TABLE 1. Characteristics of the patients at baseline

Patient no.	1	2	3	4	5	6	7	Mean ± SE
Age (yr)	11	29	19	16	15	23	33	21 ± 3
Sex	F	M	M	F	F	F	F	
Type of lipodystrophy	AGL	CGL[a]	CGL[b]	AGL	CGL[b]	CGL[b]	CGL[a]	
Body weight (kg)	31.0	32.0	52.8	34.6	43.5	52.0	53.9	42.8 ± 3.9
BMI (kg/m^2)	16.0	14.2	19.7	13.9	17.9	21.2	20.3	17.6 ± 1.1
Body fat (%)[c]	5.0	4.7	5.0	5.8	5.4	5.7	10.0	5.9 ± 0.7
Age of DM onset (yr)	9	11	10	15	6	5	14	10 ± 1
Duration of DM (yr)	2	18	9	1	9	18	19	11 ± 3
Leptin (ng/ml)	0.92	0.82	1.23	1.15	0.90	1.23	1.40	1.09 ± 0.08
Fasting glucose (mg/dl)	208	142	105	138	247	221	130	172 ± 21
HbA1c (%)	10.0	10.3	8.8	7.9	7.7	10.2	10.2	9.3 ± 0.4
Triglyceride (mg/dl)	1941	69	1031	1246	89	254	232	695 ± 273
Total cholesterol (mg/dl)	250	183	194	298	185	285	233	233 ± 18
L/S ratio	0.78	1.23	0.95	0.35	1.12	0.88	0.73	0.86 ± 0.11
Urinary albumin (mg/d)	48.4	6.9	31.0	778	20.3	359	11.3	179.3 ± 110.5
Blood pressure (mm Hg)	114/48	120/82	126/54	92/52	108/56	104/64	108/70	110 ± 4/61 ± 5
Bone mineral density (g/cm^2)[c]	0.94	0.88	1.32	0.90	1.06	1.37	1.33	1.11 ± 0.08
Diet therapy (kcal/d)	1500	1500	1800	1500	1500	1600	1500	
Antidiabetic therapy	Pioglitazone (15 mg/d)	Glibenclamide (2.5 mg/d), voglibosie (0.6 mg/d)	Insulin (60 IU/d)	Pioglitazone (30 mg/d)	None	Pioglitazone (45 mg/d)	Insulin (20 U/d)	
Lipid-lowering therapy	None	None	None	Pravastatin (40 mg/d), bezafibrate (400 mg/d)	None	None	None	

BMI, Body mass index; DM, diabetes mellitus; L/S ratio, ratio of liver to spleen for CT attenuation values.
[a] CGL due to neither *seipin* nor *AGPAT2* mutation.
[b] CGL due to *seipin* mutation.
[c] Body fat and bone mineral density were measured by dual energy x-ray absorptiometry.

was administered as twice-daily sc injection (18). The physiological replacement dose was estimated to be 0.02 mg/kg·d for men, 0.03 mg/kg·d for girls under 18 yr of age, and 0.04 mg/kg·d for women on the basis of information provided by Amgen. Patient 1 was treated with 100% of the replacement dose for the entire period. Patient 2 was treated with 100% for the first and second month and 200% thereafter. Patients 3–7 were treated with 50% for the first month, 100% for the second month, and 200% thereafter. All patients were evaluated as inpatients for the first 4 months. After discharge, patients attended local clinics for every leptin injection, and all of the leptin injections were done by medical doctors because self-injection of r-metHuLeptin, which was not approved as a drug, was not permitted in Japan. Each patient had been prescribed a diet of fixed calories indicated in Table 1 beginning at least 2 months before the initiation of leptin-replacement therapy, and this was not altered throughout the therapy. The dose of antidiabetic and lipid-lowering drugs was tapered or the treatment discontinued as needed. After 12 months of twice-daily leptin treatment, we reduced the dosing frequency to once daily without change of total daily dose. At present, total duration of leptin-replacement therapy was 36 months for patient 1 and 2, 24 months for patient 3, 18 months for patient 4, 8 months for patient 5, and 2 months for patients 6 and 7.

Biochemical analysis

Plasma leptin levels were determined by the immunoassay (Linco, St. Charles, MO). Plasma glucose, serum triglycerides, total cholesterol, alanine aminotransferase, aspartate aminotransferase, and serum and urine creatinine levels were determined according to standard methods with the use of automated equipment. Glycosylated hemoglobin (HbA1c) levels were measured by ion-exchange HPLC. Serum insulin levels were determined by immunoassays (Shibayagi Co., Ltd., Gunma, Japan). Urine albumin excretion was assayed with a human albumin ELISA kit (Sanko Junyaku Co., Ltd., Tokyo, Japan). Antibodies to leptin in serum was tested with the use of a solid-phase RIA, and the potential neutralizing effects of antibodies on leptin bioactivity were assessed in an *in vitro* bioassay developed by Amgen (Thousand Oaks, CA) (21).

Procedures

Body fat and whole-body bone mineral density were determined by dual-energy x-ray absorptiometry (QDR-2000; Hologic Inc., Bedford, MA). The oral glucose tolerance test (75 g) was performed after an overnight fast. In patients under insulin therapy, insulin injection was stopped from the previous night. The Σ values of plasma glucose (PG) levels and serum insulin (IRI) levels were calculated by the sum of the values at 0, 30, 60, 90, 120, and 180 min after administration. Insulin action on glucose uptake in peripheral tissues was evaluated using the hyperinsulinemic-euglycemic glucose clamp technique (22). Fatty liver was diagnosed by both ultrasound and computed tomography (CT) imaging. Liver volume was calculated with the use of CT imaging. Lipid contents of liver and skeletal muscle were determined by magnetic resonance imaging performed on a 1.5-T system (Magnetom Symphony; Siemens Medical System, Erlangen, Germany). The signal intensity of the same region on both the in-phase image (I_{in}) and the out-of-phase image (I_{out}) was measured. The fat index (FI) was defined by the following formulae: $FI = (I_{in} - I_{out}) / I_{in}$. Tissue lipid content was calculated using FI as previously reported (23).

Statistical analysis

Data were expressed as the mean ± SE. Comparison between baseline data and data obtained at various times was assessed by ANOVA and completed by Fisher's probable least-significant difference test, as required.

Results

Baseline characteristics

Three of five CGL patients were homozygous for the same nonsense mutation (R275X) of the *seipin* gene as we previously reported (Table 1) (24). The remaining CGL patients had neither *seipin* nor *AGPAT2* gene mutation (24, 25). All the patients had markedly decreased body fat, hypoleptinemia, and uncontrolled diabetes with high fasting glucose levels and HbA1c levels, despite the diet and exercise therapy and the use of oral antidiabetic drugs or insulin. Their age of onset and duration of diabetes are also summarized in Table 1. Three of seven patients had marked fasting hypertriglyceridemia at the level above 1000 mg/dl. The mean ± SE of the total cholesterol level was 233 ± 18 mg/dl. Five patients were diagnosed to have fatty liver, and their ratios of liver to spleen (L/S ratio) for CT attenuation values were under 0.95. Four of seven patients had elevated urine albumin excretion (>30 mg/d), and two of them had macroalbuminuria (>300 mg/d). All the patients showed normal blood pressure (mean ± SE, 110 ± 4/61 ± 5 mm Hg) and bone mineral density (mean ± SE, 1.11 ± 0.08 g/cm^2).

High compliance of leptin-replacement therapy

All of the leptin injections were done by medical doctors. For the initial 4 months, all the patients received 100% of scheduled leptin injections as inpatients. After discharge, patients attended local clinics for every leptin injection and received over 98% as outpatients thereafter.

Achievement of physiological replacement of leptin

At any dose, peak plasma levels occurred 2 h after the leptin injection. The peak plasma leptin levels at the doses of 50, 100, and 200% under the protocol of twice-daily injections were 4.05 ± 0.19, 9.80 ± 1.70, 18.95 ± 1.58 (mean ± SE) ng/ml, respectively. The peak plasma leptin level of the 400% dose under the protocol of once-daily injections was 34.48 ± 2.11 (mean ± SE) ng/ml. Thus, the elevations of plasma leptin level were dose dependent, and physiological replacement was achieved as expected.

Rapid effects on glucose and triglyceride levels

The fasting plasma glucose levels decreased day by day in all the patients, and a significant reduction was achieved within 7 d (mean ± SE, 172 ± 20 mg/dl at baseline *vs.* 120 ± 12 mg/dl after 7 d, $P < 0.05$) (Table 2). By 4 months, all the patients, except patient 6, were able to discontinue all of the antidiabetic drugs (Table 1). Patient 6 could reduce the dose of the antidiabetic drug by 2 months.

The fasting triglyceride levels also decreased day by day in all the patients, and a significant reduction was achieved within 7 d (mean ± SE, 700 ± 272 mg/dl at baseline *vs.* 260 ± 98 mg/dl after 7 d, $P < 0.05$) (Table 2). Lipid-lowering drugs of patient 4 were able to be discontinued by 4 months.

Glucose tolerance tests

As shown in Fig. 1A, the mean plasma glucose levels in response to the oral 75-g glucose load were dramatically improved already at 1 month and were maintained at 2 and 4 months in all patients. The insulin levels were distinctly low before the treatment in both AGL and CGL patients (Fig. 1, B and C). The changes after the initiation of the leptin-replacement therapy of serum insulin levels showed a marked contrast between AGL and CGL patients. Glucose-induced insulin secretion was dramatically improved already at 1

TABLE 2. Changes of fasting plasma glucose and serum triglyceride levels for first month of the leptin-replacement therapy

Patient no.	Fasting plasma glucose (mg/dl)					Fasting serum triglyceride (mg/dl)				
	Baseline	1 d	3 d	7 d	30 d	Baseline	7 d	14 d	21 d	28 d
1	208	177	160	108	76	1941	653	210	204	218
2	142	115	119	102	94	69	51	49	72	50
3	105	100	89	105	108	1031	122	108	97	115
4	138	125	126	96	108	1246	589	320	425	496
5	247	227	204	182	151	254	399	134	151	194
6	221	185	169	141	130	89	63	54	131	74
7	141	135	133	105	125	269	54	85	95	99
Mean ± SE	172 ± 20	152 ± 17	143 ± 14	120 ± 12[a]	113 ± 9[b]	700 ± 272	260 ± 98[a]	110 ± 38[b]	168 ± 46[b]	178 ± 58[b]

[a] $P < 0.05$ compared to baseline.
[b] $P < 0.01$ compared to baseline.

month in AGL patients (Fig. 1B), whereas no apparent improvement in insulin secretion was observed even after 4 months of the therapy in CGL patients (Fig. 1C). To evaluate the ability of insulin secretion, we calculated the values of ΣIRI/ΣPG in a 75-g oral glucose tolerance test. The values of ΣIRI/ΣPG were substantially increased at 1 month in two AGL patients, and additional increases were observed at 2 and 4 months, whereas those in five CGL patients remained unchanged even after 4 months of the therapy (Fig. 1D).

Hyperinsulinemic-euglycemic clamp study

The glucose infusion rates during the hyperinsulinemic-euglycemic clamp study were distinctly low at baseline in all the patients (mean ± SE, 2.5 ± 0.3 mg/kg·min; range, 1.60–3.6 mg/kg·min). The increase of glucose infusion rate was observed but not statistically significant at 1 month on the treatment (mean ± SE, 3.7 ± 0.3 mg/kg·min, $P = 0.062$ *vs.* at baseline). A significant increase was achieved at 2 months (mean ± SE, 4.4 ± 0.4 mg/kg·min, $P < 0.01$ *vs.* at baseline) and an additional increase was observed at 4 months (mean ± SE, 5.6 ± 1.0 mg/kg·min, $P < 0.001$ *vs.* at baseline). By contrast to insulin secretion, no apparent difference between AGL and CGL patients was observed on the changes of insulin sensitivity.

