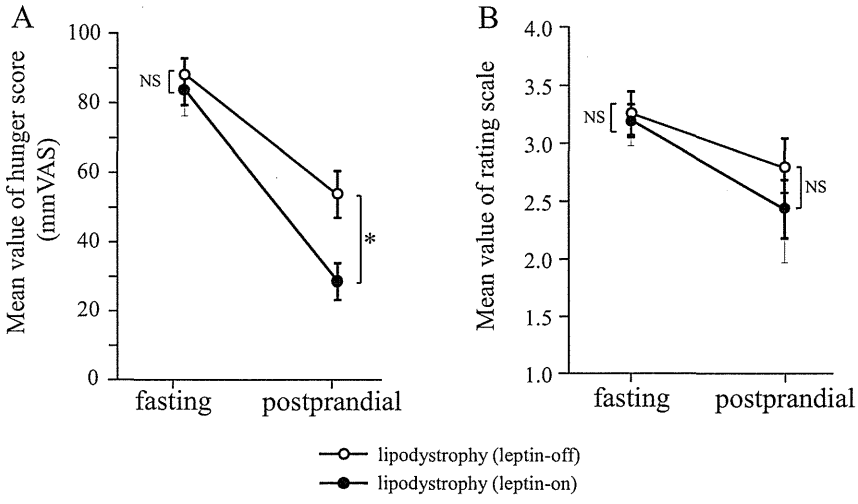

FIG. 4. Subjective feelings of appetite under fasting and postprandial conditions in patients with leptin-on and leptin-off. A, Hunger scores on the 100-mm VAS before the fMRI scan. B, Mean value of rating scores for food pictures during the fMRI scan. Data are means ± SEM (n = 10 in each group). *,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

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

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

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

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

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

been established as playing a pivotal role in regulation of fear, emotion and cognition (8,9), has been suggested to be involved in energy regulation because lesion of the amygdala has been reported to induce hyperphagia, resulting in marked obesity (10,11). Moreover, the amygdala has recently been demonstrated to be one of the brain regions regulating appetite via activation of the melanocortin system (12).

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

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

Materials and methods

Animals and diets

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

Blood sampling and analysis of metabolic parameters

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

Fear-conditioning test

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

Jumping–vocalisation response

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

Spontaneous locomotor activity

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

Elevated plus maze test

This test was performed in accordance with our previous study (27). The elevated plus maze (Muromachi Kikai Co., Ltd., Tokyo, Japan) was constructed of gray Plexiglas and consisted of four arms (length 300 mm, width 60 mm): two closed arms with high gray walls (150 mm high) and two open arms with a small raised lip (3 mm). The maze was elevated to a height of 400 mm above the ground. At least 1 h before the test, mice were transferred to a standby room (20 lux) that was separated from the test room. Experiments were performed between 13.00 and 15.00 h. Each mouse was placed on the center platform facing an open arm to initiate the test session. Mice were allowed to freely explore the apparatus under overhead fluorescent lighting (20 lux) for 5 min. Increased exploration of the relatively open arms is indicative of reduced anxiety-like behaviour in this paradigm. Open/closed arm entries and time spent in the open/closed arms were scored. Arm entries were scored upon entry of the two front paws into the arm.

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

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

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

Measurement of BDNF and NT-3 content in the brain

BDNF and NT-3 content in the brain of CD and DIO mice fed CD and HFD, respectively, for 16 weeks was measured in accordance with our previous study (3) using commercially available measurement kits for BDNF (BDNF Emax® ImmunoAssay System: Promega Inc., Madison, WI, USA) and for NT-3 (NT-3 Emax® ImmunoAssay System: Promega Inc. Madison, WI).

Western blot analysis of TrkB and TrkC

Western blotting of full-length TrkB and TrkC in the brain of CD and HFD mice was performed in accordance with our previous study (3). Full-length TrkB and TrkC were detected using rabbit polyclonal anti-TrkB antibody (sc-8316; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and rabbit polyclonal anti-TrkC antibody (sc-14025; Santa Cruz Biotechnology, Inc.), respectively. Results represent the densitometry data relative to glyceraldehyde 3-phosphate dehydrogenase detected in each sample.

Statistical analysis

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

Results

Metabolic parameters in CD and DIO mice

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

Fear-conditioning response

CD mice exhibited 93% freezing as a result of fear in the first session in the contextual conditioning response, and the freezing percentage gradually decreased during the sessions to reach 60% in the fifth session (Fig. 1). In DIO mice, the freezing percentage of the contextual fear response was significantly lower than that in CD mice in each session (Fig. 1). DIO mice exhibited 64% freezing percentage in the first session of the contextual fear response, and the freezing percentage subsequently decreased during the sessions to 23% in the fifth session (Fig. 1). Similarly, the freezing percentage of the cued fear response in DIO mice was 47% in the first session, which was much lower than the 81% in CD mice, and a significant decrease in freezing percentage of DIO mice was observed over the course of three cued sessions compared to CD mice (Fig. 1).

