

# Skeletal Muscle AMP-Activated Protein Kinase Phosphorylation Parallels Metabolic Phenotype in Leptin Transgenic Mice Under Dietary Modification

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**Leptin augments glucose and lipid metabolism independent of its effect on satiety. Administration of leptin in rodents increases skeletal muscle  $\beta$ -oxidation by activating AMP-activated protein kinase (AMPK). We previously reported that, as hyperleptinemic as obese human subjects, transgenic skinny mice overexpressing leptin in liver (LepTg) exhibit enhanced insulin sensitivity and lipid clearance. To assess skeletal muscle AMPK activity in leptin-sensitive and -insensitive states, we examined phosphorylation of AMPK and its target, acetyl CoA carboxylase (ACC), in muscles from LepTg under dietary modification. Here we show that phosphorylation of AMPK and ACC are chronically augmented in LepTg soleus muscle, with a concomitant increase in the AMP-to-ATP ratio and a significant decrease in tissue triglyceride content. Despite preexisting hyperleptinemia, high-fat diet (HFD)-fed LepTg develop obesity, insulin-resistance, and hyperlipidemia. In parallel, elevated soleus AMPK and ACC phosphorylation in regular diet-fed LepTg is attenuated, and tissue triglyceride content is increased in those given HFD. Of note, substitution of HFD with regular diet causes a robust recovery of soleus AMPK and ACC phosphorylation in LepTg, with a higher rate of body**

**weight reduction and a regain of insulin sensitivity. In conclusion, soleus AMPK and ACC phosphorylation in LepTg changes in parallel with its insulin sensitivity under dietary modification, suggesting a close association between skeletal muscle AMPK activity and sensitivity to leptin. *Diabetes* 54:2365–2374, 2005**

**L**eptin, an adipocyte-derived hormone, serves as a master regulator of energy homeostasis by suppressing food intake and enhancing glucose and lipid metabolism (1). Metabolic derangements associated with leptin deficiency and lipotrophic diabetes have been successfully treated with leptin in murine models and human subjects (2–5). However, mechanisms underlying leptin-induced enhancement in glucose and lipid metabolism have not been fully elucidated. Recent studies have implicated skeletal muscle mitochondrial dysfunction and subsequent intramyocellular lipid accumulation in the pathophysiology of insulin resistance (6). Leptin has the potency of decreasing intramyocellular lipid by enhancing mitochondrial fatty acid  $\beta$ -oxidation (7,8). It has recently been reported that skeletal muscle AMP-activated protein kinase (AMPK), a cellular fuel gauge (9), is critically involved in the process (10).

On the other hand, a growing body of evidence has suggested the presence of insensitivity to leptin in prevalent forms of human obesity and rodent models of diet-induced obesity (DIO) (11). Mechanisms whereby leptin loses its effect under such conditions still remain to be elucidated. To the best of our knowledge, there has been no report to date addressing skeletal muscle AMPK activity under leptin-insensitive states in vivo.

We previously generated transgenic skinny mice overexpressing leptin in liver (LepTg) with elevated plasma leptin concentrations comparable with those of obese human subjects (12). LepTg lack almost all white adipose tissue depots throughout the body but exhibit enhanced glucose and lipid metabolism, providing a unique experimental model to investigate the mechanisms of leptin's metabolic action (4,12–15). Notably, unlike obese hyperleptinemic humans or rodents with DIO, sustained hyperleptinemia in LepTg remains metabolically active. LepTg

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ACC, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; DIO, diet-induced obesity; EDL, extensor digitorum longus; FFA, free fatty acid; GTT, glucose tolerance test; HFD, high-fat diet; ITT, insulin tolerance test; SCD-1, stearoyl CoA desaturase-1; UCP-1, uncoupling protein-1.

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show increased insulin sensitivity with augmented liver and skeletal muscle insulin receptor signaling (12) and increased whole-body lipid clearance during lipid loading tests (16). These data led us to hypothesize that skeletal muscle AMPK is chronically activated in LepTg.

In the present study, we tested whether augmented insulin sensitivity and lowered plasma lipid levels in LepTg is accompanied by an activation of leptin-skeletal muscle AMPK axis. As long as LepTg are maintained on regular diet, phosphorylation of AMPK and its target, acetyl CoA carboxylase (ACC), is chronically augmented in soleus muscle, a representative oxidative red muscle. Augmented phosphorylation of AMPK and ACC under regular diet is attenuated when LepTg loses its lean, insulin-sensitive phenotype under high-fat diet (HFD). After diet substitution from HFD to regular, LepTg exhibit a higher rate of body weight loss and become more insulin sensitive than nontransgenic littermates (non-Tg), suggesting a rapid recovery of sensitivity to leptin. Early in the process of weight loss, AMPK and ACC phosphorylation are robustly increased in LepTg but not in non-Tg. Our data demonstrate that skeletal muscle AMPK and ACC phosphorylation are in close association with metabolic phenotype in LepTg and shed light upon the physiologic and pathophysiologic roles of the leptin-skeletal muscle AMPK axis.

## RESEARCH DESIGN AND METHODS

The generation of transgenic mice has been reported previously (12). Six- to eight-week-old male mice heterozygous for the transgene and non-Tg on C57BL/6N background were used for the experiments. Animals were maintained on regular diet (F-2, 3.73 kcal/g, 11.6% kcal fat, source: soybean; Funahashi Farm, Chiba, Japan) and on a 14-h light/10-h dark cycle at 23°C. Mice were fed HFD or regular diet for 4 or 15 weeks. In another experiment, LepTg and non-Tg maintained on HFD were subjected to diet substitution from HFD to regular. HFD (D12493, 5.24 kcal/g, 60% kcal fat, source: soybean/lard) was purchased from Research Diets (New Brunswick, NJ). Throughout the experiments, animals were allowed free access to food and water. Chloral hydrate (1.6 µg/g i.p.; Nacalai Tesque, Kyoto, Japan) was used for anesthesia. All animal experiments were undertaken in accordance with the guideline for animal experiments of Kyoto University and were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

**Metabolic parameters and locomotive activity measurements.** Body weight/food intake were recorded daily. Dual-energy X-ray absorptiometry scan (DCS-600EX-III; Aloka, Japan) was undertaken using PHA-601R (Aloka) as a phantom.  $V_{O_2}$  was measured using a two-chamber Oxymax (MK5000; Muromachi kikai, Tokyo, Japan), an open-circuit indirect calorimetry. Plasma levels for glucose (Tidex; Sankyo, Tokyo, Japan), insulin (ELISA kit; Morinaga, Yokohama, Japan), triglycerides (Triglyceride-E test; Wako, Osaka, Japan), free fatty acids (FFAs) (NEFA-C test; Wako), leptin (mouse leptin radioimmunoassay kit; Linco, St. Charles, MO), and adiponectin (enzyme-linked immunosorbent assay kit; Otsuka, Tokyo, Japan) were measured. For the glucose tolerance test (GTT), mice were fasted for 8 h and were given 1.5 g/kg glucose i.p. (Otsuka). For the insulin tolerance test (ITT), mice were fasted for 3 h and given 1.25 units/kg human regular insulin i.p. (Novo Nordisk, Bagsvaerd, Denmark). Spontaneous locomotion was measured by tracing the distance traveled using infrared camera and Ethovision version 1.9 (Noldus Information Technology). Mice were placed in a dark actometer for a 15-min recording session from 1900 to 2300. Mean values from 3 consecutive days were used.

