Original Article

Increasing Dietary Leucine Intake Reduces Diet-Induced Obesity and Improves Glucose and Cholesterol Metabolism in Mice via Multimechanisms

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Leucine, as an essential amino acid and activator of mTOR (mammalian target of rapamycin), promotes protein synthesis and suppresses protein catabolism. However, the effect of leucine on overall glucose and energy metabolism remains unclear, and whether leucine has beneficial effects as a long-term dietary supplement has not been examined. In the present study, we doubled dietary leucine intake via leucine-containing drinking water in mice with free excess to either a rodent chow or a high-fat diet (HFD). While it produced no major metabolic effects in chow-fed mice, increasing leucine intake resulted in up to 32% reduction of weight gain ($P < 0.05$) and a 25% decrease in adiposity ($P < 0.01$) in HFD-fed mice. The reduction of adiposity resulted from increased resting energy expenditure associated with increased expression of uncoupling protein 3 in brown and white adipose tissues and in skeletal muscle, while food intake was not decreased. Increasing leucine intake also prevented HFD-induced hyperglycemia, which was associated with improved insulin sensitivity, decreased plasma concentrations of glucagon and glucogenic amino acids, and downregulation of hepatic glucose-6-phosphatase. Additionally, plasma levels of total and LDL cholesterol were decreased by 27% ($P < 0.001$) and 53% ($P < 0.001$), respectively, in leucine supplemented HFD-fed mice compared with the control mice fed the same diet. The reduction in cholesterol levels was largely independent of leucine-induced changes in adiposity. In conclusion, increases in dietary leucine intake substantially decrease diet-induced obesity, hyperglycemia, and hypercholesterolemia in mice with ad libitum consumption of HFD likely via multiple mechanisms. *Diabetes* 56:1647–1654, 2007

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BAT, brown adipose tissue; G6P, glucose-6-phosphatase; HFD, high-fat diet; HOMA-IR, homeostasis model assessment of insulin resistance; mTOR, mammalian target of rapamycin; UCP, uncoupling protein; WAT, white adipose tissue.

Despite the increased awareness of health risks associated with obesity and its attendant metabolic disorders, the prevalence of obesity has doubled in the past 20 years (1). Many dieting strategies have been developed and practiced in the past two decades, with variable short-term success rates (2,3). Long-term success in maintaining weight loss has proven to be difficult, in part because energy metabolism becomes more efficient at the reduced weight as a result of the normal physiological responses to weight loss and energy deficits (4,5).

Manipulation of dietary composition of macronutrients merits careful investigation because macronutrients not only provide calories but some components may also function as signaling molecules to affect feeding behavior, energy balance, and fuel efficiency. Dietary supplementation is particularly attractive in that it may improve diet qualities and reduce cardiovascular risks associated with obesity and the metabolic syndrome without affecting the gross macronutrient compositions and the palatability of regular food, which will likely result in better long-term compliance.

Leucine is an essential branched chain amino acid that cannot be produced and can only be obtained from diet in human. Leucine serves not only as a building block for protein synthesis but is also a potent activator of the mammalian target of rapamycin (mTOR), a serine/threonine kinase involved in many cellular processes, including protein synthesis, cell growth, and metabolism (6–10). Indeed, leucine supplementation increases the net protein anabolism in various tissues (11–16). However, by activating mTOR and S6 kinase, leucine also feedback inhibits insulin signaling and decrease glucose utilization in skeletal muscle (17–20).

Several recent studies have shown that protein-rich diets produce a better glycemic control and a greater loss of body fat and less of lean tissue during weight loss than do carbohydrate-rich diets with the same fat and caloric content (21–24). Branched chain amino acids, especially leucine, have been speculated to play a key role in this process (25). Supporting this hypothesis, Donato et al. (26) reported that leucine supplementation during caloric restriction also results in more fat loss and improves protein synthesis in liver and muscle. Recently, Seeley and colleagues (10) showed that infusion of leucine into the third ventricle of the rat brain decreases the animal's food intake and body weight via activation of the mTOR pathway in the arcuate nucleus of the hypothalamus. Thus, as

leucine may function to promote fat loss while also, independently, increase insulin resistance in peripheral tissues (see above), it remains unclear whether increased leucine intake in animals affects the development of dietary obesity as well as whether it has an overall detrimental or beneficial health effect.

We sought to determine the net effects of dietary supplementation of leucine on whole-body macronutrient and energy metabolism in mice with free access to a high-fat diet (HFD) and to assess how cardiovascular risks associated with diet-induced obesity may be modified by increased dietary leucine intake. Here, we show that doubling dietary leucine intake significantly reduces HFD-induced weight gain and improves hyperglycemia and hypercholesterolemia, which is associated with a previously unrecognized effect of leucine, i.e., increased resting energy expenditure. Furthermore, modifications of the various cardiometabolic risk factors associated with increased leucine intake appear to involve multiple mechanisms that are not solely dependent on reduced adiposity.

RESEARCH DESIGN AND METHODS

Animal husbandry, diets, and leucine supplementation. Male C57BL/6J mice were purchased from The Jackson Laboratories. Animal protocols were in compliance with the accepted standards of animal care and were approved by the Columbia University institutional animal care and use committee. Mice were maintained at 22°C on a 12:12 light-dark cycle (0700–1900 h) and had ad libitum access to a regular rodent chow (Purina Rodent Diet 20 5053) or, when indicated, a HFD containing 60% fat calories (D12492; Research Diets, New Brunswick, NJ). Both diets contained 20% protein calories. Leucine was supplemented via drinking water made of 1.5% (wt/vol) L-leucine (Sigma, St. Louis, MO).

