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ORIGINAL ARTICLE

Leucine Deprivation Decreases Fat Mass by Stimulation of Lipolysis in White Adipose Tissue and Upregulation of Uncoupling Protein 1 (UCP1) in Brown Adipose Tissue

Ying Cheng, Qingshu Meng, Chunxia Wang, Houkai Li, Zhiying Huang, Shanghai Chen, Fei Xiao, and Feifan Guo

OBJECTIVE—White adipose tissue (WAT) and brown adipose tissue (BAT) play distinct roles in adaptation to changes in nutrient availability, with WAT serving as an energy store and BAT regulating thermogenesis. We previously showed that mice maintained on a leucine-deficient diet unexpectedly experienced a dramatic reduction in abdominal fat mass (Guo and Cavener, Cell Metab 2007;5:103–141). The cellular mechanisms responsible for this loss, however, are unclear. The goal of current study is to investigate possible mechanisms.

RESEARCH DESIGN AND METHODS—Male C57BL/6J mice were fed either control, leucine-deficient, or pair-fed diets for 7 days. Changes in metabolic parameters and expression of genes and proteins related to lipid metabolism were analyzed in WAT and BAT.

RESULTS—We found that leucine deprivation for 7 days increases oxygen consumption, suggesting increased energy expenditure. We also observed increases in lipolysis and expression of β-oxidation genes and decreases in expression of lipogenic genes and activity of fatty acid synthase in WAT, consistent with increased use and decreased synthesis of fatty acids, respectively. Furthermore, we observed that leucine deprivation increases expression of uncoupling protein (UCP)-1 in BAT, suggesting increased thermogenesis.

CONCLUSIONS—We show for the first time that elimination of dietary leucine produces significant metabolic changes in WAT and BAT. The effect of leucine deprivation on UCP1 expression is a novel and unexpected observation and suggests that the observed increase in energy expenditure may reflect an increase in thermogenesis in BAT. Further investigation will be required to determine the relative contribution of UCP1 upregulation and thermogenesis in BAT to leucine deprivation-stimulated fat loss.
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Obesity develops from an imbalance between calorie intake and energy expenditure (1). Excess calories are stored in the white adipose tissue (WAT) as triglyceride (TG), which are mobilized in response to increased energy demands (2). Various strategies have been proposed to treat obesity by promoting fat mobilization and/or increasing energy expenditure (3–5).

Recently, there has been a growing interest in controlling body weight by manipulating macronutrients (6–8). Recent studies have shown that dietary manipulation of essential amino acids, including leucine, arginine, and glutamine, have significant effects on lipid metabolism and glucose utilization (9–14). Most of these studies, however, have focused on the effects of increased levels of essential amino acids in the diet (4,14–18). For example, Zhang et al. (15) recently demonstrated that doubling intake of dietary leucine decreases body weight and improves glucose metabolism in mice maintained on a high-fat diet. The effect of increasing dietary leucine, however, is controversial. Additional studies have shown that dietary supplementation of leucine has no effect on lipid metabolism (16).

By contrast, our research has focused on the effect of eliminating leucine from the diet on lipid metabolism. As we recently reported, mice maintained on a leucine-deficient diet for 7 days experienced a dramatic reduction in abdominal fat mass (9). The cellular mechanisms responsible for this loss, however, are unclear. The goal of our current research is to elucidate the molecular and cellular mechanisms underlying the rapid abdominal fat loss induced by leucine deprivation.

In our current study, we observed increases in lipolysis and expression of β-oxidation genes and decreases in expression of lipogenic genes and activity of fatty acid synthase (FAS) in WAT, consistent with increased use and decreased synthesis of fatty acids, respectively. In addition, we observed for the first time that leucine deprivation increases expression of uncoupling protein (UCP)-1 in brown adipose tissue (BAT), suggesting increased thermogenesis. We hypothesize that these changes in WAT and BAT account for the significant loss of abdominal fat mass under leucine deprivation.

RESEARCH DESIGN AND METHODS

Animals and diets. Wild-type male C57BL/6J mice were obtained from Shanghai Laboratory Animal Company (Shanghai, China). Eight- to ten-week-old mice were maintained on a 12-h light/dark cycle at 25°C and were provided free access to commercial rodent food and tap water before the experiments. Control (nutritionally complete amino acid) and (−) leu (leucine-deficient)

From the Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, the Graduate School of the Chinese Academy of Sciences, Shanghai, China.

Corresponding author: Feifan Guo, ffguo@sibs.ac.cn.

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Y.C. and Q.M. contributed equally to this study.

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diets were obtained from Research Diets (New Brunswick, NJ). All diets were isocaloric and compositionally the same in terms of carbohydrate and lipid component. At the start of the feeding experiment, mice were acclimated to a control diet for 7 days and then randomly divided into either control or (−) leu diet groups with free access to either control or (−) leu diet, respectively, for 7 days. In addition, a pair-fed group was included to distinguish possible influences from a minor reduction in food intake previously observed in the (−) leu group (9). The pair-fed mice were provided with 15% less food compared with mice in the control diet group. This percentage was determined by our observation in the current study that, on average, mice maintained on a leucine-deficient diet consumed 15% less food compared with mice maintained on a control diet. Food intake and body weight were recorded daily. At the end of the experiment, the total body fat content of each mouse was quantified in vivo by a mini-spec nuclear magnetic resonance spectrometer (Bruker Corporation), following the manufacturer's protocol. Animals were killed by CO_2 inhalation. WAT weight was recorded at the time of death. Adipose tissues were isolated and either put into 4% paraformaldehyde buffer immediately for histological study or snap-frozen and stored at −80°C for future analysis. These experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences (Sibs, CAS).

Indirect calorimetry. After 6 days of feeding with either control, leucine-deficient, or pair-fed diets, mice were maintained in a comprehensive lab animal monitoring system (Columbus Instruments, Columbus, OH) for 24 h according to the instructions of the manufacturer. Volume of O_2 consumption and CO_2 production were continuously recorded over a 24-h period.

Rectal temperature measurement. The rectal temperatures of the mice were measured using a rectal probe attached to a digital thermometer (Physitemp, Clifton, NJ).