**Muscle triglyceride and adenine nucleotide content measurements.** Triglyceride content was measured as previously described (16) using isopropylalcohol/heptane for homogenization. For nucleotides, muscle samples were homogenized in 0.2 mol/l HClO<sub>4</sub> (3:80 wt/vol) in an ethanol-dry ice bath, and the supernatant was used for the assay (17). Briefly, the supernatant was neutralized, centrifuged, filtered (0.45-µm Cosmonice filter W; Nacalai Tesque), and then analyzed by high-performance liquid chromatography (DX300; Dionex, Sunnyvale, CA) equipped with an SPD-10Ai detector (Shimadzu, Kyoto, Japan) and Shodex Asahipack GS-320HQ (Showa Denko,

TABLE 1

Metabolic parameters in leptin transgenic mice under regular diet

	Non-Tg	LepTg
Leptin (ng/ml)	7.6 ± 1.0	39.4 ± 4.2*
Body weight (g)	24.9 ± 1.1	21.7 ± 0.6†
Energy intake (kcal/day)	12.5 ± 0.3	11.6 ± 0.6
$V_{O_2}$ (ml · kg <sup>-0.75</sup> · h <sup>-1</sup> )	1,257 ± 39	1,576 ± 29†
Glucose (mg/dl)	117 ± 7	114 ± 7
Insulin (ng/ml)	1.01 ± 0.09	0.43 ± 0.08†
Triglycerides (mg/dl)	131 ± 5	88 ± 14†
FFAs (mEq/l)	1.82 ± 0.10	1.43 ± 0.08†
Adiponectin (µg/ml)	20.8 ± 1.5	19.7 ± 0.4
Locomotion (m/h)	137 ± 7	177 ± 10†

Data are means ± SE. Six-week-old male mice fed regular diet for 4 more weeks. Body weight and plasma levels for leptin, glucose, insulin, triglycerides, free fatty acids, and adiponectin are from the end of 4 weeks. Blood was obtained ad libitum during 0900–1200. Energy intake is a mean daily caloric intake during 4 weeks.  $V_{O_2}$  was measured during the 1st week of the period.  $n = 10$  for leptin and adiponectin,  $n = 7$  for other parameters. \* $P < 0.01$ , † $P < 0.05$  vs. non-Tg.

Tokyo, Japan) equilibrated with 200 mmol/l sodium phosphate buffer (pH 3.0) at 1 ml/min and monitored at 254 nm.

**Western and Northern blot analyses.** Muscle samples were homogenized as previously described (17). Ten milligrams of protein per lane were run on 10% SDS-polyacrylamide gel for AMPK and on 4–10% NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA) for ACC and transferred to polyvinylidene fluoride (Perkin Elmer, Boston, MA). Thr172-phosphorylated AMPK $\alpha$  and Ser79-phosphorylated ACC were detected using phospho-specific antibodies. The antibodies are as follows: anti-phospho-Thr172 AMPK $\alpha$  and anti-AMPK $\alpha$  from Cell Signaling Technology (Wilmington, MA) and anti-phospho-Ser79 ACC and anti-ACC from Upstate Biotech (Charlottesville, VA). ECL Plus (Amersham) and an LAS-1000 image analyzer (Fujifilm) were used for the detection, and quantification was by MultiGauge version 2.0 software (Fujifilm). For Northern blotting, liver and brown adipose total RNA was extracted using Trizol reagent (Invitrogen). Northern blot analysis was performed using standard protocol (12). Membranes were hybridized with radiolabeled cDNA probe spanning the coding regions of mouse stearoyl CoA desaturase-1 (SCD-1) or uncoupling protein-1 (UCP-1).

**Statistical analyses.** Data are means ± SE unless otherwise indicated. Comparison between or among animal groups was undertaken by Student's *t* test or repeated-measures ANOVA, where applicable, and completed by Fisher's probable least significant difference test.

## RESULTS

**AMPK and ACC phosphorylation is augmented chronically in soleus muscle from LepTg.** Metabolic parameters in LepTg are summarized in Table 1. Along with hyperleptinemia, LepTg exhibit lower plasma insulin, triglyceride, and FFA levels with lower body weight and higher oxygen consumption compared with non-Tg (Table 1). Thr172 phosphorylated; thus, activated AMPK $\alpha$  was significantly increased ( $150 \pm 4\%$  of non-Tg;  $P < 0.05$  vs. non-Tg;  $n = 7$ ) in LepTg soleus muscle compared with that from non-Tg (Fig. 1A). Concomitantly, Ser79-phosphorylated ACC, an established target of AMPK, was also increased ( $268 \pm 9\%$  of non-Tg;  $P < 0.01$  vs. non-Tg;  $n = 7$ ) (Fig. 1B). Soleus muscle AMPK $\alpha$  and ACC $\beta$  protein levels in LepTg were not significantly different from those in non-Tg (Fig. 1C and D). In extensor digitorum longus (EDL) muscles, no difference in phosphorylation or protein levels of AMPK $\alpha$  or ACC $\beta$  was noted between the genotypes (Fig. 1).

Triglyceride was more abundant in soleus than in EDL muscle (Fig. 1E), as previously described (18). In parallel

