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Comparison of high-protein diets and leucine supplementation in the prevention of metabolic syndrome and related disorders in mice☆,☆☆

Anne Freudenberg*, Klaus J. Petzke, Susanne Klaus

German Institute of Human Nutrition in Potsdam-Rehbruecke (DIfE), Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany

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Abstract

High-protein diets have been shown to promote weight loss, to improve glucose homeostasis and to increase energy expenditure and fat oxidation. We aimed to study whether leucine supplementation is able to mimic the alleviating effects of high-protein diets on metabolic syndrome parameters in mice fed high-fat diet.

Male C57BL/6 mice were fed for 20 weeks with semisynthetic high-fat diets (20% w/w of fat) containing either an adequate (10% protein, AP) or high (50% protein, HP) amount of whey protein, or an AP diet supplemented with L-leucine corresponding to the leucine content of the HP diet (6% leucine, AP+L). Body weight and composition, energy expenditure, glucose tolerance, hepatic triacylglycerols (TG), plasma parameters as well as expression levels of mRNA and proteins in different tissues were measured. HP feeding resulted in decreased body weight, body fat and hepatic TG accumulation, as well as increased insulin sensitivity compared to AP. This was linked to an increased total and resting energy expenditure (REE), decreased feed energy efficiency, increased skeletal muscle (SM) protein synthesis, reduced hepatic lipogenesis and increased white fat lipolysis. Leucine supplementation had effects that were intermediate between HP and AP with regard to body composition, liver TG content, insulin sensitivity, REE and feed energy efficiency, and similar effects as HP on SM protein synthesis. However, neither HP nor AP+L showed an activation of the mammalian target of rapamycin pathway in SM. Leucine supplementation had no effect on liver lipogenesis and white fat lipolysis compared to AP. It is concluded that the essential amino acid leucine is able to mimic part but not all beneficial metabolic effects of HP diets.

Keywords: High-protein diet; Leucine supplementation; Metabolic syndrome; Diet-induced fatty liver; Skeletal muscle protein synthesis; mTOR

1. Introduction

Obesity and related disorders including insulin resistance, dyslipidemia and nonalcoholic fatty liver disease have reached epidemic proportions. Therefore, effective treatments are required to curtail this progression. High-protein (HP) restriction diets have been shown to promote weight loss without reducing skeletal muscle mass in obese humans and animal models in contrast to isocaloric high-carbohydrate diets. Furthermore, it was reported that HP diets improve glucose homeostasis, and increase energy expenditure (EE) and fat oxidation [1–4]. A recent study also shows an improvement of hepatic steatosis and plasma lipid levels in obese nondiabetic subjects consuming a whey-protein-enriched diet [5]. Furthermore, it was shown that increasing the protein:carbohydrate ratio in a high-fat diet delayed the development of obesity and improved glucose tolerance in mice even when fed *ad libitum* [6]. The underlying mechanisms of these beneficial effects are not fully understood. The branched-chain amino acid (BCAA) leucine has been suggested to mediate the metabolic advantages of HP diets by influencing energy balance and lipid metabolism, and playing a key role in skeletal muscle metabolism including protein turnover [7–9]. Leucine was suggested to act as a nutrient signal for skeletal muscle protein synthesis due to activation of the mammalian target of rapamycin (mTOR) [10,11]. However, the subject remains controversial because the results available do not unequivocally confirm the antiobese role of leucine feeding and of leucine signaling mechanisms [12,13]. Recently, we showed that mice kept on normal-fat diets with different whey protein and leucine concentrations for 14 weeks showed no differences in body weight (BW) gain [14]. In contrast, Zhang et al. [15] found a reduced weight gain and improved glucose homeostasis when high-fat diets were used and leucine was supplemented via drinking water. A very recent study reported beneficial effects of leucine supplementation via drinking water on insulin resistance without affecting body fat gain on a high-fat diet [16]. However, none of these studies included an HP-diet-fed group for comparison.

Therefore, our study was designed to directly compare the effects of high-whey-protein diets with leucine-supplemented diets to

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* Corresponding author. Deutsches Institut für Ernährungsforschung in Potsdam Rehbrücke, Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany.

E-mail address: anne.noatsch@dife.de (A. Freudenberg).

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evaluate if leucine may be the key component of HP diets for alleviating the development of high-fat-diet-induced metabolic syndrome. By supplementation of a high-fat diet with the same amount of leucine as present in the HP diet, we aimed to elucidate which of the beneficial dietary high-protein effects could be due to specific metabolic effects of leucine.

