Original Research *Feature article*

Dietary supplementation with L-leucine reduces nitric oxide synthesis by endothelial cells of rats

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Impact statement

Obesity is associated with elevated concentrations of L-leucine in plasma and metabolic syndrome. L-Leucine may increase whole-body energy expenditure and reduce obesity by activating the mechanistic target of rapamycin cell signaling and stimulating the development of brown adipose tissue (BAT). Results of this study indicate that L-leucine supplementation to diet-induced obese rats decreased nitric oxide (NO) synthesis in endothelial cells and whole-body insulin sensitivity; had no effect on adiposity, BAT mass or substrate oxidation, skeletal muscle mass, or whole-body energy expenditure; and did not exacerbate adiposity, insulin resistance, plasma glucose and lipid concentrations, or hepatic and intramuscular lipid concentrations. Dietary L-leucine supplementation is not likely to be an effective means to alleviate metabolic syndrome in obese individuals. Rather, attenuating increases in concentrations of L-leucine in plasma may be beneficial for enhancing NO production by the vasculature, thereby reducing the risk of health problems (including cardiovascular disorders) in obese mammals (including humans).

Abstract

This study tested the hypothesis that elevated L-leucine concentrations in plasma reduce nitric oxide (NO) synthesis by endothelial cells (ECs) and affect adiposity in obese rats. Beginning at four weeks of age, male Sprague-Dawley rats were fed a casein-based low-fat (LF) or high-fat (HF) diet for 15 weeks. Thereafter, rats in the LF and HF groups were assigned randomly into one of two subgroups ($n = 8$/subgroup) and received drinking water containing either 1.02% L-alanine (isonitrogenous control) or 1.5% L-leucine for 12 weeks. The energy expenditure of the rats was determined at weeks 0, 6, and 11 of the supplementation period. At the end of the study, an oral glucose tolerance test was performed on all the rats immediately before being euthanized for the collection of tissues. HF feeding reduced ($P < 0.001$) NO synthesis in ECs by 21% and whole-body insulin sensitivity by 19% but increased ($P < 0.001$) glutamine:fructose-6-phosphate transaminase (GFAT) activity in ECs by 42%. Oral administration of L-leucine decreased ($P < 0.05$) NO synthesis in ECs by 14%, increased ($P < 0.05$) GFAT activity in ECs by 35%, and reduced ($P < 0.05$) whole-body insulin sensitivity by 14% in rats fed the LF diet but had no effect ($P > 0.05$) on these variables in rats fed the HF diet. L-Leucine supplementation did not affect ($P > 0.05$) weight gain, tissue masses (including white adipose tissue, brown adipose tissue, and skeletal muscle), or antioxidative capacity (indicated by ratios of glutathione/glutathione disulfide) in LF- or HF-fed rats and did not worsen ($P > 0.05$) adiposity, whole-body insulin sensitivity, or metabolic profiles in the plasma of obese rats. These results indicate that high concentrations of L-leucine promote glucosamine synthesis and impair NO production by ECs, possibly contributing to an increased risk of cardiovascular disease in diet-induced obese rats.

Keywords: Amino acids, energy expenditure, leucine, nitric oxide, obesity, vascular function

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Introduction

Obesity is a risk factor for endothelial dysfunction and coronary artery disease.[1,2] Compared with non-obese controls, concentrations of L-leucine (Leu, a branched-chain amino acid [AA]) in the plasma of obese humans are increased, for example, by 23%[3] to 29%,[4] due primarily to increased proteolysis[5,6] and impaired catabolism of Leu[7,8] in the body. The elevated concentrations of Leu in plasma may merely serve as a biomarker for insulin resistance in obesity, and it is unknown whether Leu can adversely affect vascular metabolism or health.[9–11] There were reports that oral administration of Leu (e.g. 1.5% in drinking water) reduced white adipose tissue (WAT) and improved insulin sensitivity in

diet-induced obese (DIO) mice[12,13] and rats.[14] Similarly, adding 1.5% Leu to drinking water reduced subcutaneous (SC) and visceral fat mass as well as body weight (BW) and prevented mitochondrial dysfunction and metabolic disorders in DIO mice.[15] In contrast, dietary supplementation with 3.2% Leu for six weeks increased epididymal (EP) fat mass, while decreasing muscle mass in male rats fed a high-fat (HF) diet.[16] Ceglarek *et al*.[17] found that supplementation with 1.5% Leu in drinking water for 4 months did not prevent the development of obesity or metabolic abnormalities in rats receiving SC injections of monosodium glutamate during the neonatal period. Interestingly, oral administration of Leu at the same dose for 6 weeks exacerbated the adiposity of DIO rats.[18] Reasons for these discrepancies are not known.

Nitric oxide (NO), synthesized from L-arginine, is the major endothelium-derived relaxing factor with an important role in regulating blood flow[19,20] and nutrient metabolism.[21] Thus, either the pharmacological inhibition or the deletion of endothelial NO synthase resulted in vasoconstriction, hypertension, adiposity, and endothelial dysfunction in animal models.[21–23] Conversely, dietary supplementation with L-arginine reduced the concentrations of Leu in plasma and adiposity in DIO rats.[24] In addition, oral administration of α-ketoglutarate to DIO rats reduced circulating levels of Leu by stimulating its catabolism in the small intestine and other tissues, while enhancing the synthesis of NO by endothelial cells (ECs) and whole-body insulin sensitivity.[25] At present, it is unknown whether dietary supplementation with Leu can influence endothelial NO production and adiposity in DIO animals. This question was addressed in the present study.

Materials and methods

This study was reviewed and approved by the Texas A&M University Animal Use and Care Committee. All animal handling and care followed the Guide for the Care and Use of Laboratory Animals established by Public Health Service of the U.S. Department of Health and Human Services.

Chemicals

L-Alanine (Ala) and Leu, enzymes required for the assays of metabolites, as well as columns and reagents for AA analyses by high-performance liquid chromatography (HPLC) were obtained from Sigma Chemicals (St. Louis, MO, USA). HPLC-grade methanol and water were procured from Fisher Scientific (Houston, TX, USA). Collagenase used for the isolation of ECs and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Roche (Indianapolis, IN, USA) and Invitrogen (Grand Island, NY, USA), respectively.

Animals and diets

Forty-four 23-day-old male Sprague-Dawley rats were obtained from Charles River Laboratories and housed individually in a temperature- and humidity-controlled facility with a 12-h light (7:00 AM to 7:00 PM):12-h dark (7:00 PM to 7:00 AM) cycle and fed a regular non-purified diet (Harlan Teklad catalog no. 8604). At 28 days of age, rats were assigned randomly to receive either a low-fat (LF) diet ($n = 22$ rats; 84.1 ± 1.0 g BW, mean ± standard error of the mean [SEM]) or a HF diet ($n = 22$ rats; 83.9 ± 1.1 g BW, mean ± SEM). The casein-based LF and HF diets (Table 1) were obtained from Research Diets (New Brunswick, NJ, USA). The LF diet contained 4.3% fat and provided 10% of total energy as lipids, whereas the HF diet contained 23.6% fat and provided 40% of its total energy as lipids. Because rats fed the LF diet consumed 23% more food than rats fed the HF diet,[24] the HF diet was formulated to contain 23% more energy, protein, minerals, and vitamins than the LF diet, so that intakes of energy and all nutrients would not differ between the LF and HF groups. Rats had free access to drinking water (distilled and deionized water) and their respective LF or HF diet for 15 weeks.

Following 15 weeks of feeding the LF or HF diet between 4 and 19 weeks of age, six rats from each diet group were euthanized to obtain tissues, and the remaining rats in the LF or HF group were divided randomly into two subgroups, which continued to be fed their same respective diets and received drinking water (distilled and deionized water) containing either 1.02% Ala (isonitrogenous control) or 1.5% Leu. The concentration of Leu in the drinking water for rats was based on that for obese mice.[26] The drinking water was provided daily. The rats continued to consume their respective LF or HF diet between 19 and 31 weeks of age for 12 weeks. Water consumption was recorded daily, whereas the BW and food intake of the rats were measured weekly. We used Ala as the isonitrogenous control based on our previous work on determining the effect of dietary arginine supplementation to diet-induced or spontaneously obese rats.[21,24] In all these studies, Ala had no effect on the adiposity or plasma metabolic profiles of the rats. Results of our separate experiment indicated that adding 1.02% Ala to drinking water for LF- or HF-fed rats between 19 and 31 weeks of age did not affect body, WAT, or skeletal muscle weights; plasma metabolic profiles; endothelial NO synthesis; or endothelial glutamine:fructose-6-phosphate transaminase (GFAT) activity, compared with the absence of Ala from drinking water (Supplemental Table 1).

