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SHORT COMMUNICATION

Body weight and energy homeostasis was not affected in C57BL/6 mice fed high whey protein or leucine-supplemented low-fat diets

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Abstract

Background Leucine is suggested to act as nutrient signal of high-protein diets regulating pathways associated with an alleviation of metabolic syndrome parameters. However, the subject remains controversial.

Aim of the study The aim of this study was to assess and to compare the effects of high-protein diets with dietary leucine supplementation in mice, particularly on energy homeostasis, body composition, and expression of uncoupling protein (UCP), which are suggested to decrease food energy efficiency.

Methods Male C57BL/6 mice were exposed for 14 weeks to semi-synthetic diets containing either 20% (adequate protein content, AP) or 50% whey protein (high-protein content, HP). A third group was fed the AP diet supplemented with L-leucine (AP + L) corresponding to the leucine content of the HP diet. The total fat content was 5% (w/w).

Results Body weight gain, body composition, energy expenditure, and protein expression of UCP1 in brown adipose tissue, and UCP3 in skeletal muscle were not different between groups. In HP-fed mice, a stronger increase in blood glucose levels was detected during glucose tolerance tests compared to AP and AP + L, whereas plasma insulin was similar in all groups. Leucine supplementation did not affect glucose tolerance. Plasma cholesterol was significantly decreased in HP and AP + L when compared to AP. Plasma triglyceride concentrations were increased twofold in HP-fed mice when compared to AP + L and AP groups. Liver and skeletal muscle triglyceride and glycogen concentrations were similar in all groups. Postabsorptive plasma concentrations of branched-chain amino acids were not significantly increased after exposure to HP and AP + L diets, whereas those of lysine were decreased in HP and AP + L mice when compared to AP ($P < 0.001$). Plasma methionine concentrations were lower after HP intake when compared to AP and AP + L ($P < 0.05$).

Conclusions We suggest that an exposure of mice to HP diets or a corresponding leucine supplementation has no significant effect on energy homeostasis and UCP expression compared with AP diets when feeding a low-fat diet. The use of high-quality whey protein might at least in part explain the results obtained.

Keywords Dietary protein · Leucine supplementation · Energy expenditure · Body composition · Uncoupling protein · Mice

Abbreviations

AP	Adequate protein diet
AP + L	AP supplemented with L-leucine
BW	Body weight
EE	Energy expenditure
HP	High-protein diet
LBM	Lean body mass
NEFA	Non-esterified fatty acids

This study was presented in part at 11th International Congress on Amino Acids, Peptides and Proteins, August 3–7, 2009, Vienna, Austria [1].

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RMR	Resting metabolic rate
RQ	Respiratory quotient
TEE	Total energy expenditure
TG	Triglyceride
UCP	Uncoupling protein
QMR	Quantitative magnetic resonance

Introduction

There is plausible evidence that energy-restricted diets containing a high protein-to-carbohydrate ratio may be effective in humans to reduce the incidence of metabolic syndrome components, such as obesity and insulin resistance, by favoring weight loss, a negative fat balance, an improved glucose homeostasis, and better conservation of lean body mass (for review, see Westerterp-Platenga et al. [2]). Leucine has been suggested to mediate the metabolic advantages of high-protein diets by influencing energy balance, lipid metabolism, and playing a key role in skeletal muscle metabolism including protein turnover [3–5]. Based on rat studies, we hypothesized that an induction of uncoupling protein (UCP) homologs by high-protein diets could play a role in energy balance due to dissipation of food energy as heat [6, 7]. This view is supported by the results of Zhang et al. [8] showing increased resting metabolic rate (RMR) in mice associated with increased UCP3 protein expression in skeletal muscle, brown and white adipose tissue after leucine supplementation via drinking water. However, the subject remains controversial because the results available do not unequivocally confirm the antiobese role of leucine feeding and of leucine signaling mechanisms ([9], for review see She et al. [10]). Therefore, we explored in a feeding experiment whether a 14-week exposure to semi-synthetic high whey protein diets or a leucine supplementation could be effective in affecting energy homeostasis and metabolic syndrome components when compared to an adequate whey protein diet in mice. We hypothesized that high whey protein diets may reduce weight gain due to metabolic adaptations with the consequence of an increase in energy expenditure [11]. In addition, we wished to address the question whether leucine supplementation could influence UCP expression.