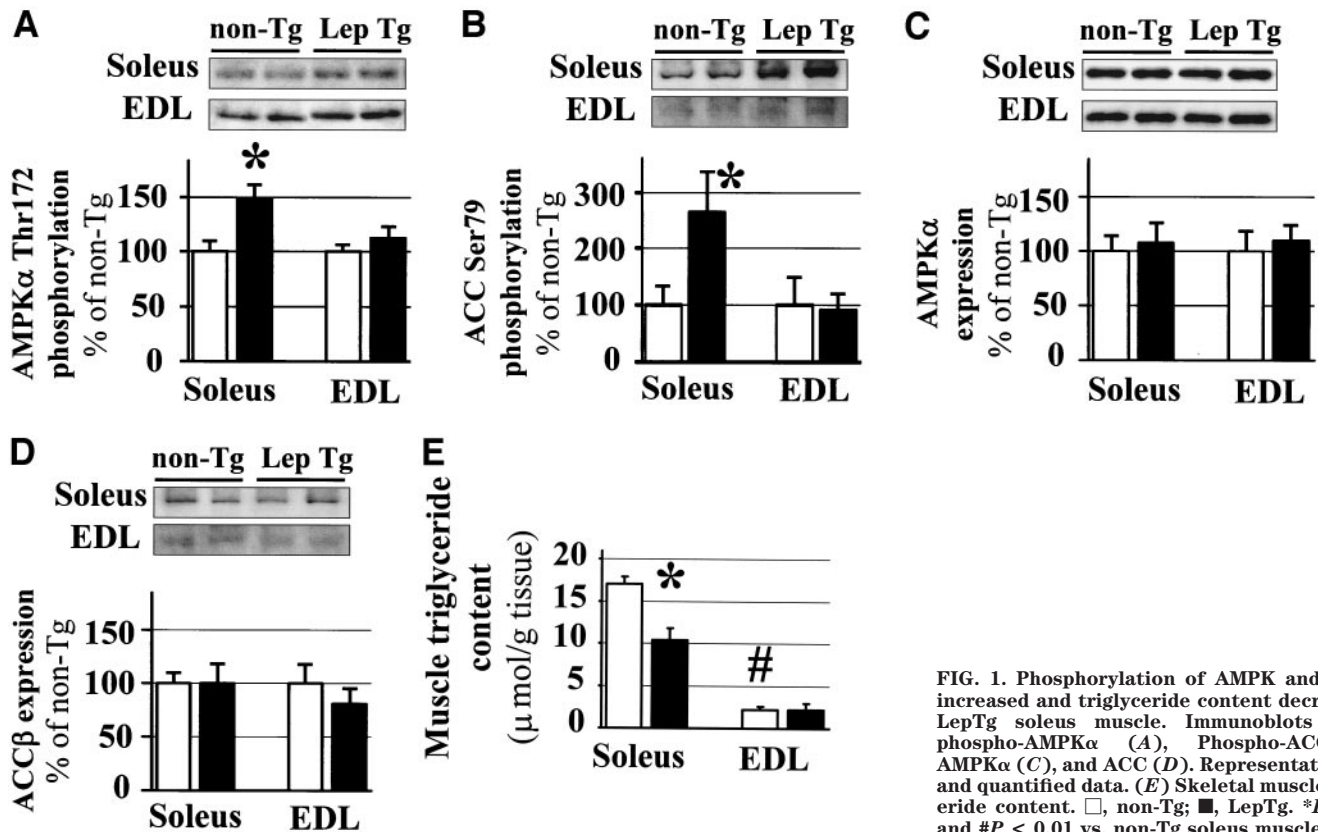


FIG. 1. Phosphorylation of AMPK and ACC is increased and triglyceride content decreased in LepTg soleus muscle. Immunoblots against phospho-AMPK $\alpha$  (A), Phospho-ACC (B), AMPK $\alpha$  (C), and ACC (D). Representative blots and quantified data. (E) Skeletal muscle triglyceride content.  $\square$ , non-Tg;  $\blacksquare$ , LepTg. \* $P < 0.05$  and # $P < 0.01$  vs. non-Tg soleus muscle.  $n = 7$ .

with increased phospho-AMPK and phospho-ACC levels, LepTg soleus muscle contained significantly less triglyceride than non-Tg ( $61.2 \pm 5.9\%$  of non-Tg;  $P < 0.05$  vs. non-Tg;  $n = 7$ ) (Fig. 1E). In EDL muscle, where phospho-AMPK and phospho-ACC levels were not altered in LepTg, triglyceride levels did not differ between the genotypes.

Apart from leptin, exercise (muscle contraction) and adiponectin are known activators of skeletal muscle AMPK (19–22). Plasma adiponectin levels in LepTg were comparable with those in non-Tg (Table 1). LepTg showed enhanced spontaneous locomotion (Table 1). Although AMPK activity elevated by exercise returns to basal level within an hour after exercise cessation (23), endurance training in humans is associated with upregulated AMPK phosphorylation and activity (24). A possible role of physical activity in enhanced AMPK phosphorylation in LepTg still awaits further investigation.

**Increased AMPK and ACC phosphorylation in LepTg soleus muscle is accompanied by increased AMP-to-ATP ratio.** AMPK is activated through multiple pathways, including its phosphorylation at Thr172 in its regulatory

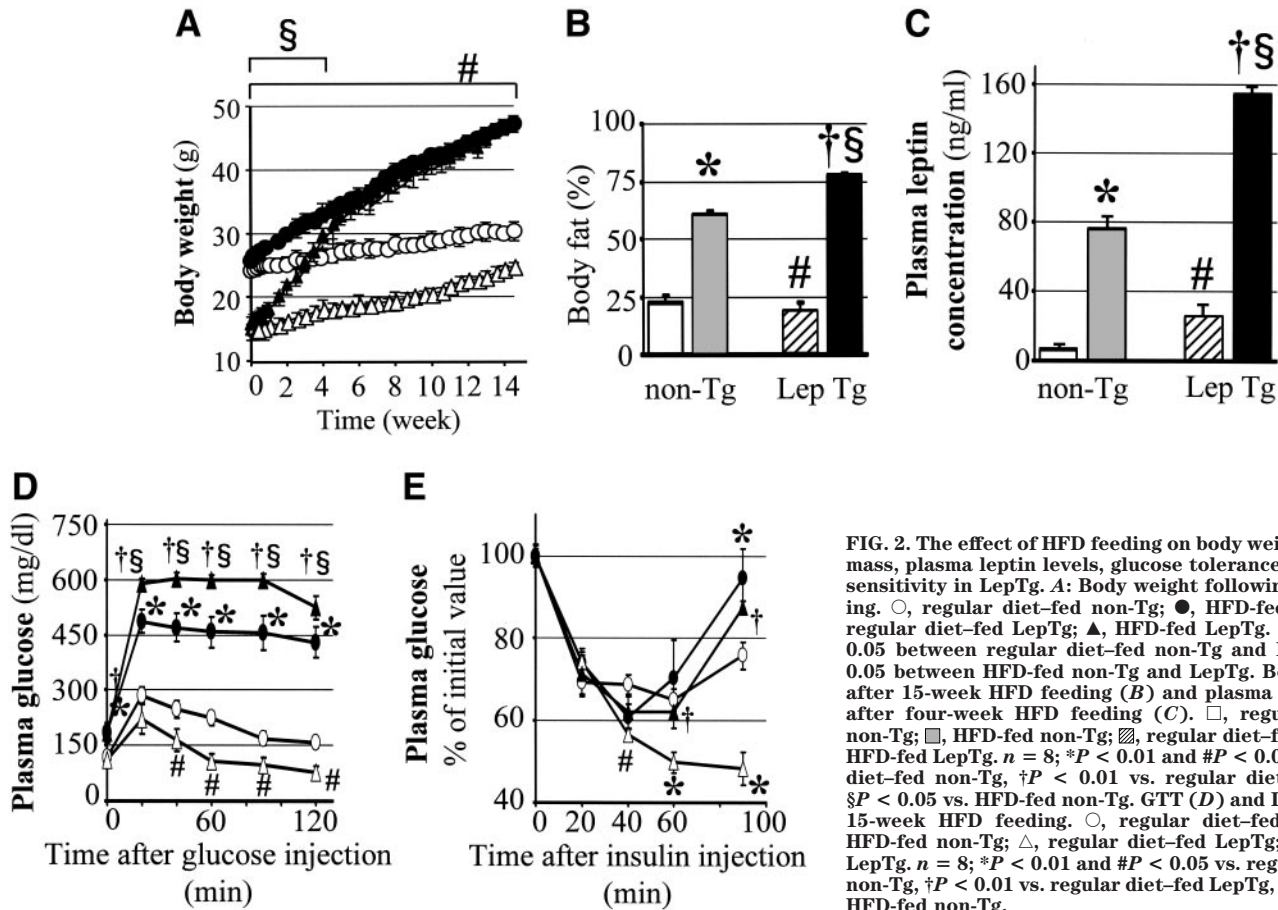
$\alpha$ -subunit by upstream AMPK kinase (9). AMPK is also allosterically activated by an increase in cellular AMP-to-ATP ratio. Furthermore, the increase in AMP-to-ATP ratio predisposes AMPK for Thr172 phosphorylation and prevents dephosphorylation (9). To unravel the mechanisms underlying chronic increase in AMPK and ACC phosphorylation in LepTg soleus muscle, adenine nucleotide levels were measured. A significant increase in the AMP-to-ATP ratio was observed in LepTg soleus muscle with increased AMP and decreased ATP levels (Table 2). In EDL muscle, the AMP-to-ATP ratio was not altered in transgenics. The increase in the AMP-to-ATP ratio may partly explain the increased AMPK and ACC phosphorylation in LepTg soleus muscle.