Blood glucose, glucose tolerance tests, and insulin tolerance tests. Blood glucose levels were measured in tail-vein blood using a Glucometer Elite (Bayer, Elkhart, IN). For glucose tolerance tests, blood glucose was measured at 0, 30, 60, and 90 min after a bolus intraperitoneal glucose administration (1 mg/g body wt) to overnight-fasted mice. For insulin tolerance tests, regular human insulin was administered intraperitoneally (0.75 mU/g) to mice after 4-h food and leucine deprivation, and blood glucose was measured at 0, 30, 60, and 90 min after insulin injection. The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated based on the conventional formula: HOMA-IR = basal glucose (mmol/l) × basal insulin (mU/l)/22.5.

Indirect calorimetry. Five-month-old male C57BL/6J mice that had been on a HFD with or without leucine supplementation for 10 weeks were implanted with E-mitters (Mini-Mitter) for temperature measurements under anesthesia and were allowed a 4-day recovery before being housed in metabolic chambers. Oxygen consumption, locomotor activity, core temperature, and respiration exchange ratio were obtained continuously during 12:12 light-dark cycles using a comprehensive laboratory animal monitoring system (Columbus Instruments, Columbus, OH) open-circuit indirect calorimetry system. The same diet and leucine regimens for the mice were continued during indirect calorimetry. Data were collected over at least 5 days following 2–4 days of adaptation to the metabolic cages.

Determination of mRNA expression. Quantitative real-time RT-PCR was used to determine mRNA expression as previously described (27,28). Briefly, total RNA was isolated using miniRNeasy columns (Qiagen, Valencia, CA) and reverse transcribed into single-stranded cDNA using random hexamers and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative amplification by PCR was carried out using Bio-Rad iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Cyclophilin A was used as a control. Cycle numbers at a set fluorescence threshold within the exponential amplification range (C_t) were used to calculate expression levels of the genes of interest (relative to cyclophilin A). Changes in gene expression were expressed as fold increase or decrease over a specified reference sample. The following primers were used in this study: uncoupling protein (UCP)1, 5′-ggtgagttcga-caacttccg-3′ (forward) and 5′-ggtgatggtccctaa-gacacc-3′ (reverse); UCP2, 5′-gtggtcggagataccagagc-3′ (forward) and 5′-gaggttggctttcaggagag-3′ (reverse); UCP3, 5′-caagggagcggaccactcc-3′ (forward) and 5′-ctc tctcctccagttcccatg-3′ (reverse); and cyclophilin A, 5′-atggcactggcggcaggtcc-3′ (forward) and 5′-ttgccattcctggacccaaa-3′ (reverse).

Protein extraction and immunoblotting. Gastrocnemius muscle (30 mg) or adipose tissues (100 mg) were powdered in liquid nitrogen and homogenized in a homogenization buffer (50 mmol/l Tris-HCl, pH 8.0, 1% NP-40, 1 mmol/l EDTA, 150 mmol/l $NaCl_2$, 50 mmol/l NaF, 0.5 mmol/l NaVO4, 5 mmol/l Na4P2O7, and 1 mmol/l DTT and protease inhibitors). Tissue homogenates were sonicated for 2 min in an ice-water bath, followed by centrifugation at 14,000 rpm for 15 min in an Eppendorf Microcentrifuge to remove insoluble cellular debris. Proteins (50–100 μg) were resolved by SDS-PAGE and then transferred to nitrocellulose membranes. Levels of specific proteins were determined by immunoblotting using enhanced chemiluminescence detection horseradish peroxidase reagents (Amersham Biosciences, Piscataway, NJ) and antibodies to mouse UCP1 and UCP3 (Sigma-Aldrich, St. Louis, MO) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblotting signals were quantified using the Quantity One program (Bio-Rad).

Determination of body composition and plasma parameters. Body composition was determined using a Minispec TD-NMR Spectrometer (Bruker Optics). Unless otherwise indicated, plasma lipids, amino acids, and hormones, including insulin, leptin, glucagon, adiponectin, corticosterone, and catecholamines, were measured at the basal state (after a 5-h food and leucine deprivation) in HFD leucine and HFD control mice. Assays were done by the Mouse Phenotyping Center, Vanderbilt University. Plasma hormone concentrations are shown in Table 1.

Statistical analysis. The effects of leucine supplementation on weight, body composition, food intake, and blood glucose levels in HFD-fed and chow-fed mice were assessed separately using one-way ANOVA (Statistica version 6; StatSoft, Tulsa, OK); Newman-Keuls tests were used for post hoc comparisons. Multiple regression analysis was used to assess direct effects of leucine versus leucine-mediated reduction in adiposity on glucose and cholesterol metabolism. A two-tailed $P < 0.05$ was considered statistically significant. For indirect calorimetry measurements, repeated-measures ANOVA (Prism; GraphPad Software, San Diego, CA) were used. Post hoc analyses were conducted using Tukey post hoc or Bonferroni comparisons. Data are expressed as means with SEs.

TABLE 1
Effects of leucine supplementation on plasma hormone levels in HFD-fed mice

	HFD-W	HFD-Leu
Insulin (ng/ml)	5.28 ± 0.78	3.26 ± 0.56*
Glucagon (pg/ml)	52.3 ± 9.9	23.9 ± 1.3*
Leptin (ng/ml)	36.3 ± 3.3	18.2 ± 5.4†
Adiponectin (mg/ml)	11.1 ± 1.4	10.3 ± 0.5
Corticosterone (ng/ml)	149 ± 39	112 ± 34
Epinephrine (ng/ml)	0.89 ± 0.19	1.11 ± 0.20
Norepinephrine (ng/ml)	0.89 ± 0.22	0.86 ± 0.16

Data are means ± SE. *$P < 0.05$ and †$P < 0.01$, HFD-W vs. HFD-Leu; $n = 5$.