Materials and methods

Experimental design and diets

The experiments were performed in accordance with the guidelines of the ethics committee of the Ministry for environment, health and consumer protection (State Brandenburg, Germany, Permission No. 23-2347-8-3-2008). Ten-week-old male C57Bl/6 mice (Charles River, Sulzfeld, Germany) were housed individually at 22 °C with a 12 h:12 h light/dark cycle. This mouse strain was shown to be a suitable model for studies of diet-induced obesity and diabetes [12]. Before the experiment, mice consumed ad libitum a standard pelletized rodent diet containing (wt:wt) 19% protein, 4% fat, and 50.5% carbohydrates (1321, Altromin GmbH, Lage, Germany). The metabolizable energy density of the diets was calculated according to the following macronutrient energy contents (kJ/g): whey protein, 15.7; carbohydrate, 16.0; fat, 38. At the start of the experiment, mice were distributed randomly into 3 experimental groups ($n = 10$–11) and assigned to three different semi-synthetic, isoenergetic low-fat diets (Table 1) that were consumed ad libitum for 14 weeks. Water was provided ad libitum. The experimental diets contained either 20% (adequate protein, AP) or 50% (high protein, HP) whey protein. A third group was exposed to an AP diet supplemented with L-leucine (AP + L) corresponding to the leucine content of the HP diet that was calculated based on measured amino acid composition of whey protein [13]. A high-quality protein content of 20% as used in AP is considered to be adequate for mice fed diets containing a balanced amino acid pattern and 5% fat [14]. In HP and AP + L diets, protein and leucine were exchanged isoenergetically for wheat starch. The experimental diets were pelleted manually at room temperature and provided in special containers that allowed the collection of spilled food. Food intake and body weight (BW) were monitored weekly. At the end of the experiment (14 weeks), mice were anesthetized between 0900 and 1000 h, 2–3 h after food withdrawal (postabsorptive state), by isoflurane inhalation and killed by cardiac puncture. Plasma (Li-heparinized) was obtained by centrifugation (9,000×g, 4 °C, and 10 min), and organs were dissected, weighed, frozen in liquid nitrogen, and stored at −80 °C until analyses.

Measurements of energy expenditure

Energy expenditure (EE) and respiratory quotient (RQ) were determined by indirect calorimetry in individual mice essentially as described [15, 16]. Gas analysis was performed using the analyzing system Advanced Optima (ABB, Mannheim, Germany; former Hartmann & Braun GmbH & Co. KG, Frankfurt/Main, Germany) containing oxygen (Magnos 16) and carbon dioxide (Uras 14) analyzers. Calorimetric measurements were performed in the 12th week of intervention after a 24-h acclimation period in 6-min intervals over a period of 23 h. Total energy expenditure (TEE), resting metabolic rate (RMR), and RQ were computed as described [7].

Table 1 Composition of semi-synthetic test diets containing different protein and leucine concentrations

Component	AP (g/100 g)	AP + L	HP
Whey protein[a]	20	20	50
Wheat starch[b]	53	48.5	23
Saccharose[c]	10	10	10
Coconut oil[d]	3	3	3
Soybean oil[e]	1	1	1
Safflower oil[f]	1	1	1
Cellulose[g]	5	5	5
L-Leucine[h]	–	4.5	–
Mineral mixture[i]	5	5	5
Vitamin mixture[j]	2	2	2
Total dietary L-leucine[k]	3	7.5	7.5
Metabolizable energy (kJ/g)	14.3	14.3	14.2
Protein (energy %)	21.3	26.0	53.2
Carbohydrates (energy %)	67.0	62.2	35.1
Fat (energy %)	11.8	11.8	11.8

AP adequate protein, *HP* high protein, *AP + L* AP supplemented with L-leucine corresponding to HP

[a] Fonterra Europe GmbH, Hamburg, Germany (93.5% crude protein, <1.0% fat, <1.0% carbohydrates)

[b] Kröner GmbH, Ibbenbüren/Westfalen, Germany

[c] Nordzucker GmbH, Uelzen, Germany

[d] Ostthüringer Nahrungsmittel GmbH, Gera, Germany

[e] Kunella Feinkost GmbH, Cottbus, Germany

[f] EUCO GmbH, Hamburg, Germany

[g] JRS Pharma GmbH & Co. KG, Weissenborn, Germany;

[h] Sigma Aldrich (Fluka), Steinheim, Germany

[i] Mineral mixture, content per 100 g diet: Ca 730.35 mg; Mg 43.92 mg; P 486.78 mg; Na 196.15 mg; K 582.48 mg; S 52.68 mg; Fe 23,32 mg; Mn 8.67 mg; Zn 1.94 mg; Cu 0.43 mg; I 0.04 mg; F 0.35 mg; Se 0.02 mg; Co 0.01 mg (Altromin GmbH, Lage, Germany)

[j] Vitamin mixture, content per 100 g diet: A 1,500 I.U.; Cholecalciferol 50 I.U.; E15 mg; Menadione 1 mg; Thiamine 2 mg; Riboflavin 2 mg; B-6 1.5 mg; B-12 0.003 mg; Niacin 5 mg; Pantothenate 5 mg; Folic acid 1 mg; Biotin 0.02 mg; Choline chloride 100 mg; *p*-Aminobenzoic acid 10 mg; Inositol 10 mg; C 1.95 mg (Altromin GmbH, Lage Germany)

[k] Theoretical values based on amino acid composition of whey protein [13]. The measured concentrations of individual amino acids of each experimental diet is represented in Table 2

Body composition analysis

Body composition was determined non-invasively without anesthetization essentially as described [15, 17] using quantitative magnetic resonance (QMR; Bruker's Minispec MQ10, Houston, TX) before the start of the experiment (d 0) and every 2 weeks, respectively. Lean body mass (LBM) was calculated by subtracting body fat values obtained by QMR from BW values obtained by weighing prior to QMR measurements.