**LepTg develop obesity, glucose intolerance, insulin resistance, and hyperlipidemia under HFD.** When LepTg is genetically crossed with obese/diabetic  $A^y$  mice and lipotrophic A-ZIP/F-1 mice, transgenic overexpression of leptin improves glucose metabolism and insulin sensitivity in these mice (4,13). In this study, we examined whether HFD-induced metabolic derangements are also

TABLE 2  
Skeletal muscle adenine nucleotide contents in LepTg mice

	non-Tg		LepTg	
	Soleus	EDL	Soleus	EDL
ATP (nmol/mg muscle)	$9.35 \pm 0.13$	$9.49 \pm 0.42$	$8.19 \pm 0.19^*$	$10.18 \pm 0.52$
ADP (nmol/mg muscle)	$6.39 \pm 0.17$	$5.67 \pm 0.20$	$6.61 \pm 0.19$	$5.94 \pm 0.28$
AMP (nmol/mg muscle)	$0.24 \pm 0.03$	$0.36 \pm 0.02$	$0.34 \pm 0.01^\dagger$	$0.33 \pm 0.01$
AMP-to-ATP ratio ( $\times 10^3$ )	$25.69 \pm 6.30$	$38.51 \pm 7.07$	$41.86 \pm 3.92^*$	$32.28 \pm 5.41$

Data are means  $\pm$  SE. Muscles are excised unilaterally from 8- to 10-week-old male mice. Muscles from three mice were pooled in one tube and nucleotide levels measured.  $n = \sim 4$ –5 pooled samples. \* $P < 0.01$ ,  $^\dagger P < 0.05$  vs. non-Tg.



**FIG. 2.** The effect of HFD feeding on body weight, body fat mass, plasma leptin levels, glucose tolerance, and insulin sensitivity in LepTg. **A:** Body weight following HFD feeding. **B:** Body fat mass after 15-week HFD feeding. **C:** Plasma leptin levels after four-week HFD feeding. **D:** GTT after 15-week HFD feeding. **E:** ITT after 15-week HFD feeding.  $\circ$ , regular diet-fed non-Tg;  $\bullet$ , HFD-fed non-Tg;  $\triangle$ , regular diet-fed LepTg;  $\blacktriangle$ , HFD-fed LepTg.  $n = 8$ ; # $P < 0.05$  between regular diet-fed non-Tg and LepTg,  $\S P < 0.05$  between HFD-fed non-Tg and LepTg. **B:** Body fat mass after 15-week HFD feeding. **C:** Plasma leptin levels after four-week HFD feeding.  $\square$ , regular diet-fed non-Tg;  $\blacksquare$ , HFD-fed non-Tg;  $\text{▨}$ , regular diet-fed LepTg;  $\blacksquare$ , HFD-fed LepTg.  $n = 8$ ; \* $P < 0.01$  and # $P < 0.05$  vs. regular diet-fed non-Tg, † $P < 0.01$  vs. regular diet-fed LepTg,  $\S P < 0.05$  vs. HFD-fed non-Tg. **D:** GTT (**D**) and ITT (**E**) after 15-week HFD feeding.  $\circ$ , regular diet-fed non-Tg;  $\bullet$ , HFD-fed non-Tg;  $\triangle$ , regular diet-fed LepTg;  $\blacktriangle$ , HFD-fed LepTg.  $n = 8$ ; \* $P < 0.01$  and # $P < 0.05$  vs. regular diet-fed non-Tg, † $P < 0.01$  vs. regular diet-fed LepTg,  $\S P < 0.05$  vs. HFD-fed non-Tg.

ameliorated in LepTg. Body weight and body fat mass measured by dual-energy X-ray absorptiometry were significantly lower in LepTg than in non-Tg under regular diet (Fig. 2A and B). However, after starting on an HFD, both LepTg and non-Tg gained weight. HFD-induced weight gain was more pronounced in LepTg, and the weight of LepTg was comparable with that of non-Tg after 4 weeks of HFD feeding (Fig. 2A). Body fat mass after 15 weeks of HFD feeding was even higher in LepTg than in non-Tg (Fig. 2B). During the weight gain, both LepTg and non-Tg exhibited an increase in plasma leptin concentration, but the level was significantly higher in LepTg, even under HFD (Fig. 2C). In parallel with the loss of a lean phenotype, HFD-fed LepTg lost metabolic advantage and demon-

strated an aggravation of glucose tolerance and insulin sensitivity in GTTs and ITTs (Fig. 2D and E). Under HFD, there was no difference in oxygen consumption or plasma insulin, triglyceride, and FFA levels between the genotypes (Table 3). The development of obesity and the impairment of glucose and lipid metabolism in HFD-fed LepTg indicates that transgenic overexpression of leptin cannot prevent HFD-induced metabolic derangements.

**Increased AMPK and ACC phosphorylation and triglyceride reduction in LepTg soleus muscle are abrogated under HFD.** We next examined whether augmented AMPK and ACC phosphorylation in LepTg soleus muscle is affected by HFD. HFD feeding for 4 weeks per se did not change phospho-Thr172 AMPK $\alpha$  and phospho-Ser79 ACC levels in non-Tg (Fig. 3A and B). However, increased phospho-AMPK and phospho-ACC levels in soleus muscle from regular diet-fed LepTg were attenuated under HFD (Fig. 3A and B). HFD feeding did not affect soleus AMPK $\alpha$  nor ACC $\beta$  protein level in both genotypes (Fig. 3C and D). In EDL muscles, phospho-AMPK, phospho-ACC, AMPK $\alpha$ , and ACC $\beta$  levels were unaltered by HFD in both genotypes (data not shown). To test whether attenuated AMPK and ACC phosphorylation under HFD is accompanied by any change in AMP-to-ATP ratio, we measured adenine nucleotide levels in soleus muscle from LepTg fed HFD for 4 weeks ( $n = \sim 4-6$  pooled samples, 3 muscles per sample). HFD per se led to a  $68 \pm 16\%$  decrease in AMP and a  $26 \pm 4\%$  decrease in ADP level in wild-type mice. ATP level was not significantly altered by HFD ( $12 \pm 12\%$  increase), while AMP-to-ATP ratio in

**TABLE 3**  
Metabolic parameters in leptin transgenic mice under HFD

	non-Tg	LepTg
Body weight (g)	32.9 $\pm$ 0.7	30.0 $\pm$ 0.6
Energy intake (kcal/day)	15.5 $\pm$ 0.9	15.5 $\pm$ 0.2
Vo <sub>2</sub> (ml $\cdot$ kg <sup>-0.75</sup> $\cdot$ h <sup>-1</sup> )	1,537 $\pm$ 8	1,576 $\pm$ 44
Glucose (mg/dl)	145 $\pm$ 4	140 $\pm$ 10
Insulin (ng/ml)	3.98 $\pm$ 0.40	3.07 $\pm$ 0.42
Triglycerides (mg/dl)	144 $\pm$ 17	143 $\pm$ 13
FFAs (mEq/l)	2.11 $\pm$ 0.13	2.17 $\pm$ 0.16

Data are means  $\pm$  SE. Six-week-old male mice were fed HFD for 4 weeks. Body weight and plasma levels for glucose, insulin, triglycerides, and free fatty acids are from the end of 4 weeks. Blood was obtained ad libitum during 0900–1200. Energy intake is a mean daily caloric intake during 4 weeks. Vo<sub>2</sub> was measured during the 2nd week of the period.  $n = 8$ .