At 19, 25, and 30 weeks of age (i.e. weeks 0, 6, and 11 of the Leu or Ala supplementation period), O_2 consumption, CO_2 production, and heat production by the rats were measured as described by Tekwe *et al*.[25,27] Specifically, rats were placed in their assigned metabolic cages (1 rat/cage) for four hours each day for three days to acclimatize them to the cages. On the day of measurement, individual rats were placed in their metabolic cages for one hour before any measurement was taken. Energy expenditure of individual rats was measured by placing them, between 9:00 AM and 11:00 AM, in a computer-controlled Oxymas Comprehensive Lab Animal Monitoring System (an open-circuit calorimeter; Columbus Instruments, Columbus, Ohio, USA). Gas analyzers were calibrated using a standard gas mixture containing known concentrations of CO_2 (0.50%), O_2 (20.5%), and N_2 (79%). One rat from each treatment group was analyzed simultaneously for variables of energy expenditure, namely O_2 consumption (L/h/kg BW), CO_2 production (L/h/kg BW), respiratory quotient (RQ), and heat production (kcal/kg BW/h).

Table 1. Composition of low-fat (LF) and high-fat (HF) diets for rats.

Ingredient	LF diet[1]		HF diet[2]	
	Composition, %	kJ/kg diet[5]	Composition, %	kJ/kg diet[5]
Casein (88.1% protein)	18.96	3173	23.31	3901
L-Cystine	0.284	48	0.350	59
Corn starch	29.85	4996	8.48	1419
Maltodextrin-10	3.32	556	11.65	1950
Sucrose	33.17	5552	20.14	3371
Cellulose	4.74	0	5.83	0
Soybean oil	2.37	892	2.91	1096
Lard	1.90	715	20.68	7788
Mineral mix S10026[3]	0.95	63	1.17	78
Dicalcium phosphate	1.23	0	1.51	0
Calcium carbonate	0.521	0	0.641	0
Potassium citrate	1.56	0	1.92	0
Vitamin mix V10001[4]	0.95	159	1.17	196
Choline bitartrate	0.19	0	0.233	0
Yellow dye	0.005	0	0	0
Red dye	0	0	0.006	0
Total	100	16155	100	19858

Ala: L-alanine; Leu: L-leucine; Arg: arginine; Asn, asparagine; Asp: aspartate; Cys: cysteine; Gln: glutamine; Glu, glutamate; Gly: glycine; His: histidine; Ile, isoleucine; Lys: lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine. Except for glycine, all amino acids are L-isomers.
[1]Containing 67.3% carbohydrate, 4.3% fat, and 17.0% protein on an as-fed basis. Based on our analysis (Dai *et al.* 2014) and calculations according to the molecular weights of intact amino acids, this diet contained the following (g/kg diet, as-fed basis): Ala, 4.34; Arg, 6.01; Asn, 4.33; Asp, 6.55; Cys, 3.56; Gln, 18.7; Glu, 15.7; Gly, 3.02; His, 4.38; 4-hydroxyproline, 0.23; Ile, 8.02; Leu, 14.7; Lys, 12.4; Met, 4.38; Phe, 8.37; Pro, 18.4; Ser, 8.97; Thr, 6.85; Trp, 2.07; Tyr, 8.79; and Val, 9.52.
[2]Containing 41.0% carbohydrate, 23.6% fat, and 20.9% protein on an as-fed basis. Based on our analysis (Dai *et al.* 2014) and calculations according to the molecular weights of intact amino acids, this diet contained the following (g/kg diet, as-fed basis): Ala, 5.34; Arg, 7.39; Asn, 5.32; Asp, 8.05; Cys, 4.38; Gln, 23.0; Glu, 19.3; Gly, 3.72; His, 5.38; 4-hydroxyproline, 0.29; Ile, 9.86; Leu, 18.1; Lys, 15.3; Met, 5.38; Phe, 10.3; Pro, 22.6; Ser, 11.0; Thr, 8.42; Trp, 2.55; Tyr, 10.8; and Val, 11.7.
[3]Containing the following (g/kg mineral mix): magnesium oxide, 41.9; magnesium sulfate.7H_2O, 257.6; sodium chloride, 259; chromium KSO_4.12H_2O, 1.925; cupric carbonate, 1.05; potassium iodate, 0.035; ferric citrate, 21; manganous carbonate, 12.25; sodium selenite, 0.035; zinc carbonate, 5.6; sodium fluoride, 0.20; ammonium molybdate.4H_2O, 0.30; sucrose, 399.105. Sucrose in the mineral mix provided 63 kJ energy/kg diet.
[4]Containing the following (g/kg vitamin mix): retinyl palmitate, 0.80; cholecalciferol, 1.0; all-*rac*-α-tocopheryl acetate, 10; menadione sodium bisulfite, 0.08; biotin (1.0%), 2.0; cyanocobalamin (0.1%), 1.0; folic acid, 0.20; nicotinic acid, 3.0; calcium pantothenate, 1.6; pyridoxine-HCl, 0.70; riboflavin, 0.60; thiamin-HCl, 0.60; and sucrose, 978.42. Sucrose in the vitamin mix provided 159 kJ energy/kg diet.
[5]Expressed on an as-fed basis.

During the last week (week 12) of the supplementation period, an oral glucose tolerance test was performed on all the rats, as we described previously.[24,25] Specifically, after a five-hour period of food deprivation, glucose (2 g/kg BW) in 5-mL water was administrated orally into stomach by gavage, and 20 µL of blood was obtained from the tail vein of conscious rats at 0, 30, 60, 90, 120, and 180 min after gavage (using plain microhematocrit capillary tubes). The blood samples were immediately centrifuged at 10,000 g for one minute to provide sera for glucose analysis.[25] The insulin sensitivity index is defined as the area of serum glucose concentrations under the response curve (h mmol glucose/L).[24] Due to insufficient volumes of the serum samples, insulin assays were not performed in conjunction with the glucose intolerance test.

Following the 12-week supplementation, after a five-hour period of food deprivation, rectal temperatures were obtained from conscious rats, rats were euthanized with CO_2, and cardiac blood samples were then withdrawn into heparinized tubes.[27] The following tissues were obtained from the euthanized rats: skeletal muscle, WAT (EP, mesenteric [MT], retroperitoneal [RP], and SC), brown adipose tissue (BAT), heart, kidney, brain, testes, lungs, kidneys, spleen, and digestive tract. The cardiac blood samples were centrifuged at 600 $\times g$ and 4 °C for 10 min to obtain plasma for analyses of metabolites (including AAs, glucose, and lipids) and insulin. The hearts were used for the isolation of coronary ECs (see the section on NO synthesis in ECs).

NO synthesis in ECs

Coronary ECs were isolated to determine NO synthesis as we described previously.[28,29] The endothelial identity of these cells was confirmed by positive staining for factor VIII–related antigen and the uptake of modified low-density lipoprotein.[28] The freshly isolated ECs (1.5×10^6) were rinsed three times with 1 mL of DMEM containing 5-mM D-glucose, antibiotics (100,000 U/L penicillin, 100 mg/L streptomycin, and 0.25 mg/L amphotericin), 0.2-mM L-arginine, 0.2-mM Leu, 0.5-mM L-glutamine, and other AAs at concentrations in the plasma of normal rats.[24] Thereafter, cells were incubated in this medium at 37 °C for six hours. At the end of the six-hour incubation period, samples of the medium were analyzed for nitrite plus nitrate (an indicator of NO production). The medium, incubated without cells, was used as the blank. Due to a limited number of ECs, the expression of endothelial NO synthase and its cofactors was not determined.