In patient 4, the hyperinsulinemic-euglycemic clamp study was performed at 10 d. A substantial increase of glucose infusion rate was detected already at 10 d (2.52 mg/kg·min at baseline and 4.63 mg/kg·min at 10 d) and again at 1 month (4.59 mg/kg·min), which was comparable to that at 4 months (5.06 mg/kg·min).

Effects on fatty liver

Five of seven patients were diagnosed to have apparent fatty liver. The L/S ratios of CT attenuation value in five of seven patients were 0.74 ± 0.10 (mean ± SE) (Table 1). The L/S ratio of CT attenuation value in these patients improved from 0.74 ± 0.10 (mean ± SE) to 1.09 ± 0.06 (mean ± SE) by 2 months and further improved thereafter. Consistent with this, in these patients, the alanine aminotransferase level decreased from 80.5 ± 24.2 to 32.3 ± 4.6 U/liter (mean ± SE), and the ASL level decreased from 42.3 ± 11.1 to 21.5 ± 4.3 U/liter (mean ± SE) by 2 months, and these values were also further improved thereafter. The liver volume also decreased in all patients who had fatty liver at baseline (mean ± SE,

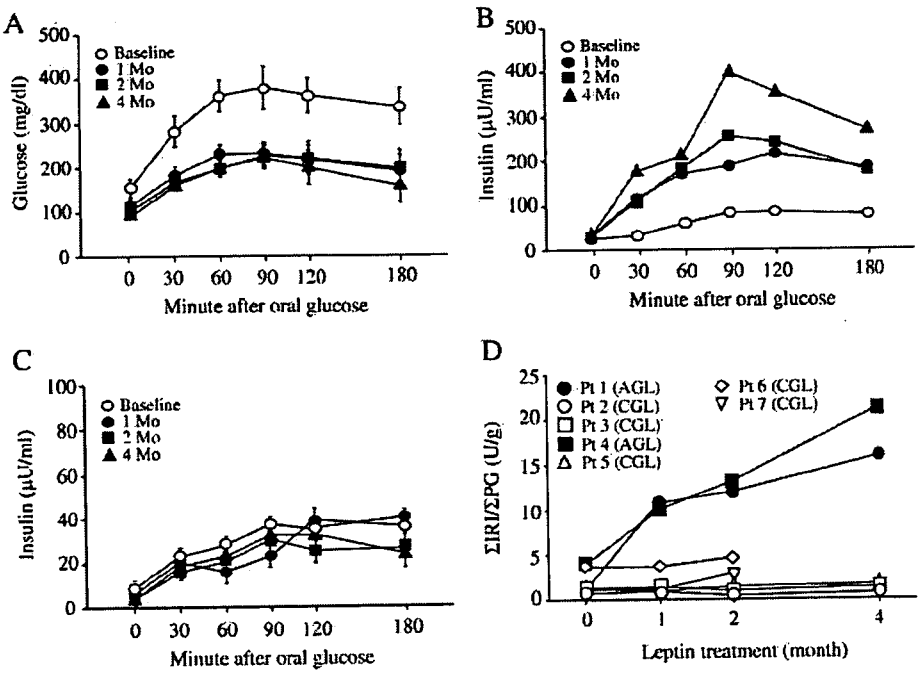

FIG. 1. Mean ± SE plasma glucose levels in all seven patients (A), mean serum insulin levels in two AGL patients (B), mean ± SE serum insulin levels in five CGL patients (C), and ΣIRI/ΣPG of each patient (D) after an oral 75-g glucose-tolerance test before and after 1, 2, and 4 months of the leptin-replacement therapy. ΣIRI, Sum of the plasma insulin levels before and 30, 60, 90, 120, and 180 min after an oral 75-g glucose load; ΣPG, sum of the plasma insulin levels before and 30, 60, 90, 120, and 180 min after an oral 75-g glucose load; ΣIRI/ΣPG, value of ΣIRI divided by ΣPG.

1.88 ± 0.12 l at baseline to 1.50 ± 0.10 l at the end of the second month).

In patient 4, measurements of tissue lipid content were performed using magnetic resonance imaging before and after 3 and 10 d and 1, 2, and 4 months of the leptin-replacement therapy. At baseline, lipid content in her liver was clearly increased (29.0%), whereas that in her skeletal muscle was not increased (4.3%). After the leptin-replacement therapy, a distinct change of lipid content in the liver was not detected at 3 and 10 d (31.5 and 28.4%, respectively), but a substantial and gradual decrease was detected at 1 month and again at 2 and 4 months (23.5, 17.5, and 9.6%, respectively). On the other hand, in the skeletal muscle, no distinct change of lipid content was detected even at 4 months (4.2%).

Metabolic controls after discharge for 8 months

After the initial 4 months of hospitalization, the patients were continuously followed as outpatients on the protocol of twice-daily injection. Their fasting glucose levels (Fig. 2A), HbA1c levels (Fig. 2B), glucose infusion rates during the hyperinsulinemic-euglycemic clamp study (Fig. 2C), triglyceride levels (Fig. 2D), total cholesterol levels (Fig. 2E), and liver volumes (Fig. 2F) at 8 and 12 months were almost unchanged when compared with those at 4 months, the end of the hospitalization.

Once-daily leptin injection

After 12 months of twice-daily leptin injection, the treatment protocol was altered to once-daily dosing without change of total daily dose in patient 1–4. The alteration of leptin injection protocol did not affect the plasma glucose levels before breakfast, lunch, and dinner in four patients (Fig. 3, A–C). Consistent with these results, HbA1c (Fig. 2B) levels and results of the 75-g oral glucose tolerance test (data not shown) in these patients did not change after the protocol alteration. Likewise, glucose infusion rates during the hyperinsulinemic-euglycemic clamp study, triglyceride levels, total cholesterol levels, and liver volumes were unchanged after the alteration of the treatment protocol (Fig. 2, C–F).

Long-term effects

The duration of leptin-replacement therapy was 36 months for patients 1 and 2, 24 months for patient 3, and 18 months for patient 4. The fasting plasma glucose levels and HbA1c levels were well controlled throughout the therapy period (Fig. 2, A and B). The improved glucose infusion rates during the hyperinsulinemic-euglycemic clamp study, decreased triglyceride and total cholesterol levels, and liver volumes after 4 months of leptin-replacement therapy as inpatients

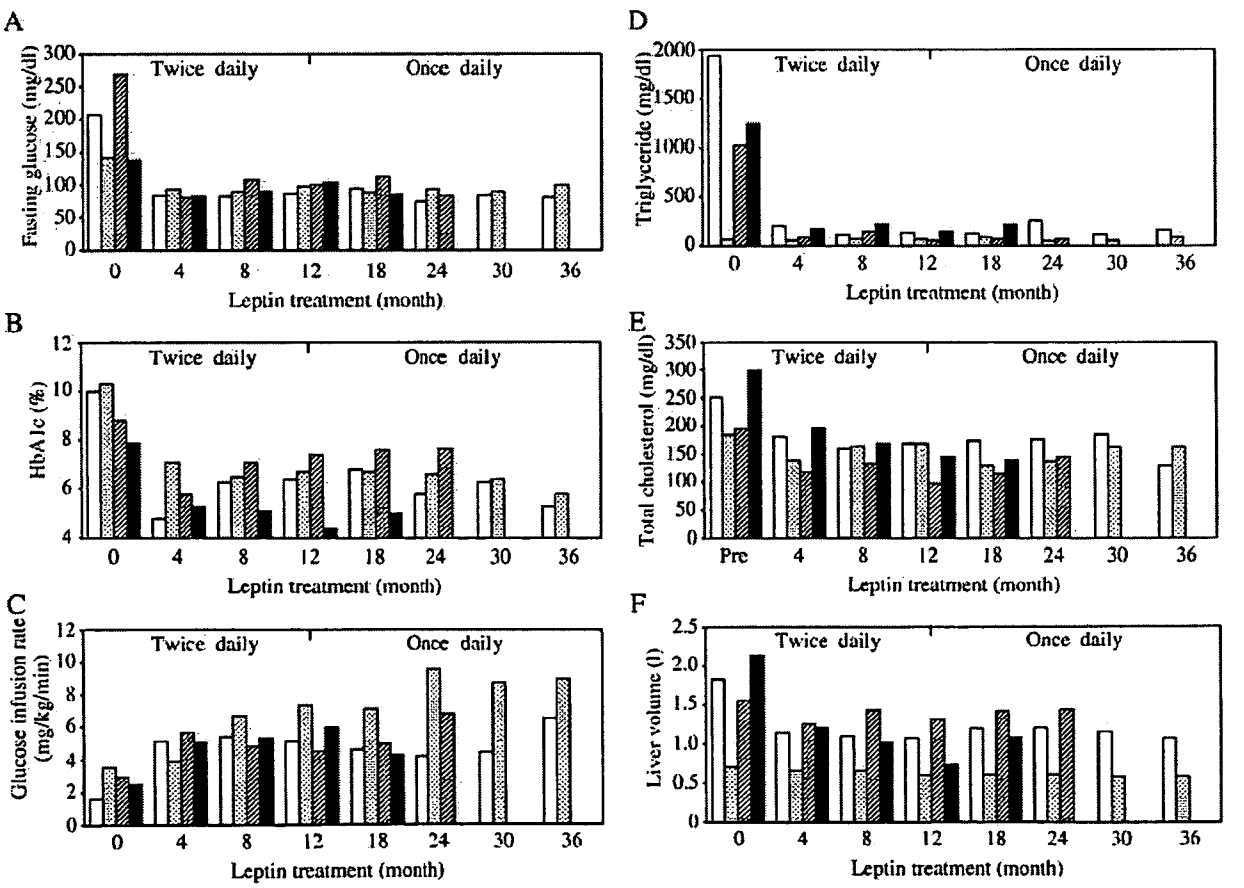

FIG. 2. Fasting plasma glucose levels (A), HbA1c levels (B), glucose infusion rates during the hyperinsulinemic euglycemic clamp study (C), triglyceride levels (D), total cholesterol levels (E), and liver volumes (F) before and after 4, 8, 12, 18, 24, 30, and 36 months of the leptin-replacement therapy in patient 1 (*white bars*), patient 2 (*dotted bars*), patient 3 (*hatched bars*), and patient 4 (*black bars*).