Oxygen consumption measurement. Brown adipocytes were isolated and oxygen consumption was measured using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, U.K.), as previously described (19), with minor modifications. Each sample was analyzed by incubating 1×10^6 cells in a magnetically stirred chamber at 37°C. After the basal respiration was recorded, 5 mmol/l oleate was added to determine the maximal oxygen consumption.

Serum measurements. Serum was obtained by centrifugation of clotted blood and then snap-frozen in liquid nitrogen and stored at −20°C. Serum free fatty acids and glycerol were determined enzymatically using a NEFA C Reagent (Wako) and Glycerol Assay kit (SinoPCR, China), respectively. Serum norepinephrine, thyroid hormone 3,5,3′-triiodothyronine (T3), epinephrine, and glucocorticoid levels were determined using enzyme-linked immunosorbent assay kits from R&D. All of these assays were performed according to manufacturer's instructions.

Analysis of cell volume and DNA content in WAT. WAT was fixed in 4% paraformaldehyde overnight and stained with hematoxylin and eosin. The WAT cell volumes were analyzed as described previously (20). DNA content in WAT samples was quantified as previously reported (21).

Western blot analysis. Whole-cell lysates from frozen tissues were isolated using RIPA lysis buffer (150 mmol/l Tris-HCl, 50 mmol/l NaCl, 1% NP-40, 0.1% Tween-20). Protease and phosphatase inhibitors were added to all buffers before experiments. Western blot was performed as previously described (9). Protein concentrations were assayed using a BCA Kit (Pierce). Primary antibodies (anti-FAS antibody [BD Scientific], anti-PPARα, anti-p-HSL, anti-HSL, anti-p-PKA substrate antibodies [Cell Signaling], anti-actin antibody [Sigma], and anti-SREBP1c and anti-UCP1 antibodies [Santa Cruz Biotechnology]) were incubated overnight at 4°C, and specific proteins were visualized by ECL Plus (Amersham Biosciences). Band intensities were measured using Quantity One (Bio-Rad Laboratories) and normalized to actin.

FAS enzyme activity assay. FAS activity was determined as described by Kim et al. with minor modifications (22). The rate of NADPH oxidation was measured at 340 nm before and after addition of the substrate malonyl-CoA. The concentration of enzyme was adjusted to assure a linear reaction rate. Protein concentration in the homogenate was determined by the BCA Kit (Pierce).

RNA isolation and relative quantitative RT-PCR. Total RNA was prepared from frozen tissues with TRIZOL (Invitrogen) reagent. One microgram of RNA was reverse-transcribed with random primer (Invitrogen) and M-MLV Reverse Transcriptase (Invitrogen). Quantitative amplification by PCR was carried out using SYBR Green I Master Mix reagent by an ABI 7500 system (Applied Biosystem). PCR products were subjected to a melting curve analysis. Cycle numbers of both GAPDH (as an internal control) and cDNAs of interest at a specific threshold within the exponential amplification range were used to calculate relative expression levels of the genes of interest. The sequences of primers used in this study are available upon request.

Glycerol release assay. WAT was removed and incubated in Krebs-Ringer HEPES buffer containing 1 mg/ml collagenase (Sigma) and 2% bovine serum albumin as previously described (23). Freshly isolated adipocytes were incubated in Krebs-Ringer HEPES buffer containing adenosine deaminase (Sigma) in the absence or presence of isoproterenol (1 μmol/l), followed by glycerol assay with the Glycerol assay kit (SinoPCR, China).

Statistical analysis. All data are expressed as means ± SE. Significant differences among the control, (−) leu, and pair-fed groups were assessed using a one-way ANOVA followed by the Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

RESULTS

Leucine deprivation results in significant reduction in fat mass and increase of energy expenditure. We previously showed that mice maintained on a leucine-deficient diet undergo rapid loss of abdominal fat (9). The goal of the present study is to elucidate the underlying molecular and cellular mechanisms of this loss. For this purpose, mice were fed control, leucine-deficient, or pair-fed diets for 7 days. Consistent with a previous report (9), leucine deprivation for 7 days resulted in an ~15% reduction in food intake and body weight compared with mice maintained on the control diet (Fig. 1*A* and *B*). The extent of abdominal fat loss in mice fed a leucine-deficient diet (Fig. 1*C*) was also similar to that reported in our previous study (9). By contrast, body weight was reduced <5% (Fig. 1*B*) and abdominal fat was not significantly reduced (Fig. 1*C*) in pair-fed mice, compared with control diet–fed mice. In addition, the total body fat was significantly decreased by leucine deprivation in comparison with the control and pair-fed mice, whereas there was no difference in the proportion of lean mass among the groups as measured by nuclear magnetic resonance (Fig. 1*D* and *E*).

The rapid fat loss induced by leucine deprivation suggested a possible increase in energy expenditure. We therefore measured energy expenditure by indirect calorimetry, rectal temperature, and physical activity. The total energy expenditure (24-h O_2 consumption, normalized to lean body mass) was markedly increased (Fig. 2*A*) in leucine-deprived mice, but not in pair-fed mice, compared with mice maintained on a control diet. The respiratory exchange ratio (RER) (VCO_2/VO_2) was low in leucine-deprived mice during both dark phases and light phases. By contrast, pair-fed mice exhibited lower RER only during the light phase (Fig. 2*B*). Rectal temperatures measured at 3:00 P.M. in the afternoon (basal metabolic state) were significantly higher in leucine-deprived mice, but were lower in pair-fed mice, compared with mice maintained on a control diet (Fig. 2*C*). We did not, however, see significant differences in rectal temperatures at other times examined (morning or evening, data not shown). We also did not see increased physical activity in leucine-deprived mice, measured in a metabolic cage (Fig. 2*D*).

Serum free fatty acid (FFA) and glycerol levels were much lower in leucine-deprived mice than in pair-fed and control diet–fed groups (Table 1). Levels of serum hormones including norepinephrine, T3, epinephrine, and glucocorticoids were also examined in mice under different diets (Table 1). Serum norepinephrine and T3 levels were higher in mice maintained on a leucine-deficient diet compared with pair-fed and control groups. Serum epinephrine levels were increased in both pair-fed and leucine-deprived mice, and there was no difference between these two groups. Levels of serum glucocorticoids were not affected by leucine deprivation.