Measurements of oxidation of glucose, leucine, glutamine, and oleic acid in BAT

BAT was used to determine the oxidation of glucose, leucine, glutamine, and oleic acid, as we described previously.[27] BAT was cut into small pieces (~10 mg per piece and ~1 mm in thickness). The tissue slices (~60 mg) were rinsed with oxygenated (95% O_2/5% CO_2) Krebs bicarbonate buffer (pH 7.4) and then incubated at 37 °C for two hours in 2 mL of

Table 2. Intakes of energy and nutrients by rats during the 12-week period of consuming a low-fat or high-fat diet and water containing either 1.5% Leu or 1.02% L-alanine.[1,2]

Intake	Low-fat diet		High-fat diet		*P* value		
	Control	Leucine	Control	Leucine	Diet	Leucine	Diet × Leucine
Water, mL/kg BW/day	69.7 ± 2.5	72.4 ± 3.1	66.4 ± 2.6	68.2 ± 3.0	0.187	0.434	0.506
Food, g/kg BW/day	38.3 ± 0.4[a]	38.0 ± 0.7[a]	32.0 ± 0.6[b]	31.8 ± 0.8[b]	<0.001	0.669	<0.001
Energy, kJ/kg BW/day[3]	632 ± 7.6	644 ± 12	648 ± 13	660 ± 16	0.191	0.605	0.588
Protein, g/kg BW/day	6.52 ± 0.08	6.47 ± 0.12	6.69 ± 0.13	6.64 ± 0.16	0.169	0.706	0.578
Digestible Ala from food, mg/kg BW/day[4]	169 ± 1.9	167 ± 3.1	173 ± 3.4	171 ± 4.1	0.202	0.705	0.633
Ala from drinking water, mg/kg BW/day	711 ± 25	0.0	677 ± 27	0.0	0.384	NA	0.384
Digestible Leu from food, mg/kg BW/day[4]	570 ± 6.6	566 ± 11	584 ± 11	580 ± 14	0.206	0.704	0.638
Leu from drinking water, mg/kg BW/day	0.0	1,085 ± 47	0.0	1,023 ± 45	0.350	NA	0.350
Digestible Ile from food, mg/kg BW/day[4]	311 ± 3.6	309 ± 5.8	318 ± 6.2	316 ± 7.6	0.205	0.704	0.638
Digestible Val from food, mg/kg BW/day[4]	369 ± 4.3	367 ± 6.9	378 ± 7.4	376 ± 9.0	0.205	0.704	0.638
Minerals,[5] mg/kg BW/day	219 ± 2.5	217 ± 4.1	225 ± 4.4	224 ± 5.4	0.128	0.708	0.496
Vitamins,[6] mg/kg BW/day	8.02 ± 0.09	7.95 ± 0.15	8.24 ± 0.16	8.18 ± 0.20	0.144	0.707	0.530

Ala: L-alanine; BW: body weight; Ile, isoleucine; Leu: L-leucine; NA: not applicable; SEM: standard error of the mean; Val: valine.
[1]Beginning at 4 weeks of age, male Sprague-Dawley rats were fed a low-fat or high-fat diet for 15 weeks. Between 19 and 31 weeks of age, lean or obese rats were fed their respective diets and received drinking water containing either 1.02% Ala (isonitrogenous control) or 1.5% Leu.
[2]Values are means ± SEM, $n = 8$.
[3]From diet plus drinking water.
[4]Calculated on the basis of the true digestibility of casein in adult rats (96%).
[5]Provided from mineral mix S10026 in the diet.
[6]Provided from the vitamin mix V10001 in the diet.
[a,b]Within a row, means not sharing the same superscript letter differ ($P < 0.05$).

oxygenated (95% O_2/5% CO_2) Krebs bicarbonate buffer (pH 7.4) containing 2.5 ng/mL insulin, 5-mM D-glucose, 0.5-mM L-glutamine, 0.5-mM Leu, and 1-mM oleic acid. The medium also contained 0.1 μCi D-[U-^{14}C]glucose, L-[U-^{14}C]glutamine, L-[1-^{14}C]leucine, L-[U-^{14}C]leucine, or [1-^{14}C]oleic acid for the determination of oxidation of each of these substances. L-[1-^{14}C]Leucine was used to determine the oxidative decarboxylation of Leu and the release of α-ketoisocaproate (KIC) by BAT, whereas L-[U-^{14}C]leucine was used to determine the oxidation of all carbons of Leu.[30] The concentrations of the substrates were similar to those in the plasma of DIO rats.[24] In all experiments, the medium incubated in duplicate without tissues was included as the blank. At the end of the two-hour incubation period, 0.2 mL of 1.5 M $HClO_4$ was added through a rubber stopper into the medium, and $^{14}CO_2$ was collected in 0.2 mL Soluene-350 (Perkin Elmer, Shelton, CT, USA) for the determination of its radioactivity using a liquid scintillation counter.[31] The specific activity of each tracer in the incubation medium was used to calculate the rate of production of CO_2 from its corresponding tracee.[27,32]

Biochemical measurements

The plasma of blood taken from the heart was analyzed for AAs, insulin, glucose, triacylglycerols, cholesterol, free fatty acids, ammonia (NH_3 plus NH_4^+), and urea, as we described previously.[24,25,27] The enzymatic activity of GFAT in ECs was determined as the formation of glucosamine-6-phosphate from glutamine and fructose-6-phosphate, as we described previously.[33] Concentrations of the reduced form of glutathione (GSH) and glutathione disulfide (oxidized glutathione; GSSG) in tissues were determined using our established HPLC method.[34] Concentrations of protein and lipids in the liver and gastrocnemius muscle were analyzed by the Lowry *et al.*[35] and Folch *et al.*[36] methods, respectively.

Statistical analysis

Data are expressed as means ± SEM. All data, except for concentrations of AAs in plasma, were normally distributed as assessed by the Shapiro-Wilk test.[37] Thus, the data on concentrations of AAs in plasma were log-transformed prior to the statistical analysis. Two-way analysis of variance was used to determine the effects of HF feeding and Leu supplementation on all the measured variables. Differences among treatment means were assessed using the Student-Newman-Keuls multiple comparison test. The data on the BW, tissue weights, and metabolic profiles of LF- and HF-fed rats at 19 weeks of age were analyzed by the unpaired *t*-test. Probability values ≤ 0.05 were taken to indicate statistical significance. All the statistical analyses were performed using the R Statistical Software (v4.1.2; R Core Team 2021; R Foundation for Statistical Computing, Vienna, Austria).

Results

Intakes of food, energy, protein, leucine, and water

Leu supplementation for 12 weeks between 19 and 31 weeks of age did not affect ($P > 0.05$) the food intake of rats fed either the LF or HF diet (Table 2). There were interaction effects of diet × Leu ($P < 0.001$) on food intake by the rats. Intakes of energy, protein, minerals, and vitamins from the enteral diet did not differ ($P > 0.05$) between the LF and HF groups of rats. Consumption of water did not differ ($P > 0.05$) among the four groups of rats. The total intake of digestible Leu (from the enteral diet plus drinking water) by rats

Table 3. Effects of high-fat feeding and oral administration of leucine on water consumption, food intake, and BW of rats.[1,2]

Variable	Low-fat diet		High-fat diet		*P* value		
	Control	Leucine	Control	Leucine	Diet	Leucine	Diet × Leucine
Initial BW[3], g	627 ± 13	620 ± 26	676 ± 28	672 ± 36	0.072	0.848	0.960
Final BW[4], g	715 ± 17	710 ± 30	793 ± 33	785 ± 40	0.020	0.833	0.978
BW gain[5], g/12 weeks	88.4 ± 5.6[a]	89.3 ± 6.3[a]	117 ± 7.1[b]	113 ± 7.5[b]	0.001	0.838	0.739
Rectal temperature, °F	97.5 ± 0.06	97.5 ± 0.05	97.4 ± 0.08	97.4 ± 0.07	0.181	0.449	0.502

BW: body weight; SEM: standard error of the mean.
[1]Beginning at four weeks of age, male Sprague-Dawley rats were fed a low-fat or high-fat diet for 15 weeks. Between 19 and 31 weeks of age, lean or obese rats were fed their respective diets and received drinking water containing either 1.02% L-alanine (isonitrogenous control) or 1.5% L-leucine.
[2]Values are means ± SEM, $n = 8$.
[3]The age of rats was 19 weeks.
[4]The age of rats was 31 weeks.
[5]Between 19 and 31 weeks of age.
[a,b]Within a row, means not sharing the same superscript letter differ ($P < 0.05$).

receiving Leu supplementation was 182% greater ($P < 0.001$) than that for rats fed the isonitrogenous (Ala) control diets.

Body and tissue weights and rectal temperature

At the end of a 15-week period of LF or HF feeding between 4 and 19 weeks of age, the BW of the 19-week-old rats fed the LF and HF diets were 623 ± 11 and 674 ± 17 g (means ± SEM, $n = 22$; $P = 0.016$), respectively. The absolute and relative (% of BW) weights of the major white-fat depots (i.e. RP, EP, SC, and SC fats) were 48% and 37% greater, respectively, in the 19-week-old rats fed the HF diet, compared with the 19-week-old rats fed the LF diet (Supplemental Table 2).

During the 12-week period of Leu or Ala supplementation (i.e. between 19 and 31 weeks of age), rats fed the HF diet gained 29% more ($P < 0.01$) BW than rats fed the LF diet, but Leu supplementation did not affect ($P > 0.05$) the BW gain of rats fed either the LF or HF diet (Table 3). Neither HF feeding nor Leu supplementation affected ($P > 0.05$) the rectal temperature of rats. Compared with the LF group, HF feeding increased the weights of the heart (+20%), lungs (+9%), RP fat (65%), EP fat (52%), and SC fat (50%) ($P < 0.001$), as well as kidneys (+8%), MT fat (18%), extensor digitorum longus muscle (+3%), and soleus muscle (11%) ($P < 0.05$), but had no effect ($P > 0.05$) on the weights of liver, spleen, BAT, small and large intestines, pancreas, testes, brain, and stomach (Table 4). Leu supplementation had no effect ($P > 0.05$) on the weights of the measured tissues (Table 4). However, there were interaction effects of diet × Leu on the weights of the heart ($P = 0.004$), lungs ($P = 0.008$), kidneys ($P = 0.031$), RP fat ($P < 0.001$), EP fat ($P < 0.001$), SC fat ($P < 0.001$), and MT fat ($P = 0.038$) (Table 4).