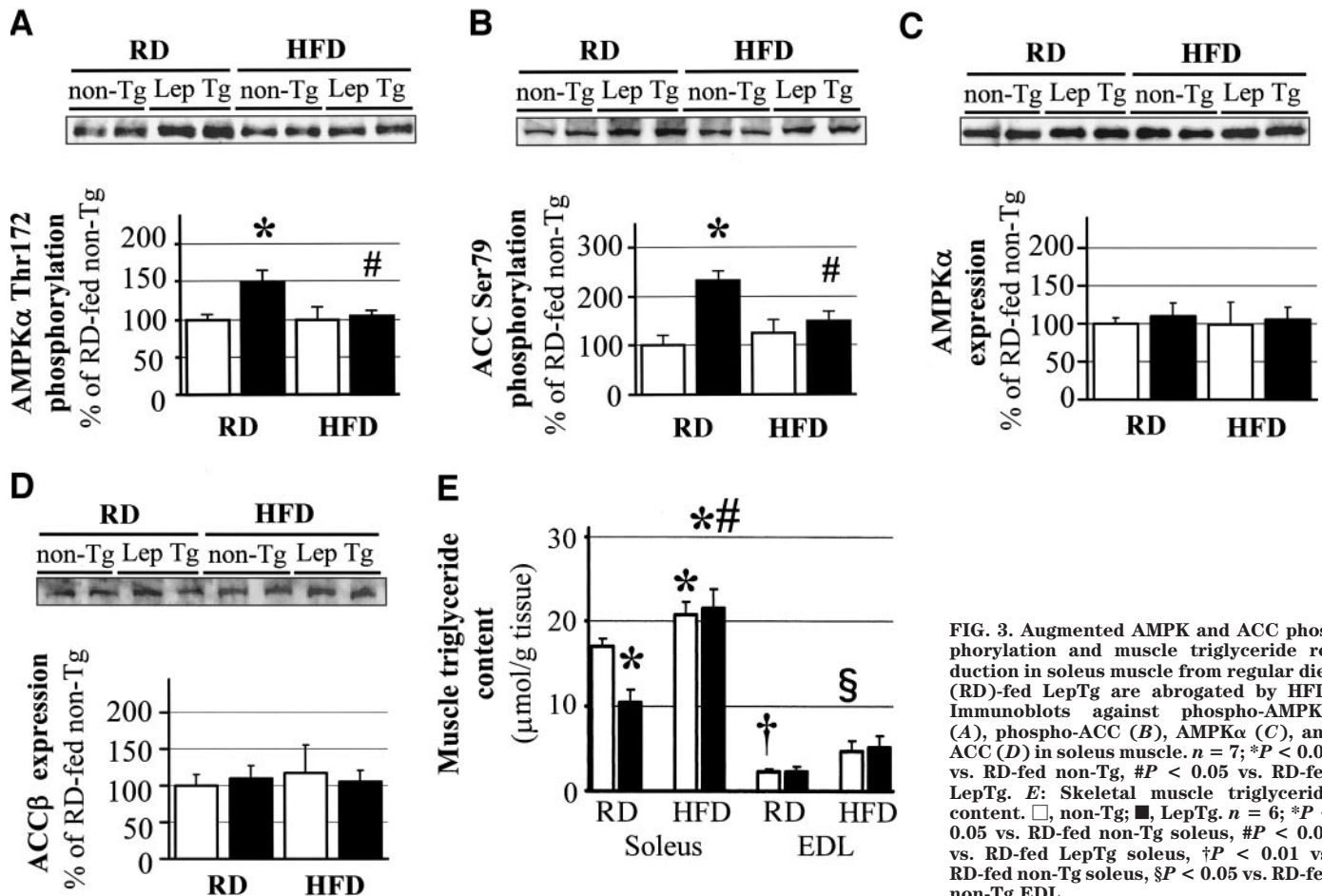


FIG. 3. Augmented AMPK and ACC phosphorylation in soleus muscle from regular diet (RD)-fed LepTg are abrogated by HFD. Immunoblots against phospho-AMPK $\alpha$  (A), phospho-ACC (B), AMPK $\alpha$  (C), and ACC (D) in soleus muscle.  $n = 7$ ; \* $P < 0.05$  vs. RD-fed non-Tg, # $P < 0.05$  vs. RD-fed LepTg. E: Skeletal muscle triglyceride content.  $\square$ , non-Tg;  $\blacksquare$ , LepTg.  $n = 6$ ; \* $P < 0.05$  vs. RD-fed non-Tg soleus, # $P < 0.05$  vs. RD-fed LepTg soleus, † $P < 0.01$  vs. RD-fed non-Tg soleus, § $P < 0.05$  vs. RD-fed non-Tg EDL.

HFD-fed wild-types tended to be lower than in regular diet-fed wild-types. In HFD-fed LepTg, the AMP level was still significantly higher than in HFD-fed non-Tg ( $0.43 \pm 0.03$  vs.  $0.16 \pm 0.08$  nmol/mg muscle,  $P < 0.05$ ). However, there was no significant difference in ATP level or AMP-to-ATP ratio between HFD-fed LepTg and non-Tg. In stark contrast to soleus muscle, AMP, ADP, and ATP levels and AMP-to-ATP ratio in EDL muscle were not altered by HFD, nor was there any difference in these parameters between the genotypes even under HFD. These data raise the possibility that HFD-induced attenuation in soleus muscle AMP-to-ATP ratio may be associated with attenuated AMPK and ACC phosphorylation in HFD-fed LepTg.

HFD feeding led to an increase in tissue triglyceride content both in soleus and EDL muscles (Fig. 3E). In contrast to reduced triglyceride content in soleus muscle from regular diet-fed LepTg, the content in HFD-fed LepTg was comparable with that from HFD-fed non-Tg (Fig. 3E). In our experimental settings, plasma adiponectin levels increased in response to 4 weeks of HFD feeding, and no difference was observed between HFD-fed LepTg and non-Tg ( $27.8 \pm 3.3$   $\mu$ g/ml in HFD-fed LepTg vs.  $23.8 \pm 5.2$   $\mu$ g/ml in HFD-fed non-Tg;  $n = 6$ ; not significant).