Glucose tolerance tests

Oral glucose tolerance tests were performed by oral glucose applications (2 mg glucose/g BW, Glucosteril 20%, Fresenius, Germany) at 9 weeks after mice were fasted overnight for 16 h. Blood samples were taken from tail vein before and after glucose treatment for the determination of insulin concentrations using an ELISA assay (insulin mouse ultrasensitive ELISA, DRG Instruments, Marburg, Germany). Blood glucose was measured using glucose test strips before 15, 30, 60,120, and 240 min after glucose challenge.

Blood and tissue measurements

Circulating non-esterified fatty acids (NEFA) were determined using the NEFA C kit (Wako Chemicals GmbH, Neuss, Germany). Plasma cholesterol and triglyceride (TG) concentrations were measured using colorimetric and enzymatic standard methods (Cholesterol liquicolor, Human GmbH, Wiesbaden, Germany, and Serum TG Determination Kit, Sigma-Aldrich, Inc., Steinheim, Germany) according to the instructions of the manufacturers. The TG concentrations of liver and skeletal muscle were measured after extraction with 10 mmol/L sodium phosphate buffer (pH 7.4) containing 1 mmol/L EDTA and 1% polyoxyethylene (10) tridecyl ether using the TG Determination Kit (Sigma-Aldrich). Concentrations of glycogen in liver and skeletal muscle were determined (Starch Kit; R-Biopharm, Darmstadt, Germany) after extraction with 0.1 mol/L sodium hydroxide. TG and glycogen concentrations in tissues were normalized to protein content (detergent compatible protein assay; Bio-Rad, Hercules, CA, USA). The amino acid concentrations in plasma and test diets were determined by HPLC essentially as described [13].

Immunoblots

Protein was prepared from brown adipose tissue and skeletal muscle (quadriceps and gastrocnemius combined). Briefly, about 50 mg of frozen tissue samples were homogenized in 1 mL of homogenizing buffer (50 mmol/L Tris–HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 0,25% SDS, 1% Triton X-100) containing protease and phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) for 3 min in a Speed Mill P12 (Analytik Jena AG, Jena, Germany). The homogenates were stored on ice for 30 min. After centrifugation steps at 18,400 and 23,000×g (30 min, 4 °C), respectively, the supernatant was assayed for protein concentration and then used for Western blotting. Samples were run on a 10% SDS–polyacrylamide gel and transferred to PVDF membranes (Roti-PVDF, Carl Roth GmbH, Karlsruhe, Germany). Unspecific

Table 2 Amino acid composition of semi-synthetic test diets containing different protein and leucine concentrations

Amino acid	AP g/100 g	AP + L	HP
Isoleucine	1.12	1.17	3.13
Leucine	3.54	11.88	10.70
Lysine	2.39	2.47	7.52
Phenylalanine	0.75	0.77	1.91
Threonine	1.40	1.41	3.32
Valine	1.07	1.13	2.68
Alanine	1.13	1.14	2.89
Arginine	0.51	0.52	1.28
Aspartic acid + Asparagine	2.52	2.55	6.60
Glutamic acid + Glutamine	3.99	4.11	10.38
Glycine	0.38	0.38	0.93
Histidine	0.37	0.38	0.97
Proline	0.90	0.95	2.22
Serine	0.82	0.82	1.94
Tyrosine	0.44	0.65	1.56

Values are means of three different hydrolyses. Tryptophan and sulfur-containing amino acids were not determined

AP adequate protein, *HP* high protein, *AP + L* AP supplemented with L-leucine corresponding to HP

antibody binding was blocked with 5% milk powder (Carl Roth GmbH) in 0.1% Tween-20/Tris-buffered saline (TBS-T; 20 mmol/L Tris, 137 mmol/L NaCl, 0.1% (v/v) Tween-20, pH 7.6) for 60 min at room temperature. The membranes were incubated in 1% BSA/TBS-T with rabbit anti-UCP1 and anti-UCP3 antibodies (Abcam, Cambridge, UK, final dilution 1:2,000) overnight at 4 °C. After washing three times with TBS-T, membranes were subsequently incubated for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody, anti-rabbit IgG (Cell Signaling Technology, Danvers, MA, USA; final dilution 1:2,000). Antibody bindings were visualized by chemiluminescence with Super Signal West Femto Sensitivity Substrate (Thermo Scientific, Waltham, MA, USA). Signals were detected with the UVIprochemi imaging system (Biometra, Goettingen, Germany) and quantified using the BioDocAnalyze 2.46.8.1 Software (Biometra) and normalized to α-tubulin (Sigma-Aldrich, Saint Louis, Missouri, USA, final dilution 1:500) signals.