RESULTS

Leucine supplementation decreases diet-induced obesity by increasing resting energy expenditure associated with increased UCP3 expression in thermogenic tissues. Eight week-old male C57BL/6J mice on a regular rodent chow diet (chow) or on a HFD were supplemented with leucine via drinking water (chow-Leu and HFD-Leu, respectively). HFD and leucine supplementation were started simultaneously. Control mice on the respective diets were provided with regular tap water (chow-W or HFD-W). The amounts of regular or leucine-supplemented water consumed by the mice were the same. Leucine-supplemented mice ingested an additional ~55 mg leucine daily via water consumption, which nearly doubled the total daily leucine intake from food (~56 mg from chow; ~63 mg from HFD) (Fig. 1*A*). Leucine supplementation increased plasma leucine concentrations by ~30% during feeding but decreased overall plasma amino acid levels, particularly Asp, Glu, Gln, and Gly, in postabsorptive states and Asn, Pro, and Lys during feeding (Fig. 1*C* and *D*), suggesting a decreased protein catabolism in leucine-supplemented mice.

Mice on the HFD gained significantly more weight, mainly as fat, than did those on the chow diet as was

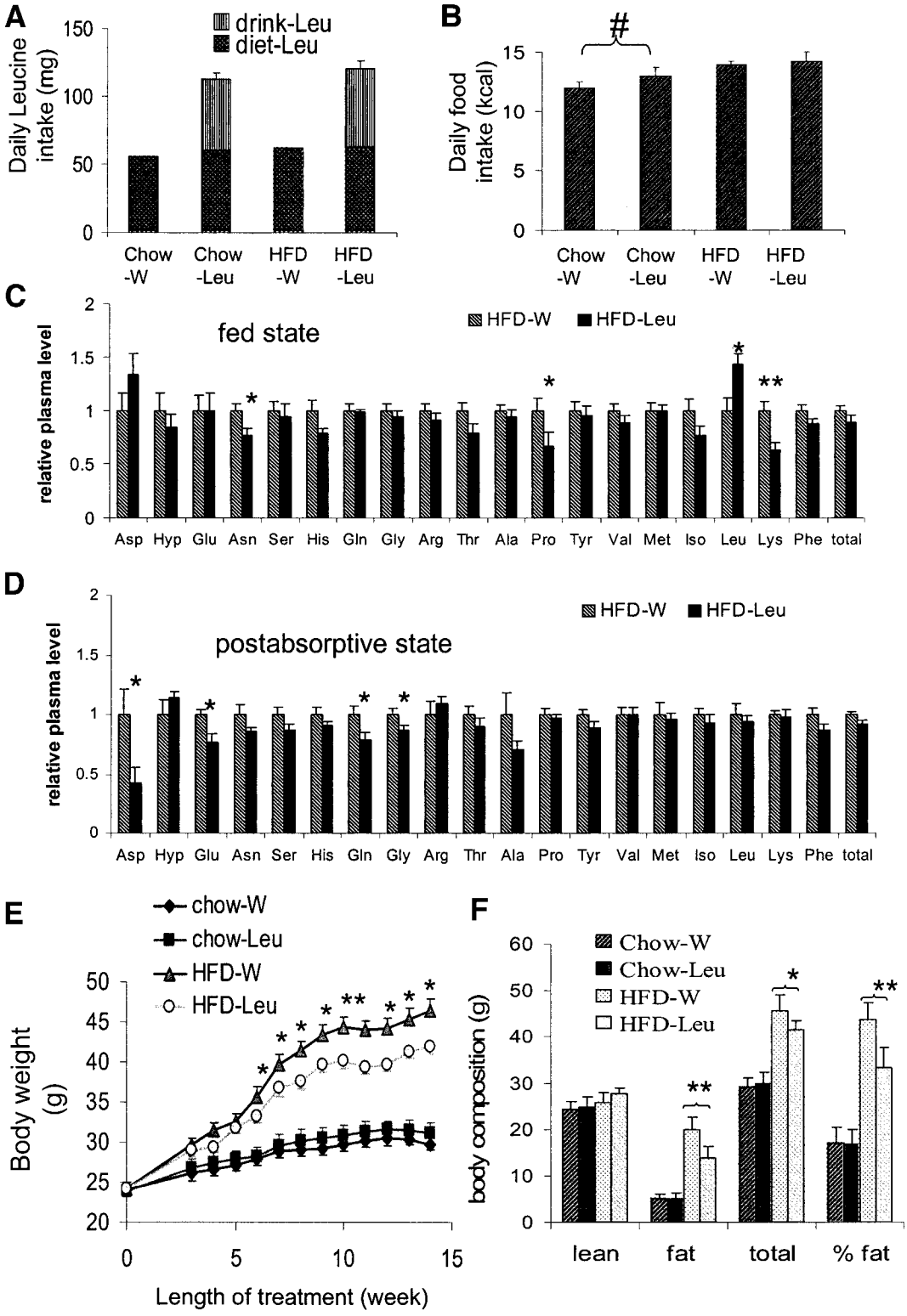


FIG. 1. Leucine supplementation decreases protein catabolism and reduces HFD-induced weight gain and adiposity. *A* and *B*: Daily leucine intake and caloric intake, respectively, calculated from the amount of ingested food and drinking water measured in individually caged mice over 14 days. Chow-Leu and HFD-Leu designate leucine-supplemented mice on regular chow and HFD, respectively; Chow-W and HFD-W designate the control mice supplied with regular