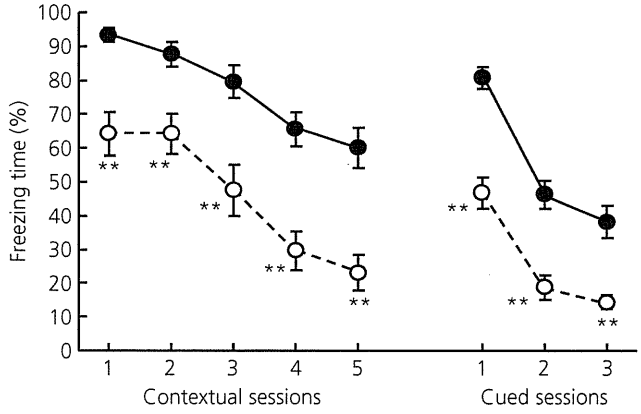

Fig. 1. Fear-conditioning responses in control diet (CD) and diet-induced obese (DIO) mice. Fear-conditioning responses in CD (closed circles) and DIO (open circles) mice. Freezing percentages of CD and DIO mice in the contextual conditioning test were measured every minute for 5 min. Freezing percentages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

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

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

BDNF and NT-3 content in the brain areas

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

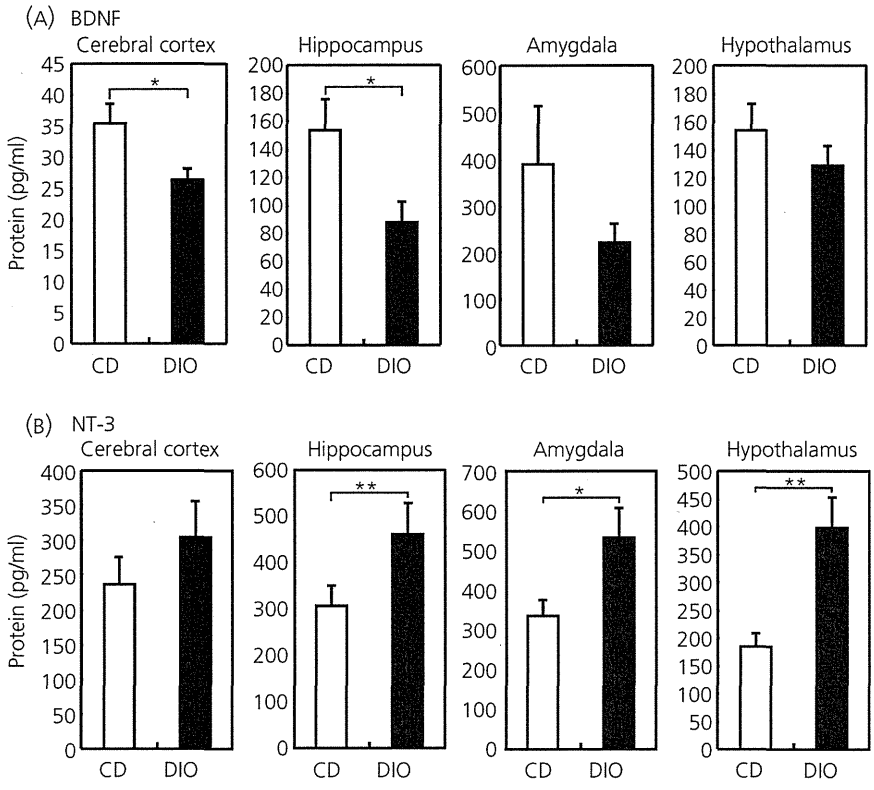

Fig. 2. Content of (A) brain-derived neurotrophic factor (BDNF) and (B) neurotrophin-3 (NT-3) in the cerebral cortex, hippocampus, amygdala and hypothalamus in control diet (CD) and diet-induced obese (DIO) mice. Results are presented as the mean ± SEM (n = 18–29). Significantly different from CD mice: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

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

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

Discussion

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

Chronic dietary fat intake, especially saturated fatty acid intake, is reported to contribute to deficits of hippocampus-dependent spatial cognition in the water maze test of rats (5,6,28). The adverse effects of high-dense diets on learning and memory have been associated with impaired hippocampal synaptic plasticity and suppressed neurogenesis (29–31).

Long-term structural alterations of synapses, so-called neuronal plasticity, are regulated by several synaptic molecules including neurotrophic factors, such as BDNF (15), and have been demonstrated to be essential for spatial learning performance, which is dependent primarily on hippocampal functions (15). Animals lacking BDNF show deficits in LTP related to processes of learning and memory, and in hippocampus-dependent spatial learning, which can be amended by exogenous BDNF (15). Although the mechanisms by which a HFD can affect BDNF expression are largely unknown, in the present study, the feeding of HFD or obesity led to a reduction of BDNF content in the hippocampus and cerebral cortex to the extent that cognitive performance was compromised. By contrast to the decrease in BDNF content, the present study demonstrated that NT-3 content was significantly increased in the hippocampus, amygdala and hypothalamus of DIO mice compared to that in CD mice. BDNF and NT-3 oppose one another in regulating