Statistics

Data are reported as means ± SEM. Differences between groups were assessed by ANOVA (one-way or repeated measurements when appropriate) followed by comparison using the Newman–Keuls multiple-range test (GraphPad Prism$^{®}$, vers. 4.03, GraphPad Software, Inc. La Jolla, CA 92037 USA). Differences with $P < 0.05$ were considered statistically significant.

Results

BW and composition were not significantly different ($P > 0.05$, Table 3) between mice fed AP, AP + L, and HP at any time of dietary exposure. However, the mean body weight gain was slightly ($P > 0.05$) lower in mice fed AP + L and HP diets compared to AP by 1.7 and 1.6 g/14 weeks, respectively. Compared to the AP group, mice exposed to AP + L and HP consumed about 7 and 5% less food ($P < 0.05$), respectively, but feed energy efficiency was not significantly influenced by HP or AP + L feeding ($P > 0.05$). Epididymal fat pad, brown adipose tissue, and m. quadriceps weights were not significantly different between feeding groups at the end of the experiment. Kidney weights were significantly higher in HP diet–fed mice when compared to AP and AP + L. Liver weights were significantly higher in HP mice when compared to AP + L (Table 3).

Overall daily TEE and RMR did not differ ($P > 0.05$) between dietary groups (Table 3). The RQ of HP mice was significantly reduced compared to the AP group. This is indicative of lower carbohydrate oxidation in the HP diet group [18] and consistent with the reduced carbohydrate content of the HP diet.

Fasting plasma glucose in 9 weeks was significantly increased in HP compared to controls (Table 4). After oral glucose applications, the blood glucose levels of HP-fed mice were also consistently elevated in comparison with AP and AP + L groups (Fig. 1A), whereas plasma insulin concentrations were not significantly different between groups throughout the glucose tolerance test (Fig. 1B).

TG and glycogen concentrations in liver and skeletal muscle (m. quadriceps and m. gastrocnemius) were not significantly different between the groups. However, plasma TG concentrations were found to be twice as high in HP-fed mice when compared to AP + L- and AP-fed mice (Table 4). Conversely, plasma total cholesterol concentrations were significantly lower in AP + L- and in HP-fed mice when compared to AP-fed controls. The plasma indispensable amino acid concentrations were not significantly different between groups except significantly lower methionine levels in the HP group and decreased lysine levels in both HP and AP + L groups when compared to the AP group (Table 4).

Western blot analysis revealed no significant influences of experimental diets on UCP1 expression in brown adipose tissue and on UCP3 expression in skeletal muscle (m. quadriceps and m. gastrocnemius) after 14 weeks (Fig. 2).

Table 3 Body weight (BW), food intake, body composition, energy homeostasis, and organ weights of C57Bl/6 mice fed semi-synthetic test diets containing different protein and leucine concentrations for 14 weeks

Variable	AP	AP + L	HP
BW, food intake, and leucine intake			
BW at d 0 (g)	27.1 ± 0.5	27.1 ± 0.4	27.1 ± 0.5
BW at d 98 (g)	37.8 ± 0.9	36.1 ± 0.9	36.1 ± 1.1
BW gain (g/14 weeks)	10.7 ± 0.8	9.0 ± 0.6	9.1 ± 0.8
Body fat mass at 14 weeks (g)	11.5 ± 0.7	10.9 ± 0.9	11.7 ± 0.7
LBM at wk 14 (g)	24.1 ± 0.4	24.5 ± 0.6	24.7 ± 0.3
Food intake (g/14 weeks)	380 ± 5^{a}	354 ± 6^{b}	360 ± 6^{b}
Food intake (MJ/14 weeks)	5.45 ± 0.07^{a}	5.14 ± 0.09^{b}	5.04 ± 0.08^{b}
Feed energy efficiency (g/MJ)[1]	1.64 ± 0.12	1.39 ± 0.09	1.34 ± 0.12
L-leucine intake (g/14 weeks)[2]	13.5 ± 0.2^{a}	42.7 ± 0.8^{c}	37.9 ± 0.6^{b}
Energy homeostasis at 12 weeks			
TEE (kJ/d)	50.2 ± 0.7	51.4 ± 1.1	50.1 ± 1.3
TEE (kJ/g d)	1.45 ± 0.04	1.46 ± 0.02	1.41 ± 0.05
TEE (kJ/g LBM d)	2.19 ± 0.05	2.21 ± 0.04	2.12 ± 0.04
RMR (kJ/d)	37.5 ± 1.0	38.4 ± 1.26	39.1 ± 1.3
RMR (kJ/g d)	1.09 ± 0.05	1.09 ± 0.03	1.10 ± 0.05
RMR (kJ/g LBM d)	1.63 ± 0.05	1.65 ± 0.05	1.65 ± 0.05
RQ	0.917 ± 0.009^{b}	0.906 ± 0.014^{a,b}	0.876 ± 0.008^{a}
Organ weights			
Liver weight (g)	1.69 ± 0.06^{a,b}	1.55 ± 0.08^{a}	1.90 ± 0.08^{b}
M. quadriceps weight (g)	0.44 ± 0.02	0.43 ± 0.01	0.41 ± 0.01
Kidney weight (g)	0.40 ± 0.01^{a}	0.45 ± 0.02^{b}	0.52 ± 0.01^{c}
Epididymal fat pad weight (g)	1.72 ± 0.09	1.57 ± 0.09	1.70 ± 0.14
Brown adipose tissue weight (g)	0.25 ± 0.02	0.24 ± 0.03	0.22 ± 0.02