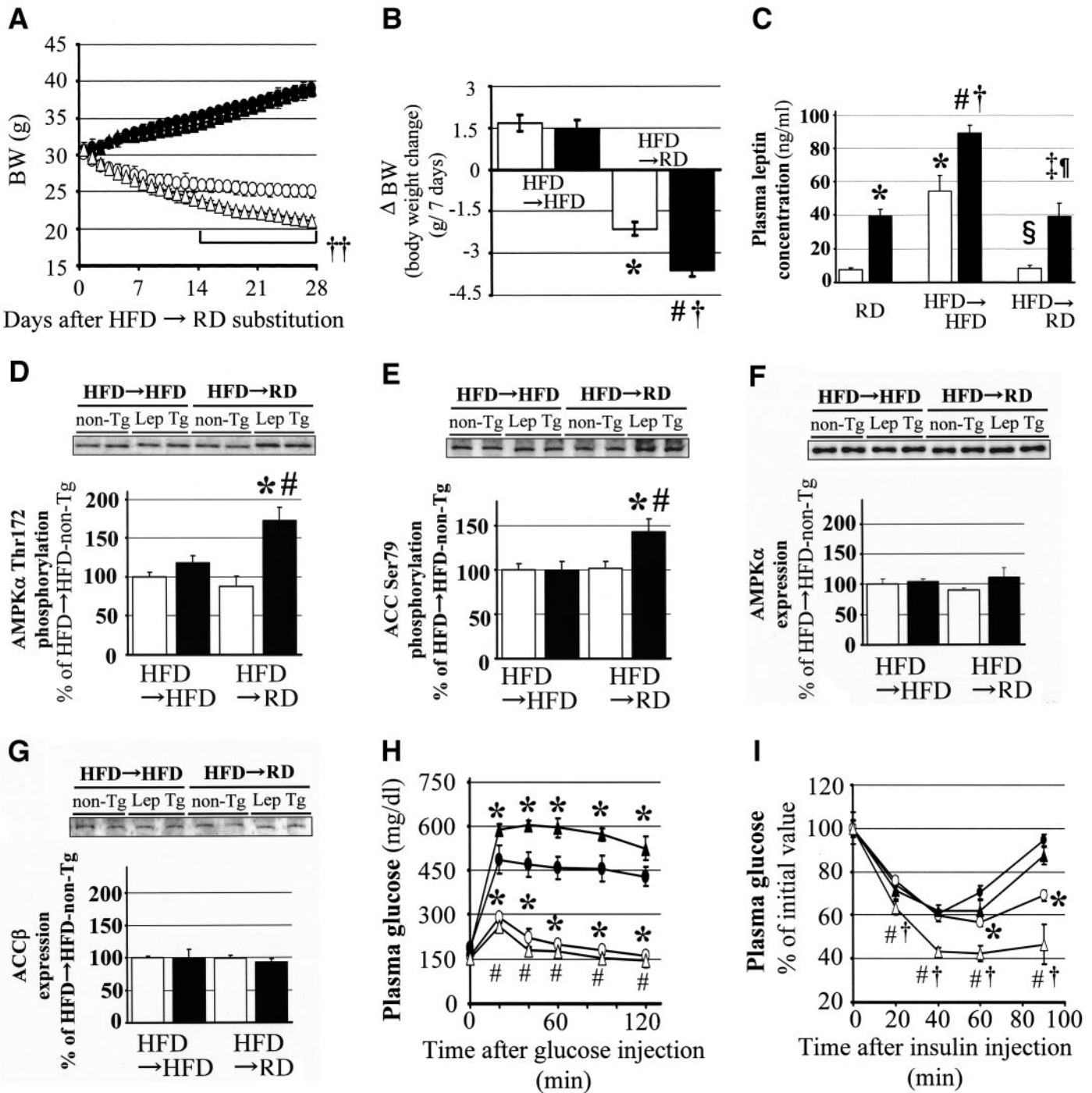
**LepTg lose weight more rapidly after substitution of HFD with regular diet in parallel with a restored AMPK and ACC phosphorylation in soleus muscle.** After the 4-week HFD feeding, HFD was substituted with regular diet. Mice were fed ad libitum throughout the study. Diet substitution per se led to a weight loss both in

LepTg and non-Tg (Fig. 4A). Of note, LepTg exhibited a significantly higher rate of weight loss than non-Tg during the first 7 days (Fig. 4B), and LepTg weighed significantly less than non-Tg 14 days after diet substitution (Fig. 4A). Plasma leptin concentration returned to pre-HFD levels within 7 days of regular diet feeding in both genotypes, with LepTg maintaining its hyperleptinemia (Fig. 4C).

AMPK and ACC phosphorylation in soleus muscle increased only in LepTg and were significantly higher than in similarly treated non-Tg 7 days after diet substitution (Fig. 4D and E). Of note, restoration in AMPK and ACC phosphorylation preceded the complete resumption of lean phenotype in LepTg (Fig. 4A). Neither AMPK $\alpha$  nor ACC $\beta$  protein levels were altered by HFD to regular diet substitution (Fig. 4F and G). Phospho-AMPK, phospho-ACC, AMPK $\alpha$ , and ACC $\beta$  levels in EDL muscles remained constant even after diet substitution (data not shown).

GTTs and ITTs were performed following HFD to regular diet substitution. Glucose profiles in GTTs were comparable between the genotypes after diet substitution (Fig. 4H). Notably, however, insulin sensitivity was drastically recovered and was enhanced in LepTg compared with similarly treated non-Tg (Fig. 4I).

**mRNA levels for liver SCD-1 and brown adipose UCP-1 are not associated with metabolic phenotype in LepTg.** Leptin-induced downregulation of hepatic SCD-1 expression and activity is reported as an important component of leptin's metabolic action (25–27). SCD-1 is an enzyme that catalyzes the biosynthesis of monounsaturated



**FIG. 4.** HFD to regular diet (RD) substitution leads to an accelerated body weight loss and augmented insulin sensitivity in LepTg with a robust recovery of AMPK and ACC phosphorylation. Body weight during the 4 weeks (A) and the rate of body weight change during the first 7 days (B) following HFD to RD substitution.  $n = 10$ ; \* $P < 0.001$  vs. HFD to HFD non-Tg, # $P < 0.001$  vs. HFD to HFD LepTg, † $P < 0.01$  and ‡ $P < 0.05$  vs. HFD to RD non-Tg. C: Plasma leptin levels under RD or after 4-week HFD plus 1-week HFD or RD.  $n = 10$ ; \* $P < 0.01$  vs. RD non-Tg, # $P < 0.01$  vs. RD LepTg, † $P < 0.05$  vs. HFD to HFD non-Tg, § $P < 0.01$  vs. HFD to HFD non-Tg, ‡ $P < 0.01$  vs. HFD to HFD LepTg, ¶ $P < 0.01$  vs. HFD to RD non-Tg. Immunoblots against phospho-AMPKα (D), phospho-ACC (E), AMPKα (F), and ACC (G) in soleus muscle (4-week HFD plus 1-week HFD or RD).  $n = 7$ ; \* $P < 0.05$  vs. HFD to HFD LepTg, # $P < 0.05$  vs. HFD to RD non-Tg. □, non-Tg; ■, LepTg. GTT (H) and ITT (I) after 15-week HFD feeding followed by 4-week RD. ●, HFD to HFD non-Tg; ○, HFD to RD non-Tg; ▲, HFD to HFD LepTg; △, HFD to RD LepTg.  $n = 8$ ; \* $P < 0.05$  vs. HFD to HFD non-Tg, # $P < 0.05$  vs. HFD to HFD LepTg, † $P < 0.05$  vs. HFD to RD non-Tg.

urated fatty acids. Unexpectedly, SCD-1 mRNA expression in liver was not different between the genotypes (Fig. 5A). SCD-1 expression was markedly suppressed by HFD and was not recovered 7 days after HFD to regular diet substitution. These data are consistent with a report that SCD-1 expression is suppressed by polyunsaturated fatty

acids (28). mRNA levels were comparable between the genotypes throughout dietary modification (Fig. 5B), suggesting that liver SCD-1 expression is not associated with metabolic phenotype in LepTg.