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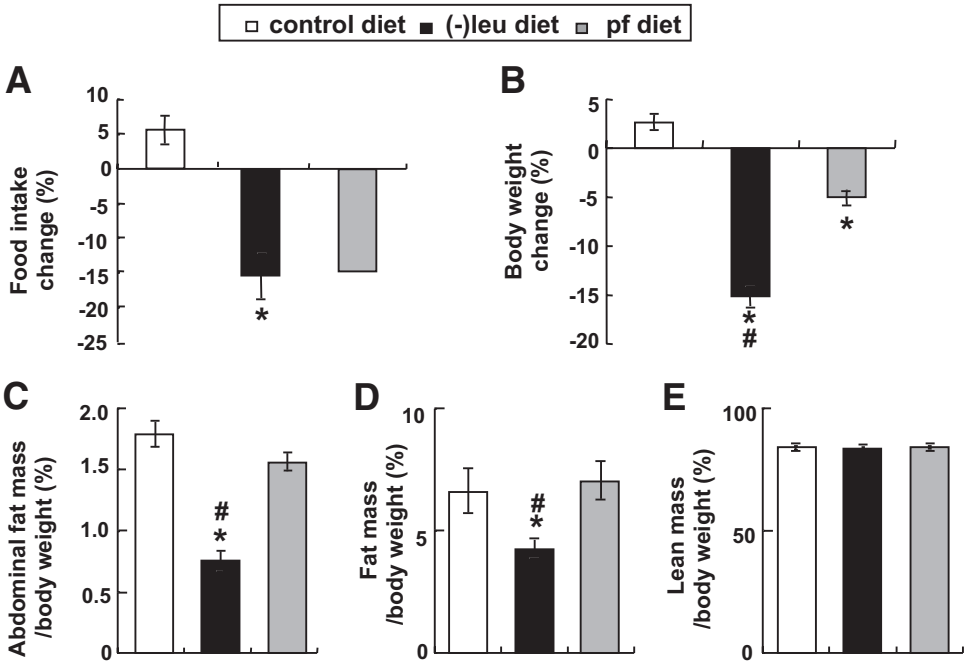


FIG. 1. Body weight and fat mass decreases in leucine-deprived mice. Mice were fed a control, leucine-deficient, or pair-fed diet for 7 days. Body weight and food intake were monitored daily. Data are means ± SE of at least two independent experiments with mice of each diet for each experiment (control diet, $n = 6$; (−) leu diet, $n = 6$; pair-fed diet, $n = 6$). Statistical significance was determined by one-way ANOVA followed by the Student-Newman-Keuls test for the effect of either (−) leu or pair-fed diet versus control diet (*$P < 0.01$) or (−) leu diet versus pair-fed diet (#$P < 0.01$). *A*: Food intake change. (In every case, pair-fed mice consumed all of this food every day. For this reason, there are no error bars for food intake in this group.) *B*: Body weight reduction. *C*: Adipose tissue mass in proportion to body weight. *D*: Body composition measured with nuclear magnetic resonance.

Leucine deprivation reduces WAT cell volume. Leucine deprivation–induced abdominal fat loss may result from decreased adipocyte volume and/or number. Histological analysis of WAT showed that leucine deprivation resulted in a 42% reduction in adipocyte volume compared with mice fed a control diet (Fig. 3*A* and *B*). By contrast, the adipocyte volume was only slightly reduced in pair-fed mice (Fig. 3*A* and *B*). However, cell numbers were the same in mice maintained on each of the three diets, as demonstrated by DNA content (Fig. 3*C*). Consistent with these findings, no apoptosis was detected by Tdt-mediated dUTP nick end labeling (TUNEL) staining in mice maintained on a leucine-deficient diet (data not shown).

Leucine deprivation accelerates triglyceride lipolysis and expression of β-oxidation genes in WAT. To investigate whether the diminished adipocyte volume observed in leucine-deprived mice was the result of increased triglyceride lipolysis and/or fatty acid β-oxidation, we examined changes in levels of phosphorylated proteins related to each of these processes. Levels of total HSL in WAT were not significantly different among the three groups. By contrast, levels of phosphorylated HSL (p-HSL) were significantly increased in WAT of leucine-deprived mice, but were not increased in pair-fed mice, compared with mice fed a control diet (Fig. 4*A*). Consistent with increased levels of phosphorylated HSL, levels of phosphorylated substrate for PKA, the kinase that phosphorylates HSL (24), were also elevated in WAT of leucine-deprived mice, but not in pair-fed mice (Fig. 4*A*).

To further investigate the effect of leucine deprivation on lipolysis, we compared basal and stimulated glycerol release in WAT isolated from mice under the three different diets. Rates of glycerol release, under basal conditions and after stimulation with the β-adrenoceptor agonist isoproterenol, were significantly higher in adipocytes isolated from leucine-deprived mice, but were not significantly changed in pair-fed mice, compared with mice maintained on control diet (Fig. 4*B*). Increased lipolysis in these mice was accompanied by increased cAMP levels (Fig. 4*C*) and expression of β3-adrenoceptors (*Adrb3*) mRNA (Fig. 4*D*).

To examine if genes and proteins related to fatty acid β-oxidation are differentially regulated in WAT of mice fed control, leucine-deficient, or pair-fed diets, we examined the expression levels of mRNAs encoding the transcription factor peroxisome proliferator–activated receptor (PPAR)-α and its target genes carnitine palmitoyltransferase 1 (*Cpt1*) and fatty acyl-CoA oxidase (*Aco*). These genes were significantly increased in WAT of mice maintained on a leucine-deficient diet compared with mice maintained on control diet, whereas mice maintained on a pair-fed diet exhibited no significant increase in expression (Fig. 4*E*). Increased expression of *Pparα* mRNA in leucine-deprived mice was accompanied by an almost 200% increase in PPAR-α protein compared with mice fed a control diet, whereas it was unchanged in mice maintained on a pair-fed diet (Fig. 4*F*).