Values are means ± SEM, $n = 9–11$. Within a row, values without a common superscript differ, $P < 0.05$; For diet composition, see Table 1

AP adequate protein, *HP* high protein, *AP + L* AP supplemented with L-leucine corresponding to HP

[1] BW gain (g) divided by energy intake (MJ)

[2] Calculations are based on measured L-leucine concentration of each experimental diet (Table 2)

Discussion

Our study shows that EE, BW gain, and body composition was not significantly modified in C57Bl/6 mice exposed for 14 weeks to AP + L or HP diets that were approximately 3 times higher in leucine concentration when compared to the AP control diet. Likewise, we could not detect significant changes in brown adipose tissue UCP1 and skeletal muscle UCP3 expressions. These results seem to contradict the suggested role of HP diets and of leucine supplementation in reducing energy balance, BW gain, and body fat content [2, 3]. However, in contrast to other studies, we elucidated the effect of increased dietary leucine without a simultaneous challenge with adipogenic diets. Such a challenge is usually generated by feeding high-fat diets and was shown to induce obesity in mice accompanied by an elevated feed efficiency, increased BW gain, and higher body fat accumulation [19, 20]. Using adipogenic conditions, Zhang et al. [8] observed substantial decreases in weight gain with increased RMR in mice after doubling the daily leucine intake from approximately 63 to 118 mg by supplementation to the drinking water for 15 weeks. Interestingly, despite the remarkable effects of leucine observed in their high-fat-diet–fed mice, there was no influence of an additional leucine intake notably on BW and EE in relatively low-fat-chow-diet–fed mice [8]. In contrast, Donato et al. [21] showed that even under conditions of 50% dietary energy restriction, a dietary leucine supplementation (5.91 g/kg diet) could increase body fat loss in rats. Unlike this, Nairizi et al. [9] did not find a significant reduction in BW, body fat content, and effects on insulin tolerance in mice by more than doubling the daily leucine ingestion from approximately 50 to 120 mg by supplementation to drinking water when fed a high-fat diet comparable to the one used by Zhang et al. [8]. Lopez et al. [22] reported subtle positive effects of dietary leucine

Table 4 Postabsorptive plasma parameters and concentrations of TG and glycogen in liver and skeletal muscle (m. quadriceps and m. gastrocnemius combined) of C57Bl/6 mice fed semi-synthetic test diets containing different protein and leucine concentrations for 14 weeks

Variable	AP	AP + L	HP
Glucose (mmol/l)	7.6 ± 0.19	7.3 ± 0.48	8.5 ± 0.55
Glucose, week 9, fasted 16 h overnight (mmol/l)[1]	8.2 ± 0.2^{a}	8.9 ± $0.3^{a,b}$	9.3 ± 0.2^{b}
Cholesterol (mg/dl)	149 ± 4^{c}	124 ± 9^{b}	92 ± 6^{a}
TG (mmol/l)	3.95 ± 0.47^{a}	3.61 ± 0.28^{a}	7.23 ± 0.6^{b}
NEFA (mmol/l)	0.71 ± 0.05	0.78 ± 0.07	0.74 ± 0.05
Indispensable amino acids (μmol/l)			
Isoleucine	76 ± 3	80 ± 5	82 ± 6
Leucine	142 ± 6	182 ± 37	153 ± 10
Lysine	259 ± 19^{b}	178 ± 17^{a}	153 ± 9^{a}
Methionine	48 ± 4^{b}	46 ± 6^{b}	33 ± 2^{a}
Phenylalanine	63 ± 4	65 ± 4	59 ± 4
Threonine	124 ± 15	118 ± 6	97 ± 5
Tryptophan	54 ± 3	55 ± 5	46 ± 4
Valine	167 ± 6	169 ± 13	195 ± 16
TG_{liver} (mg/g protein)	241 ± 31	199 ± 22	182 ± 31
$Glycogen_{liver}$ (μg/mg protein)	303 ± 13	237 ± 29	251 ± 14
$TG_{skeletal\ muscle}$ (mg/g protein)	258 ± 49	185 ± 34	185 ± 42
$Glycogen_{skeletal\ muscle}$ (μg/mg protein)	1.66 ± 0.35	1.71 ± 0.07	2.12 ± 0.32