water, on chow and HFD, respectively. *C* and *D*: Plasma concentrations of amino acids during feeding (fed state; measured at the fourth hour of the dark cycle) and at the postabsorptive state (measured at the seventh hour of the light cycle after a 5-h food and leucine deprivation), respectively. Values were determined from mice at the 14th week of indicated diet/supplement treatments. *E*: Body weights and growth curves during 14 weeks of indicated diet and supplementation treatments. The indicated diet/supplement regimens were started in 8-week-old weight-matched male mice, designated as week 0. *F*: Body composition at the end of 14 weeks of diet/supplement treatments. Lean body mass (Lean), fat mass (Fat), and total body mass (Total) were measured by nuclear magnetic resonance spectrometry, and the percentage of fat mass (%Fat) was calculated as fat mass/(lean + fat mass). Data are expressed as mean ± SE, $n = 5$ in each group. *$P < 0.05$ and **$P = 0.01$, HFD-Leu vs. HFD-W; #$P < 0.05$, Chow-Leu vs. Chow-W. Results shown in this figure are from a single representative experiment (from a total of three experiments, $n = 5$ for each treatment group in each experiment).

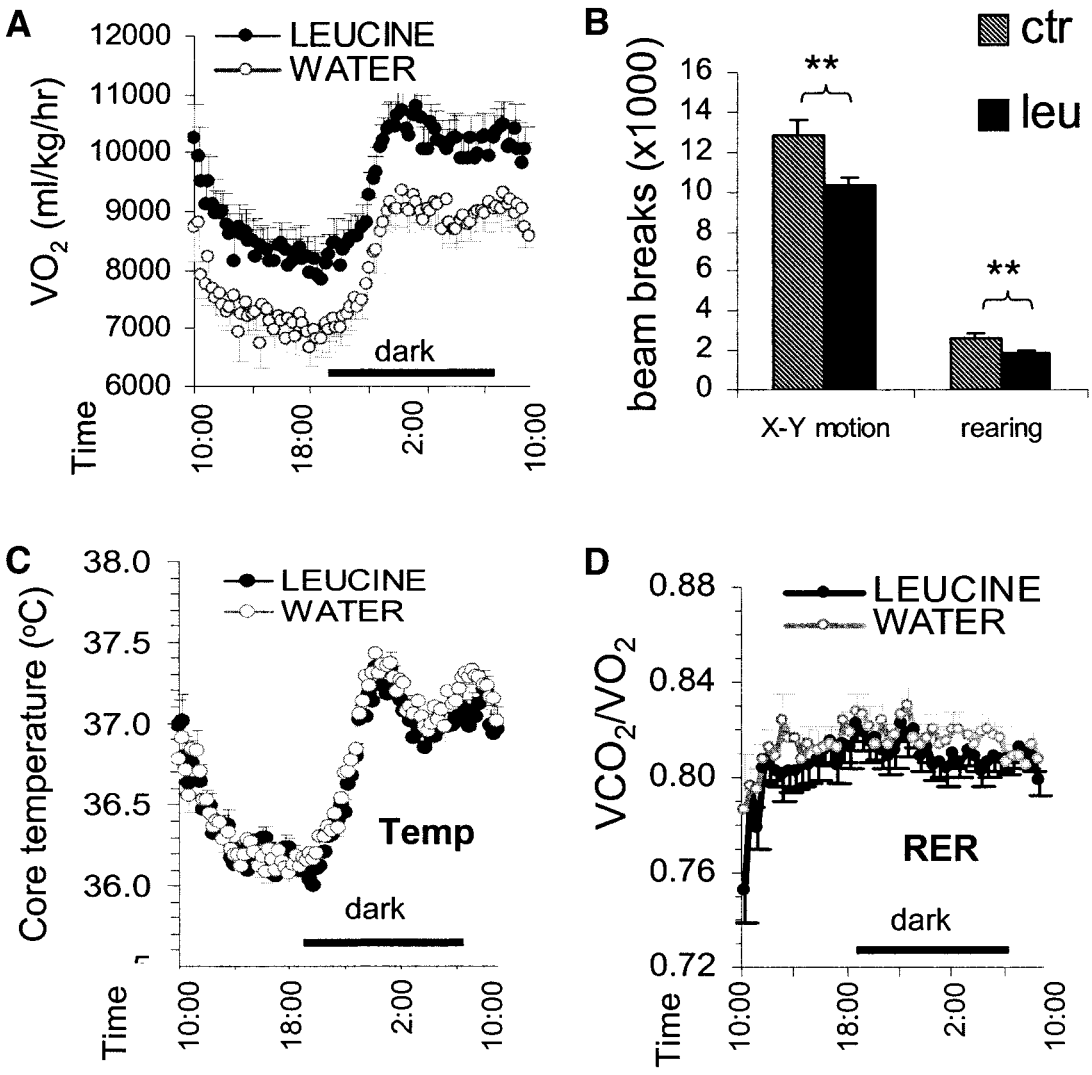


FIG. 2. Leucine supplementation increases resting energy expenditure. Four pairs of HFD-Leu and HFD-W mice were studied by indirect calorimetry after 10 weeks of the indicated diet/supplement regimens. *A*: Twenty-four hour oxygen consumption. *B*: Locomotor activity. ctr, control; leu, leucine. *C*: Core body temperature. *D*: Respiratory exchange ratio (RER). Date shown are average values measured over a 5- to 8-day period after a 4-day adaptation to the metabolic chamber.