Leucine deprivation represses lipogenesis in WAT. Impaired lipogenesis is another potential cause of diminished fat mass under leucine deprivation. For this reason, we investigated whether genes underlying the synthesis of fatty acids were differentially regulated in mice under different diets. These proteins included acetyl CoA carboxylase 1 (ACC1), FAS, and stearoyl CoA desaturase (SCD)-1. Our previous study demonstrated that leucine deprivation significantly decreased FAS activity in liver (9). Similarly, in this study, we found that levels of *Fas* mRNA and protein were greatly reduced in WAT of mice maintained on a leucine-deficient diet, but not in pair-fed mice, compared with mice maintained on a control diet (Fig. 5*A* and *B*). Consistent with these changes, FAS enzyme activity was

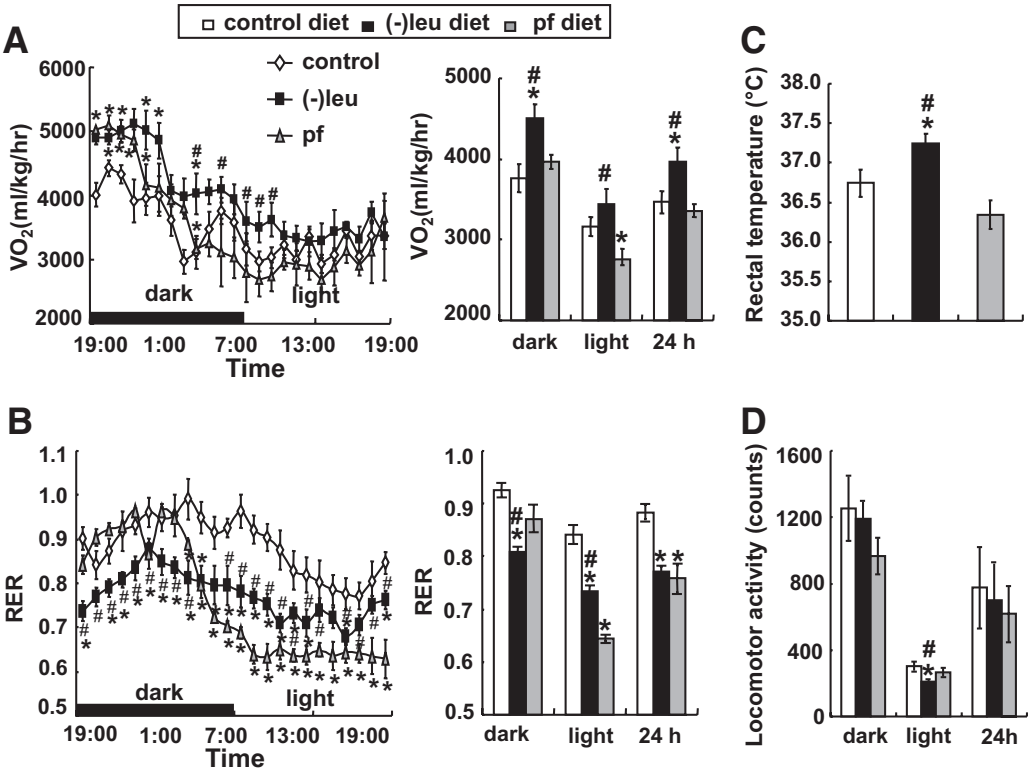


FIG. 2. Leucine deprivation increases energy expenditure. The energy expenditure was measured by indirect calorimetry in mice fed a control, leucine-deficient, or pair-fed diet for 7 days. *A*: 24-h oxygen consumption. *B*: RER. An RER of 0.70 indicates that fat is the predominant fuel source; a RER of 0.85 suggests a mix of fat and carbohydrates, and a value of ≥1.00 is indicative of carbohydrate being the predominant fuel source. *C*: Rectal temperature. *D*: Physical activity. Data are means ± SE of at least two independent experiments with mice of each diet for each experiment (control diet, $n = 6$; (−) leu diet, $n = 6$; pair-fed diet, $n = 6$) over 24–48 h after 6 h acclimation to the metabolic chamber. Statistical significance was determined by one-way ANOVA followed by the Student-Newman-Keuls test for the effect of either (−) leu or pair-fed diet versus control diet (*$P < 0.01$) or (−) leu diet versus pair-fed diet (#$P < 0.01$).

significantly reduced in WAT of mice fed a leucine-deficient diet, but was not reduced in pair-fed mice (Fig. 5*C*). Leucine deprivation also resulted in a large decrease in the mRNA levels of *Acc*I and *Me* in WAT of leucine-deprived mice, but not in pair-fed mice (Fig. 5*A*). Only *Scd1* mRNA, among the fatty acid synthesis genes, was reduced in both leucine-deprived and pair-fed mice (Fig. 5*A*). Levels of SREBP-1c protein, the transcription factor regulating transcription of genes for fatty acids synthesis such as FAS (25), were also downregulated in WAT of leucine-deprived mice (Fig. 5*D*).

Leucine deprivation increases expression of β-oxidation genes in BAT. Many studies have demonstrated that increased lipolysis increases levels of FFA in serum. Despite increased lipolysis, however, levels of FFA in serum are low in leucine-deprived mice (Table 1), suggesting possible increased fatty acid utilization by other tissues, including liver, BAT, and skeletal muscle. We have previously shown that β-oxidation is not upregulated in livers of mice fed a leucine-deficient diet (9). Fatty acid β-oxidation–related genes were significantly increased in BAT of leucine-deprived mice, but not in pair-fed mice, compared with mice maintained on control diet (Fig. 6*A*). Levels of fatty acid transport protein (*Fatp*) and lipoprotein lipase (*Lpl*) mRNA were also significantly increased in BAT of leucine-deprived mice (Fig. 6*A*). Fatty acid β-oxidation–related genes were also increased in muscle of leucine-deprived mice (data not shown). F6

Leucine deprivation increases expression of UCP1 in BAT. The main function of BAT is thermogenesis, which is mediated by upregulation of UCP1. Levels of *Ucp1* mRNA and protein were significantly increased in BAT of mice