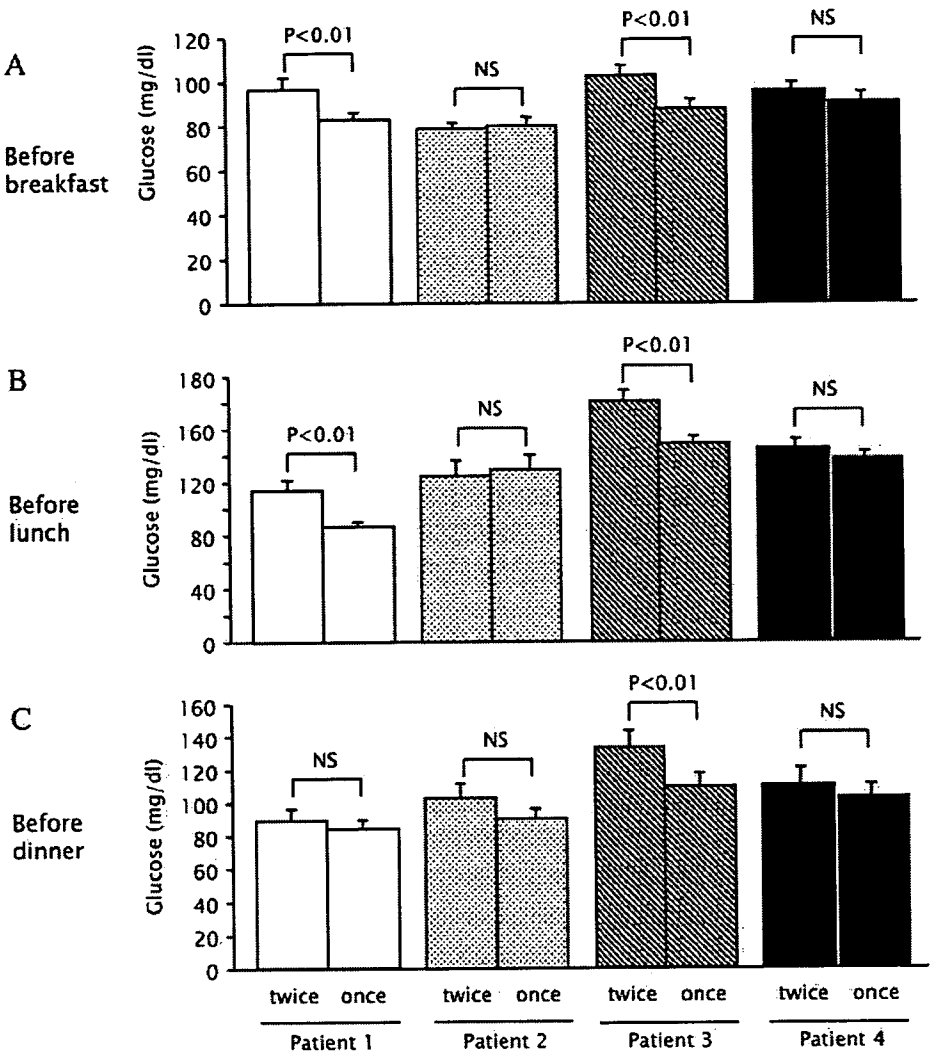

FIG. 3. Comparison of the mean (± SE) plasma glucose levels during the 12th month under the protocol of twice-daily leptin injection and during the 13th month under the protocol of once-daily leptin injection before breakfast (A), lunch (B), and dinner (C) in patient 1 (*white bars*), patient 2 (*dotted bars*), patient 3 (*hatched bars*), and patient 4 (*black bars*). NS, No significance difference ($P > 0.05$) between groups.

were well controlled throughout the therapy period (Fig. 2, C–F).

Antileptin antibodies

Patients 2 and 3, both CGL patients, showed elevations of basal plasma leptin levels, 75.0 and 42.4 ng/ml at the end of the 12th month, respectively. We detected antileptin antibodies in both patients. Antibodies from these patients did not neutralize the action of leptin at all in a bioassay.

Diabetes and other complications

All seven patients had normal renal functions at baseline; however, two patients had microalbuminuria (>30 mg/d), and two patients had macroalbuminuria (>300 mg/d) (Table 1). In addition, five of seven patients had elevated creatinine clearance (mean ± SE, 206.5 ± 22.0 ml/min·1.73 m^2) at the level above 125 ml/min·1.73 m^2. After the initiation of leptin-replacement therapy, urine albumin excretion of patients 1 and 3 with microalbuminuria began to decrease gradually within 1 month and was normalized within 2 months (Fig. 4A). Macroalbuminuria of patients 4 and 6 was also regressed to microalbuminuria within 3 and 1 month, respectively (Fig. 4B). In parallel, the creatinine clearance of the five patients with glomerular hyperfiltration significantly decreased to 129.5 ± 24.5 ml/min·1.73 m^2 (mean ± SE) for the 4-month leptin-replacement therapy ($P < 0.05$). These beneficial effects of leptin on urine albumin excretion and glomerular hyperfiltration were stable for up to 36 months.

Six of seven patients showed no diabetic retinopathy, but patient 7 had a nonproliferative retinopathy at baseline. No deterioration of her retinopathy was observed during the therapy. Six of seven patients had no diabetic neuropathy at baseline, although patient 6 showed neurogenic bladder. During the therapy period, her neurogenic bladder did not worsen, and no patients developed diabetic retinopathy or diabetic neuropathy.

Five of seven patients had moderate to severe acanthosis nigricans at baseline, but the acanthosis nigricans was improved in five patients after the leptin-replacement therapy.

Four of five female patients who were of reproductive age had hypogonadotropic amenorrhea at baseline as previously reported (26, 27) but resumed and sustained normal menses

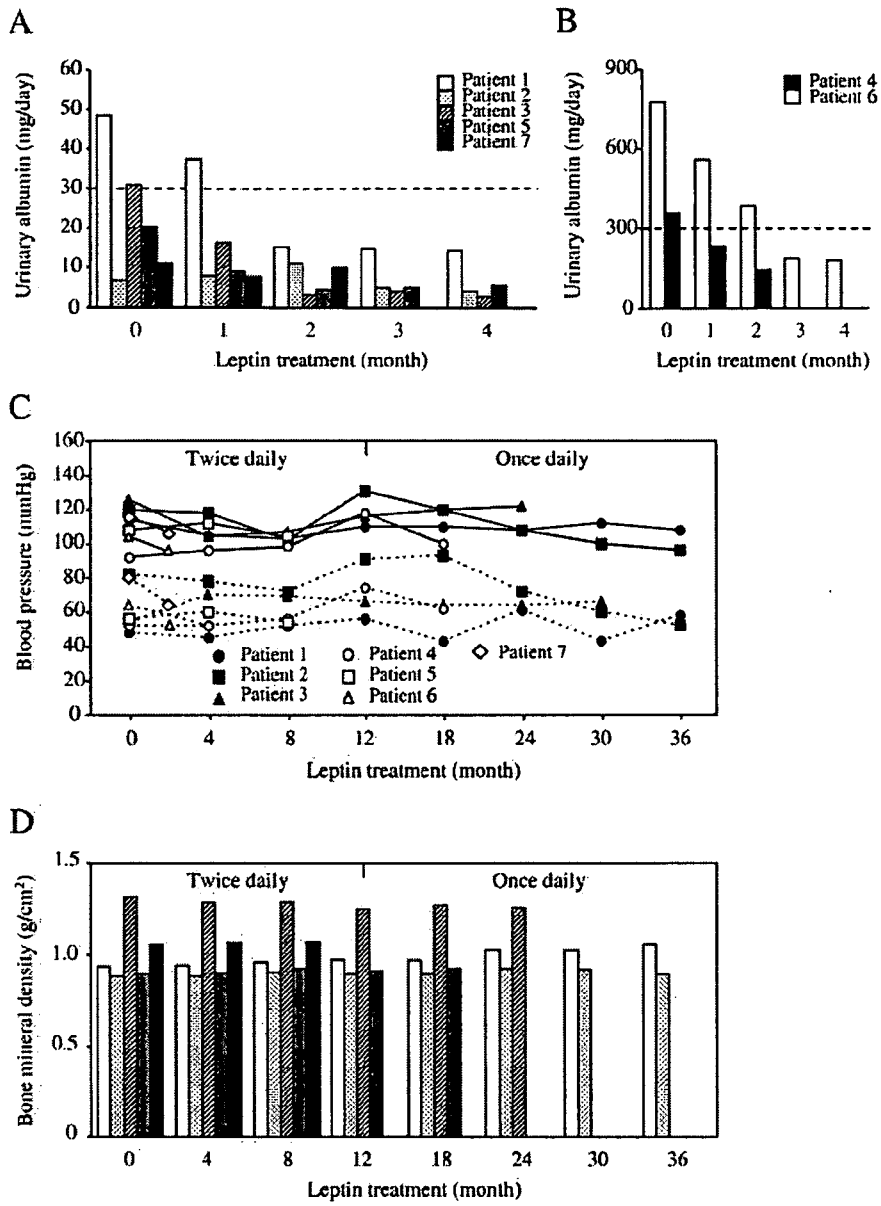

FIG. 4. A and B, Daily excretion levels of urinary albumin before and after 1, 2, 3, and 4 months of the leptin-replacement therapy in patients 1, 2, 3, 5, and 7 and in patients 4 and 6. C, Blood pressure before and after 4, 8, 12, 18, 24, 30, and 36 months of the leptin-replacement therapy in each patient. *Solid lines* indicate systolic blood pressure, and *broken lines* indicate diastolic blood pressure. D, Whole-body bone mineral density before and after 4, 8, 12, 18, 24, 30, and 36 months of the leptin-replacement therapy in patient 1 (*white bars*), patient 2 (*dotted bars*), patient 3 (*hatched bars*), patient 4 (*gray bars*), and patient 5 (*black bars*).

after the initiation of the leptin therapy. In an 11-yr-old girl, the menarche was observed after 12 months of the leptin therapy.

All the patients indicated an improvement in feeling of satisfaction after a meal within 1 or 2 d after the initiation of leptin therapy. This effect was sustained throughout the leptin therapy. For the first 4 months, a tendency of body weight reduction was observed in all the patients, but this change was not significant (mean ± SE, 40.9 ± 3.5 to 38.1 ± 3.1 kg, $P = 0.55$). After the first 4 months, the body weight was almost unchanged throughout the leptin therapy.

Adverse effects

We carefully observed blood pressure in the patients. At baseline, no patients showed hypertension (Table 1), and no distinct elevation of blood pressure was observed at any time throughout the therapy period (Fig. 4C).

No patients showed abnormal bone mineral density (Table 1). Whole-body bone mineral densities of the patients were unchanged for up to 36 months (Fig. 4D).

In all the patients, no other adverse effects of the leptin-replacement therapy including skin reactions at injection sites were detected for up to 36 months.

Discussion

In the present study, all of the leptin injections were done by medical doctors, because self-injection of leptin, which is not approved as a drug, is not permitted in Japan. In addition, all the patients were evaluated as inpatients during the initial 4 months of the leptin-replacement therapy. After leaving the hospital, the patients attended local clinics every day for every leptin injection. This allowed close supervision of leptin-replacement therapy, and the patients' lifestyles including diet and exercise were maintained constant. This

condition could minimize the influences of compliance of leptin injection and changes of diet and exercise. Although we could not include a randomized, placebo-treated control group in the present study because of the rarity and clinical diversity of generalized lipodystrophy, it is highly likely that the improved metabolic control is due to the leptin therapy rather than to an improvement in general compliance associated with participation in the study.

In previous reports, improvements of glucose and triglyceride levels, glucose tolerance, and insulin sensitivity were reported at 1 month (18, 25, 28). The present study clearly shows that significant reductions of fasting glucose levels are achieved within 7 d after the initiation of the leptin-replacement therapy, and substantial reductions of the triglyceride levels are also gained within 7 d (Table 2). These rapid and powerful effects of leptin-replacement therapy were further confirmed with the glucose tolerance test and hyperinsulinemic-euglycemic glucose clamp study performed after 7 or 10 d in patient 4. These rapid effects on glucose and lipid metabolism in the present study are comparable to the rapid effects of leptin administration in two different mouse models of generalized lipodystrophy (16, 17).

After 12 months of the twice-daily leptin treatment, we tried to alter the leptin injection protocol to a once-daily injection without change of total daily dose. This protocol alteration did not affect the controls of glucose and lipid metabolism, and these controls were maintained for up to 24 months (Figs. 2 and 3). These observations demonstrate that a once-daily leptin injection is sufficient to control glucose and lipid metabolism in patients with generalized lipodystrophy.

In the present study, we detected antileptin antibodies in two of four tested patients. Both of them were CGL patients, whereas we did not detect antileptin antibodies in AGL patients. This observation raises the possibility that antileptin antibodies more easily develop in CGL patients than AGL patients. Antibodies from both of our CGL patients did not neutralize the leptin action *in vitro* bioassay. In at least one child with congenital leptin deficiency, the transient appearance of neutralizing antibodies against leptin was reported (21). It is possible to speculate that neutralizing antibodies against leptin more easily develop in patients with congenital leptin deficiency than CGL patients, who have a little leptin levels.

The leptin-replacement therapy substantially ameliorated or did not worsen diabetic complications. Amelioration of proteinuria in the present study is consistent with our and other's previous reports that leptin-replacement therapy significantly alleviates the glomerular injury and proteinuria of lipoatrophic diabetes in mice and humans (29, 30). Although we could not perform renal biopsies, it is highly likely that proteinuria observed in our patients is due to diabetic nephropathy because their proteinuria and hyperfiltration were evidently improved in parallel with the metabolic improvement. These findings indicate that leptin is useful to treat, at least, a certain type of diabetic nephropathy.