2. Methods and materials

2.1. 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 light/dark cycle. Before the experiment, mice received a standard pelletized rodent diet containing (w:w) 19% protein, 4% fat and 50.5% carbohydrates (1321, Altromin GmbH, Lage, Germany) *ad libitum*. Mice were distributed randomly into three experimental groups (n=9–10) and assigned to the different semisynthetic, isoenergetic high-fat (20%) diets (Supplement 1) fed for 20 weeks *ad libitum*. The experimental diets contained either 10% w/w (adequate protein, AP) or 50% w/w (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 (6% w/w), which was calculated based on the measured amino acid composition of whey protein [17]. AP was defined as the control diet. Amino acid concentrations in test diets were determined by high-performance liquid chromatography (HPLC) essentially as described [17]. Water was provided *ad libitum*.

The metabolizable energy content of used diets was calculated according to following macronutrient energy contents (kJ/g): whey protein, 15.7; carbohydrate, 16.0; fat, 38.0.

Food intake and BW were monitored weekly. Feed energy efficiency was calculated as average weight gain per MJ energy intake. In week 20, mice were sacrificed between 9.00 and 10.00 a.m., 2 h after food withdrawal. Li-heparinized plasma was obtained, and organs were snap frozen in liquid nitrogen and stored at −80°C until analyses.

2.2. Measurements of EE

In week 12, EE and respiratory quotient (RQ) were determined by indirect calorimetry over a period of 23 h, according to published protocols [18,19]. Total EE (TEE) was normalized to 24 h and body mass. Resting EE (REE) and RQ were computed as described [20].

2.3. Body composition analysis

Body composition was determined noninvasively essentially as described [18,21] using quantitative magnetic resonance (QMR) every 2 weeks. Lean body mass (LBM) was calculated by subtracting body fat mass obtained by QMR from BW obtained by weighing prior to QMR measurements.

2.4. Oral glucose tolerance test (OGTT)

OGTT was performed by oral glucose applications (2 mg glucose/g BW; Glucosteril 20%, Fresenius, Germany) at week 14 after mice were fasted overnight for 16 h. Blood samples were taken from tail vein before and after glucose treatment for determination of plasma insulin concentrations using an enzyme-linked immunosorbent assay (ELISA) (Insulin mouse ultrasensitive ELISA, DRG Instruments, Marburg, Germany). Blood glucose was measured using glucose test strips before and 15, 30, 60, 120 and 240 min after glucose challenge.

2.5. Blood and tissue measurements

Postabsorptive plasma glucose and insulin concentrations in week 7 were measured in blood removed from tail vain 3 h past food withdrawal at the beginning of the light phase as described above. Circulating nonesterified fatty acids (NEFA), plasma cholesterol and triacylglycerol (TG) concentrations in plasma and liver were determined as previously described [14]. Free amino acid concentrations in plasma were determined by HPLC according to published protocols [17].

2.6. Histological analysis

Frozen 5-μm liver sections were fixed in 4% formaldehyde and air dried, and oil red O staining was performed using standard protocols [22].

2.7. Immunoblots

Protein was extracted from brown adipose tissue, liver and skeletal muscle (*Musculus quadriceps* and *gastrocnemius*) as previously described [14]. Detailed procedures from sodium dodecyl sulfate polyacrylamide gel electrophoresis until incubation of different antibodies as well as chemiluminescence detection and quantification of protein bands were described [14]. The following primary antibodies were used: uncoupling protein (UCP) 1 and UCP3 (Abcam, Cambridge, UK); UCP2 (Alpha Diagnostics, San Antonio, TX, USA); p-mTOR, total mTOR, p-4EB-P1 and p-rS6protein (Cell Signaling Technology, Danvers, MA, USA); CD36 (R&D Systems, Wiesbaden, Germany) and alpha-tubulin (Sigma-Aldrich, St. Louis, MO, USA). Horseradish-peroxidase-conjugated secondary antibodies were used: anti-rat (R&D

Table 1
Body weight, food intake, body composition, RQ, EE, and plasma and tissue parameter of mice fed AP, HP or AP+L for 20 weeks[1,2]