TABLE 1
Serum measurements in mice maintained on different diets

	Control	(−) leu	Pair-fed
Norepinephrine (ng/l)	219.15 ± 16.64	301.63 ± 15.61*†	193.52 ± 11.83
Epinephrine (μg/l)	100.07 ± 3.76	140.22 ± 11.49*	117.88 ± 3.46*
Glucocorticoid (nmol/l)	134.03 ± 11.76	109.23 ± 14.41	108.36 ± 14.25
T3 (pmol/l)	14.53 ± 1.18	39.39 ± 3.14*†	26.67 ± 2.98*
Glycerol (mmol/l)	0.54 ± 0.07	0.23 ± 0.05*†	0.56 ± 0.06
FFA (mmol/l)	0.50 ± 0.09	0.27 ± 0.03*†	0.44 ± 0.04

Data are means ± SE. Two- to three-month-old mice were maintained on either nutritionally complete amino acid diet (control), diet devoid of leucine [(−) leu], or pair-fed diet for 7 days. Numbers of mice used: $n = 6$ in each group. Statistical significance is calculated by one-way ANOVA followed by the Student-Newman-Keuls test for the effect of either (−) leu or pair-fed diet versus control diet (*$P < 0.05$) or (−) leu diet versus pair-fed diet (†$P < 0.05$).

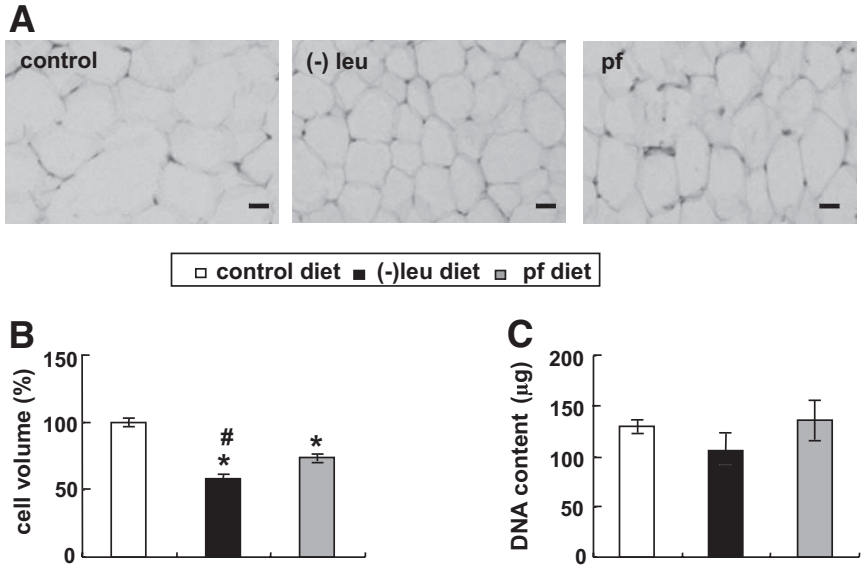


FIG. 3. Cell volume diminishes in WAT of leucine-deprived mice. WAT histology for mice fed a control, leucine-deficient, or pair-fed diet for 7 days is shown. *A*: WAT sections from mice in each group were stained with hematoxylin and eosin (20× magnification). Images shown are representative of several animals for each group. *B*: Analysis of WAT cell volume. *C*: DNA content of total abdominal WAT. Data are means ± SE of at least two independent experiments with mice of each diet for each experiment (control diet, $n = 6$; (−) leu diet, $n = 6$; pair-fed diet, $n = 6$). Statistical significance was determined by one-way ANOVA followed by the Student-Newman-Keuls test for the effect of either (−) leu or pair-fed diet versus control diet (*$P < 0.01$) or (−) leu diet versus pair-fed diet (#$P < 0.01$).

maintained on a leucine-deficient diet, but not in pair-fed mice, compared with mice maintained on a control diet (Fig. 6*B* and *C*), consistent with increased thermogenesis in these mice. Oxygen consumption was also significantly increased in BAT isolated from mice maintained on a leucine-deficient diet, under basal conditions and after stimulation with oleate, compared with pair-fed or control diet–fed mice (Fig. 6*D*).