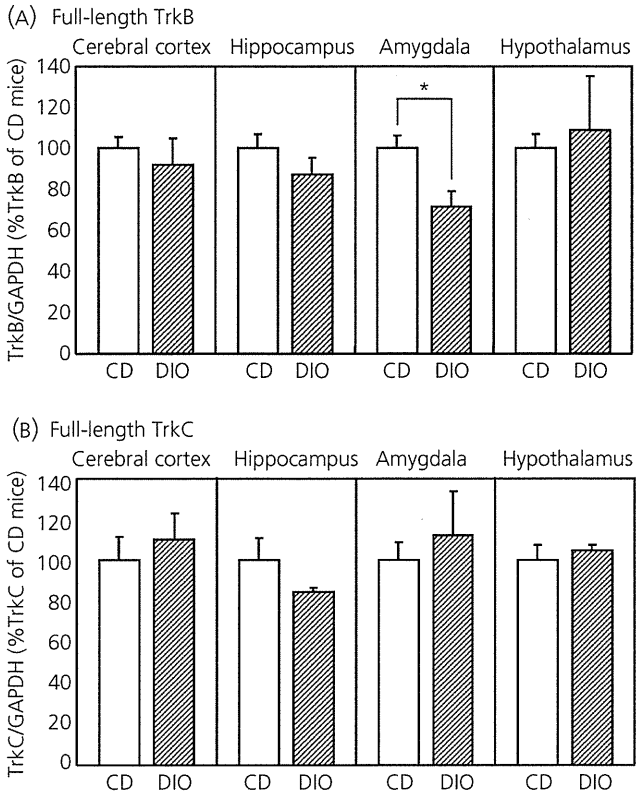

Fig. 3. Expression of full-length TrkB (A) and TrkC (B) in the cerebral cortex, hippocampus, amygdala and hypothalamus in control diet (CD) and diet-induced obese (DIO) mice. Results are presented as the mean ± SEM (n = 3–7). Significantly different from CD mice: *P < 0.05. GAPDH, glyceraldehyde 3-phosphate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

the dendritic growth of pyramidal neurones in the hippocampus and neural activity (22,23). NT-3 was reported to inhibit the dendritic growth stimulated by BDNF (22). The amygdala, which is well established as playing a pivotal role in regulation of fear, emotion and cognition (8,9), is suggested to be involved in energy regulation because lesion of the amygdala has been reported to induce hyperphasia, resulting in marked obesity (10,11). Moreover, the amygdala has recently been demonstrated to be one of the brain regions regulating appetite via activation of the melanocortin system (12). Taken together, these findings suggest that the impaired fear-conditioning response in DIO mice is attributed to the decrease of BDNF, which facilitates memory processes and the antagonistic actions of NT-3 against BDNF in the hippocampus and amygdala, although the present study did not address the mechanisms responsible for changes in BDNF and NT-3 content in the brain of DIO mice.

Several lines of electrophysiological and behavioural evidence demonstrate that leptin and insulin enhance hippocampal synaptic plasticity and improve learning and memory (31,32). Electrophysiological studies in genetically obese Zucker rats with leptin-receptor deficiency demonstrated that LTP of the hippocampal CA1 region, which is closely related to learning and the formation of memory and is regulated by NMDA and AMPA receptors (6), is markedly impaired compared to lean rats (7). Streptozotocin-treated insulin-deficient rats are reported to exhibit impaired cognition in the water maze test, which is dependent on the hippocampus (33). Therefore, it is likely that impairment of actions of leptin or insulin might be attributable to cognitive deficits in obesity and diabetes mellitus (34,35). Although there is no direct evidence for the impairment of cognition in DIO mice, the impaired cognitive behaviours of fear-conditioning tests observed in the present study may be partly mediated by decreased inherent functions of leptin and insulin in the brain, despite high plasma levels of leptin and insulin, giving rise to the so-called leptin resistance or insulin resistance associated with obesity.

The present study has shown that DIO mice exhibit impairment of both hippocampus-dependent contextual and amygdala-dependent cued responses of the fear-conditioning test. Moreover, BDNF content decreases in the hippocampus and cerebral cortex of DIO mice, whereas NT-3 content increases in the hippocampus, amygdala and hypothalamus of DIO mice, compared to CD mice. The expression of TrkB in the amygdala of DIO mice decreases compared to CD mice. These findings suggest that consumption of a HFD may contribute to aspects of dysfunction in the central nervous system.