Concentrations of glucose in plasma at the end of the 12-week supplementation period between 19 and 31 weeks of age were 15% greater ($P < 0.001$) for HF-fed rates than for LF-fed rats, but did not differ ($P > 0.05$) between Leu- and Ala-supplemented rats (Table 5). Concentrations of lipids in liver (+25%) and skeletal muscle (+26%) were greater ($P < 0.001$) in HF-fed rats than in LF-fed rats, but concentrations of protein in those two tissues did not differ ($P > 0.05$) between the two groups of rats (Table 5). Leu supplementation had no effect ($P > 0.05$) on concentrations of protein or lipids in liver and skeletal muscles (Table 5). There were interaction effects of diet × Leu on concentrations of lipids in the liver ($P < 0.05$) and skeletal muscles ($P < 0.001$), but a diet × Leu interaction was not detected ($P > 0.05$) for intramuscular concentrations of protein (Table 5).

Concentrations of AAs in plasma

Data on the concentrations of AAs in the plasma of rats at the end of the 12-week period of Leu or Ala supplementation between 19 and 31 weeks of age are summarized in Table 6. Compared with the LF group, HF feeding increased ($P < 0.001$) concentrations of the following AAs in plasma: β-alanine (+18%; $P < 0.001$), asparagine (+12%; $P = 0.010$), glutamine (+14%; $P < 0.001$), glycine (+17%; $P < 0.001$), isoleucine (+18%; $P < 0.001$), Leu (+9%; $P < 0.001$), phenylalanine (+16%; $P < 0.001$), threonine (+18%; $P < 0.001$), tryptophan (+16%; $P < 0.001$), tyrosine (+19%; $P < 0.001$), and valine (+17%; $P < 0.001$), while reducing concentrations of serine (–8%) in plasma (Table 6). In control rats without Leu supplementation, HF feeding increased ($P < 0.001$) the concentrations of Leu, isoleucine, and valine in plasma by 19%, 16%, and 18%, respectively. The concentrations of other AAs in plasma did not differ ($P > 0.05$) between the LF and HF groups.

Compared with the Ala group, Leu supplementation increased concentrations of arginine (+ 19%) and Leu (+ 69%) in plasma but reduced ($P = 0.003$) concentrations of glycine (–13%) in plasma (Table 6). Concentrations of Ala in the plasma of control rats were 58% greater ($P < 0.001$) than those for Leu-supplemented rats. There were interaction effects of diet × Leu on concentrations of glutamine ($P < 0.001$), glycine ($P < 0.001$), arginine ($P < 0.001$), β-Alanine ($P < 0.001$), Ala ($P < 0.001$), tyrosine ($P = 0.007$), tryptophan ($P = 0.001$), valine ($P < 0.001$), phenylalanine ($P = 0.001$), isoleucine ($P < 0.001$), and Leu ($P < 0.001$) in plasma.

NO synthesis and GFAT activity in ECs, whole-body insulin sensitivity, and concentrations of metabolites in plasma and tissues

These data for rats at the end of the 12-week period of Leu or Ala supplementation between 19 and 31 weeks of age are summarized in Table 5. Compared with the LF group, HF feeding reduced ($P < 0.001$) NO synthesis in ECs by 21%

Table 4. Effects of high-fat feeding and oral administration of leucine on tissue weights of rats at 31 weeks of age.[1,2]

Tissue	Low-fat diet		High-fat diet		*P* value		
	Control	Leucine	Control	Leucine	Diet	Leucine	Diet × Leucine
Heart	1.82 ± 0.04[b]	1.89 ± 0.06[b]	2.19 ± 0.08[a]	2.27 ± 0.15[a]	<0.001	0.508	0.004
Lungs	2.04 ± 0.04[b]	2.01 ± 0.05[b]	2.16 ± 0.06[a]	2.25 ± 0.06[a]	<0.001	0.558	0.008
Liver	29.6 ± 1.2	29.4 ± 1.3	29.0 ± 0.8	28.8 ± 1.4	0.595	0.885	0.961
Spleen	0.91 ± 0.02	0.92 ± 0.04	0.93 ± 0.04	0.91 ± 0.04	0.924	0.976	0.976
Kidneys	2.17 ± 0.06[b]	2.20 ± 0.05[b]	2.28 ± 0.08[a,b]	2.43 ± 0.05[a]	0.013	0.206	0.031
RP fat	35.6 ± 1.2[b]	36.4 ± 1.1[b]	63.5 ± 1.9[a]	55.0 ± 5.1[a]	<0.001	0.443	<0.001
EP fat	19.1 ± 1.1[b]	18.2 ± 0.8[b]	29.8 ± 0.5[a]	26.8 ± 2.1[a]	<0.001	0.356	<0.001
SC fat	17.8 ± 0.9[b]	18.9 ± 0.5[b]	29.5 ± 0.7[a]	25.7 ± 2.2[a]	<0.001	0.528	<0.001
MT fat	21.4 ± 0.8[b]	21.0 ± 0.6[b]	26.6 ± 1.6[a]	23.5 ± 2.2[a]	0.012	0.276	0.038
Fat depots	93.9 ± 3.4[b]	94.5 ± 1.8[b]	149.4 ± 3.1[a]	130.9 ± 9.6[a]	<0.001	0.381	<0.001
Brown fat	1.06 ± 0.06	1.17 ± 0.07	1.13 ± 0.06	1.08 ± 0.07	0.915	0.599	0.629
Small intestine	7.08 ± 0.33	6.52 ± 0.23	6.93 ± 0.35	6.52 ± 0.26	0.806	0.104	0.443
Pancreas	1.40 ± 0.07	1.32 ± 0.06	1.42 ± 0.06	1.32 ± 0.05	0.891	0.113	0.482
EDL muscle	0.238 ± 0.002	0.242 ± 0.007	0.253 ± 0.008	0.243 ± 0.011	0.028	0.361	0.121
Soleus muscle	0.211 ± 0.003[c]	0.221 ± 0.006[b,c]	0.237 ± 0.011[a,b]	0.241 ± 0.005[a]	0.008	0.398	0.050
Testes	3.94 ± 0.11	3.67 ± 0.16	3.85 ± 0.12	3.83 ± 0.07	0.769	0.250	0.481
Brain	2.09 ± 0.05	1.91 ± 0.14	2.04 ± 0.06	2.02 ± 0.08	0.745	0.244	0.533
Large intestine	2.60 ± 0.11	2.46 ± 0.10	2.40 ± 0.12	2.51 ± 0.09	0.498	0.852	0.595
Stomach	2.31 ± 0.08	2.24 ± 0.08	2.28 ± 0.15	2.13 ± 0.09	0.483	0.280	0.623

EDL: extensor digitorum longus; EP: epididymal; MT: mesenteric; RP: retroperitoneal; SC: subcutaneous; SEM: standard error of the mean.
[1]Beginning at four weeks of age, male Sprague-Dawley rats were fed a low-fat or high-fat diet for 15 weeks. Between 19 and 31 weeks of age, lean or obese rats were fed their respective diets and received drinking water containing either 1.02% L-alanine (isonitrogenous control) or 1.5% L-leucine. At 31 weeks of age (i.e. week 12 of the supplementation period), rats were euthanized to obtain tissues for analyses.
[2]Values, expressed as g, are means ± SEM, $n = 8$.
[a,b]Means in a row without common superscript letters differ, $P < 0.05$.