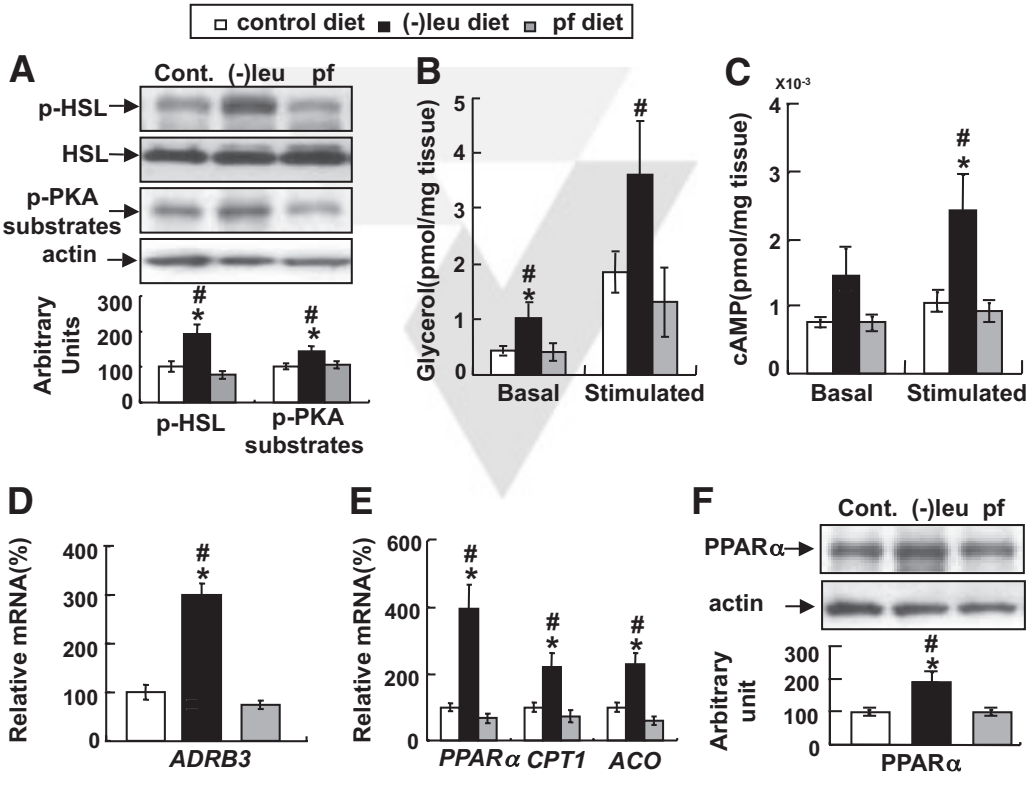


FIG. 4. Triglyceride lipolysis and fatty acid β-oxidation genes increases in WAT of leucine-deprived mice. Expression of triglyceride lipolysis in WAT of mice fed a control, leucine-deficient, or pair-fed diet for 7 days is shown. Data are means ± SE of at least two independent real-time PCR experiments (*D* and *E*) or Western blot (*A* and *F*) with mice of each diet for each experiment (control diet, $n = 6$; (−) leu diet, $n = 6$; pair-fed diet, $n = 6$). Statistical significance was determined by one-way ANOVA followed by the Student-Newman-Keuls test for the effect of either (−) leu or pair-fed diet versus control diet (*$P < 0.01$) or (−) leu diet versus pair-fed diet (#$P < 0.01$). *A*: p-HSL, HSL, and p-PKA substrate proteins. *B*: Glycerol release assay. *C*: cAMP content. *D*: *Adrb3* mRNA. *E*: *Pparα*, *Cpt-1*, and *Aco1* mRNA. *F*: PPARα protein.

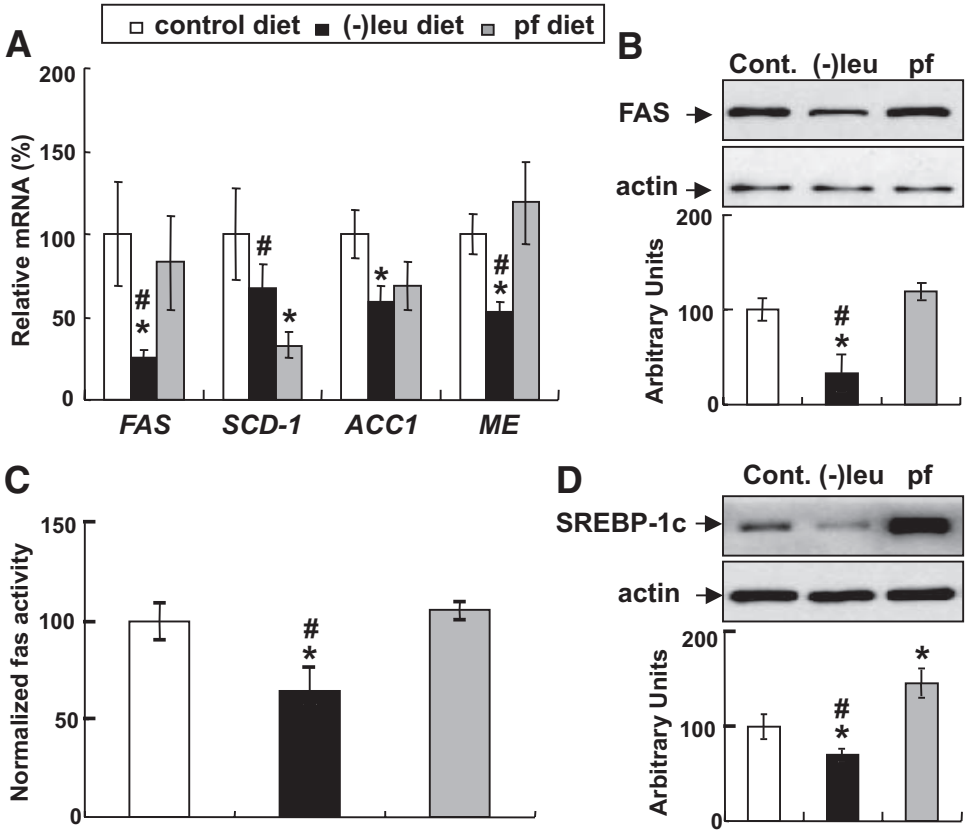


FIG. 5. Lipogenic genes are repressed in WAT of leucine-deprived mice. Expression of lipogenic genes in WAT of mice fed a control, leucine-deficient, or pair-fed diet for 7 days is shown. Data are means ± SE of at least two independent real-time PCR experiments (*A*) or Western blot (*B* and *D*) with mice of each diet for each experiment (control diet, $n = 6$; (−) leu diet, $n = 6$; pair-fed diet, $n = 6$). Statistical significance was determined by one-way ANOVA followed by the SNK test for the effect of either (−) leu or pair-fed diet versus control diet (*$P < 0.01$) or (−) leu diet versus pair-fed diet (#$P < 0.01$). *A*: *Fas, Scd1*, *Acc*I, and *Me* mRNAs. *B*: FAS protein (*top*, Western blot; *bottom*, FAS protein relative to actin and normalized to control diet group). *C*: FAS enzyme activity. *D*: SREBP-1c protein (*top*, Western blot; *bottom*, SREBP1C protein relative to actin and normalized to control diet group).

To investigate the mechanisms by which leucine deprivation on UCP1 expression, we examined expression levels of transcription factors that regulate UCP1 gene expression, including peroxisome proliferator–activated receptor γ coactivator gene (PGC1)-α, PPAR-γ, and CCAAT enhancer binding protein (c/EBP)-α (26,27). Expression of *Pgc1α* and *Pparγ* mRNA in BAT was significantly increased by leucine deprivation, but unchanged by pair-fed diet, compared with mice maintained on control diet. By contrast, *c/EBPα* mRNA was not changed in BAT from mice maintained on either diet (Fig. 6*E*). Increased UCP1 is regulated by the sympathetic nervous system through the activation of Adrb1 and Adrb3. Our results showed that levels of *Adrb1* and *Adrb3* mRNA in BAT were significantly increased in leucine-deprived mice compared with mice maintained on control diet, whereas pair-fed mice exhibited no induction of *Adrb1* and *Adrb3* mRNA (Fig. 6*F*). Consistent with increased serum T3 levels, mRNA level of *Dio2*, encoding type II deiodinasese, which generates T3 via deiodination of its precursor thyroxine (28,29), was also increased in BAT of mice maintained on leucine-deficient diet compared with pair-fed or control diet–fed mice (Fig. 6*F*).