expected (Fig. 1*E* and *F*). However, this diet-induced weight gain and expansion of fat mass were significantly attenuated in leucine-supplemented HFD-fed mice (Fig. 1*E* and *F*). Eight weeks into the diet/supplement regimens, HFD-W mice weighed 41.4 ± 1.2 g, whereas HFD-Leu mice weighed 37.6 ± 1.0 g. This represents a 32% ($P < 0.05$) reduction of HFD-induced weight gain (12.1 g in HFD-W vs. 8.3 g in HFD-Leu, $P < 0.05$) (Fig. 1*E*) and a 25% decrease in adiposity (41.3% fat in HFD-W vs. 31.0% fat in HFD-Leu, $P < 0.01$). Differences in body weight, fat mass, and adiposity persisted throughout the 14-week study (Fig. 1*E* and *F*). Leucine supplementation had no effect on body weight or composition in mice on the chow diet (Fig. 1*E* and *F*). Total caloric intake was increased by 8.4% in chow-Leu mice, compared with chow-W mice, but was not significantly different between HFD-Leu and HFD-W mice (Fig. 1*B*).

Since weight loss without decreased food intake (the HFD group) and increased food intake without weight gain (the chow group) both suggested a leucine-mediated increase in energy expenditure, we directly measured energy metabolism in four pairs of HFD-W and HFD-Leu mice by indirect calorimetry. Total energy expenditure (24-h V_{O_2}) normalized to body weight was markedly increased (~15%, $P < 0.01$) in HFD-Leu mice relative to HFD-W mice (Fig. 2*A*), despite a decrease in locomotor activity in HFD-Leu mice (Fig. 2*B*). Core body temperature was not significantly different between HFD-Leu and HFD-W mice (Fig. 2*C*). The respiratory exchange ratio (V_{CO_2}/V_{O_2}) was slightly lower in HFD-Leu mice than in HFD-W mice (an average 0.808 vs. 0.814, respectively, $P < 0.01$) (Fig. 2*D*), suggesting increased fat oxidation in HFD-W mice.

To gain insight into molecular mechanisms for increased resting energy expenditure in HFD-Leu mice, we examined mRNA and/or protein levels of UCP1, -2, and -3, three major uncoupling proteins, in skeletal muscle and in brown adipose tissue (BAT) and white adipose tissue (WAT). Messenger RNA levels for UCP3, a major uncoupling protein in skeletal muscle and BAT, were increased by 1.51-fold ($P < 0.05$) in gastrocnemius muscle, 2.08-fold ($P < 0.001$) in perigonadal adipose tissue (WAT), and 1.67-fold ($P < 0.05$) in BAT of HFD-Leu mice, relative to the respective tissues of HFD-W mice (Fig. 3*A*). At the

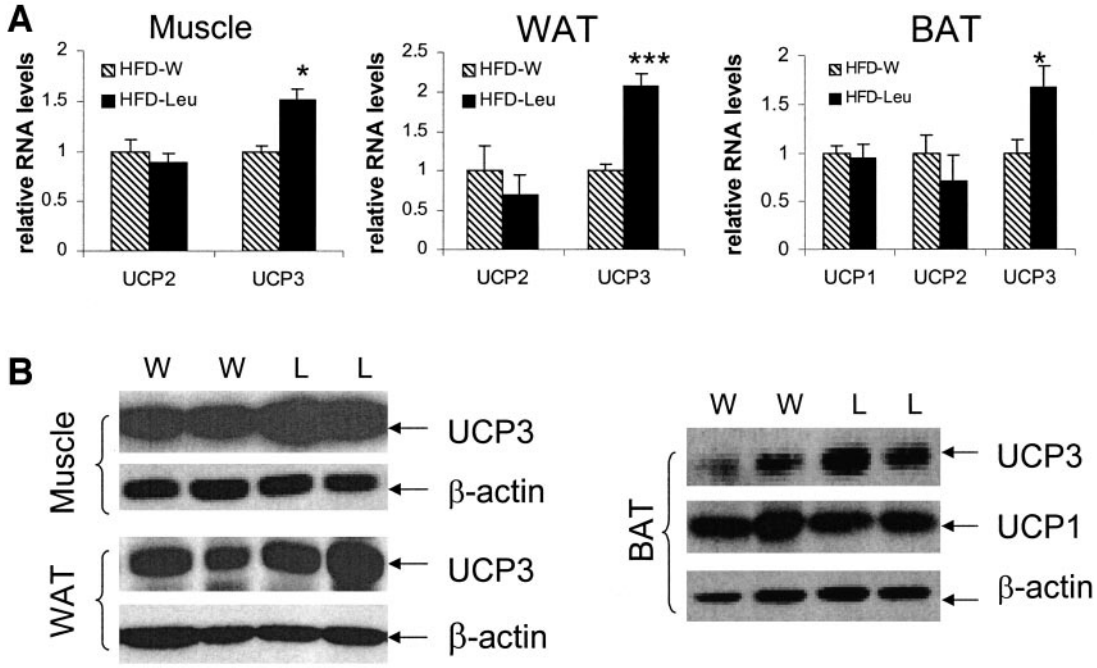


FIG. 3. Leucine supplementation increases UCP3 expression in skeletal muscle and adipose tissues. *A*: Messenger RNA levels of UCP2 and -3 in gastrocnemius muscle, perigonadal adipose tissue (WAT), and intrascapular BAT and UCP1 in BAT of HFD-Leu (L) and HFD-W (W) mice described in Fig. 1 ($n = 5$). Data are shown as means ± SE. *$P < 0.05$ and ***$P = 0.001$, HFD-Leu vs. HFD-W. *B*: Representative immunoblotting results for UCP3 protein expression in the same tissues as indicated. UCP1 protein levels were also shown in BAT.