All data were obtained from mice killed between 0900 and 1000 h and 2–3 h after food withdrawal except for glucose values at week 9

Values are means ± SEM, $n = 9$–11 (for skeletal muscle $n = 4$). Within a row, values without a common superscript differ: for glucose at week 9, fasted 16 h overnight, $P < 0.01$; for methionine $P < 0.05$; for cholesterol, TG, and lysine $P < 0.001$; For diet composition, see Table 1

AP adequate protein, *HP* high protein, *AP + L* AP supplemented with L-leucine corresponding to HP

[1] Baseline values of glucose tolerance tests (Fig. 1)

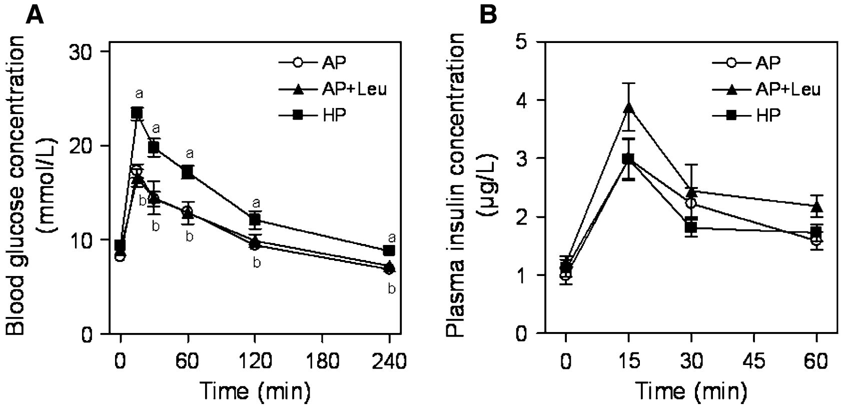

Fig. 1 Glucose tolerance test at week 9 after fasting for 16 h in C57Bl/6 mice fed semi-synthetic test diets containing different protein and leucine concentrations (*AP* adequate protein, *HP* high protein, *AP + L* AP supplemented with L-leucine corresponding to HP). **A** Blood glucose concentrations before ($t = 0$) and after oral glucose application (2 mg/kg BW). **B** Plasma insulin concentrations before ($t = 0$) and after oral glucose application (2 mg/kg BW). Data are means ± SEM, $n = 9$–11, $^{a,b}P < 0.05$ (with ^{b}referring to AP and AP + L groups; the P-values for blood glucose at time point 0 were not indicated, but are given in Table 4)

supplementation (2%) on energy balance and body composition in lactating rats without using adipogenic high-fat diets and without induction of UCPs.

As opposed to other mice studies [8, 9], we aimed at increasing the leucine consumption by using high leucine containing whey protein for the experimental diets. As a result, the daily leucine ingestion was already about 135 mg in the AP group and about 400 mg in the AP + L and HP groups. Therefore, the leucine intake of mice of the AP group is comparable with those of the supplemented