The leptin-replacement therapy did not induce elevation of blood pressure in any patients throughout the therapy period (Fig. 4C). We previously demonstrated that a high plasma leptin level that is 10 times of that in normal controls elevates blood pressure through the activation of the sympathetic nervous system in mice (31). It is highly likely that the leptin-replacement therapy at the physiological replacement dose does not affect blood pressure.

Bone mineral density of the patients was within normal range at baseline and was unchanged during the therapy period for up to 36 months (Fig. 4D), consistent with the study reported previously (32). We also previously demonstrated that leptin is a powerful inhibitor of bone formation in mice (33). Although the present study indicates that the leptin-replacement therapy at the physiological replacement dose does not affect bone mineral density in humans, careful follow-up is necessary for young patients.

The effect of leptin on β-cell function remains unclear. Leptin treatment decreased serum insulin levels in mouse models of lipodystrophy (16, 17) and human lipoatrophic patients in the United States (18). These decreases of insulin levels were explained by the reduction of glucose levels rather than the suppressive effect of leptin. Indeed, insulin levels peaked earlier in lipoatrophic patients in the United States, although their overall amounts of insulin secreted in response to the glucose load were less after the leptin therapy than at baseline (28). On the other hand, we here demonstrate that leptin-replacement therapy dramatically improves insulin secretion in Japanese AGL patients. Because glucose-lowering therapy often leads to the restoration of β-cell function in patients with diabetes, this effect can be explained at least in part by the cancellation of glucotoxicity (34). The different responses of insulin secretion to leptin-replacement therapy between AGL and CGL patients could be accounted for by the different duration of diabetes. The impaired insulin responses to the glucose load in CGL patients suggests that their β-cell functions were already exhausted before leptin-replacement therapy. Although whether leptin has an additional effect on β-cell is unknown, we here demonstrate that leptin-replacement therapy is beneficial to the treatment of impaired β-cell function.

The mechanisms through which leptin exerts its insulin-sensitizing actions are unclear at present. Fat accumulation in the insulin target organs, which causes so-called lipotoxicity, is considered to be one of the mechanisms for insulin resistance in patients with lipodystrophy (35). Because in patient 4 with AGL, the improvement of insulin sensitivity was observed before a substantial decrease of tissue lipid content in the liver and muscle, additional studies are necessary to clarify the relationship between insulin resistance and the tissue lipid content in humans.

Based on the effect of the leptin-replacement therapy, it is highly likely that leptin deficiency is the main cause of the metabolic abnormalities associated with lipodystrophy. However, the adipose tissue is recognized as the largest endocrine organ. Therefore, it is possible to speculate that these hormones other than leptin may be involved to some degree in the pathogenesis of lipoatrophic diabetes.

Using leptin-overexpressing transgenic skinny mice (8), we previously reported that leptin treatment is useful for treatment of not only lipoatrophic diabetes mice (17) but also other diabetic mice models (36, 37). These observations along with dramatic effects and safety of the leptin therapy in the

present study indicate possible application of the leptin therapy to diabetes and its complications.

In summary, under strict control of lifestyle and an extremely high compliance of leptin injection, we demonstrate that the leptin-replacement therapy improves both insulin sensitivity and insulin secretion dramatically and rapidly improves glucose and lipid metabolism in patients with generalized lipodystrophy, and its effects are maintained for up to 36 months without any adverse effects. In addition, the leptin-replacement therapy is beneficial to diabetic complications and lipodystrophic ones. The once-daily leptin injection is sufficient to control glucose and lipid metabolism for a long time. It is concluded that leptin-replacement therapy is an effective and safe treatment for long-term improvement of glucose and lipid metabolism and complications in generalized lipodystrophy.

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Cell Metabolism
Short Article

Central Melanocortin Signaling Restores Skeletal Muscle AMP-Activated Protein Kinase Phosphorylation in Mice Fed a High-Fat Diet

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SUMMARY

Little is known about the role of the central melanocortin system in the control of fuel metabolism in peripheral tissues. Skeletal muscle AMP-activated protein kinase (AMPK) is activated by leptin and serves as a master regulator of fatty acid β-oxidation. To elucidate an unidentified role of the central melanocortin system in muscle AMPK regulation, we treated conscious, unrestrained mice intracerebroventricularly with the melanocortin agonist MT-II or the antagonist SHU9119. MT-II augmented phosphorylation of AMPK and its target acetyl-CoA carboxylase (ACC) independent of caloric intake. Conversely, AMPK/ACC phosphorylation by leptin was abrogated by the coadministration of SHU9119 or in KKA^y mice, which centrally express endogenous melanocortin antagonist. Importantly, high-fat-diet-induced attenuation of AMPK/ACC phosphorylation in leptin-overexpressing transgenic mice was not reversed by central leptin but was markedly restored by MT-II. Our data provide evidence for the critical role of the central melanocortin system in the leptin-skeletal muscle AMPK axis and highlight the system as a therapeutic target in leptin resistance.

INTRODUCTION

Leptin augments fatty acid β-oxidation in skeletal muscle and enhances whole-body insulin sensitivity, thereby serving as a promising therapeutic candidate for the treatment of insulin resistance and dyslipidemia (Shimabukuro et al., 1997; Minokoshi et al., 2002). In agreement with this notion, we and others have demonstrated the clinical efficacy of leptin in the treatment of diabetes, dyslipidemia, and steatosis in patients with lipodystrophy (Oral et al., 2002; Ebihara et al., 2007). The clinical application of leptin has been hampered, however, by the fact that leptin does not fully exert its metabolic effect in prevalent forms of human obesity (Maffei et al., 1995) and in diet-induced obese rodents (El-Haschimi et al., 2000).

Using transgenic skinny mice overexpressing leptin in liver (LepTg mice), we recently demonstrated that enhanced lipid metabolism and insulin sensitivity in LepTg mice are attenuated on a high-fat diet (HFD) (HFD-LepTg) despite persistent hyperleptinemia, compared with HFD-fed nontransgenic (HFD-non-Tg) littermates (Tanaka et al., 2005). Even with pronounced hyperleptinemia, skeletal muscle AMPK activity is attenuated in HFD-LepTg mice to the level of HFD-non-Tg mice (Tanaka et al., 2005). Noteworthy is the fact that switching HFD back to a standard diet (STD) leads to a significant recovery of muscle AMPK activity in LepTg mice before they regain their skinny phenotype (Tanaka et al., 2005), suggesting the reversible nature of the dietary lipid-induced leptin resistance.

AMPK is activated by decreased energy stores and orchestrates energy-sparing reactions in a cell-specific manner (Hardie et al., 2006). In skeletal muscle cells, AMPK activation stimulates glucose uptake, glycolysis, fatty acid β-oxidation, and mitochondrial biogenesis (Hardie et al., 2006) and critically mediates leptin-induced fatty acid β-oxidation (Minokoshi et al., 2002). Our previous work demonstrated that skeletal muscle AMPK activity closely parallels insulin sensitivity and inversely correlates with energy efficiency in LepTg mice under STD or HFD feeding (Tanaka et al., 2005), indicating that AMPK activity should be a novel biochemical marker of leptin sensitivity in vivo.

The central melanocortin system consists of endogenous melanocortin agonists and receptors. Endogenous agonist is synthesized as pro-opiomelanocortin (POMC) preprohormone and is proteolytically cleaved to produce melanocyte-stimulating hormones (MSHs). In the brain,

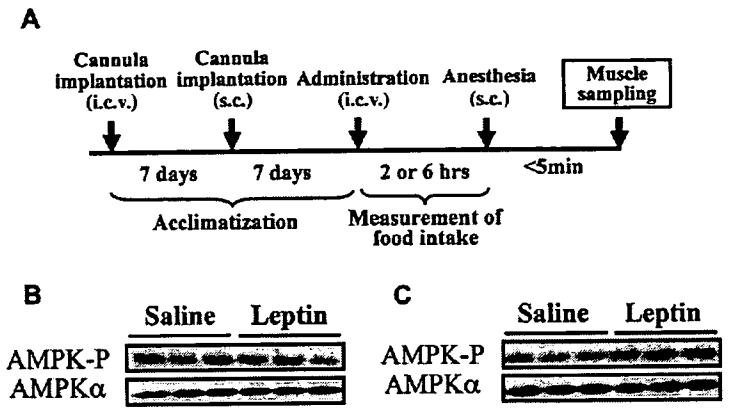

Figure 1. Establishment of the Experimental Protocol

(A) After intracerebroventricular (i.c.v.) and subcutaneous (s.c.) cannula implantation, mice were acclimatized and trained for handling. On the day of sampling, agents were administered i.c.v. in free-moving nonanesthetized mice. Food intake was measured and anesthesia was introduced s.c.

(B) Representative blots for phospho-AMPK and AMPKα in soleus muscle after leptin (0.5 μg) i.c.v. under intraperitoneal (i.p.) anesthesia. Muscles were sampled after another i.p. injection of the anesthetic agent.

(C) Representative blots for phospho-AMPK and AMPKα using the protocol in (A).

POMC is principally expressed in the arcuate nucleus of the hypothalamus (ARC) and the nucleus of the solitary tract (NTS) of the brainstem (Schwartz et al., 1997). The majority of POMC-expressing neurons in the ARC coexpress functional leptin receptor and mediate the anorectic effect of leptin (Cowley et al., 2001; Seeley et al., 1997). Although mouse models and human subjects with defective melanocortin signaling develop obesity (Huszar et al., 1997; Fan et al., 1997; Kobayashi et al., 2002; Farooqi et al., 2003), it remains largely unknown whether the central melanocortin system regulates fuel metabolism in skeletal muscle. In this context, we demonstrate here that the central melanocortin system is a critical mediator of leptin-induced skeletal muscle AMPK activation. In sharp contrast to leptin, melanocortin agonist retains its AMPK-activating potency even in mice fed a HFD.

RESULTS

Assessment of Skeletal Muscle AMPK Phosphorylation after Intracerebroventricular Administration in Conscious, Unrestrained Mice

AMPK activity is readily altered by various stimuli such as muscle contraction and ischemia (Hardie et al., 2006). To obtain reproducible results, establishment of an elaborately organized protocol was indispensable to minimize nonspecific AMPK activation. Intracerebroventricular (i.c.v.) and subcutaneous (s.c.) cannulae were implanted 14 and 7 days prior to the experiment, respectively, so that body weight recovered to the level of nonoperated mice on the sampling day (data not shown). Subcutaneous instead of intravenous cannulation was used to avoid a major weight loss. Agents were injected i.c.v. in well-acclimatized, nonanesthetized, free-moving mice. Anesthesia was introduced gently through s.c. cannula, and samples were obtained within 5 min (Figure 1A).

We first administered leptin i.c.v. under intraperitoneal (i.p.) anesthesia and sampled muscle after another i.p. injection of the anesthetic agent. By this method, we could not see a reproducible increase in phospho-AMPK levels by leptin (Figure 1B). In contrast, by the protocol in Figure 1A, leptin-induced increase in AMPK phosphorylation was clear (Figure 1C). In addition, we could also measure food intake, which was unaffected by anesthesia or by the nervousness of the mouse, precisely.

Melanocortin Agonist Increases AMPK Phosphorylation in Skeletal Muscle

Melanotan II (MT-II) is a potent melanocortin 3/4 receptor (MC3R/4R) agonist (Fan et al., 1997). Although it is known that central treatment with MT-II reduces food intake (Fan et al., 1997), its effect on skeletal muscle fuel metabolism has not been thoroughly addressed. We treated 8-week-old male C57BL/6 mice with leptin (0.5 μg) or MT-II (3.5 μg) i.c.v. and sampled the soleus muscle 6 hr later. Leptin i.c.v. significantly increased AMPK phosphorylation in the soleus muscle (Figure 2A). Likewise, MT-II i.c.v. increased phospho-AMPK levels by 69% ± 19% ($p < 0.05$ versus vehicle, $n = 7$). Neither leptin nor MT-II altered AMPKα protein levels, resulting in a 78% ± 14% ($p < 0.05$ versus vehicle, $n = 7$) and 64% ± 20% ($p < 0.05$ versus vehicle, $n = 7$) increase in phospho-AMPK/AMPKα ratio by leptin and MT-II, respectively (Figure 2A).