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

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

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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*

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

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

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

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

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

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

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

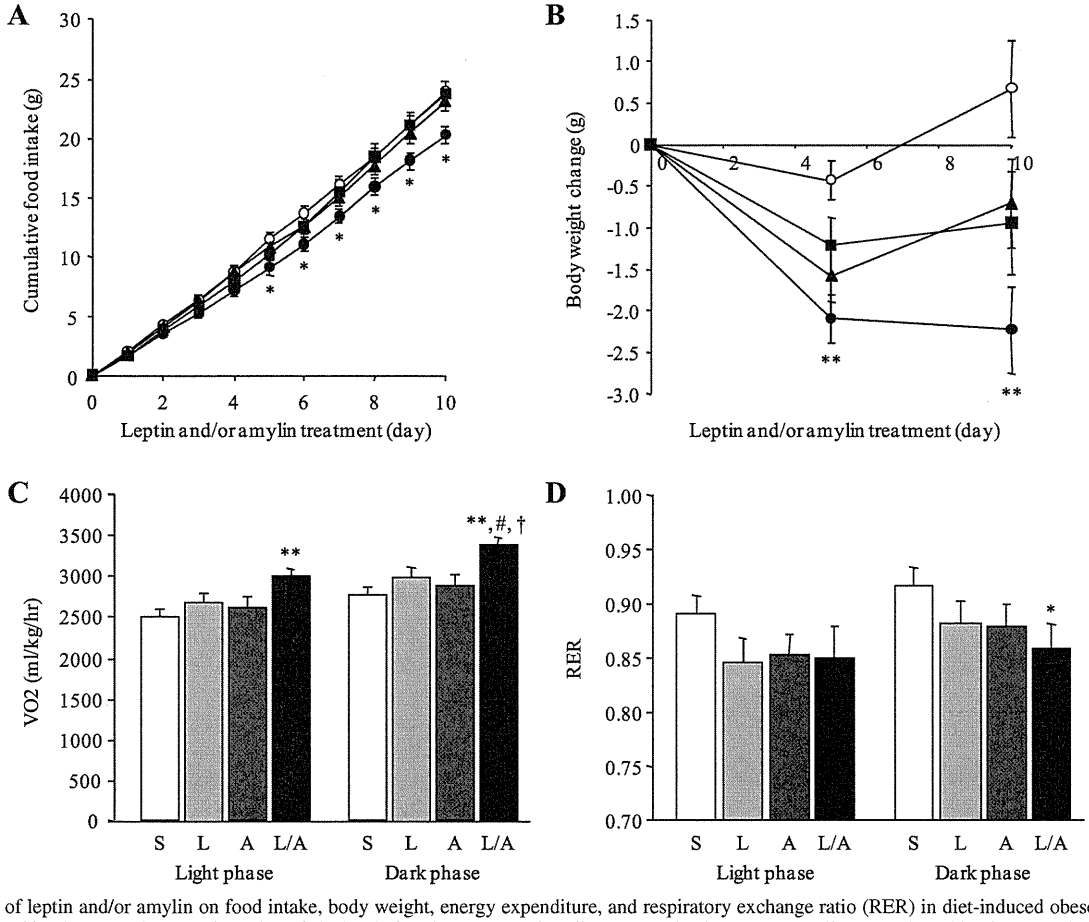

Fig. 1. Effect of leptin and/or amylin on food intake, body weight, energy expenditure, and respiratory exchange ratio (RER) in diet-induced obese (DIO) mice. Cumulative food intake (*A*) and change in body weight (*B*) during the treatment in saline- (S; ○), leptin- (L; ■), amylin- (A; ▲), and leptin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