Table 5. Effects of high-fat feeding and oral administration of leucine to rats on NO synthesis and GFAT activity in ECs, whole-body insulin sensitivity, and metabolites in plasma and tissues.[1,2]

Variable	Low-fat diet		High-fat diet		*P* value		
	Control	Leucine	Control	Leucine	Diet	Leucine	Diet × Leucine
NO synthesis by ECs[3]	212 ± 8.0[a]	183 ± 7.3[b]	159 ± 6.1[c]	152 ± 5.4[c]	<0.001	0.093	<0.001
GFAT activity in ECs[4]	109 ± 5.2[c]	147 ± 6.5[b]	178 ± 8.3[a]	186 ± 9.4[a]	<0.001	0.079	<0.001
Whole-body ISI[5]	1083 ± 31[c]	1256 ± 38[b]	1419 ± 44[a]	1460 ± 47[a]	<0.001	0.104	<0.001
Insulin and metabolites in plasma							
Insulin, pM	121 ± 6.8	133 ± 7.4	136 ± 8.2	139 ± 9.4	0.201	0.343	0.427
Glucose, mM	8.13 ± 0.06[b]	8.27 ± 0.08[b]	9.48 ± 0.12[a]	9.41 ± 0.13[a]	<0.001	0.891	<0.001
Triacylglycerols, mM	0.90 ± 0.04	0.92 ± 0.05	0.98 ± 0.06	0.95 ± 0.05	0.303	0.990	0.752
Cholesterol, mM	4.60 ± 0.15[b]	4.73 ± 0.17[b]	6.25 ± 0.19[a]	6.12 ± 0.32[a]	<0.001	0.994	<0.001
Free fatty acids, mM	0.82 ± 0.04	0.84 ± 0.03	0.87 ± 0.05	0.81 ± 0.06	0.929	0.673	0.836
Ammonia, μM	101 ± 1.7	102 ± 3.3	105 ± 5.4	109 ± 6.1	0.212	0.559	0.588
Urea, mM	2.68 ± 0.14	2.77 ± 0.11	2.56 ± 0.13	2.83 ± 0.16	0.806	0.188	0.539
Protein and lipids in liver							
Protein, g/100 g	15.1 ± 0.34	15.3 ± 0.37	14.6 ± 0.40	14.8 ± 0.45	0.205	0.623	0.615
Lipids, g/100 g	3.76 ± 0.15[b]	3.88 ± 0.19[b]	4.70 ± 0.31[a]	4.84 ± 0.35[a]	0.001	0.676	0.012
Protein and lipids in gastrocnemius muscle							
Protein, g/100 g	18.1 ± 0.26	18.2 ± 0.24	17.6 ± 0.20	17.8 ± 0.29	0.078	0.577	0.343
Lipids, g/100 g	1.60 ± 0.04[b]	1.62 ± 0.03[b]	2.02 ± 0.06[a]	2.05 ± 0.07[a]	<0.001	0.770	<0.001

NO: nitric oxide; ECs: endothelial cells; GFAT: glutamine:fructose-6-phosphate transaminase; ISI: insulin sensitivity index; SEM: standard error of the mean.
[1]Beginning at four weeks of age, male Sprague-Dawley rats were fed a low-fat or high-fat diet for 15 weeks. Between 19 and 31 weeks of age, lean or obese rats were fed their respective diets and received drinking water containing either 1.02% L-alanine (isonitrogenous control) or 1.5% L-leucine. At 31 weeks of age (week 12 of the supplementation period), an oral glucose tolerance test was performed on all rats to assess whole-body insulin sensitivity.
[2]Values are means ± SEM, $n = 8$.
[3]pmol/10^6 cells/h.
[4]pmol/mg protein/min.
[5]Insulin sensitivity index. The greater the value, the lower the whole-body insulin sensitivity.
[a–c]Means in a row without a common superscript letter differ, $P < 0.05$.

Table 6. Effects of high-fat feeding and oral administration of leucine on amino acid (AA) concentrations in the plasma of rats.[1,2]

Plasma AA	Low-fat diet		High-fat diet		*P* value		
	Control	Leucine	Control	Leucine	Diet	Leucine	Diet × Leucine
Alanine	665 ± 16[b]	419 ± 13[d]	732 ± 18[a]	465 ± 15[c]	0.262	<0.001	<0.001
β-Alanine	11 ± 0.3[b]	11 ± 0.5[b]	13 ± 0.3[a]	13 ± 0.4[a]	<0.001	0.746	<0.001
Arginine	149 ± 3.1[b]	170 ± 3.4[a]	146 ± 3.3[b]	182 ± 4.1[a]	0.470	<0.001	<0.001
Asparagine	49 ± 2.6	52 ± 1.8	56 ± 1.6	57 ± 2.4	0.010	0.434	0.058
Aspartate	39 ± 1.6	40 ± 2.4	43 ± 2.9	41 ± 1.9	0.155	0.989	0.454
Citrulline	67 ± 1.8	64 ± 2.2	66 ± 2.5	65 ± 2.8	0.957	0.514	0.907
Cysteine[3]	144 ± 4.3	141 ± 4.8	136 ± 3.7	139 ± 4.6	0.258	0.876	0.625
Glutamate	96 ± 4.7	101 ± 8.0	93 ± 6.0	95 ± 5.7	0.435	0.551	0.807
Glutamine	577 ± 9.4[b]	607 ± 18[b]	669 ± 12[a]	681 ± 16[a]	<0.001	0.327	<0.001
Glycine	238 ± 5.4[b]	202 ± 9.9[c]	271 ± 8.1[a]	243 ± 6.2[b]	<0.001	0.003	<0.001
Histidine	72 ± 3.5	71 ± 2.2	73 ± 3.9	75 ± 5.1	0.509	0.835	0.899
Isoleucine	127 ± 3.2[b]	125 ± 3.2[b]	147 ± 2.8[a]	149 ± 3.3[a]	<0.001	0.986	<0.001
Leucine	205 ± 4.0[c]	373 ± 5.6[a]	243 ± 4.3[b]	386 ± 7.4[a]	<0.001	<0.001	<0.001
Lysine	234 ± 14	229 ± 8.2	226 ± 9.0	231 ± 13	0.791	0.960	0.965
Methionine	39 ± 1.4	38 ± 2.2	40 ± 1.8	39 ± 1.5	0.487	0.562	0.845
Ornithine	73 ± 2.7	75 ± 3.6	71 ± 3.6	72 ± 3.0	0.505	0.639	0.886
Phenylalanine	117 ± 4.0[b]	115 ± 3.2[b]	134 ± 4.4[a]	136 ± 4.9[a]	<0.001	0.968	0.001
Proline	278 ± 15	282 ± 12	292 ± 9.3	295 ± 13	0.273	0.774	0.743
Serine	210 ± 8.2	213 ± 9.7	184 ± 7.2	205 ± 6.0	0.046	0.168	0.068
Taurine	396 ± 18	390 ± 12	382 ± 16	398 ± 13	0.850	0.732	0.870
Threonine	220 ± 9.2[b]	231 ± 7.5[b]	264 ± 12[a]	267 ± 10[a]	<0.001	0.559	0.003
Tryptophan	74 ± 1.8[b]	72 ± 2.4[b]	86 ± 2.6[a]	84 ± 2.9[a]	<0.001	0.572	0.001
Tyrosine	106 ± 5.4[b]	103 ± 4.9[b]	125 ± 5.8[a]	123 ± 4.2[a]	<0.001	0.678	0.007
Valine	186 ± 4.9[b]	193 ± 6.8[b]	220 ± 6.3[a]	223 ± 7.1[a]	<0.001	0.552	<0.001

SEM: standard error of the mean.
[1]Beginning at four weeks of age, male Sprague-Dawley rats were fed a low-fat or high-fat diet for 15 weeks. Between 19 and 31 weeks of age, lean or obese rats were fed their respective diets and received drinking water containing either 1.02% L-alanine (isonitrogenous control) or 1.5% L-leucine. At 31 weeks of age (week 12 of the supplementation period), rats were euthanized, and plasma obtained for the analysis of AAs.
[2]Values for the concentrations of AAs in plasma (μM) are means ± SEM, $n = 8$.
[3]Total cysteine (1/2 cystine + cysteine).
[a–c]Means in a row without a common superscript letter differ, $P < 0.05$.

and whole-body insulin sensitivity by 19% but increased ($P < 0.001$) GFAT activity in ECs by 42%. In rats fed the LF diet, Leu supplementation decreased ($P < 0.05$) NO synthesis in ECs by 14%, increased ($P < 0.05$) GFAT activity in ECs by 35%, and reduced ($P < 0.05$) whole-body insulin sensitivity by 14%. By contrast, in rats fed the HF diet, Leu supplementation did not affect ($P > 0.05$) NO synthesis in ECs, GFAT activity in ECs, or whole-body insulin sensitivity. However, there were diet × Leu interactions for NO synthesis in ECs ($P < 0.001$), GFAT activity in ECs ($P < 0.001$), and whole-body insulin sensitivity ($P < 0.001$).

Compared with the LF group, HF feeding increased ($P < 0.001$) concentrations of glucose and cholesterol in plasma, as well as concentrations of lipids in liver and skeletal muscle, but had no effect ($P > 0.05$) on concentrations of insulin, triacylglycerols, free fatty acids, ammonia, or urea in plasma (Table 6). Leucine supplementation had no effect ($P > 0.05$) on any of these variables. There were diet × Leu interactions ($P < 0.001$) for concentrations of glucose and cholesterol in plasma, as well as concentrations of lipids in liver and skeletal muscle.