ACC is an established target of AMPK in muscle (Hardie et al., 2006). Phosphorylation of ACC by AMPK inhibits ACC enzyme activity and reduces the production of malonyl-CoA, thereby activating fatty acid β-oxidation (Minokoshi et al., 2002). In the present study, ACC phosphorylation was also augmented by MT-II by 90% ± 28% ($p < 0.05$ versus vehicle, $n = 7$) an increase comparable to that by leptin (83% ± 12%) ($p < 0.05$ versus vehicle, $n = 7$) (Figure 2B). Neither leptin nor MT-II i.c.v. altered ACC protein levels (see Figure S1A in the Supplemental Data available with this article online).

LepTg mice exhibit a more than 10-fold increase in plasma leptin levels, a paucity of adipose tissue, and enhanced glucose and lipid metabolism (Ogawa et al., 1999). In LepTg mice, soleus muscle AMPK phosphorylation and ACC phosphorylation were substantially increased compared with wild-type littermates (insets in Figures 2A and 2B). The levels of AMPK and ACC phosphorylation in MT-II or leptin i.c.v.-treated mice were comparable to those in LepTg mice.

AMPK phosphorylation was increased 2 hr after leptin i.c.v. by 63% ± 11% ($p < 0.05$ versus vehicle, $n = 7$) and maintained this level up to 6 hr (75% ± 10% increase)

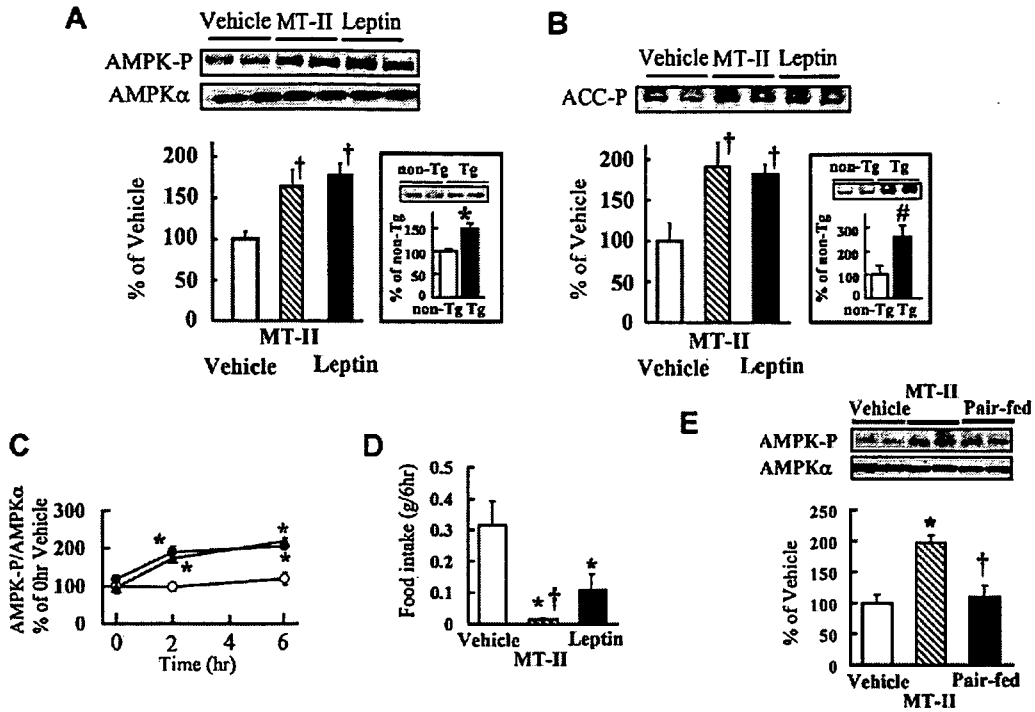

Figure 2. Increased Skeletal Muscle AMPK and ACC Phosphorylation after Intracerebroventricular MT-II Treatment

(A) Representative blots for phospho-AMPK and AMPKα in soleus muscle sampled 6 hr after MT-II (3.5 μg) or leptin (0.5 μg) i.c.v. The graph shows quantification of phospho-AMPK divided by that of AMPKα (phospho-AMPK/AMPKα ratio). †$p < 0.05$ versus vehicle, n = 7. Inset: Blots for phospho-AMPK and phospho-AMPK/AMPKα in LepTg mice. *$p < 0.05$ versus non-Tg, n = 6. In this and all other figures, error bars represent ± SEM.

(B) Representative blots for phospho-ACC in soleus muscle 6 hr after MT-II or leptin i.c.v. †$p < 0.05$ versus vehicle, n = 7. The graph shows the quantified data. Inset: Phospho-ACC in LepTg. #$p < 0.05$ versus non-Tg, n = 6.

(C) Phospho-AMPK/AMPKα before and 2 and 6 hr after MT-II or leptin i.c.v. ○, vehicle; ●, leptin; ▲, MT-II; *$p < 0.05$ versus vehicle, n = 6.

(D) Cumulative food intake over 6 hr. *$p < 0.05$ versus vehicle, †$p < 0.05$ versus leptin, n = 7.

(E) Blots for phospho-AMPK and AMPKα, and graph showing phospho-AMPK/AMPKα in MT-II-treated mice and vehicle-treated mice pair-fed with MT-II-treated counterparts. *$p < 0.05$ versus vehicle, †$p < 0.05$ versus MT-II, n = 6.

($p < 0.05$ versus vehicle, n = 7) (Figure 2C). Likewise, MT-II i.c.v. led to a significant increase in AMPK phosphorylation over 2 hr, by 100% ± 7% ($p < 0.05$ versus vehicle, n = 7), and this level was maintained until the 6 hr point (125% ± 12% increase) ($p < 0.05$ versus vehicle, n = 7) (Figure 2C). To determine whether increased AMPK phosphorylation is paralleled by any change in plasma glucose or insulin levels, mice were fasted for 3 hr, MT-II was injected i.c.v., and blood samples were obtained 6 hr later. No difference was observed in plasma glucose (vehicle 122 ± 12 mg/dl versus MT-II 125 ± 9 mg/dl; not significant [NS], n = 6) or insulin (vehicle 1.84 ± 0.10 ng/ml versus MT-II 1.77 ± 0.08 ng/ml; NS, n = 6) levels between vehicle- and MT-II-treated mice.

Leptin or MT-II i.c.v. suppressed food intake (Figure 2D). Over 6 hr, mice treated with leptin i.c.v. consumed 0.11 ± 0.05 g of food ($p < 0.05$ versus vehicle), while vehicle-treated mice consumed 0.32 ± 0.08 g. Mice treated with MT-II i.c.v. consumed 0.02 g with a standard deviation of less than 0.01 g ($p < 0.05$ versus vehicle, $p < 0.05$ versus leptin, n = 7). To rule out possible involvement of the anorectic effect of MT-II in increased AMPK phosphorylation in the muscle, we pair-fed vehicle-treated mice with mice treated with MT-II. In contrast to MT-II-treated mice, AMPK phosphorylation was not altered in the pair-fed mice ($p < 0.05$ versus vehicle, n = 6) (Figure 2E), showing that MT-II-induced AMPK phosphorylation is independent of suppression of food intake.

We next examined whether peripheral MT-II administration has a similar effect on skeletal muscle AMPK. We administered a single dose of MT-II (10 μg, s.c.) and examined AMPK and ACC phosphorylation 6 hr later. Peripheral treatment with MT-II at this dose also significantly increased AMPK (61% ± 19%) and ACC (69% ± 20%) phosphorylation compared with vehicle ($p < 0.05$ versus vehicle, n = 5) (data not shown).

Leptin-Induced AMPK and ACC Phosphorylation Is Attenuated by Melanocortin Antagonism

Leptin activates the hypothalamic melanocortin pathway (Schwartz et al., 1997; Cowley et al., 2001) and enhances skeletal muscle AMPK activity (Minokoshi et al., 2002). To test whether the central melanocortin system is involved in leptin-induced AMPK activation in the muscle, we injected

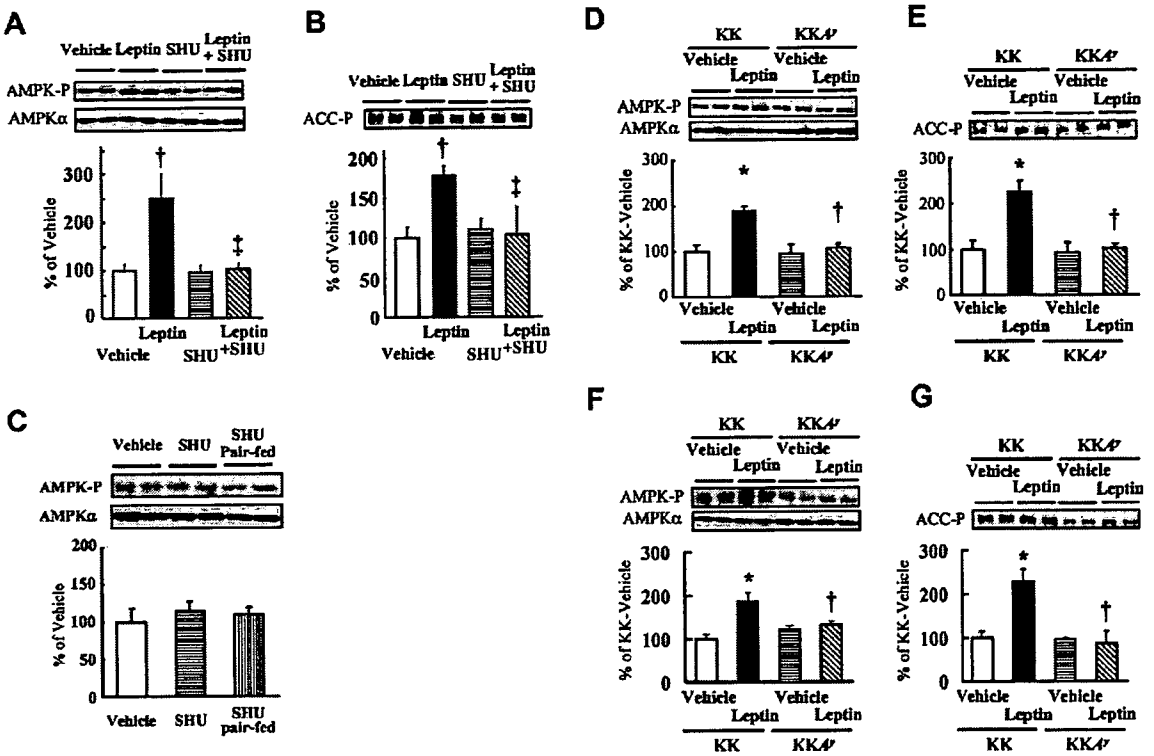

Figure 3. Attenuation of Leptin-Induced Muscle AMPK and ACC Phosphorylation by Pharmacologic or Genetic Melanocortin Blockade

(A, C, D, and F) Blots for phospho-AMPK and AMPKα, and graph showing phospho-AMPK/AMPKα.

(B, E, and G) Blots for phospho-ACC and quantified data.

