correlated with each other. So, generally, the higher the previous BMI, the higher the current BMI. When we analyzed the effects of previous obesity, we had to separate the effects of current obesity from those of previous obesity. This was the reason we analyzed the effect of previous obesity according to the categories defined as previous maximum BMI and current BMI. As a result, we could elucidate the effect of previous obesity on diabetic nephropathy.

Whether nephropathy in type 2 diabetes really derives from diabetes has always been a point of discussion. However, type 2 diabetes *per se* is a disease so closely linked with obesity and the metabolic syndrome that we cannot strictly distinguish the cause among the components of the syndrome. We here found that the effect of obesity was independent of the

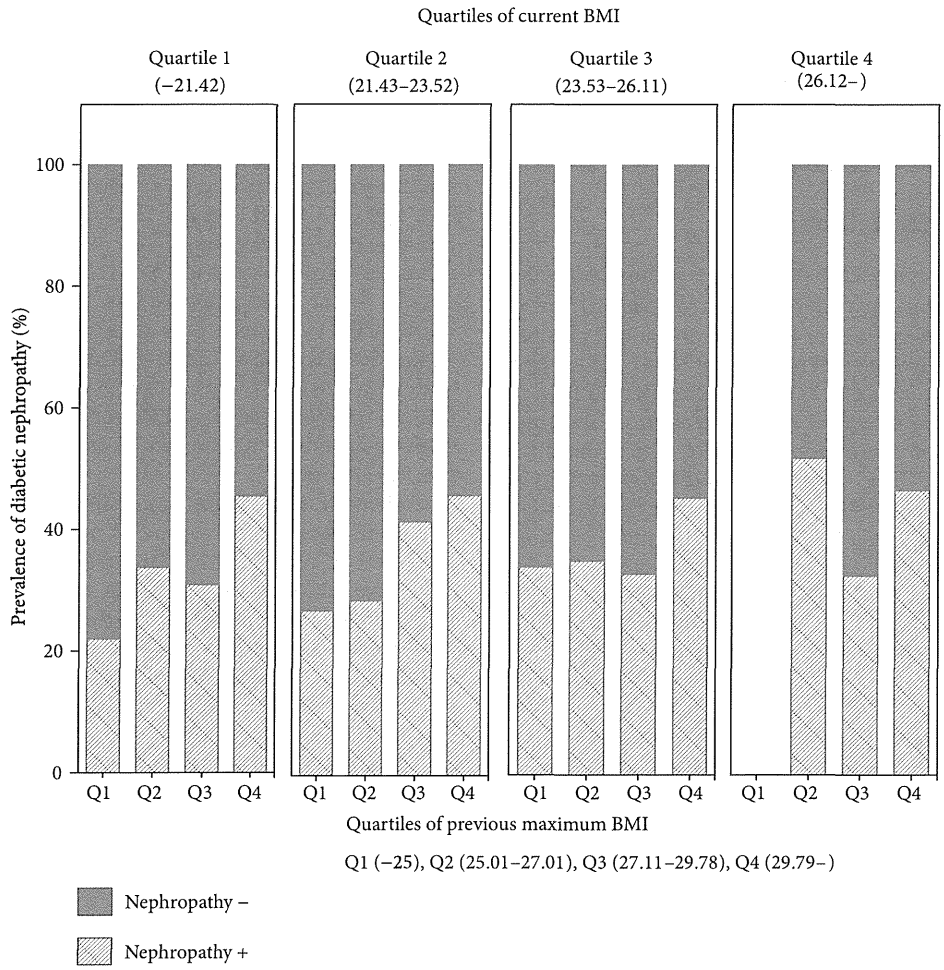

FIGURE 1: Prevalence of diabetic nephropathy divided by quartiles of current BMI and quartiles of previous maximum BMI. As for quartiles of current BMI, Quartile 1; BMI ≦ 21.42, Quartile 2; BMI: 21.43–23.52, Quartile 3; BMI: 23.53–26.11, and Quartile 4; BMI > 26.11. As for quartiles of previous maximum BMI, Quartile 1 (Q1); BMI ≦ 25.00, Quartile 2 (Q2); BMI: 25.01–27.10, Quartile 3 (Q3); BMI: 27.11–29.78, and Quartile 4 (Q4); BMI > 29.78. Prevalence of diabetic nephropathy was significantly different among the quartile groups of current BMI ($P < 0.001$ by chi-squared analysis). Prevalence of diabetic nephropathy was significantly different among the quartile groups of previous maximum BMI ($P < 0.001$ by chi-squared analysis).

existence of diabetic retinopathy, and we expect this result can be applied to all type 2 diabetes patients.

Several groups, including us, have reported that albuminuria was the strongest predictor of the progression of diabetic nephropathy [18–22]. We did not chronologically follow the decline of glomerular filtration rate (GFR) in this study. However, as we defined diabetic nephropathy by albuminuria, obesity, either current or past, might relate to the GFR decline through albuminuria.

Recently, the concept of obesity-related nephropathy has been advocated [23]. Although this concept is strictly defined with exclusion of both nephrosclerosis and diabetic nephropathy, the suspected mechanisms, including the contraction of efferent glomerular arterioles by the activated renin-angiotensin system (RAS) and glomerular hyperfiltration, as well as glomerular hypertrophy due to insulin resistance, are very similar to those of diabetic nephropathy. The border between the concepts of diabetic nephropathy and obesity-related nephropathy is unclear, and they cannot be distinguished clinically if a patient has both. Vivante et al. reported that overweight state and obesity in adolescents were associated with significantly increased risk for both diabetic and nondiabetic ESRD during a 25-year period [24]. If obesity even before the diagnosis of diabetes influences kidney function later, these concepts are continuous and indivisible.

There are some limitations of this study. One is that this study was retrospective, based on self-reported body weight in the past. As for the other limitation, we might have to consider how they lost their body weight because the study subjects required metabolic interventions for poor glycemic status. The duration of obesity must be a factor of interest affecting the results. However, we only have the data of maximum body weight and the body weight on admission but