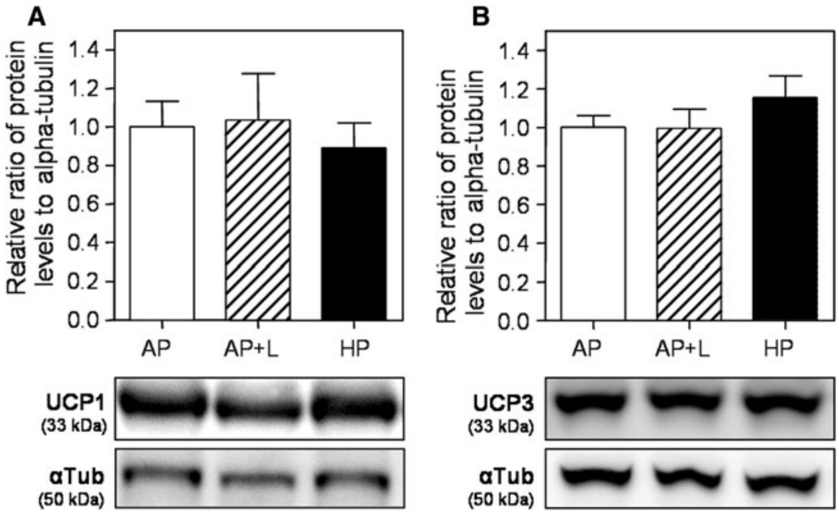

Fig. 2 Relative ratio of protein levels of UCPs to alpha-tubulin (aTub) in C57Bl/6 mice fed semi-synthetic test diets containing different protein and leucine concentrations (*AP* adequate protein, *HP* high protein, *AP + L* AP supplemented with L-leucine corresponding to HP) for 14 weeks. **A** UCP1 protein level in brown adipose tissue. **B** UCP3 protein level in skeletal muscle (quadriceps and gastrocnemius combined). All values are expressed as mean ± SEM, *n* = 4 per group

groups as used by others [8, 9]. Consequently, the use of high leucine containing whey protein could have affected our results. It remains to be elucidated whether the effects on body mass and fat mass in AP + L- and HP-fed mice could only be obtained when compared with a control diet containing either less whey protein or a lower-quality protein, which both would result in lower dietary leucine exposures. However, it was not expected that the remarkable and intended increase in daily leucine ingestion by about 200% in the AP + L and HP groups compared to AP did not result in significant increases of postabsorptive plasma leucine concentrations (Table 3) and in changes of energy homeostasis parameters (Table 3).

Thus, the results remain controversial. One may speculate that differences in study design other than the fat content of the experimental diets could contribute to these inconsistent results. For example, while Nairizi et al. [9] and we housed mice individually during the entire period, in the study of Zhang et al. [8], mice were housed individually only for the last 14 days. Stress due to single housing could thus explain the different results. Another important aspect in interpreting postabsorptive plasma free leucine levels is whether leucine was ingested as dietary protein or supplemented via drinking water. This could have consequences on the time course of changes of plasma leucine concentrations [8]. It is not clear whether the rise in plasma leucine concentration by supplementation of leucine in diets or in drinking water [9] is great enough to cause metabolic effects. For mice in which the mitochondrial branched-chain aminotransferase isozyme was deleted, a 5- to 30-fold increase in circulating leucine concentrations was observed depending on the diet provided [10]. These mice were shown to be resistant to diet-induced obesity [10]. In contrast to such non-physiological conditions, we were not able to detect significantly higher circulating leucine concentrations in mice fed either HP or the leucine-supplemented AP + L diet 2–3 h after food removal in the postabsorptive state (Table 4). This was surprising since we could calculate approximately three times higher intakes of leucine in the AP + L and HP groups compared with AP (Table 3). On the other hand, we have shown previously in rats that even in the postabsorptive state, plasma leucine concentrations are approximately doubled when increasing the dietary protein (casein) exposure from 13.8 to 51.3% [6]. According to the concept of "slow" and "fast" proteins of Boirie et al. [23], it was observed that the type of dietary protein or the ingestion of amino acids as dietary protein as opposed to mixtures of free amino acids [24] can influence post-meal aminoacidemia and postprandial kinetics of dietary amino acids. The results [23, 24] not only suggest differences in the sites of catabolism of amino acids but also in the efficiency of use of whether the amino acids were fed in the free or protein bound form. Therefore, it can be speculated that leucine ingested in forms of a "fast" protein (whey) or in crystalline supplement can be oxidized relatively fast. As a result, it seems not surprising that dietary leucine supplementation can increase the circulating leucine levels only moderately and no more than about twofold [8, 9, 22]. Probably, this also applies to high leucine–containing whey protein diets of our study. For comparison, doubling the leucine intake via drinking water can elevate the average plasma leucine concentrations in high-fat-diet–fed mice by 76% compared to controls [9]. Interestingly, this elevation was not significant in food-deprived mice compared to mice with free access to food. Furthermore, a doubling of leucine intake of high-fat-diet–fed mice via supplementation in drinking water increased plasma leucine concentrations by about 30% only during feeding and not in the postabsorptive state [8]. Obviously, the rise in plasma leucine concentrations after feeding of either HP diets or leucine supplementations should be transient and limited to the fed and postprandial states, which could have consequences for the corresponding metabolic effects [8, 25]. Even a physiological rise in circulating leucine levels was shown to prevent diet-induced obesity and to increase the lean/fat mass ratio in mice [8]. Taken together, future studies will be necessary to examine whether and under which conditions a HP diet exposure or a dietary leucine supplementation can affect energy homeostasis, BW gain, and body composition.