Acute administration of leptin increases brown adipose UCP-1 expression (29). UCP-1 is a crucial factor for

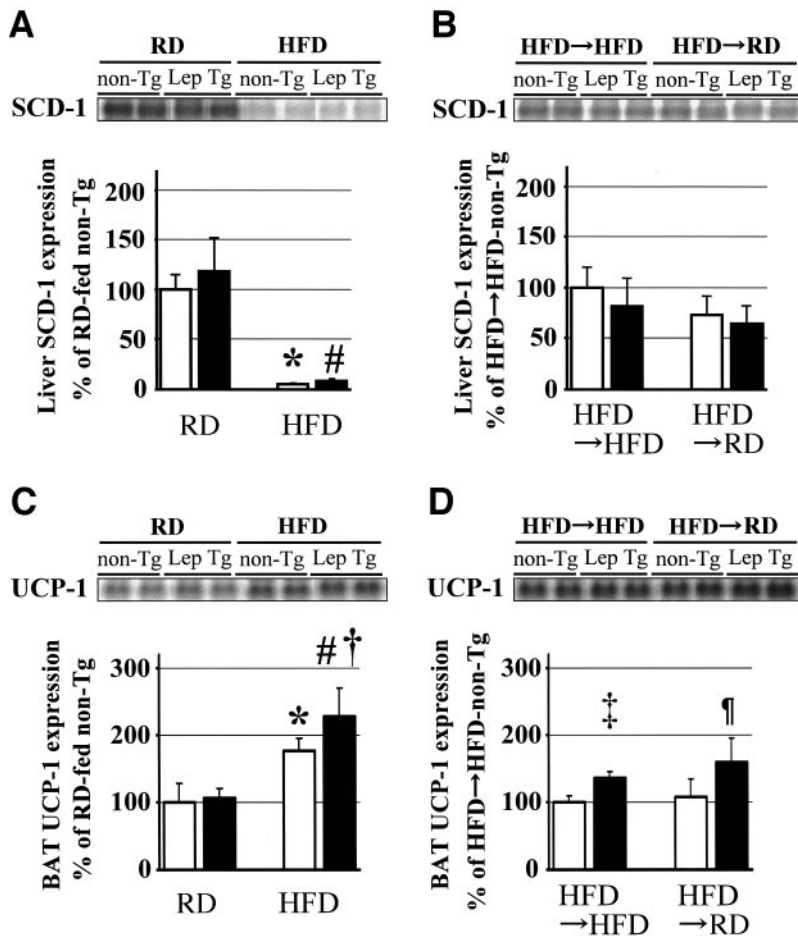


FIG. 5. Northern blot analysis of liver SCD-1 and brown adipose UCP-1 mRNA expression. Liver SCD-1 (A and B) and brown adipose UCP-1 (C and D) expression under regular diet (RD), HFD (4 weeks) (A and C) and 4-week HFD followed by 1-week RD (B and D). Representative blots and quantified data normalized by 18S rRNA expression. □, non-Tg; ■, LepTg.  $n = 5$  (mean  $\pm$  SD); \* $P < 0.01$  vs. RD-fed non-Tg, # $P < 0.01$  vs. RD-fed LepTg, † $P < 0.05$  vs. HFD-fed non-Tg, ‡ $P < 0.05$  vs. HFD→HFD non-Tg, ¶ $P < 0.01$  vs. HFD→RD non-Tg.

nonshivering thermogenesis and energy dissipation in rodents (30) and is suggested as one of the mediators of leptin's insulin-sensitizing action (31). Although LepTg possess a smaller amount of brown adipose tissue than non-Tg (12), brown adipose UCP-1 mRNA expression in LepTg was equivalent to that in non-Tg (Fig. 5C). Interscapular brown adipose tissue from LepTg maintains histological characteristics of brown adipose tissue (4). Brown adipose UCP-1 expression was slightly higher in LepTg under HFD and after HFD to regular diet substitution (Fig. 5D).

## DISCUSSION

Here, we demonstrate that soleus muscle AMPK and ACC phosphorylation is augmented chronically in LepTg. Despite predetermined hyperleptinemia, LepTg is not protected from HFD-induced metabolic derangements, consistent with a previous report that transgenic mice overexpressing leptin in adipocytes are not protected against DIO (32). Noteworthy is that, on an HFD, soleus AMPK and ACC phosphorylation in LepTg is decreased to the level of regular diet-fed or HFD-fed non-Tg. Substitution of HFD with regular diet led to increased AMPK and ACC phosphorylation only in LepTg, with accelerated body weight loss and restored insulin sensitivity. These data suggest that soleus muscle AMPK activity changes in parallel with metabolic action of leptin under nutritional modification. To the best of our knowledge, the present study is the first in addressing the leptin-skeletal muscle AMPK axis under leptin-sensitive and -insensitive states.

AMPK activity is regulated by intracellular energy status as well as upstream AMPK kinase (9,33). Besides allosteric activation of AMPK by AMP, increased AMP-to-ATP ratio predisposes AMPK to be phosphorylated and inhibits its dephosphorylation. In LepTg soleus muscle, AMP-to-ATP ratio was chronically elevated, suggesting a possibility that leptin may chronically regulate skeletal muscle AMPK activity through modulation of cellular energy status in this animal model. Recent studies have suggested LKB1 as a promising candidate for AMPK kinase (34–36). Possible regulation of LKB1 activity by leptin may be an interesting problem to be solved in the future.

In the present study, we used Western blot to examine AMPK phosphorylation. Although increased AMPK phosphorylation cannot be interpreted as an equivalent of AMPK activation as measured by *in vitro* AMPK assay, data from the AMPK assay performed with immunoprecipitated samples in the presence of abundant AMP and ATP (37) may not necessarily reflect AMPK activity *in vivo*. The level of AMPK $\alpha$  phosphorylation at Thr172 is closely associated with AMPK activity in many experimental conditions, including leptin-induced AMPK activation in skeletal muscle (10). Our data showing that AMP-to-ATP ratio, AMPK phosphorylation, and ACC phosphorylation were all increased in parallel in LepTg soleus muscle highly suggest enhanced AMPK activity.

Adiponectin activates AMPK in liver and skeletal muscle (21,22). Despite an apparent loss of white adipose tissue in LepTg, there is no significant difference in plasma adiponectin levels between the genotypes. This is in striking

contrast to murine models and human patients with generalized lipodystrophy, where plasma adiponectin levels are extremely low (21,38,39). A lack of difference in plasma adiponectin levels between the genotypes argues against a role of adiponectin in increased AMPK and ACC phosphorylation in LepTg.