Concentrations of GSH and GSSG in tissues

Data on the effects of HF feeding and oral administration of Leu between 19 and 31 weeks of age on concentrations of GSH and GSSG in tissues are summarized in Table 7. Compared with the LF group, feeding the HF diet reduced ($P < 0.001$) concentrations of GSH in the heart (–8%), liver (–25%), RP-WAT (–26%), and skeletal muscles (–18%), while increasing ($P < 0.001$) concentrations of GSSG (+17 to +48%) and the ratio of GSSG/GSH (+50% to +100%) in these tissues. By contrast, Leu supplementation had no effect ($P > 0.05$) on any of these variables, as compared to the Ala group. There were diet × Leu interactions for concentrations of GSH ($P < 0.001$ for all tissues, except for $P = 0.013$ for GSH concentration in the heart) and GSSG ($P < 0.001$ for all tissues), as well as the ratio of GSSG/GSH ($P < 0.001$ for all tissues).

O_2 consumption, CO_2 production, RQ, and heat production

We assessed the impacts of HF diet, Leu, and the combined administration of diet and Leu on O_2 consumption, CO_2, and heat production by the rats at 19, 25, and 30 weeks of age, namely weeks 0, 6, and 11 of the supplementation period (Table 8). Rates of O_2 consumption, CO_2 production, RQ, or heat production at 19 weeks of age (i.e. week 0 of the supplementation period) did not differ ($P > 0.05$) among all the four groups of rats. Compared with the LF group, HF feeding reduced O_2 consumption (–7%; $P < 0.05$), CO_2 production

Table 7. Effects of high-fat feeding and oral administration of leucine on concentrations of GSH and GSSG in tissues of rats.[1,2]

Tissue	Low-fat diet		High-fat diet			*P* value	
	Control	Leucine	Control	Leucine	Diet	Leucine	Diet × Leucine
Heart							
GSH[3]	1708 ± 40[a]	1750 ± 37[a]	1568 ± 31[b]	1604 ± 34[b]	<0.001	0.454	0.013
GSSG[4]	121 ± 4.4[b]	118 ± 5.6[b]	162 ± 4.6[a]	168 ± 4.2[a]	<0.001	0.864	<0.001
GSSG/GSH[5]	0.071 ± 0.001[b]	0.068 ± 0.002[b]	0.103 ± 0.004[a]	0.106 ± 0.005[a]	<0.001	0.941	<0.001
Liver							
GSH[3]	6139 ± 178[a]	6212 ± 245[a]	4598 ± 135[b]	4722 ± 133[b]	<0.001	0.764	<0.001
GSSG[4]	484 ± 10[b]	473 ± 12[b]	563 ± 16[a]	552 ± 21[a]	<0.001	0.595	<0.001
GSSG/GSH[5]	0.079 ± 0.002[b]	0.077 ± 0.003[b]	0.123 ± 0.003[a]	0.117 ± 0.005[a]	<0.001	0.640	<0.001
RP-WAT							
GSH[3]	724 ± 18[a]	716 ± 13[a]	525 ± 15[b]	538 ± 17[b]	<0.001	0.941	<0.001
GSSG[4]	84.8 ± 2.7[b]	82.1 ± 2.0[b]	126 ± 3.8[a]	121 ± 4.8[a]	<0.001	0.651	<0.001
GSSG/GSH[5]	0.118 ± 0.005[b]	0.115 ± 0.002[b]	0.241 ± 0.008[a]	0.227 ± 0.011[a]	<0.001	0.707	<0.001
Skeletal muscle							
GSH[3]	722 ± 15[a]	708 ± 28[a]	581 ± 21[b]	593 ± 24[b]	<0.001	0.968	<0.001
GSSG[4]	54.3 ± 1.5[b]	53.0 ± 1.9[b]	66.3 ± 2.9[a]	64.8 ± 3.2[a]	<0.001	0.672	<0.001
GSSG/GSH[5]	0.075 ± 0.003[b]	0.076 ± 0.003[b]	0.115 ± 0.005[a]	0.112 ± 0.009[a]	<0.001	0.871	<0.001

GSH: glutathione; GSSG: glutathione disulfide; RT-WAT: retroperitoneal white adipose tissue; SEM: standard error of the mean.
[1]Beginning at four weeks of age, male Sprague-Dawley rats were fed a low-fat or high-fat diet for 15 weeks. Between 19 and 31 weeks of age, lean or obese rats were fed their respective diets and received drinking water containing either 1.02% L-alanine (isonitrogenous control) or 1.5% L-leucine. At 31 weeks of age (week 12 of the supplementation period), rats were euthanized to obtain the heart, liver, RT-WAT, and skeletal muscles (gastrocnemius muscle) for the analyses of GSH and GSSG.
[2]Values are means ± SEM, $n = 8$.
[3]Values are expressed as nmol/g of wet tissue.
[4]Values are expressed as nmol/g of wet tissue.
[5]Values are expressed as mol/mol.
[a,b]Means in a row without a common superscript letter differ, $P < 0.05$.

Table 8. Effects of high-fat feeding and oral administration of leucine on oxygen consumption, CO_2 production, and heat production by rats.[1,2]

Variable	Low-fat diet		High-fat diet			*P* value	
	Control	Leucine	Control	Leucine	Diet	Leucine	Diet × Leucine
19 weeks of age (week 0 of the supplementation period)							
Body weight, g	630 ± 13	623 ± 26	635 ± 24	627 ± 34	0.859	0.773	0.990
O_2 consumption[3]	1.32 ± 0.035	1.35 ± 0.032	1.30 ± 0.034	1.32 ± 0.037	0.487	0.442	0.784
CO_2 production[3]	1.15 ± 0.031	1.17 ± 0.029	1.10 ± 0.032	1.12 ± 0.034	0.106	0.596	0.419
RQ	0.873 ± 0.008	0.868 ± 0.016	0.848 ± 0.013	0.846 ± 0.014	0.070	0.775	0.351
Heat production[4]	6.37 ± 0.17	6.50 ± 0.11	6.24 ± 0.16	6.34 ± 0.18	0.357	0.461	0.711
25 weeks of age (week 6 of the supplementation period)							
Body weight, g	672 ± 13	665 ± 26	682 ± 24	674 ± 34	0.707	0.776	0.975
O_2 consumption[3]	1.22 ± 0.033	1.24 ± 0.030	1.14 ± 0.027	1.16 ± 0.029	0.011	0.575	0.084
CO_2 production[3]	1.04 ± 0.028[a]	1.06 ± 0.026[a]	0.874 ± 0.021[b]	0.881 ± 0.025[b]	<0.001	0.712	<0.001
RQ	0.853 ± 0.013[a]	0.861 ± 0.009[a]	0.768 ± 0.022[b]	0.759 ± 0.018[b]	<0.001	0.988	<0.001
Heat production[4]	5.87 ± 0.15[a]	5.95 ± 0.14[a]	5.36 ± 0.11[b]	5.45 ± 0.13[b]	0.001	0.592	0.009
30 weeks of age (week 11 of the supplementation period)							
Body weight, g	703 ± 19	697 ± 30	730 ± 32	723 ± 38	0.373	0.822	0.846
O_2 consumption[3]	1.16 ± 0.034[a]	1.18 ± 0.031[a]	1.06 ± 0.030[b]	1.07 ± 0.029[b]	0.002	0.686	0.019
CO_2 production[3]	0.948 ± 0.017[a]	0.956 ± 0.026[a]	0.801 ± 0.016[b]	0.814 ± 0.012[b]	<0.001	0.744	<0.001
RQ	0.817 ± 0.012[a]	0.810 ± 0.011[a]	0.756 ± 0.014[b]	0.760 ± 0.014[b]	<0.001	0.934	0.002
Heat production[4]	5.54 ± 0.15[a]	5.61 ± 0.14[a]	4.98 ± 0.13[b]	5.04 ± 0.12[b]	<0.001	0.689	0.003

RQ: respiratory quotient (volume of CO_2 produced/volume of O_2 consumed); SEM: standard error of the mean.
[1]Beginning at four weeks of age, male Sprague-Dawley rats were fed a low-fat or high-fat diet for 15 weeks. Between 19 and 31 weeks of age, lean or obese rats were fed their respective diets and received drinking water containing either 1.02% L-alanine (isonitrogenous control) or 1.5% L-leucine. At 19, 25, and 30 weeks of age (weeks 0, 6, and 11 of the supplementation period), oxygen consumption, CO_2 production, and heat production by rats were measured.
[2]Values are means ± SEM, $n = 8$.
[3]Values are expressed as L/kg body weight/h.
[4]Values are expressed as kcal/kg body weight/h.
[a,b]Means in a row without a common superscript letter differ, $P < 0.05$.