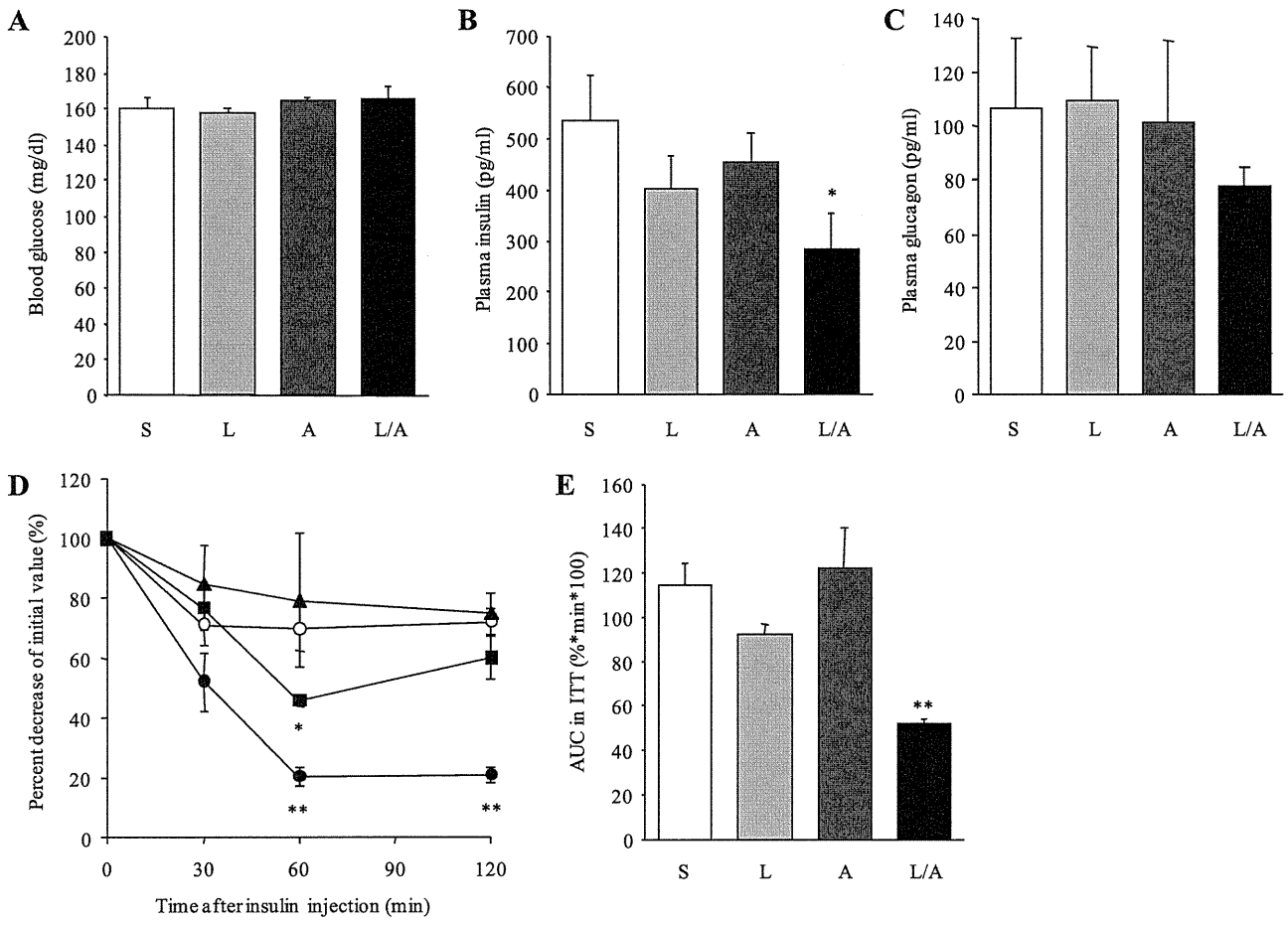

Fig. 2. Effect of L and/or A on glucose metabolism in DIO mice. Blood glucose (*A*), plasma insulin (*B*), and plasma glucagon levels (*C*) under ad libitum feeding on *day 14* in S, L, A, and L/A-treated mice. Values are means ± SE (n = 8–9/group). %Change of initial value of blood glucose levels (*D*)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