Spontaneous locomotive activity is increased in LepTg. Mechanisms whereby hyperleptinemia alters locomotion must await further investigation. A human study (24) using vastus lateralis muscle shows that 3-week supervised endurance training increases AMPK protein levels and basal AMPK activity. Thus, it does not necessarily exclude the possibility that chronically increased locomotion in LepTg may partly contribute to augmented AMPK phosphorylation. However, contraction activates AMPK more readily in white rather than red muscle fibers (40), which marks a sharp contrast to our data indicating that increased AMPK and ACC phosphorylation in LepTg were preferential in soleus muscle. Acute administration of leptin also activates AMPK more readily in red muscles than in white muscles (10). Red muscles have higher rates for oxidation, while white muscles have higher rates for glycolysis (41). The finding that increased AMP-to-ATP ratio, increased AMPK and ACC phosphorylation, and reduced tissue triglyceride content in LepTg all occur preferentially in red muscle further highlights the importance of red muscle as a target for leptin-mediated fuel metabolism. It is interesting to note that muscle fiber-type composition of soleus muscle was not altered in LepTg as assessed by histochemical ATPase activity and also by SDS-PAGE analysis of myosin heavy chain isoforms (T.Ta., T. Ishii, S. Masuda, S. Taguchi, unpublished observation). This suggests that AMPK activation and triglyceride reduction in LepTg soleus muscle is independent of muscle fiber-type switch.

Steinberg et al. (42) have reported that 2-week subcutaneous leptin infusion in rats leads to increased AMPK $\alpha$ 2 protein levels along with AMPK phosphorylation in both soleus and white gastrocnemius muscle. They have also found increased ATP level in white gastrocnemius from leptin-treated rats but unaltered ATP and AMP levels in soleus muscle (42). These data are in contrast to ours, showing that AMPK $\alpha$  protein levels were not increased, AMP-to-ATP ratio was increased in LepTg soleus muscle, and AMPK phosphorylation and ATP levels were not changed in EDL muscle. The discrepancy may partly be explained by a difference in species, muscles used, and the extent and duration of hyperleptinemia.

Very recently, a study (43) using human rectus abdominis muscle has demonstrated that in muscle from obese subjects, fatty acid esterification is augmented, while AMPK activity is not downregulated. Consistent with the report, our data clearly show that HFD per se did not change AMPK nor ACC phosphorylation but did increase triglyceride content in non-Tg soleus muscle. In this context, our results may indicate that leptin-induced upregulation of AMPK phosphorylation (but not its basal level) is attenuated in DIO. Treatment of HFD-fed rats and insulin-resistant obese Zucker fatty rats with an AMP mimetic, 5-aminoimidazole-4-carboxamide riboside, enhances AMPK activity and augments liver and muscle insulin

sensitivity (40,44), thereby suggesting a therapeutic implication of AMPK activators in leptin-insensitive states.

Under HFD, LepTg exhibit higher rates of body weight gain and higher feed efficiency (milligrams of weight gain per kilocalories of energy intake) than non-Tg ( $28.2 \pm 1.2$  mg/kcal in LepTg vs.  $20.8 \pm 1.6$  mg/kcal in non-Tg;  $P < 0.05$ ;  $n = 8$ ). Body fat mass is also higher and GTT profile further deteriorated in HFD-fed LepTg than in non-Tg. These data cannot simply be explained by an abrogation of metabolic action of leptin, but would rather suggest a possible role of predetermined hyperleptinemia in exacerbating metabolic derangements under HFD. In accordance with this notion, transgenic mice overexpressing leptin in adipocytes are more prone to HFD-induced obesity (32). The investigators of that study proposed the hypothesis that on an HFD, hyperleptinemia predisposes adipocytes to be hypercellular and hypertrophic (32). Our observation is that skeletal muscle AMPK activity and brown adipose UCP-1 expression in HFD-fed LepTg are comparable with and higher than HFD-fed non-Tg, respectively. Under HFD, no difference was found in energy intake and O<sub>2</sub> consumption between the genotypes as well. The possible role of predetermined hyperleptinemia as a precipitating factor of DIO must await further investigation.

We have previously shown that hyperleptinemia can accelerate the recovery from diabetes in *A<sup>y</sup>* mice during caloric restriction, suggesting the potential usefulness of leptin in combination with a caloric restriction for the treatment of obesity-associated diabetes (13). To examine whether hyperleptinemia can augment recovery from HFD-induced obesity and insulin resistance, HFD was substituted with regular diet. After HFD to regular diet substitution, the rate of body weight loss and amelioration in insulin sensitivity were more pronounced in LepTg than in non-Tg. Notably, skeletal muscle AMPK and ACC phosphorylation was increased only in LepTg, suggesting a close association between AMPK activation and insulin sensitivity. Increased AMPK and ACC phosphorylation was observed before LepTg fully regained the lean phenotype. Although LepTg ate less than non-Tg after HFD to regular diet substitution, pair-fed non-Tg weighed significantly heavier than LepTg (T.Ta., unpublished observation). Further studies are necessary to prove a causal role of AMPK in the recovery of insulin sensitivity in LepTg following HFD to regular diet substitution.

Suppression of liver SCD-1 by leptin has been suggested to play a beneficial role in leptin-mediated weight loss in *ob/ob* mice and improvement of steatosis in a murine model of lipoatrophic diabetes (25,26). The finding that leptin improves hepatic insulin resistance in lipoatrophic mice at a low dose that does not alter SCD-1 expression suggests the presence of SCD-1-independent mechanisms (26). In the present study, hepatic SCD-1 expression was not different between the genotypes under any dietary condition, demonstrating that chronic hyperleptinemia in LepTg is not associated with suppressed hepatic SCD-1 expression. A possible role of liver AMPK as an effector molecule of leptin's metabolic action has not been fully investigated and must await further studies.

Unexpectedly, brown adipose UCP-1 expression was not altered in LepTg. This is in contrast to data from acute administration of leptin (29). A recent study (45) has



shown that adenoviral overexpression of leptin in rats increases AMPK and ACC phosphorylation and UCP-1 expression in white adipose tissue, transforming white adipocytes into unique fat-burning cells. In LepTg, white adipose tissue has almost completely disappeared (12), and we could not find such cells even microscopically (4,12). Taken together, brown adipose UCP-1 and other adipocyte molecules are less likely to be associated with leptin sensitivity in LepTg under dietary modification.

In summary, we show that soleus muscle AMPK and ACC phosphorylation is chronically augmented in LepTg, in which hyperleptinemia is associated with enhanced glucose and lipid metabolism. Although HFD feeding attenuates AMPK and ACC phosphorylation and deteriorates insulin sensitivity and plasma lipid profile in LepTg, diet substitution from HFD to regular diet leads to a pronounced restoration of AMPK and ACC phosphorylation and results in an accelerated body weight reduction and recovery of insulin sensitivity. We demonstrate that soleus AMPK and ACC phosphorylation closely parallels metabolic phenotype in LepTg under dietary modification, suggesting a potential link between skeletal muscle AMPK and leptin sensitivity.

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