(–16%; $P < 0.01$), RQ (–11%; $P < 0.01$), and heat production (–9%; $P = 0.01$) at 25 weeks of age (i.e. week 6 of the supplementation period). Similar results were obtained for rats at 30 weeks of age (i.e. week 11 of the supplementation period). Leu supplementation did not influence ($P > 0.05$) O_2 consumption, CO_2 production, RQ, or heat production at 25 or

30 weeks of age (i.e. week 6 or 11 of the supplementation period), in comparison to the Ala group. A diet × Leu interaction was detected ($P < 0.001$) only for O_2 consumption at week 11 but not at week 6 of the supplementation period. In contrast, there were diet × Leu interactions ($P < 0.01$) on CO_2 production, RQ, and heat production at 25 or 30 weeks of age (i.e. week 6 or 11 of the supplementation period).

Oxidation of glucose, leucine, glutamine, and oleic acid in BAT

Data on the effects of HF feeding and oral administration of Leu between 19 and 31 weeks of age on substrate oxidation in BAT are summarized in Table 9. Compared with the LF group, HF feeding reduced ($P < 0.001$) rates of oxidation of glucose (–15%), glutamine (–15%), Leu carbon-1 (–17%), Leu carbons 1–6 (–17%), and oleic acid (–19%) in BAT. Compared with the Ala group, Leu supplementation did not affect ($P > 0.05$) the catabolism of any of these substrates, including the oxidative decarboxylation of Leu (i.e. CO_2 production from carbon-1), the release of KIC, and CO_2 production from carbons 1–6. There were no diet × Leu interactions ($P > 0.05$) for the oxidation of these substrates in the BAT.

Discussion

Leu, a branched-chain AA, has simple metabolite fates (i.e. oxidation and protein synthesis) in animals[38] but is a potent activator of the mechanistic target of rapamycin (MTOR) to enhance protein synthesis in tissues including skeletal muscles.[39] Thus, this AA has been used to stimulate energy expenditure in the whole body to reduce energy deposition as WAT and to improve insulin sensitivity in obese mice[12,13] and rats.[14,15] However, inconsistent results have been reported for the effect of Leu supplementation on obesity in rodents.[17,18,40] The major findings of the present study are that oral administration of Leu (1.5% in drinking water) to rats increased concentrations of Leu in plasma by 69% and GFAT activity in ECs by 29%, while decreasing the synthesis of NO in ECs by 13% and whole-body insulin sensitivity by 8%. In addition, the following variables did not differ between Ala- and Leu-supplemented rats: (1) food and energy intakes; (2) masses of WAT, BAT, skeletal muscle, heart, lungs, kidneys, brain, testes, or the digestive tract; (3) rates of oxidation of energy substrates in BAT; (4) whole-body energy expenditure; (5) concentrations of glucose, fatty acids, triacylglycerols, and cholesterol in plasma; and (6) ratios of GSSG/GSH in tissues. Based on the concentrations of branched-chain AAs in the plasma (Table 5) and feed intake (Table 2) of rats, the addition of 1.5% Leu to drinking water did not induce antagonisms among these AAs. The amount of supplemental Leu was 84% greater than the amount of digestible Leu from the enteral diet (Table 2). This value was equivalent to Leu supplementation in adult humans, as the mean intake of Leu (6.56 g/day) by U.S. adults in the National Health and Nutrition Examination Survey (NHANES) 2011–2014 study,[41] and the dose of Leu supplementation for adult humans was 10–15 g/day.[8,42] Both humans and rats have high rates of Leu catabolism through interorgan cooperation and, therefore, tolerate a large amount of supplemental Leu.[38]

Results of the present study confirm those from our previous finding that feeding a HF diet to adult rats increased concentrations of Leu in plasma[24,25] and GFAT enzymatic activity, while reducing NO synthesis in ECs and whole-body insulin sensitivity (Table 5). Greater concentrations of Leu in the plasma of HF-fed rats without Leu supplementation as compared to LF-fed rats without Leu supplementation are a highly consistent hallmark of obesity in mammals including humans and rodents.[11,26] This is likely due to impaired catabolism of Leu via interorgan metabolism involving skeletal muscle and other tissues that may exert effects on AA metabolism in the whole body.[8] Such a view is supported by our results on changes in concentrations of glutamine, glycine, and arginine in plasma (Table 6) and the generation of NO in ECs (Table 5). The increase in plasma concentrations of Leu likely stimulated GFAT expression by activating the MTOR cell signaling pathway to enhance the synthesis of specific proteins in ECs.[10] Glucosamine-6-phosphate, a product of GFAT, competitively inhibits glucose metabolism via the pentose cycle, thereby decreasing the generation of nicotinamide adenine dinucleotide phosphate (reduced form, an essential cofactor of NO synthase) and, therefore, the production of NO from arginine by ECs.[33] This may explain why NO synthesis by the vasculature is reduced in obese people and animals[1,2,19] that usually exhibit elevated concentrations of Leu, isoleucine, and valine in plasma.[11,26] Leu may also be a major AA contributing to hyperaminoacidemia-induced insulin resistance in the skeletal muscle of healthy humans[43] and may promote heart failure in animals (e.g. mice) with elevated concentrations of branched-chain AAs in plasma.[44] A decrease in NO availability in the cardiovascular system contributes to reductions in blood flow,[19] glucose uptake and oxidation by skeletal muscle,[45] fatty acid oxidation by skeletal muscle and liver,[31,32,46] and whole-body insulin sensitivity.[19,47] Thus, reducing the circulating concentrations of Leu in obese rats through oral administration of α-ketoglutarate, which stimulates Leu transamination and oxidative catabolism in the small intestine, skeletal muscle, and other tissues, can reverse the adverse effects of HF feeding on endothelial NO production and whole-body insulin sensitivity.[25] Furthermore, consistent with the view that intramuscular fat deposition is a major factor affecting insulin sensitivity in mammals including humans and rats,[21] an increase in intramuscular lipid concentrations was associated with reduced whole-body insulin sensitivity in HF-fed rats (Table 5). However, in LF-fed rats, dietary Leu supplementation decreased whole-body insulin sensitivity and endothelial NO synthesis without changes in intramuscular lipid concentrations (Table 5), further supporting a role for factors (e.g. NO) other than intramuscular fat concentrations in modulating the action of insulin under such nutritional conditions. In response to a regular intake of digestible carbohydrates, Leu supplementation did not affect glucose homeostasis (indicated by no change in the concentration of glucose in plasma) in rats (Table 5), as reported for obese humans receiving oral administration of 20 g Leu per day for four weeks[48] and for obese mice fed either a high-fat high-sucrose diet or a high-fat diet and drinking water with or without branched-chain AAs (0.75% Leu, 0.48% isoleucine, and 0.53% valine).[49]

Table 9. Effects of high-fat feeding and oral administration of leucine on the oxidation of energy substrates in the brown adipose tissue of rats.[1,2]

Substrate	Low-fat diet		High-fat diet		*P* value		
	Control	Leucine	Control	Leucine	Diet	Leucine	Diet × Leucine
5 mM Glucose	1871 ± 79[a]	1808 ± 64[a]	1570 ± 53[b]	1543 ± 58[b]	<0.001	0.577	0.002
0.5 mM Glutamine	858 ± 45[a]	846 ± 41[a]	731 ± 24[b]	720 ± 22[b]	0.001	0.783	0.011
0.5 mM Leucine							
Carbon 1	318 ± 15[a]	324 ± 17[a]	265 ± 11[b]	270 ± 13[b]	0.001	0.728	0.007
α-Ketoisocaproate	401 ± 22[a]	411 ± 26[a]	327 ± 16[b]	335 ± 18[b]	0.001	0.724	0.012
Carbons 1–6	1214 ± 56[a]	1296 ± 61[a]	1022 ± 45[b]	1058 ± 49[b]	<0.001	0.369	0.003
1 mM Oleic acid[3]	2039 ± 131[a]	1971 ± 115[a]	1647 ± 95[b]	1593 ± 82[b]	0.001	0.631	0.012

SEM: standard error of the mean.
[1]Beginning at four weeks of age, male Sprague-Dawley rats were fed a low-fat or high-fat diet for 15 weeks. Between 19 and 31 weeks of age, lean or obese rats were fed their respective diets and received drinking water containing either 1.02% L-alanine (isonitrogenous control) or 1.5% L-leucine. At 31 weeks of age (week 12 of the supplementation period), rats were euthanized to obtain brown adipose tissue for the measurement of substrate oxidation.
[2]Values, expressed as pmol/mg tissue/2 h, are means ± SEM, $n = 8$.
[3]Assuming that all the carbons of oleic acid were oxidized to CO_2.
[a,b]Means in a row without a common superscript letter differ, $P < 0.05$.