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

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

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

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

DISCUSSION

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

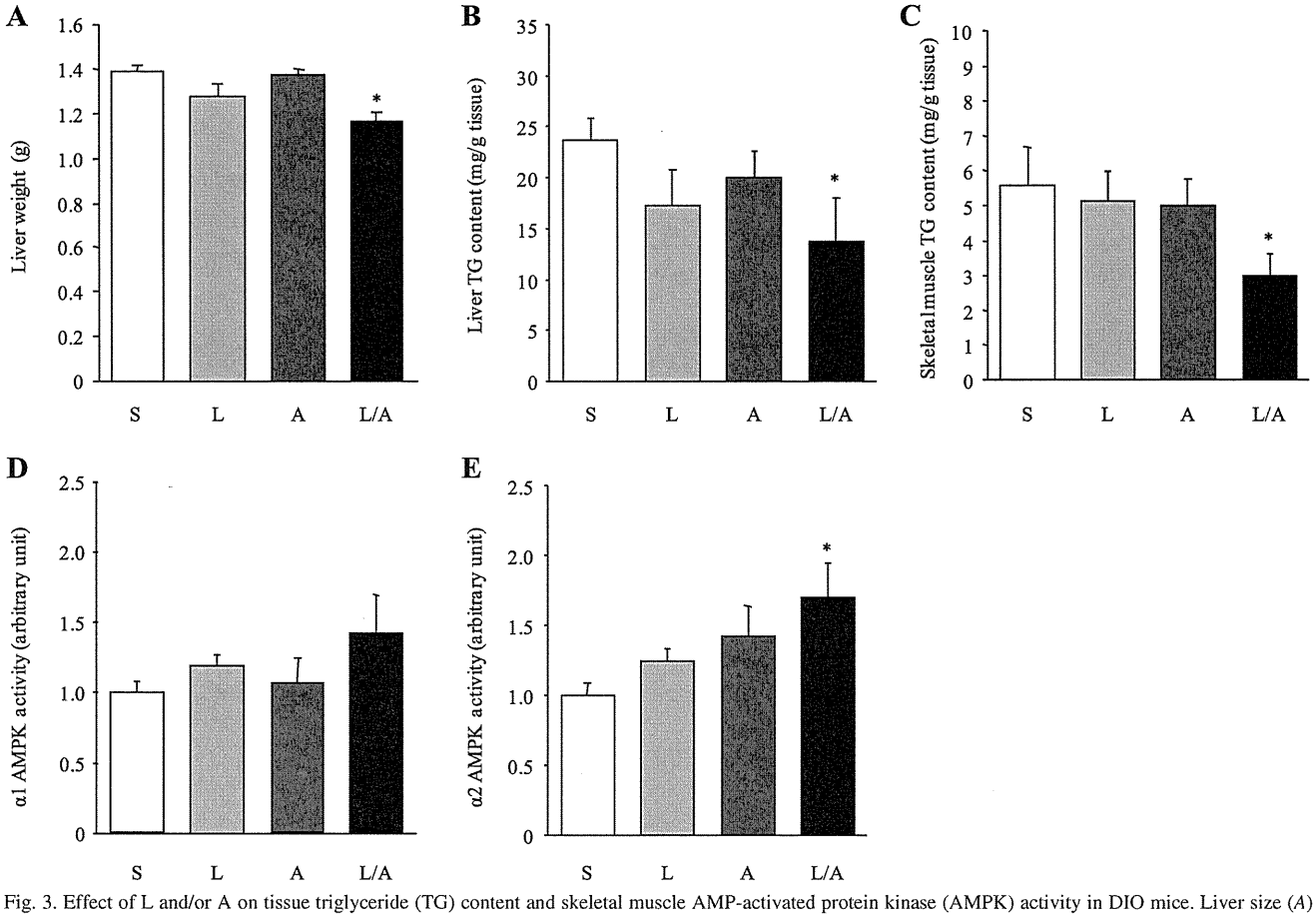

Fig. 3. Effect of L and/or A on tissue triglyceride (TG) content and skeletal muscle AMP-activated protein kinase (AMPK) activity in DIO mice. Liver size (*A*) and liver (*B*) and gastrocnemius muscle (*C*) TG contents on *day 14* in S, L, A, and L/A-treated mice. AMPKα1 (*D*) and AMPKα2 activity (*E*)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

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

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

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

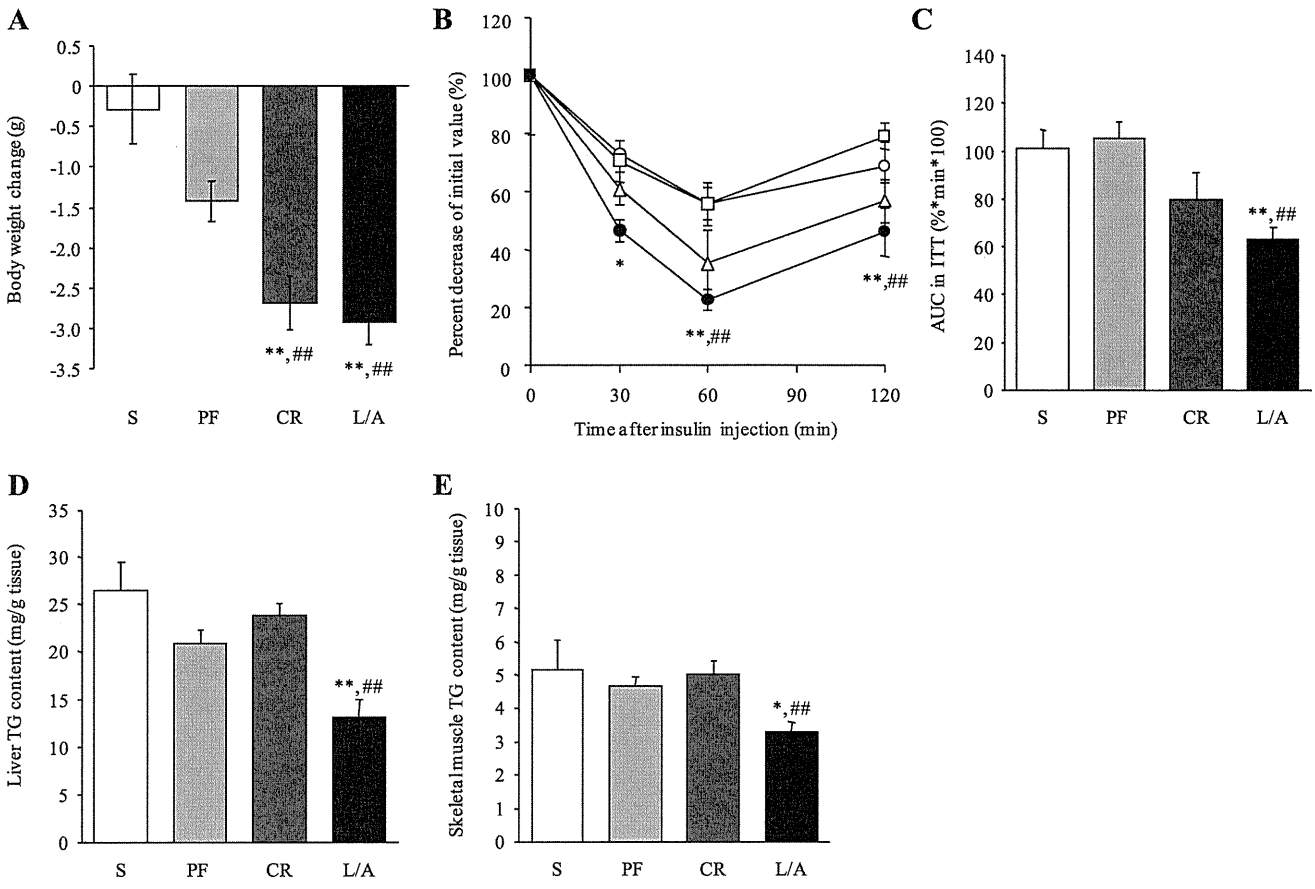

Fig. 4. Pair-feeding and weight-matched calorie restriction experiments. *A*: change in body weight on *day 10* in S, saline + pair-fed L/A-treated (PF), weight-matched DIO (CR), and L/A-treated mice. %Decrease of initial value of blood glucose levels (*B*) and AUC (*C*) during the ITT on *day 10* in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