protein level, UCP3 was increased by 22.7% ($P < 0.05$), 28.9% ($P < 0.01$), and 20.1% ($P < 0.05$) in gastrocnemius muscle, perigonadal adipose tissue, and BAT, respectively, in HFD-Leu mice compared with control tissues (Fig. 3*B*). However, the levels of the ubiquitously expressed UCP2 and BAT UCP1 were not significantly different (Fig. 3*A* and *B*). These results suggest that leucine may specifically regulate UCP3 gene expression in skeletal muscle, BAT, and WAT and that increased UCP3 expression may account, at least in part, for the increased resting energy expenditure in HFD-Leu mice.

Leucine supplementation improves glucose metabolism, reduces diet-induced insulin resistance, and lowers plasma glucagon levels and hepatic glucose-6-phosphatase expression. Four weeks after HFD treatment, fasting blood glucose levels became elevated in HFD-W mice (112.5 ± 7.0 mg/dl) compared with chow-W mice (74.2 ± 3.6 mg/dl, $P < 0.001$) (Fig. 4*A*), but leucine supplementation largely prevented this HFD-induced hyperglycemia (89.2 ± 7.6 vs. 112.5 ± 7.0 mg/dl, $P < 0.05$). The glucose-lowering effect of leucine persisted throughout the study period, and HFD-Leu mice remained euglycemic at week 10 (Fig. 4*A*). Basal plasma insulin concentrations were significantly decreased in HFD-Leu mice compared with HFD-W mice (Table 1 and Fig. 4*E*). Glucose tolerance tests and insulin tolerance tests performed at weeks 10 and 11, respectively, confirmed that HFD-Leu mice were significantly more glucose tolerant and insulin sensitive than HFD-W mice (Fig. 4*B* and *C*). The HOMA-IR index was >50% lower in HFD-Leu mice than in HFD-W mice (34.2 ± 7.6 vs. 67.7 ± 11.7, $P < 0.05$) (Fig. 4*D*). A forward stepwise regression analysis shows that leucine treatment ($F = 5.6$, $P < 0.05$) is a better predicting factor than adiposity ($F = 3.5$, $P = 0.09$) for HOMA-IR, suggesting that the glucose-lowering effect of leucine may not be entirely dependent on the decrease in adiposity.

Basal plasma glucagon levels were decreased by ~50% ($P < 0.05$) in HFD-Leu mice (23.9 ± 1.3 pg/ml) compared with HFD-W mice (52.3 ± 9.9 pg/ml) (Fig. 4*E* and Table 1). Messenger RNA levels for glucose-6-phosphatase (G6P), a key enzyme in regulating hepatic glucose production, were also significantly lower in the livers of HFD-Leu mice, although mRNA levels for PEPCK and PGC-1α (peroxisome proliferator–activated receptor-γ coactivator), another two genes involved in hepatic gluconeogenesis (29,30), were not significantly different (Fig. 4*F*). G6P mRNA levels were significantly correlated with plasma glucagon levels ($r = 0.85$, $P < 0.01$) but not with insulin levels in HFD-fed mice (data not shown). Unlike HFD-fed mice, chow-fed mice remained euglycemic over the 14-week study period, and leucine supplementation had no additional effect on these values (Fig. 4*A*).

Leucine supplementation reduces diet-induced hypercholesterolemia independent of adiposity. Leucine supplementation for 14 weeks decreased total cholesterol levels by 27% ($P < 0.001$) and LDL-cholesterol by 53% ($P < 0.001$) in HFD-fed mice (Fig. 5*A*). The cholesterol-reducing effect of leucine remained significant when adiposity was also included as an independent variable in a multiple regression analysis (Fig. 5*B*). This finding contrasts with the effect of leucine on plasma leptin levels. Leucine supplementation lowered plasma leptin levels (18.2 ± 5.4 ng/ml in HFD-Leu mice vs. 36.3 ± 3.3 ng/ml in HFD-W mice, $P < 0.01$) (Table 1), but this effect was essentially lost when the decrease of adiposity was taken into account (Fig. 5*B*). No significant differences were detected in HDL cholesterol, total triglyceride, or free fatty acid levels (Fig. 5*A*). Taken together, these data suggest that leucine has a selective