Coadministration of SHU9119 (1.0 μg) i.c.v. attenuated leptin (0.5 μg)-induced increase in AMPK (A) and ACC (B) phosphorylation (†$p < 0.05$ versus vehicle, ‡$p < 0.05$ versus leptin, n = 7). Phospho-AMPK/AMPKα was not affected in mice treated with SHU9119 alone or in SHU9119-treated mice pair-fed with vehicle-treated mice (A and C). Leptin i.c.v. significantly increased AMPK (D and F) and ACC phosphorylation (E and G) in KK mice, while the increase was attenuated in both 6-week-old (D and E) and 10-week-old KKA^y mice (F and G) (*$p < 0.05$ versus KK-vehicle, †$p < 0.05$ versus KK-leptin, n = 6; data are % of KK-vehicle).

SHU9119, a MC3R/4R antagonist (Fan et al., 1997). Coadministration of SHU9119 i.c.v. (1.0 μg) suppressed leptin-induced increase in AMPK (Figure 3A) and ACC phosphorylation (Figure 3B) to the vehicle level (phospho-AMPK/AMPKα 150% ± 49% increase by leptin versus 4% ± 10% increase by leptin + SHU9119; phospho-ACC 79% ± 13% increase by leptin versus 6% ± 34% increase by leptin + SHU9119; $p < 0.05$, n = 7). Intracerebroventricular injection of SHU9119 alone did not affect AMPK (5% ± 9% decrease) or ACC (10% ± 12% increase) phosphorylation (Figures 3A and 3B). Central administration of SHU9119 alone or in conjunction with leptin did not change ACC protein levels (Figure S1B). SHU9119 i.c.v. significantly increased food intake over the following 6 hr (vehicle 0.35 ± 0.08 g versus SHU9119 0.49 ± 0.09 g; $p < 0.05$, n = 6). To test the hypothesis that increased food intake caused by SHU9119 may play a role in skeletal muscle AMPK regulation, we compared AMPK/ACC phosphorylation in vehicle-treated mice, SHU9119-treated mice, and SHU9119-treated mice pair-fed with vehicle-treated mice. Neither treatment with SHU9119 only nor treatment with SHU9119 plus pair-feeding had any effect on AMPK (SHU9119 15% ± 12% increase, SHU9119 plus pair-feeding 10% ± 10% increase versus vehicle) (Figure 3C) or ACC phosphorylation (SHU9119 8% ± 10% decrease, SHU9119 plus pair-feeding 13% ± 5% versus vehicle; NS, n = 6) (data not shown).

KKA^y mice (A^y mutants on a KK background) ectopically express agouti protein, an endogenous melanocortin receptor antagonist, throughout the body, including in the hypothalamus, and exhibit progressive obesity in addition to yellow coat color (Lu et al., 1994). Here we used preobese (6 weeks old, KK 29.0 ± 0.8 g versus KKA^y 29.1 ± 1.0 g; NS, n = 10) and obese (10 weeks old, KK 35.7 ± 1.4 g versus KKA^y 39.9 ± 1.0 g; $p < 0.05$, n = 14) male KKA^y mice to examine leptin-induced AMPK activation. The levels of AMPK and ACC phosphorylation were not significantly different between control KK and KKA^y mice at either age (Figures 3D–3G). Leptin i.c.v. led to a significant increase in AMPK (phospho-AMPK/AMPKα 90% ± 10% increase versus KK-vehicle; $p < 0.05$, n = 5) and ACC phosphorylation (125% ± 25% increase versus

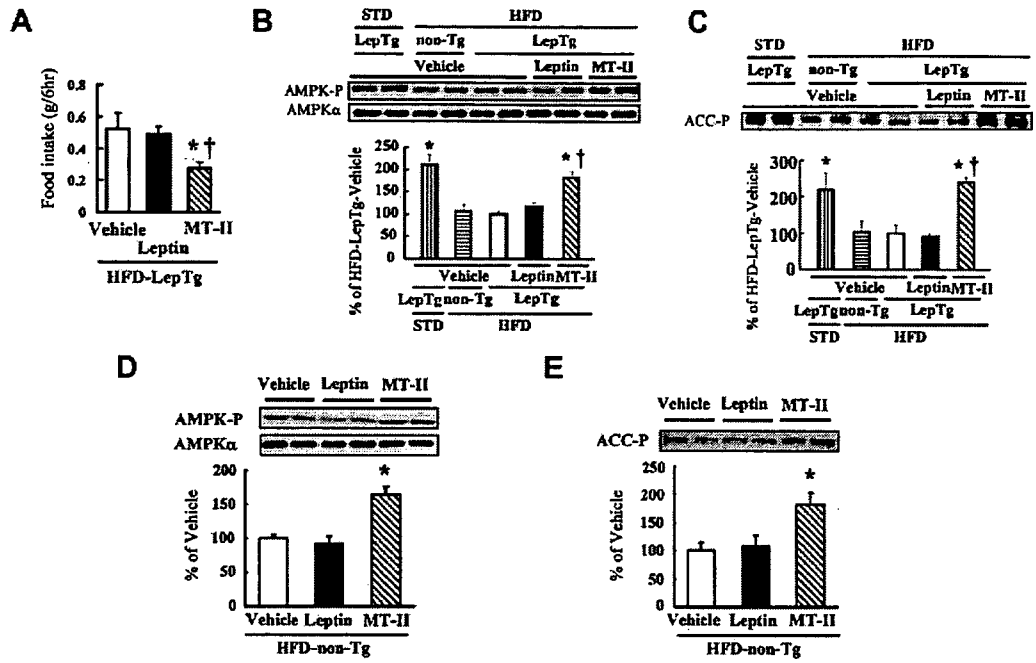

Figure 4. Recovery of Skeletal Muscle AMPK and ACC Phosphorylation by Intracerebroventricular MT-II Treatment in Mice Fed a High-Fat Diet

(A) Food intake over the 6 hr after i.c.v. injection in HFD-LepTg mice. *p < 0.05 versus vehicle, †p < 0.05 versus leptin, n = 7.

(B and D) Blots for phospho-AMPK and AMPKα, and graph showing phospho-AMPK/AMPKα ratio.

(C and E) Blots for phospho-ACC and quantified data.

(B–E) In HFD-LepTg mice, AMPK phosphorylation (B) and ACC phosphorylation (C) were decreased in comparison to STD-LepTg mice and were comparable to HFD-non-Tg mice. In HFD-LepTg mice, leptin i.c.v. did not alter AMPK (B) or ACC phosphorylation (C). MT-II (3.5 μg) i.c.v. restored AMPK (B) and ACC phosphorylation (C) in HFD-LepTg mice (*p < 0.05 versus HFD-LepTg-vehicle, †p < 0.05 versus HFD-LepTg-leptin, n = 7; data are % of HFD-LepTg vehicle) and HFD-non-Tg mice (D and E) (*p < 0.05 versus HFD-non-Tg-vehicle, n = 5).

KK-vehicle, p < 0.05, n = 5) in 6-week-old KK mice, while the increase was attenuated in 6-week-old KKA^y mice (phospho-AMPK/AMPKα 15% ± 21% increase, phospho-ACC 10% ± 11% increase; p < 0.05 versus KK-leptin, n = 5) (Figures 3D and 3E). ACC protein levels were not different between 6-week-old KK and KKA^y mice (Figure S1C). The results were similar in 10-week-olds, with a significant leptin-induced increase in AMPK (phospho-AMPK/AMPKα 85% ± 17% increase versus KK-vehicle; p < 0.05, n = 7) and ACC phosphorylation (133% ± 28% increase versus KK-vehicle; p < 0.05, n = 7) in KK mice and a lack of change in KKA^y mice by leptin (phospho-AMPK/AMPKα 18% ± 3% increase, phospho-ACC 21% ± 32% decrease; p < 0.05 versus KK-leptin, n = 7) (Figures 3F and 3G).

Central Treatment with MT-II Leads to Recovery of HFD-Induced Attenuation in AMPK and ACC Phosphorylation

LepTg mice exhibit decreased caloric intake and increased energy expenditure (Ogawa et al., 1999; Tanaka et al., 2005). Enhanced glucose tolerance, increased insulin sensitivity, and lower plasma triglyceride in LepTg mice are independent of reduced food intake (Ogawa et al., 1999). Over 4 weeks on HFD, LepTg mice, which remain significantly more hyperleptinemic than non-Tg mice (176 ± 4 versus 78 ± 7 ng/ml), become as obese, glucose intolerant, insulin resistant, and hyperlipidemic as non-Tg mice (Tanaka et al., 2005). Muscle AMPK/ACC phosphorylation is also attenuated (Tanaka et al., 2005).

To address the effect of central melanocortin activation under HFD, we treated HFD-LepTg mice with MT-II. MT-II i.c.v., but not leptin i.c.v., suppressed food intake in HFD-LepTg mice (vehicle 0.52 ± 0.10 g, leptin 0.49 ± 0.05 g, MT-II 0.28 ± 0.04 g; MT-II p < 0.05 versus vehicle or leptin, n = 7) (Figure 4A). In HFD-LepTg mice, muscle AMPK phosphorylation and ACC phosphorylation were significantly decreased compared with STD-LepTg mice and were comparable to HFD-non-Tg mice (Figures 4B and 4C). Of note, leptin i.c.v. in addition to transgenic hyperleptinemia did not augment AMPK (21% ± 14% increase, NS) or ACC phosphorylation (8% ± 6% decrease, NS) in HFD-LepTg mice (Figures 4B and 4C). In contrast, MT-II i.c.v. effectively augmented AMPK (by 85% ± 13%; p < 0.05 versus HFD-LepTg vehicle) and ACC phosphorylation (by 139% ± 16%; p < 0.05 versus HFD-LepTg vehicle) in HFD-LepTg mice (Figures 4B and 4C), suggesting that MT-II is a potent AMPK activator in muscle even under HFD. To confirm the results with LepTg mice, we treated HFD-fed wild-type (non-Tg) mice with leptin or MT-II

i.c.v. As expected, MT-II i.c.v., but not leptin i.c.v., led to a significant increase in muscle AMPK (leptin 8% ± 10% decrease, NS; MT-II 64% ± 12% increase versus vehicle; $p < 0.05$, $n = 5$) (Figure 4D) and ACC phosphorylation (leptin 19% ± 25% increase, NS; MT-II 96% ± 20% increase versus vehicle; $p < 0.05$, $n = 5$) (Figure 4E) in the wild-type mice. ACC expression was not altered by HFD or by HFD plus leptin or MT-II i.c.v. (Figure S1D).

DISCUSSION

Despite vigorous research, the question of why leptin loses its lipid-mobilizing potency under HFD has not been fully answered. Hypothalamic and peripheral induction of SOCS-3 (Bjorbaek et al., 1998; Howard et al., 2004; Wang et al., 2005; Kievit et al., 2006) and decreased permeability of the blood-brain barrier (El-Haschimi et al., 2000; Oh-I et al., 2005) have been implicated in attenuated leptin receptor signaling and metabolic efficacy. A recent study has demonstrated blunted hypothalamic AMPK as well as STAT3 signaling in HFD-fed mice (Martin et al., 2006). In the present study, neither transgenic hyperleptinemia nor central leptin treatment increased skeletal muscle AMPK phosphorylation under HFD. Notably, however, central MT-II administration did increase skeletal muscle AMPK phosphorylation in mice fed a HFD, indicating a mechanism upstream of the central melanocortin system that is responsible for the leptin resistance.

AMPK is a cellular fuel gauge activated by a variety of stresses. To stably assess AMPK activity in skeletal muscle, samples must be obtained quickly and deliberately. Furthermore, surgical interventions such as i.c.v. or intravenous cannulation cause substantial weight loss during the following week, making it more difficult to interpret the results in terms of energy homeostasis. In this sense, implementation of our devised protocol was instrumental in showing that skeletal muscle AMPK phosphorylation is regulated by the central melanocortin system. Phosphorylation of the α subunit of AMPK at Thr172 is tightly correlated with AMPK activity in many experimental conditions, including leptin-induced AMPK activation in the skeletal muscle (Minokoshi et al., 2002). Increased phosphorylation of AMPK and ACC in parallel firmly suggests increased AMPK activity in vivo.