DISCUSSION

In our previous study, we showed for the first time that leucine deprivation for 7 days results in a significant reduction in abdominal adipose mass accompanied by various metabolic changes (9). The molecular and cellular mechanisms responsible for these changes, however, were unclear. The goal of our current study is to elucidate the mechanisms underlying fat loss induced by leucine deprivation.

Consistent with our previous observations (9), the results of the present study show that leucine deprivation significantly reduces body weight and abdominal adipose mass without affecting proportion of lean body mass. Mice maintained on a leucine-deficient diet, however, reduced their food intake by 15%. These results are consistent with previous studies showing that mice consume less food when provided with a diet deficient in essential amino acids (9,14). It is unclear, however, why these mice consume less food compared with control diet.

To distinguish the influence of reduced food intake from that of leucine deprivation, we included a pair-fed group in the current study. Although a minor decrease in average body weight was observed in these mice, abdominal adipose mass was similar to that in control mice, suggesting that the observed reductions in leucine-deprived mice are primarily due to deficiency of leucine, rather than the small reduction in food intake.

It has been established that leucine plays an important role in regulation of metabolism (9,14,30). The direct link between leucine and lipid metabolism is demonstrated by our previous (9) and current work, which showed that a leucine-deficient diet resulted in significant reduction in

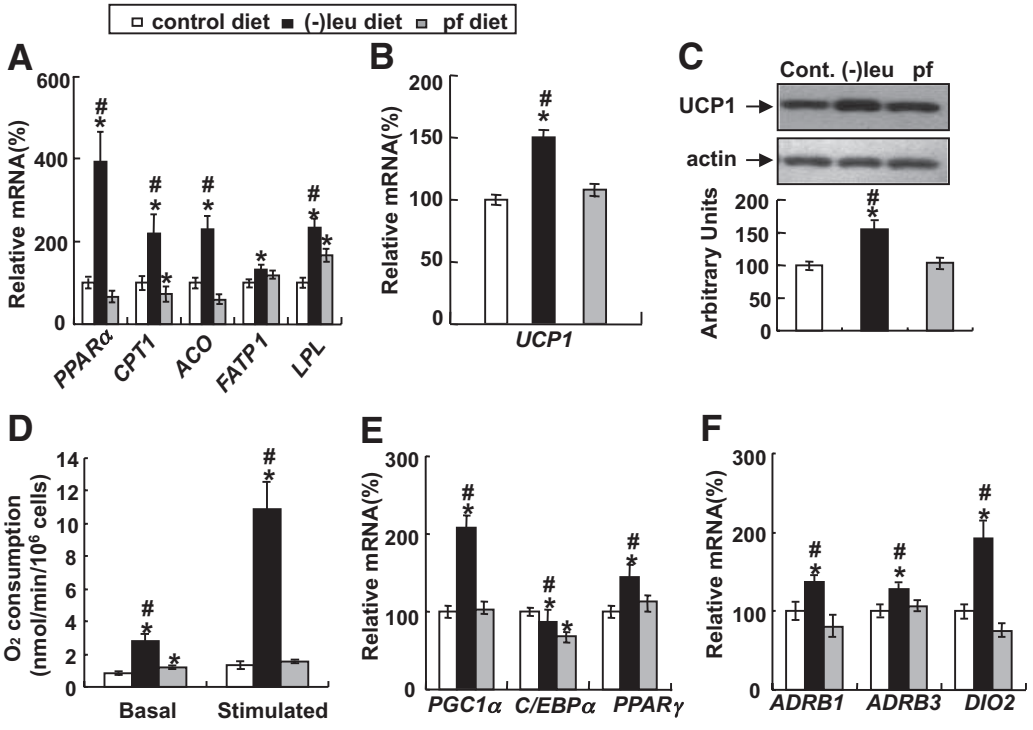


FIG. 6. Expression of β-oxidation genes and *UCP1* mRNA and protein increases in BAT of leucine-deprived mice. Expression of β-oxidation genes and *UCP1* mRNA and protein in BAT of mice fed a control, leucine-deficient, or pair-fed diet for 7 days. Data are means ± SE of at least two independent real-time PCR experiments with mice of each diet for each experiment (control diet, $n = 4$; (−) leu diet, $n = 4$; pair-fed diet, $n = 4$). Statistical significance was determined by one-way ANOVA followed by the Student-Newman-Keuls test for the effect of either (−) leu or pair-fed diet versus control diet (*$P < 0.01$) or (−) leu diet versus pair-fed diet (#$P < 0.01$). *A*: *Pparα*, *Cpt1α*, *Aco*, *Fatp1*, and *Lpl* mRNA. *B*: *Ucp1* mRNA. *C*: UCP1 protein. *D*: Oxygen consumption in isolated BAT. *E*: *Pgc1α*, *C/ebpα*, and *Pparγ* mRNA. *F*: *Adrb1*, *Adrb3*, and *Dio2* mRNA.

adipose mass in mice. Furthermore, in our current study, we show that this reduction is caused by a reduction in cell volume, rather than cell number. Because adipocyte volume is determined by the content of intracellular lipids (31), we hypothesized that reduction of adipocyte volume resulted from increased mobilization of intracellular fat. As predicted, we found that triglyceride lipolysis in WAT was substantially stimulated by leucine deprivation.

Fat mobilization in response to increased energy requirements is normally mediated via increased activities of the sympathetic nerve system (32). Increased release of norepinephrine from sympathetic nerves innervating adipose tissue activates Adrb3 on the surface of adipocytes, which interacts with Gα to stimulate adenylate cyclase. The resulting increase in intracellular levels of cAMP activates PKA, which phosphorylates and activates HSL (33). We found that leucine deprivation also increased levels of cAMP and phosphorylated-PKA substrates including HSL, suggesting that the leucine deprivation–induced fat mobilization is mediated by the cAMP-PKA-HSL pathway. Consistent with these results, the expression of *Adrb3*, the main isoform of β-adrenoceptors in WAT, was dramatically upregulated by leucine deprivation.