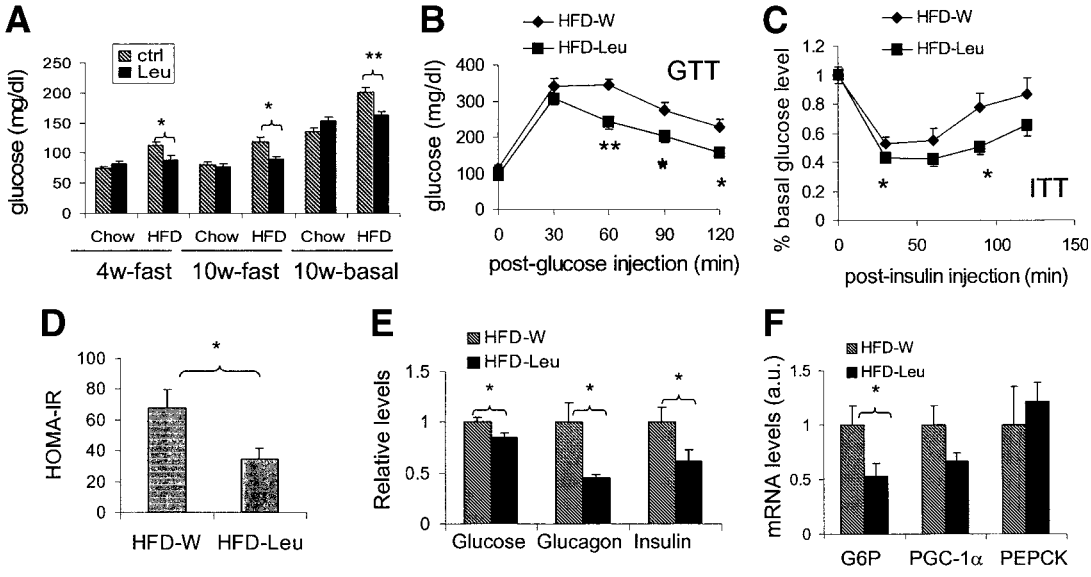
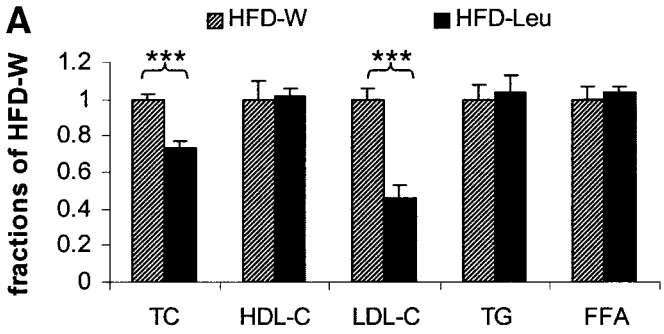


FIG. 4. Leucine supplementation improves glucose metabolism by increasing insulin sensitivity and decreasing glucagon secretion in HFD-fed mice. Study design is as in Fig. 1*E*. *A*: Fasting (overnight food and leucine deprivation, regular water allowed) blood glucose concentrations were measured at weeks 4 and 10, and basal (after a 5-h food and leucine deprivation) blood glucose concentrations were measured at week 10. Diets are as indicated. ctrl, control mice supplied with regular water; Leu, leucine supplementation. The data are representative of three similar experiments. *B* and *C*: Intraperitoneal glucose tolerance tests (ITT) (1 mg glucose per g body wt) and insulin tolerance tests (0.75 mU insulin per g body wt), respectively, performed at weeks 10 and 11 of the diet/supplement treatments. *D*: HOMA-IR index calculated from basal plasma glucose and insulin levels at the end of a 14-week study period. *E*: Basal plasma glucose, insulin, and glucagon levels at the end of 14 weeks of diet/supplement study. *F*: Relative hepatic mRNA levels of G6P, peroxisome proliferator–activated receptor-γ coactivator (PGC)-1α, and PEPCK in the same mice as in *E*. All data are shown as means ± SE, $n = 5$ in each group. *$P < 0.05$ and **$P < 0.01$, HFD-Leu vs. HFD-W.



B

Dependent variables	Independent variables			
	Leu treatment		Adiposity	
	β	p	β	p
Total C	-0.69	<0.05	0.23	0.46
LDL-C	-0.63	<0.05	0.34	0.21
leptin	0.12	0.665	1.01	<0.01

FIG. 5. Leucine supplementation decreases plasma levels of total and LDL cholesterol in HFD-fed mice. Study design is as in Fig. 1*E*. *A*: Fasting plasma levels of total cholesterol (TC), HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), triglyceride (TG), and free fatty acids (FFA), measured at week 14 of the diet/supplement treatments as described. Data are shown as means ± SE, $n = 5$ in each group. ***$P < 0.001$. *B*: Multiple regression analyses using β, standardized correlation coefficient, and *P* values to describe the outcomes, as shown. Leucine treatment and adiposity are independent variables; total cholesterol, LDL cholesterol, and leptin are dependent variables.

effect on LDL cholesterol reduction, independent of its effect on adiposity.

DISCUSSION

We have shown that doubling dietary leucine intake substantially reduced diet-induced weight gain and improved glucose and cholesterol metabolism in mice. The attenuation of HFD-induced weight gain by leucine supplementation resulted from increased resting energy expenditure associated with increased UCP3 protein expression in skeletal muscle, BAT, and WAT. Food intake was not reduced by the increased leucine intake. Both we and others have observed that mice on chronic HFD become highly fuel efficient; they gain more weight per unit of calorie consumed compared with chow-fed mice (31–34). In HFD-Leu mice, increased UCP3-mediated mitochondrial uncoupling of oxidative phosphorylation in thermogenic tissues likely accounts for the increased resting energy expenditure with reduced fuel efficiency. Our result is consistent with the reports that increased UCP3 expression reduces HFD-induced weight gain in transgenic mice (35) and is associated with the obesity-resistant phenotype in rats (34). Additionally, increases in muscle UCP3 expression level are generally seen in obese rats and humans (36,37), and weight loss results in decreases in muscle UCP3 expression in humans (37,38). However, HFD-Leu mice, while having lower adiposity relative to HFD-W mice, express higher levels of UCP3 protein in both skeletal muscle and adipose tissues. Thus, the effect of increased leucine intake on tissue UCP3 expression appears to be independent of the attenuated adiposity in HFD-Leu mice. How leucine increases UCP3 expression is unclear, it may depend on leucine-mediated activation of the mTOR-S6K pathway, since insulin also both activates mTOR-S6K signaling pathway and increases UCP3 expression in skeletal muscle (39).