As another important observation, we could not detect significantly increased protein levels of UCP homologs in HP- or AP + L-fed mice compared with AP controls. This contrasts to our previous observation in rats of increased brown adipose tissue UCP1 and liver UCP2 mRNA expressions after long-term HP casein diet exposure [7]. Interestingly, Zhang et al. [8] reported an upregulation of UCP3 in brown and white adipose tissue and in skeletal muscle of mice when leucine was supplemented to adipogenic high-fat diets. However, comparable to our results, they did not detect leucine-induced increases in UCP expressions when feeding a non-adipogenic chow-diet. Although the functions of UCP homologs are not entirely known, we and others have suspected a role in thermogenesis, EE, and fatty acid oxidation [6, 7, 26]. The lack of any changes in energy homeostasis found in our study is thus consistent with the lack of changes in UCP expression.

Interestingly, we noticed about twice as high TG concentrations in plasma of our C57Bl/6 mice model after HP diet exposure compared to AP and AP + L groups. This effect is remarkable and remains to be clarified as it was assumed that with increasing the dietary protein concentrations simultaneously reducing the proportion of carbohydrates, the plasma TG levels can be reduced [27] and that daily fat oxidation was increased after long-term HP exposure in rats [7]. However, because we could not detect an effect on TG concentrations in the AP + L group, it does not seem to be related to dietary leucine supplementation and would rather suspect effects of a higher intake of other amino acids with the HP diet. On the other hand, significantly reduced concentrations of total plasma cholesterol levels were observed after feeding HP and AP + L diets when compared to the AP group, suggesting a mechanism responsive to dietary leucine. A similar plasma total cholesterol- and LDL cholesterol-lowering effect was detected when leucine was applied via drinking water in a C57Bl/6 mice study when fed high-fat diets [8]. As discussed by Blachier et al. [28], the underlying mechanisms involved in the effects of dietary protein and of specific amino acids on lipid metabolism in general are still elusive.

We observed a reduction in postabsorptive plasma lysine concentrations in both AP + L and HP groups compared to AP, which was similar to results of Zhang et al. [8]. However, decreased lysine concentrations were detected only in the fed state in the 4th h of the dark cycle and not in postabsorptive plasma of high-fat-diet–fed mice when the leucine intake was increased via drinking water [8]. It is not clear why HP diets and leucine supplementation decrease plasma lysine, but it suggests alterations in whole-body protein balance. Furthermore, the exact mechanisms or direct effectors that bring about lower plasma concentrations of methionine after HP compared to AP and AP + L diets have yet to be determined. Significant effects of dietary protein on liver sulfur amino acid metabolism were described [29].

The plasma of mice fed HP displayed elevated glucose concentrations when fasted for 16 h overnight compared to AP and AP + L groups, suggesting increased rates of gluconeogenesis [30]. On the other hand, we did not find increased liver glycogen values in HP-fed mice 2–3 h after food withdrawal compared with AP and AP + L. Moreover, we have measured mRNA levels of phosphoenolpyruvate carboxykinase, pyruvate kinase, and glucose-6-phosphatase but did not detect significant differences between groups (results not shown). The increased blood glucose after HP feeding agrees with data of several previous studies as discussed by Lacroix et al. [31], possibly resulting from higher conversion rates of amino acids to glucose when the proportion of carbohydrate in the diet is low. However, in rats, long-term HP feeding was not associated with higher plasma glucose, which may be attributed to the reduced carbohydrate content of HP diets compared to normal protein diets [31]. Nevertheless, the effect on blood glucose seems not to be triggered by dietary leucine as we could not find differences in postabsorptive plasma glucose values and during glucose tolerance tests between AP + L and AP groups. This contrasts with data obtained in high-fat-diet–fed mice showing a significantly improved glucose tolerance by leucine supplementations via drinking water [8]. Most probably, leucine is only effective in preventing high-fat-diet–induced development of glucose intolerance but does not affect normal glucose homeostasis.

Both HP and AP + L diets reduced food intake significantly when compared to the AP diet, which corresponds to numerically lower ($P > 0.05$) gains in BW during the feeding study. This is in contrast to other studies [6, 8, 9, 22] that could not show lower food consumption in mice and rats fed long term with HP or leucine-supplemented diets. HP diets have been suggested to reduce food intake involving complex pathways of protein and amino acid signaling to the brain [32, 33]. But it is assumed that the effect occurs transiently and only during adaptation to HP diets [33].

Conclusions

Our results show that adaptation of mice to diets with high leucine content produced either by the use of whey protein or by leucine supplementation does not significantly affect energy homeostasis, BW gain, brown adipose tissue UCP1 and skeletal muscle UCP3 expression compared with an adequate whey protein diet when low-fat contents were used. Noteworthy are significantly reduced plasma cholesterol concentrations in response to a HP diet or leucine supplementation. We suspect that an effect of HP diets or of leucine supplementations on energy homeostasis of mice can only be detected against the background of high-fat, adipogenic diets with likewise moderate leucine concentrations.

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