In addition to the sympathetic nervous system, fat mobilization can also be regulated by hormones, such as insulin, which acts to inhibit lipolysis (34). The significant reduction in plasma insulin in leucine-deprived mice may attenuate its inhibitory effect on lipolysis (9), resulting in increased fat mobilization. However, further investigation will be required to determine the relative contributions of the SNS and insulin or other hormones in the regulation of leucine deprivation–induced fat mobilization.

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Consistent with previous observations in liver (9,35), we found that leucine deficiency, but not pair-feeding, substantially suppressed lipogenesis in WAT, suggesting that impaired lipogenesis could be another important contributor to fat loss. Taken together, these results suggest that leucine deprivation–induced fat loss in WAT is due to both activation of triglyceride lipolysis and suppression of fatty acids synthesis. These changes are consistent with the observed low RER, which implies that fat is the major source of energy in leucine-deprived mice. RER was also low in pair-fed mice, but this likely reflects the fact that food was usually completely gone by this time. It has been previously shown that lipolysis of triglyceride is stimulated during the initial phase of food deprivation, releasing fatty acids as a source of energy (36). This may explain why fat was the major source of energy during the light phase, resulting in a decrease in RER in pair-fed mice.

Decreased serum FFA levels in leucine-deprived mice suggest that either most of the released FFA undergo β-oxidation in WAT or is rapidly taken up from the serum and metabolized by other tissues, such as liver, BAT, or skeletal muscle. In fact, β-oxidation–related genes are upregulated in WAT, BAT, and skeletal muscle (data not shown) in leucine-deprived mice. Because fatty acid oxidation in WAT makes only a minor contribution to low serum FFA (37), we hypothesize that increased β-oxidation in BAT and skeletal muscle is responsible for the major portion of this decrease. Furthermore, expression of genes encoding proteins for fatty acid uptake are increased in BAT in mice maintained on a leucine-deficient diet, which is also consistent with an increased uptake of fatty acids into BAT.

BAT oxidizes fat to produce heat (19,38), which increases concomitantly with the expression of UCPs (39).

The role of UCP1, the major isoform expressed in BAT, in thermogenesis regulation of body weight is demonstrated by the development of obesity in UCP1-ablated mice (40). Furthermore, upregulation of UCP1 expression results in increased thermogenesis and energy expenditure, which helps to protect from fat accumulation and obesity (41). Consistent with increased UCP1 expression, rectal temperature and oxygen consumption measured in isolated BAT are increased in leucine-deprived mice, suggesting increased thermogenesis. Increased thermogenesis in leucine-deprived mice could account for increased energy expenditure, as shown by increased oxygen consumption. However, it remains unclear how expression of UCP1 is upregulated in these mice.

The expression of UCP1 is under a complex control by SNS and hormones. It has been reported that *Ucp1* mRNA expression in response to overfeeding or cold exposure is upregulated because of increased activities of sympathetic nerves (39,42,43). This upregulation is mediated by the transcription factor PGC-1α (26,44). In our current study, we observed that PGC-1α expression is also increased in mice maintained on a leucine-deficient diet, suggesting that increased UCP1 expression is mediated by PGC-1α.

In addition to SNS, multiple hormones and factors have been shown to affect UCP1 transcription. Most of these, including norepinephrine and T3, increase UCP1 expression, whereas glucocorticoids decrease UCP1 expression (45). The increased serum levels of norepinephrine are consistent with increased SNS activity in leucine-deprived mice. The increased levels of serum T3 and expression of *dio2* in BAT of leucine-deprived mice suggest a potential role for T3 in regulation of UCP1 expression. Glucocorticoids do not seem to be involved in UCP1 regulation under leucine deprivation.

It remains unclear, however, if leucine deprivation has a direct or indirect effect on UCP1 regulation in BAT. One possibility is that leucine deprivation increases UCP1 expression indirectly by increasing lipolysis in WAT, leading to increased serum FFAs. FFAs in serum would be rapidly taken up by BAT, where they would stimulate expression of UCP1 to increase thermogenesis and energy expenditure, as previously shown in another study (19). Another possibility is that leucine deprivation first upregulates UCP1 expression, thereby increasing energy expenditure by increasing thermogenesis. Increased energy expenditure would be expected to consume more fatty acids released from WAT, therefore indirectly stimulating fat loss. It is possible of course that leucine deprivation affects both WAT and BAT. Further investigation will be required to distinguish these models.

We did not see increased physical activity in leucine-deprived mice, measured in a metabolic cage. We could not, however, rule out the possibility that muscle contributes to leucine deprivation–increased energy expenditure by increasing thermogenesis. Consistent with this idea, expression of β-oxidation genes, *Ucp3*, and T3 target gene *Serca1* (46) was increased in leucine-deprived mice compared with pair-fed or control diet–fed groups (data not shown). Further studies will be required to determine the relative contribution of BAT and skeletal muscle for leucine deprivation–stimulated fat loss.

It will also be interesting to determine whether deficiency of other essential amino acids have the same effect as leucine deficiency on WAT, BAT, and muscle that we observed in this study. A previous study by the Semenkovich group (35) showed that expression of FAS mRNA is suppressed in HepG2 cells in medium deficient of any essential amino acids, but not by nonessential amino acids. Based on this result, we speculate that deficiency of other essential amino acids may produce similar effects.

In summary, we show that the rapid abdominal fat loss by leucine deprivation is caused by increased fat mobilization and suppressed fatty acid synthesis in WAT, as well as increased energy expenditure, most likely through increased thermogenesis. Our data strongly indicate a role for BAT in this increased thermogenesis. Our observations provide a rationale for the (short-term) use of dietary deprivation or restriction of leucine for the treatment of obesity and associated metabolic diseases. Independent studies will be required, however, to determine the safety of dietary leucine restriction in therapeutic applications.

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