An interesting observation from the present study is that the masses of skeletal muscle, WAT, and other tissues in adult rats were not affected by dietary Leu supplementation (Table 2). In addition, the content of protein in skeletal muscle and liver did not differ between control- and Leu-supplemented rats (Table 4). Thus, oral administration of Leu did not appear to affect the rate of net protein turnover in the whole body of rats despite a 69% increase in concentrations of Leu in plasma (Table 6). Such a notion is consistent with our results that concentrations of ammonia and urea did not differ between control and Leu-supplemented rats (Table 5). At present, there is no evidence that Leu increases the rate of protein degradation or decrease the rate of protein synthesis in skeletal muscle, liver, or other tissues under physiological conditions.[39,50–52] Thus, it is unlikely that oral administration of Leu (via its addition to drinking water at 1.5%) either increased or decreased simultaneously the rates of protein synthesis and proteolysis in tissues to the same extent. Collectively, these results can explain why Leu supplementation did not affect whole-body energy expenditure in lean or obese rats (Table 8). Tracer studies are required in future research to determine the rates of intracellular protein turnover in tissues of obese rats supplemented with or without Leu.

There has been growing interest in the role of Leu in the development of BAT over the last decade. There were reports that dietary supplementation with Leu to HF-induced obese mice increased the expression of uncoupling protein-1 in WAT and the browning of this tissue.[12,53] Interestingly, Wang *et al.*[54] found that either a lack of Leu in the RPMI 1640 medium (Invitrogen, CA) for seven days or the feeding of a Leu-free diet to adult male mice for three days stimulated the browning of the mouse WAT, compared with the presence of Leu (0.38 mM) in the RPMI 1640 medium. However, the physiological significance of such *in vitro* experiments is not clear because Leu is always present in the plasma of living mammals (ranging from 0.15 to 0.25 mM, depending on species and nutritional state).[38] Likewise, a long-term absence of Leu (which is not synthesized *de novo* in animal cells) from diets is fatal to mammals including humans and rats[38] and should not be considered as a normal intervention method for treatment of obesity. In addition, cells cannot survive when cultured in the absence of Leu for a prolonged period of time (e.g. days).[55] Furthermore, there is a suggestion that the catabolism of branched-chain AAs in BAT regulates energy homeostasis in mice.[56] Although we did not determine mitochondrial biogenesis in BAT, results of our present work indicated that oral administration of Leu did not affect the rates of oxidation of energy substrates in the BAT of lean or obese rats (Table 9). Likewise, Leu supplementation did not affect BAT mass (Table 4) or whole-body energy expenditure (based on heat production; Table 8) in lean and obese rats. Our results are not consistent with those of Bishop *et al.*,[57] who found that adding 5% Leu to a casein-based diet reduced both BAT and WAT in obese mice. Such a high dose of Leu may induce AA imbalances among branched-chain AAs to impair protein synthesis,[58] and unfortunately, the researchers did not determine the food intake of the mice. In addition, the intake of starch by mice differed markedly between the HF group and the Leu-supplemented HF group,[57] thereby confounding the interpretation of the experimental data. Results of the present study indicate that Leu does not promote BAT development in adult rats or the oxidation of energy substrates by BAT. Thus, it is likely that Leu catabolism in BAT makes only a minor contribution to whole-body energy expenditure in adult rats.

Both dietary protein deficiency and obesity are associated with oxidative stress in animals.[38] Dietary supplementation with Leu has been proposed to improve antioxidative capacity in animals. For example, based on increases in the activities of antioxidative enzymes (e.g. superoxide dismutase and glutathione peroxidase), Chen *et al.*[59] suggested that adding 0.25% Leu to the diet of young pigs enhanced antioxidant activity and mitochondrial biogenesis in the liver and skeletal muscle. Similar results were also reported for hypercholesterolemic male adult rats receiving dietary supplementation with Leu (70 mg/kg BW/day) for 60 days.[60] In contrast, results from *in vitro* studies revealed that culture medium containing branched-chain AAs (a mixture of 10 mM Leu, 10 mM isoleucine, and 10 mM valine) promoted oxidative stress and inflammation in human peripheral blood mononuclear cells, as compared with control medium

lacking branched-chain AAs.[61] However, the exceedingly high concentrations of branched-chain AAs used in the culture medium (about 50 times of those in the plasma of mammals)[38] and the experimental control without Leu are unphysiological. Results of our present study revealed that dietary supplementation with Leu did not affect either concentrations of GSH and GSSG or the ratio of GSSG/GSH (an indicator of oxidative stress in tissues)[62] in the heart, liver, WAT, and skeletal muscle of lean and obese rats (Table 7). These discrepancies among studies may be due to different animal models and supplemental dosages of Leu.

In recent years, there have been suggestions that Leu increases whole-body energy expenditure and reduces obesity in animals by activating the MTOR pathway for protein synthesis (an energy-dependent process) and stimulating BAT development. This is based on findings that oral administration of Leu (e.g. 1.5% in drinking water) reduced WAT and improved whole-body insulin sensitivity in HF-fed mice[13,15,53,63] and rats.[64] Interestingly, there are also reports that Leu supplementation exacerbated the adiposity of DIO rats.[16,18] However, in those studies, the control diet was either not isonitrogenous to the Leu-supplemented diet or inappropriately supplemented with glycine (e.g. 3.2% glycine as the control for 3.2% Leu).[16] Glycine stimulates protein synthesis and inhibits proteolysis in skeletal muscle cells,[65] while reducing fat accumulation in the WAT of rats.[66] By contrast, results of the present study indicated that Leu supplementation to DIO rats did not enhance whole-body energy expenditure (Table 8) or reduce obesity (Table 3); did not exacerbate adiposity or impair insulin sensitivity (Table 5); and did not affect concentrations of glucose and lipids (Table 5) in plasma. Because the same dosage of Leu was added to drinking water (1.5% Leu), discrepancies among studies may have resulted from differences in animal models (including genetics and breeding locations) and diets (e.g. intakes of energy and nutrients). Consistent with our results, Lee *et al.*[49] found that adding branched-chain AAs (0.75% Leu, 0.48% isoleucine, and 0.53% valine) to drinking water for 38 weeks did not exacerbate insulin resistance in mice with a mild increase (~15%) in concentrations of Leu and isoleucine and no change in concentrations of valine in plasma. Nonetheless, in contrast to previous studies involving DIO rodents,[13–18,53,63] our experimental design ensured that intakes of energy and nutrients did not differ between LF- and HF-fed rats (Table 2), and therefore, there were no confounding factors (e.g. different intakes of energy and nutrients such as AAs, vitamins, and minerals) influencing the interpretation of data. Collectively, it is unlikely that Leu supplementation is an effective means to alleviate metabolic syndrome in obese rats (Table 3) or prevent the development of HF-induced obesity in mice[67] and rats.[16] Rather, attenuating concentrations of Leu in plasma may be beneficial by enhancing NO production and glucose utilization in the vasculature,[10,68] thereby combatting health problems (e.g. cardiovascular disorders) in obese mammals (including humans).

Conclusions

HF feeding increased concentrations of branched-chain AAs, glucose, and cholesterol in plasma, as well as WAT mass, weight gain, concentrations of lipids in liver and skeletal muscle, and GFAT activity in ECs, while reducing NO synthesis, antioxidative capacity (indicated by the ratio of GSH/GSSG) in tissues, the oxidation of energy substrates in BAT, whole-body insulin sensitivity, RQ, and whole-body energy expenditure. Oral administration of Leu decreased NO synthesis in ECs, increased GFAT activity in ECs, and reduced whole-body insulin sensitivity in rats fed the LF diet but had no effect on any of these variables in rats fed the HF diet. Dietary Leu supplementation did not affect food intake; BW gain; masses of tissues including WAT, BAT, and skeletal muscle or antioxidative capacity in tissues; concentrations of insulin, glucose, free fatty acids, cholesterol, triacylglycerols, ammonia, and urea in plasma; or hepatic and intramuscular concentrations of lipids in rats fed LF or HF diets. Adiposity, whole-body insulin sensitivity, and plasma metabolic profiles in obese rats were not adversely affected by Leu supplementation. Thus, results of the present study do not support the use of oral administration of Leu as a nutritional method to treat obesity in animals.

AUTHORS' CONTRIBUTIONS

GW conceived, designed, and supervised this project. CDT, CJM, and GW performed experiments. CDT and YL analyzed the data. CJM and FWB aided in data interpretation. CDT and GW wrote the first version of the manuscript. All authors contributed to discussions and revisions of the article. GW had the primary responsibility for the content of the article. All authors read and approved this manuscript.

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SUPPLEMENTAL MATERIAL

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