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

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

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

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

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

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

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

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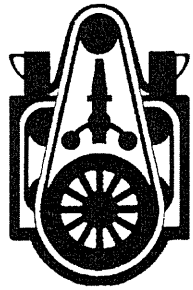

PLOS ONE

Direct Immunochemiluminescent Assay for proBNP and Total BNP in Human Plasma proBNP and Total BNP Levels in Normal and Heart Failure

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Abstract

Background: Recent studies have shown that in addition to brain (or B-type) natriuretic peptide (BNP) and the N-terminal proBNP fragment, levels of intact proBNP are also increased in heart failure. Moreover, present BNP immunoassays also measure proBNP, as the anti-BNP antibody cross-reacts with proBNP. It is important to know the exact levels of proBNP in heart failure, because elevation of the low-activity proBNP may be associated with the development of heart failure.

Methodology/Principal Findings: We therefore established a two-step immunochemiluminescent assay for total BNP (BNP+proBNP) and proBNP using monoclonal antibodies and glycosylated proBNP as a standard. The assay enables measurement of plasma total BNP and proBNP within only 7 h, without prior extraction of the plasma. The detection limit was 0.4 pmol/L for a 50-μl plasma sample. Within-run CVs ranged from 5.2%–8.0% in proBNP assay and from 7.0%–8.4% in total BNP assay, and between-run CVs ranged from 5.3–7.4% in proBNP assay and from 2.9%–9.5% in total BNP assay, respectively. The dilution curves for plasma samples showed good linearity (correlation coefficients = 0.998–1.00), and analytical recovery was 90–101%. The mean total BNP and proBNP in plasma from 116 healthy subjects were 1.4±1.2 pM and 1.0±0.7 pM, respectively, and were 80±129 pM and 42±70 pM in 32 heart failure patients. Plasma proBNP levels significantly correlate with age in normal subjects.

Conclusions/Significance: Our immunochemiluminescent assay is sufficiently rapid and precise for routine determination of total BNP and proBNP in human plasma.

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Competing Interests: Hiroyuki Okamoto, Masahiro Nakamura, Naoko Ogawa, Kazukiyo Horii and Kiyoshi Nagata are employed by Shionogi& Co., Ltd. Shionogi Company previously developed the BNP kit and they may develop a new assay kit like a proBNP in the future. There are no further patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Brain (also known as B-type) natriuretic peptide (BNP) has been used as a biomarker of heart failure for more than a decade [1]. Indeed, guidelines for the treatment of heart failure recommend measurement BNP before making a diagnosis [2,3]. During the process by which BNP is secreted from cardiac myocytes, its 108-amino acid precursor, proBNP, is cleaved to form the 32-amino acid peptide BNP and the 76-amino acid peptide N-terminal proBNP fragment (NT-proBNP) [4]. Recent studies have shown that in addition to BNP and the NT-proBNP, levels of uncleaved proBNP are also considerably increased in plasma of patients with heart failure [5,6,7]. This is noteworthy in part because the immunoassay system currently being used to measure BNP levels also detects proBNP, as the anti-BNP antibody cross-reacts with proBNP. Consequently, the present assay system actually measures not the active BNP level, but the total BNP (BNP+proBNP) level [8].

It is important to know the proBNP level and/or proBNP/total BNP ratio in heart failure, because proBNP has much less ability to induce cGMP production (about 13–17%) than BNP, and higher levels of the low-activity proBNP may be associated with the development of heart failure [7]. Consistent with that idea, we recently used the combination of gel-filtration and a fluorescent immunoenzyme assay with BNP extracted from plasma to show