In contrast to the effects of the central melanocortin system on satiety, little is known about its impact on fuel metabolism in the peripheral tissues. A recent study has shown that MT-II i.c.v. increases basal and insulin-stimulated glucose disposal and basal hepatic glucose production (Heijboer et al., 2005). However, MT-II does not seem to alter insulin-dependent suppression of hepatic glucose production (Heijboer et al., 2005). Our data here demonstrate that the administration of melanocortin agonist augments skeletal muscle AMPK and ACC phosphorylation. Considering the crucial role of skeletal muscle AMPK in the regulation of fatty acid β-oxidation (Minokoshi et al., 2002), our data strongly suggest a metabolic link between the central melanocortin system and skeletal muscle fatty acid mobilization. On the other hand, we did not observe any change in fasting plasma glucose or insulin levels 6 hr after MT-II i.c.v. Heijboer et al. (2005) also report an absence of change in basal plasma glucose and insulin levels following MT-II i.c.v. Further studies utilizing a combination of glucose clamp and measurement of AMPK activity may give a more accurate view of the temporal relationships between AMPK activation and skeletal muscle glucose utilization. POMC neurons are chiefly present in the ARC and NTS, while MC4R, a major melanocortin receptor involved in energy homeostasis, is widely distributed and present in the paraventricular hypothalamic nucleus (PVN) and the dorsal motor nucleus of the vagus (DMV) (Liu et al., 2003). Further studies are necessary to specify which nuclei within the central melanocortin system mediate skeletal muscle AMPK activation.

Leptin is a pleiotropic hormone, serving as a critical regulator of energy homeostasis, reproduction, blood pressure, and bone metabolism (Masuzaki et al., 1997; Aizawa-Abe et al., 2000; Ducy et al., 2000). Although a crucial role of the hypothalamic melanocortin system has been recognized in the anorexigenic effect of leptin, the matter of which functions of leptin are melanocortin dependent or independent still remains controversial. We previously reported that hypertension in LepTg mice is not ameliorated by SHU9119 i.c.v., implicating a melanocortin-independent pathway in blood pressure control by leptin (Aizawa-Abe et al., 2000). In terms of effects on glucose homeostasis, a recent study has shown that enhancement in hepatic gluconeogenesis by leptin is blocked by SHU9119 i.c.v., whereas leptin-dependent reduction in glycogenolysis is not (Gutierrez-Juarez et al., 2004). Taking these previous studies together, it is reasonable to postulate that metabolic regulation by leptin is mediated by both melanocortin-dependent and -independent pathways. Here we show that pharmacological (SHU9119) or genetic (KKA^y) blockade of the melanocortin receptor attenuates leptin-dependent AMPK and ACC phosphorylation. These data provide evidence that the leptin-skeletal muscle AMPK axis is mediated by the central melanocortin system.

Leptin-induced augmentation of AMPK and ACC phosphorylation was attenuated in both 6-week-old and 10-week-old KKA^y mice. In 10-week-old obese KKA^y mice, decreased AMPK response to leptin may be partly attributable to the secondary effect of obesity. However, absence of muscle AMPK activation was observed even in lean 6-week-old KKA^y mice, further supporting the notion that melanocortin signaling is necessary for leptin-induced AMPK activation. In KKA^y mice, skeletal muscle phospho-AMPK levels were not altered compared with KK mice despite reported hyperphagia (Fan et al., 1997). Central administration of SHU9119 alone also causes hyperphagia (Fan et al., 1997) but did not alter skeletal muscle AMPK phosphorylation in our present study. The discrepancy between food intake and AMPK phosphorylation may suggest diverging pathways regulating satiety and skeletal muscle AMPK activity.

A recent study has shown that peripheral, but not central, administration of ciliary neurotrophic factor (CNTF), another potent anorectic agent in HFD-fed mice, activates

skeletal muscle AMPK (Watt et al., 2006). Although CNTF, like leptin, increases phospho-STAT3 in the ARC, the anorectic effect of CNTF remains intact in *Mc4r* knockout mice (Marsh et al., 1999). Noting that the anorectic effect of leptin is substantially attenuated in *Mc4r* knockouts (Marsh et al., 1999), these data suggest that central signaling cascades of leptin and CNTF are independent at the level of the melanocortin system and that centrally mediated AMPK activation in muscle is unique to the leptin-melanocortin pathway.

A previous work (Pierroz et al., 2002) and ours here demonstrate that MT-II suppresses food intake in mice fed a HFD. Another paper published recently has shown that leptin-induced α-MSH secretion from the ARC is abrogated in obese mice fed a HFD (Enriori et al., 2007). In the Enriori et al. study, the authors also showed that *Mc4r* expression is reciprocally upregulated in PVN from obese mice. These data further support our results showing that MT-II remains effective in suppressing food intake and activating skeletal muscle AMPK even under HFD. Taken together, our findings reinforce the notion that MT-II may be beneficial for the treatment of insulin resistance, a pathology characterized by a myocellular lipid excess. However, since the results of the present study are based mainly on data from i.c.v. injections, further studies are necessary to explore the clinical efficacy of melanocortin agonists.

In conclusion, our data demonstrate that leptin-induced skeletal muscle AMPK activation is at least partly mediated by the central melanocortin system. In contrast to leptin, AMPK activation by melanocortin agonist is preserved even under HFD. Our data provide an insight into the central regulation of skeletal muscle AMPK activity and suggest a possible recovery of skeletal muscle fatty acid β-oxidation by melanocortin agonists under dietary lipid overload.

EXPERIMENTAL PROCEDURES

Animal Experiments

C57BL/6 (B6), KK, and KKA^y mice were obtained from CLEA Japan. Heterozygous LepTg and non-Tg littermates on a B6 background (Ogawa et al., 1999) were used. Animals were maintained on STD (F-2, 3.7 kcal/g, 12% of kcal from fat, source soybean, Funahashi Farm) and a 14 hr light/10 hr dark cycle at 23°C. HFD was from Research Diets (D12493, 5.2 kcal/g, 60% of kcal from fat, source soybean/lard). Animals were given free access to food and water unless otherwise mentioned. Body weight and food intake were monitored for the animals' well-being. Experiments were started between 6 and 8 weeks of age, except for KK and KKA^y experiments. HFD was administered for 4 weeks. Cannulae (Plastics One) were inserted stereotactically into lateral ventricles and fixed. On the sampling day, leptin (0.5 μg), MT-II (3.5 μg), SHU9119 (1.0 μg), or a combination was administered in 0.5 μl saline solution at around the start of the light period. The weight of the food pellet at the start and at the end of the experiment was measured with a microbalance (A&D). Five to ten g/kg chloral hydrate (Nakalai Tesque) was administered through s.c. cannula (PE20, Becton Dickinson) 2 or 6 hr after i.c.v. injection, and the soleus muscle was sampled. Successful i.c.v. delivery of the reagents was ensured by injecting dye to every animal postmortem and omitting data from those with inadequate distribution of the dye. Plasma glucose and insulin levels were measured by Glucose C-II Test Wako (Wako Pure Chemical Industries) and Insulin ELISA Kit (Morinaga). Animal experiments were performed in accordance with the Kyoto University guidelines for animal experiments and were approved by the Animal Research Committee, Kyoto University Graduate School of Medicine.

Western Blots

Muscle samples were homogenized as described (Tanaka et al., 2005). After denaturing, 15 μg per lane of protein was loaded on 10% and 4%–20% SDS-polyacrylamide gels for AMPK and ACC, respectively, and transferred to PVDF membrane (PerkinElmer). Phosphospecific antibodies were used for the detection of phospho-AMPK and phospho-ACC. Antibodies were anti-phospho-Thr172 AMPKα, anti-AMPKα (Cell Signaling Technology), and anti-phospho-Ser79 ACC (Upstate). ECL Plus (Amersham), a LAS-1000 image analyzer, and MultiGauge version 2.0 (Fujifilm) were used for detection and quantification.

Statistical Analyses

Data are presented as means ± SEM. Comparisons between or among animal groups were performed by Student's t test or repeated analysis of variance (ANOVA), where applicable, and completed by Fisher's probable least-significant-difference test.

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/5/5/395/DC1/.

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Pathway for Differentiation of Human Embryonic Stem Cells to Vascular Cell Components and Their Potential for Vascular Regeneration

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Objective—We demonstrated previously that mouse embryonic stem (ES) cell–derived vascular endothelial growth factor receptor-2 (VEGF-*R*2)–positive cells can differentiate into both vascular endothelial cells and mural cells. This time, we investigated kinetics of differentiation of human ES cells to vascular cells and examined their potential as a source for vascular regeneration.

Methods and Results—Unlike mouse ES cells, undifferentiated human ES cells already expressed VEGF-*R*2, but after differentiation, a VEGF-*R*2-positive but tumor rejection antigen 1-60 (TRA1-60)–negative population emerged. These VEGF-*R*2-positive but tumor rejection antigen 1-60–negative cells were also positive for platelet-derived growth factor receptor α and β chains and could be effectively differentiated into both VE-cadherin$^+$ endothelial cell and α-smooth muscle actin$^+$ mural cell. VE-cadherin$^+$ cells, which were also CD34$^+$ and VEGF-*R*2$^+$ and thought to be endothelial cells in the early differentiation stage, could be expanded while maintaining their maturity. Their transplantation to the hindlimb ischemia model of immunodeficient mice contributed to the construction of new blood vessels and improved blood flow.

Conclusions—We could identify the differentiation process from human ES cells to vascular cell components and demonstrate that expansion and transplantation of vascular cells at the appropriate differentiation stage may constitute a novel strategy for vascular regenerative medicine. (***Arterioscler Thromb Vasc Biol.* 2007;27:2127-2134.**)

Key Words: angiogenesis ■ developmental biology ■ embryonic stem cells ■ vascular biology ■ endothelium

Pluripotent embryonic stem (ES) cells are gaining attention as promising cell sources for regenerative medicine, especially after the establishment of human ES cells.[1] Because the knockout animal research approach is not applicable to humans, investigating human cell development/differentiation using human ES cells is more helpful. These cells possess a number of characteristics distinct from those of mouse ES cells, such as surface antigens, leukemia inhibitory factor independency, and long doubling time.[1] We demonstrated previously that mouse ES cell–derived vascular endothelial growth factor (VEGF) receptor-2 (VEGF-*R*2)–positive cells can differentiate into both vascular endothelial cells (ECs) and mural cells (MCs), the latter composed of pericytes and vascular smooth muscle cells. We termed these mouse VEGF-*R*2$^+$ cells "vascular progenitor cells" (VPCs).[2] We also showed that VEGF and the vasodilating peptide adrenomedullin, which, as we reported previously, enhances angiogenesis,[3] play important roles in EC differentiation from these mouse VEGF-*R*2$^+$ cells.[4] However, recent studies have shown that, in undifferentiated human ES cells, unlike in mouse ES cells, VEGF-*R*2 is expressed and continues to be expressed during differentiation associated with embryoid body formation.[5,6] To further clarify the vascular differentiation process in human beings and to determine the possible clinical application of ES cells to vascular regeneration, investigation of human ES cells is essential. It was also found that CD31$^+$ cells could be isolated from human embryoid bodies, indicating that they can act as ECs.[6] However, a precise analysis of the differentiation process

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from human ES cells to EC or other vascular cell components, such as MC, in the embryoid body differentiation system has so far not been possible.

In the study reported here, we identified the differentiation kinetics of human ES cells to vascular cell components by using our in vitro 2D differentiation system. Furthermore, we succeeded in establishing new human cell sources derived from human ES cells, which may be used for therapeutically effective transplantation in vivo.

Methods

Cell Culture

ES cells were maintained as described.[1,7] OP9 feeder cell lines were established and maintained as described,[8] whereas their growth was inactivated by mitomycin C.