It is of interest to note that while increased UCP3 expression in skeletal muscle and in adipose tissues is consistent with the observed increased in resting energy expenditure, core body temperatures were not significantly different between HFD-Leu and HFD-W mice. Normal core body temperatures were associated with normal diurnal fluctuations in both HFD-Leu and HFD-W mice, suggesting that the thermoregulation mechanism was intact and that excess heat generated by increased energy expenditure was effectively dissipated in HFD-Leu mice. A similar result has also been observed in mice with genetic ablation of MCH (melanin-concentrating hormone), a hypothalamic neuropeptide involved in regulating food intake and energy expenditure; a 20% increase in the rate of oxygen consumption in $MCH^{-/-}$ mice has no effect on core body temperature (40).

Cota et al. (10) showed that centrally administered leucine activates the mTOR pathway in the hypothalamic arcuate nucleus and suppresses food intake in rats. Orally administered leucine in our study, however, had no significant suppressive effect on food intake. Possible explanations for the apparent discrepancy include the doses and routes by which leucine was introduced into animals (intracerebroventricular infusion versus oral administration). Orally administered leucine may be substantially metabolized in the liver before it reaches other tissue targets. Plasma leucine concentrations were increased by 30% during feeding in HFD-Leu mice and were not significantly different in the postabsorptive state. Thus, it is possible that leucine concentrations in the hypothalamus of HFD-Leu mice did not reach a level comparable with that achieved by intracerebroventricular leucine infusion reported by Cota et al. However, this does not preclude the possible involvement of the central nervous system in mediating leucine's effects on energy expenditure, whether in animals receiving centrally administered leucine or in animals with increased oral intake of leucine, such as HFD-Leu mice. Cota et al. did not report the status of energy expenditure, but it is possible that changes in centrally mediated energy expenditure may require a lower central nervous system level of leucine than what is necessary for suppressing food intake. Another potential explanation for the apparent discrepancy between our study and that of Cota et al. is the species difference. Mice (ours) and rats (Cota et al.) may have species-specific difference in ways in which they respond to leucine.

Locomotor activity was decreased in HFD-Leu mice compared with HFD-W mice. The cause for this change is unclear. However, this change was not due to toxicity from leucine supplementation. Leucine is among the most tolerated amino acids as no adverse effects of increased leucine intake (usually three times the daily requirement) were reported in various animal and human studies (16,41). In our study, leucine-supplemented mice consumed normal amounts of food and water and had a normal growth curve compared with controls. In fact, on the rodent chow diet group, leucine-supplemented mice consumed more food than control mice. Thus, the decreased locomotor activity does not appear to be related to food-seeking activity either.

Increasing dietary leucine intake significantly improves glucose metabolism in HFD-fed mice. It appears that better glycemic control is primarily due to the improved insulin sensitivity that is associated with decreased adiposity in HFD-Leu mice. However, the involvement of other mechanisms cannot be ruled out. In fact, multiple regression analyses suggest that leucine's effect on the HOMA-IR index was only partially accounted for by the reduced adiposity. Two important players in the regulation of hepatic glucose production, plasma glucagon concentrations and hepatic G6P expression levels, were both lower in HFD-Leu mice. Additionally, plasma concentrations of glycogenic amino acids, which provide substrates for hepatic gluconeogenesis in the postabsorptive state (42), were also lower in HFD-Leu mice. Taken together, these results suggest that hepatic glucose production may be decreased in HFD-Leu mice, which could contribute to the improved glycemic control.

Leucine has a biphasic effect on glucagon secretion in the perfused rat pancreas: stimulatory in low glucose states (3.3 mmol/l) but inhibitory in high glucose states (8.3 mmol/l) (43) and may suppress glucagon secretion indirectly by lowering concentrations of glucagon secretagogues such as glutamine, glycine, and alanine in HFD-Leu mice (44,45). Leucine may also affect glucose metabolism by stimulating insulin secretion (43–45). Although fasting insulin levels were lower in HFD-Leu mice, most likely secondary to the improved insulin sensitivity, it cannot be ruled out that leucine may transiently stimulate insulin secretion in HFD-Leu mice. A direct action of leucine in skeletal muscle and liver may involve mTOR-mediated feedback inhibition of insulin signaling to affect glucose disposal and glucose production, respectively (17–20). However, our data show that increasing dietary leucine intake resulted in a net improvement of whole-body insulin sensitivity and glycemic control, along with reduced adiposity in mice on a chronic HFD.

Leucine markedly reduced plasma total and LDL cholesterol levels, independent of weight reduction. Cholesterol-reducing effects of L-arginine and keto and amino acid supplementation in diabetic rats and in patients on low protein diets have also been reported by others (46,47). Further elucidation of leucine signaling, via mTOR and/or other potential mediators, will be required to uncover the relevant molecular mechanisms.

Finally, despite the remarkable effects of leucine observed in HFD-fed mice, increased leucine intake had no notable effects on chow-fed mice, except that leucine-supplemented mice had slightly increased food intake. Since the increased food intake in these mice was not associated with increases in body weight, adiposity, or blood glucose levels, we speculate that this behavioral change was only secondary to a leucine-induced increase in basal metabolic rates, as a homeostatic mechanism to prevent downward excursion from the "normal" body weight.

In summary, we have shown that doubling dietary leucine intake on a chronic basis produces a net health benefit that includes reduction of diet-induced weight gain, hyperglycemia, and hypercholesterolemia in mice on a HFD. The reduction in adiposity results from a leucine-induced increase in resting energy expenditure and is associated with increased UCP3 protein expression in skeletal muscle and adipose tissues. The overall beneficial effects on lipid and glucose metabolism produced by leucine supplementation appear to involve multiple mechanisms that need to be further defined.

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