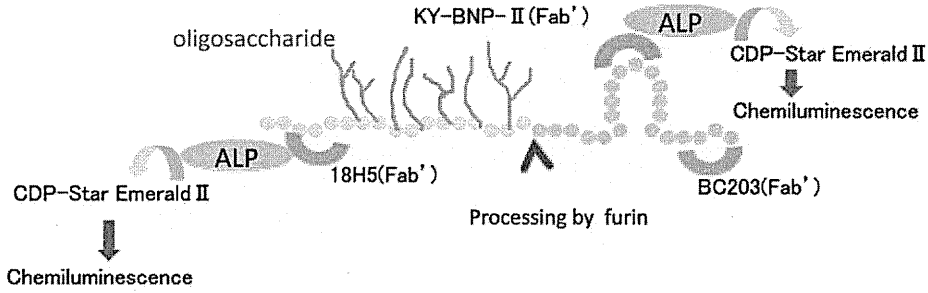

Figure 1. Schematic diagram of the total BNP and proBNP assay systems. BC203(Fab') is a common capture antibody in both systems. KY-BNP-II(Fab') is the detection antibody for the total BNP assay, and 18H5(Fab') is the detection antibody for the proBNP assay. ALP: Alkaline phosphatase; CDP-Star EmeraldII (Chemiluminescent Substrate): Disodium 2-chloro-5-(4-methoxy-spiro{1,2-dioxetane-3,2′-(5′-chloro)-tricyclo [3,3,1,13,7]decan}-4-yl)-1-phenyl phosphate.
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that although proBNP/total BNP ratios vary widely in heart failure, they are higher in cases of ventricular overload than in atrial overload [6]. Unfortunately, the method used in that study requires a great deal of time and effort, and extraction of the peptide from plasma may cause underestimation of the proBNP levels due to its high adsorptive property [9].

To overcome those shortcomings, we developed a sensitive method to more quickly and easily measure levels of proBNP and total BNP. Our idea was to make a sandwich immunoassay using a common capture antibody recognizing the C-terminal region of both BNP and proBNP and detection antibodies that recognize different epitopes: the N-terminal region of proBNP and the ring structure of BNP (Figure 1). Using this approach, we were able to develop a sensitive immunochemiluminescent assay for proBNP and total BNP in plasma. Here, we report on the assay's performance and its use to compare plasma levels of total BNP and proBNP in healthy subjects and patients with heart failure. In addition, we measured NT-proBNP and compared it with total BNP and proBNP.

Materials and Methods

All patients provided written informed consent for all blood sample analyses, and the protocol was approved by the Ethical Committee of Kyoto University Graduate School of Medicine. Sample analyses were also conducted in accordance with the policies and procedures of the Institutional Review Board for the use of human subjects in research at the Diagnostics Division of Shionogi & Co., Ltd.

Peptides and Reagents

Glycosylated proBNP and recombinant proBNP were purchased from Hytest Ltd. (Turk, Finland). The protein content was determined by amino acid analysis. BNP was from Peptide Institute, Inc. (Osaka, Japan). EZ-Link-sulfo-NHS-biotinylation kits were from Pierce (Rockford, IL). Sulfo-HMCS (N-(8-maleimidocapryloxy) sulfosuccinimide) was from Dojindo (Kumamoto, Japan). CDP/E (Disodium 2-chloro-5-(4-methoxy-spiro{1,2-dioxetane-3,2′-(5′-chloro)-tricyclo [3,3,1,13,7]decan}-4-yl)-1-phenyl phosphate) was from Applied Biosystems (Foster City, CA).

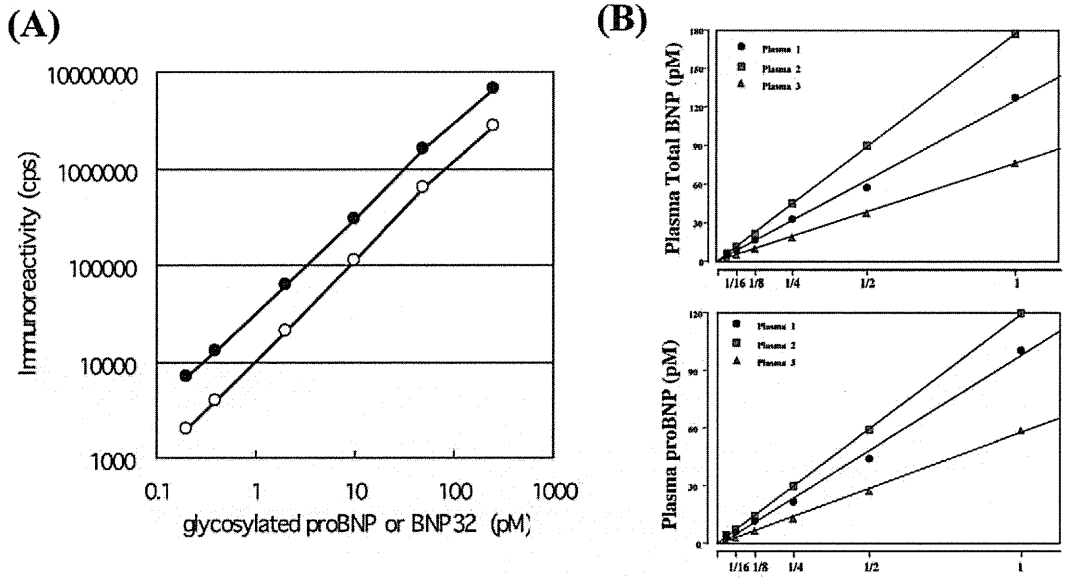

Figure 2. Standard curves for the proBNP (open circle) and total BNP (closed circle) assays (A). Plasma dilution curves (B). Three plasma samples collected from three heart failure patients were serially diluted with buffer.
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