To induce differentiation, ES cells were dissociated into small colonies with the aid of 0.1% collagenase (Wako) and cultured on an OP9 feeder layer in a differentiation medium (minimal essential medium, GIBCO) supplemented with 5×10^{-5} M 2-mercaptoethanol with 10% FCS. Sorted cells were recultured on a collagen IV–coated dish in the differentiation medium with the addition of 10% FCS, VEGF (100 ng/mL; PeproTech EC Ltd), or platelet-derived growth factor (PDGF)-BB (10 ng/mL) (PeproTech EC Ltd).

Flow Cytometry and Cell Sorting

Cells were detached by cell dissociation buffer (GIBCO) with or without collagenase and labeled with various fluorescence-conjugated monoclonal antibodies (please see http://atvb.ahajournals.org). Flow cytometry analysis and cell sorting were performed as described.[2,4,8]

Immunohistochemistry

Cultured cells were stained with various monoclonal antibodies (please see http://atvb.ahajournals.org) as described.[2,4] The immunofluorescence photographs were taken with a confocal laser-scanning microscope (LSM5-Pascal, Carl Zeiss).

Hindlimb Ischemia Model

Eight-week–old KSN/Slc and BALB/c Slc nude mice were purchased from SLC Japan. After anesthetization with pentobarbital (80 mg/kg IP), the right femoral vein was ligated. We injected 5×10^5 cells in 100 μL of PBS or 100 μL of PBS only into the right femoral artery. Immediately after the cell injection, the right femoral artery was ligated and excised.[9] Experimental procedures were performed in accordance with Kyoto University standards for animal care. Hindlimb blood flow was measured with a laser Doppler perfusion image analyzer (Moor Instruments Ltd), as described.[9] Biotin-conjugated Griffonia simplicifolia lectin I-isolectin B_4 (Vector Laboratories) was injected into the portal vein 15 minutes before sacrifice. After fixation with 4% paraformaldehyde, the ischemic lower legs were embedded in optimal cutting temperature compound (Sakura Finetechnical Co Ltd) and frozen. Capillary numbers were examined by counting the number of capillaries stained with anti-human and/or mouse CD31 antibody. Ten random fields on 2 different sections (~3 mm apart) from each mouse were photographed and analyzed by National Institutes of Health imaging as described.[9] The vessel area and length were measured quantitatively with the Kurabo angiogenesis image analyzer (Kurabo).

Teratoma Formation Study

We transplanted 5×10^5 cells in 50 μL of PBS under the dorsal back skin of SCID mice (CLEA Japan, Inc), which are commonly used for teratoma formation for human ES cells.[1,7] After 5 months, 1 cm^2 of the skin around the point of injection was harvested, and the excised tissue was serially sectioned (10 μm) at 200-μm intervals.

Analysis of Angiogenic Factor mRNA Expression

Total cellular RNA was isolated from VPCs or ECs in the early differentiation stage (eECs) with RNAeasy Mini kit (Qiagen KK). The mRNA expression was analyzed with the One-Step RNA PCR kit (TaKaRa). Primer pairs were purchased from R&D Systems Inc. PCRs were performed as manufacturers protocols.

Measurement of Angiogenic Factors in VPC- or eEC-Conditioned Media

Cells (1×10^6) of VPCs or eECs were plated on 10-cm dishes and incubated with 5 mL of media (minimal essential medium with 0.5% bovine serum) for 72 hours. The concentrations of human VEGF, basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) were measured by SRL, Inc. Human PDGF-BB was measured with the Human PDGF-BB Quantikine ELISA kit (R&D Systems Inc).

Statistical Analysis

Results are presented as mean±SEM. In the hindlimb ischemia model, the statistical significance was evaluated using ANOVA followed by Fisher's analysis for comparisons between 2 means. $P<0.05$ was considered significant.

Results

Differentiation Pathway of Human ES Cells to Vascular Cell Components

First, we examined the expression of VEGF-*R*2 and some putative stem cell markers on a human ES cell line, HES3, which was established at Monash University in Australia.[1] Approximately 50% of undifferentiated human ES cells expressed VEGF-*R*2, whereas these cells were also positive for AC133 and c-Kit but negative for CD34, respectively (Figure 1A). We also analyzed the expression of tumor rejection antigen (TRA) 1-60 on these human ES cells. The TRA1 antigen is expressed on the surface of human tetracarcinoma stem cells, human embryonic germ cells, and human ES cells. Thus, we used it as a marker of the human ES cell. The VEGF-$R2^+$ population of human ES cells was also positive for TRA1-60 (Figure 1B).

Next, we induced differentiation of human ES cells in an in vitro 2D culture on an OP9 stromal cell line. Although monkey ES cells have effectively differentiated as single cells on an OP9 layer,[10] human ES cells have not survived as single cells. We, therefore, plated small human ES cell colonies on OP9 to induce differentiation. Under these conditions, the TRA1-60^+ cell population gradually decreased in number during differentiation. On the other hand, a VEGF-$R2^+$ TRA1-60^- population emerged and accounted for ≈15% of all of the cells on day 8 (Figure 1C). We confirmed the differentiation kinetics of human ES cells by using another human ES cell line, KhES-1, established by us.[7] Similar to the HES3 cell line, VEGF-*R*2 was low positive, and the VEGF-$R2^+$ cells were also TRA1-60^+ in undifferentiated KhES-1 (Figure 1D). After differentiation on an OP9 feeder layer, VEGF-$R2^+$ TRA1-60^+ cells decreased, and VEGF-$R2^+$ TRA1-60^- cells appeared on days 8 (Figure 1E). Next, we analyzed the expression of several cell surface markers on the VEGF-$R2^+$ TRA1-60^- population on day 8 of HES3. Flt1 was positive, c-Kit and CXCR4 were both negative, PDGR receptor (PDGFR)α and PDGFRβ were positive, AC133

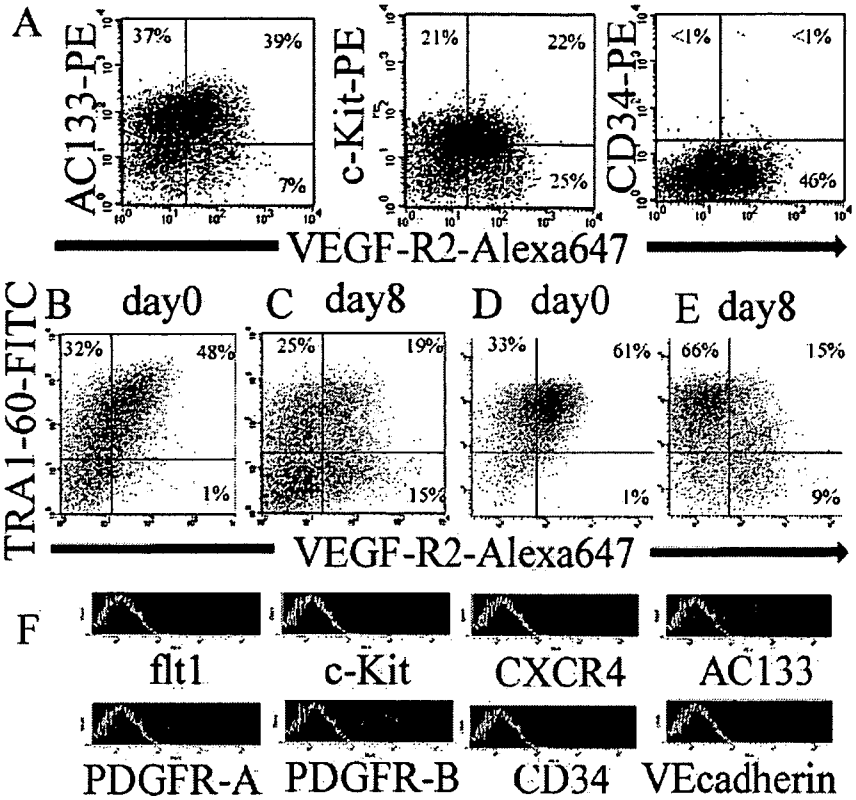

Figure 1. Flow cytometry analysis of differentiation kinetics of human ES cells. A, Expression of cell surface markers on undifferentiated human ES cells (HES3). B through E, TRA1-60 and VEGF-*R2* expression on 2 human ES cell lines (HES3 and KhES-1) during differentiation on an OP9 feeder layer. B and C, HES3; D and E, KhES-1. F, Cell surface marker expression on VEGF-*R2*$^+$ TRA1-60$^-$ cells on day 8.

was still positive, and CD34 and vascular endothelial cadherin (VE-cadherin) were negative on a large population of the VEGF-*R2*$^+$ TRA1-60$^-$ cells (Figure 1F). Monocyte markers, such as CD45, Cd11b, and CD14, were negative.

The VEGF-*R2*$^+$ TRA1-60$^-$ and VE-cadherin–negative cells were sorted by flow cytometry on day 8 and cultured on a collagen IV–coated dish without a feeder cell layer for an additional 8 days in the presence of 10% FCS and VEGF. This cell culturing condition induced the emergence of CD34$^+$, VE-cadherin$^+$, CD31$^+$, and endothelial NO synthase–positive cells (Figure 2A through 2D), which can be categorized as ECs. The rest of the cells negative for CD31 were polygonal in shape and showed α-smooth

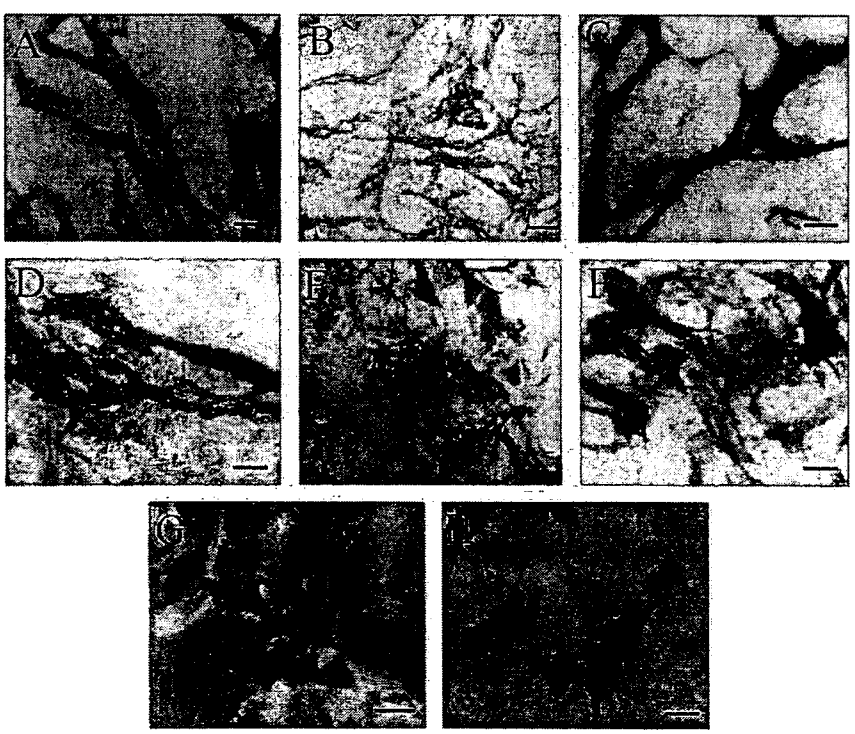

Figure 2. Immunocytological analysis of differentiation of vascular progenitor cells into vascular cells. A through D, Immunostaining for endothelial cell markers on VEGF-*R2*$^+$ TRA1-60$^-$ cells recultured with VEGF and FBS. A, CD34; B, VE-cadherin; C, CD31; D, endothelial NO synthase; E, Double immunostaining for CD31 (blue) and α-smooth muscle actin (brown). F and G, Immunostaining for MC markers on VEGF-*R2*$^+$TRA1-60$^-$ cells recultured with FBS. F, α-Smooth muscle actin; G, Calponin; H, Immunostaining for α-smooth muscle actin with treatment of PDGF-BB on VEGF-*R2*$^+$ TRA1-60$^-$ cells. Scale bars, 50 μm.