	AP	AP+L	HP	P value
BW gain (g)	20.7±0.9^{a}	14.8±1.4^{b}	9.7±0.8^{c}	<.01
Cumulative energy intake (MJ)	8.47±0.09^{a}	7.89±0.22^{b}	7.02±0.07^{c}	<.01
Daily energy intake (kJ/d)	63.2±0.7^{a}	58.9±1.7^{b}	52.4±0.5^{c}	<.01
Feed energy efficiency (g/MJ)[3]	2.14±0.08^{a}	1.57±0.10^{b}	1.12±0.08^{c}	<.01
$RQ_{23\ h}$	0.860±0.006^{a}	0.845±0.009^{a}	0.820±0.004^{b}	<.05
TEE (kJ/d)	54.0±1.4	51.8±1.8	49.4±0.9	NS
TEE (kJ/d/g BW)	1.54±0.02^{a}	1.62±0.04^{ab}	1.68±0.04^{b}	<.05
REE (kJ/d)	37.9±1.4	37.8±1.8	35.1±0.7	NS
REE (kJ/d/g BW)	1.08±0.02^{a}	1.18±0.03^{b}	1.19±0.03^{b}	<.05
Plasma insulin (pmol/L) week 7	269±22^{a}	213±29^{a}	138±18^{b}	<.01
Blood glucose (mmol/L) week 7	8.07±0.14	8.16±0.32	7.67±0.36	NS
Blood glucose (mmol/L) week 20	9.24±0.32	8.96±0.27	8.99±0.50	NS
Plasma cholesterol (mmol/L)	3.61±0.17^{a}	3.32±0.16^{a}	2.19±0.16^{b}	<.001
Plasma TG (mmol/L)	0.44±0.06^{ab}	0.32±0.03^{a}	0.59±0.06^{b}	<.01
Plasma NEFA (mmol/L)	0.86±0.05	0.79±0.04	0.76±0.04	NS
Plasma isoleucine (μmol/L)	59.6±2.8^{a}	64.8±3.1^{a}	80.1±3.4^{b}	<.01
Plasma leucine (μmol/L)	117±3^{a}	133±4^{b}	146±5^{c}	<.01
Plasma valine (μmol/L)	135±4^{a}	133±7^{a}	183±7^{b}	<.001
Liver (g/100 g BW)	3.82±0.09^{a}	3.22±0.14^{b}	4.41±0.13^{c}	<.01
Kidney (g/100 g BW)	0.97±0.04^{a}	1.09±0.04^{a}	1.75±0.07^{b}	<.001
Epididymal WAT (g/100 g BW)	5.90±0.29^{a}	6.16±0.40^{a}	3.70±0.27^{b}	<.001
M. quadriceps (g/100 g BW)	0.68±0.04^{a}	0.80±0.02^{b}	0.94±0.03^{c}	<.05
$Glycogen_{liver}$ (μg/mg protein)	385±41	309±57	294±43	NS
$Glycogen_{muscle}$ (μg/mg protein)	11.1±0.7	11.0±0.9	10.8±0.9	NS

NS, not significant.
[1] Values are means±S.E.M., n=9–11. Within a row, values without a common superscript differ, P<.05.
[2] For diet composition, see Supplement 1.
[3] BW gain (g) divided by energy intake (MJ). RQ, TEE and REE were determined in week 10.

Systems, Wiesbaden, Germany), anti-rabbit IgG or anti-mouse IgG (Cell Signaling Technology, Danvers, MA, USA).

2.8. Real-time polymerase chain reaction (PCR)

Total RNA from homogenized liver was extracted as described before [23] with modifications described by Weber et al. [24]. Quality and integrity of the RNA were checked by calculating $A_{260}/_{280}$ ratio and agarose gel electrophoresis. One microgram of RNA was treated with Turbo DNA-free Kit (Applied Biosystems, Foster City, CA, USA; Ambion, Austin, TX, USA) and reverse-transcribed using the RevertAid H Minus First Strand Complementary DNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Quantitative real-time PCR was performed on the Applied Biosystems 7900 HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan Universal PCR Master Mix or SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 5 ng of RNA and gene-specific primers (Supplement 2). Gene expression was calculated as ΔCT using 18S rRNA as reference gene and expressed relative to the AP group normalized to a value of 1.

2.9. ^{15}N-lysine incorporation into skeletal muscle and analysis of ^{15}N-lysine enrichment

One hour after food deprivation, mice were injected intraperitoneally with a bolus of isotonic ^{15}N-lysine solution (50 μl, 150 μmol/kg BW). Two hours after ^{15}N-lysine application, mice were sacrificed and tissues were stored in liquid N_2. After homogenization (Ultra Turrax Tube Dispenser, IKA-Werke GmbH & Co. KG, Staufen, Germany) and lyophilization (ALPHA 1-4 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany), tissue proteins (~1 mg) were precipitated with trichloroacetic acid (0.6 mol/L, final concentration) and centrifuged (2000g, 10 min, at 4°C). Precipitates were washed with acetone and ether. Dried protein pellets were hydrolyzed (24 h, in 2 ml of 6 mol/L hydrochloric acid, 110°C) in capped Pyrex vials. Amino acid purification by cation exchange and derivatization to *N*-pivaloyl-*i*-propyl esters were performed as described previously [25]. The ^{15}N-enrichment of lysine was determined by gas chromatography–combustion–isotope ratio mass spectrometry (Delta S, Thermo Scientific, Bremen, Germany) [17,25], and ^{15}N-enrichments were expressed as atom% excess above baseline (APE). The baseline atom% values were determined using samples of mice without ^{15}N-lysine injection. The APE values were normalized relative to plasma lysine concentrations.

2.10. Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 4.03, GraphPad Software, Inc., La Jolla, CA, USA). Data are reported as means±S.E.M. Statistical significance was assessed by one-way analysis of variance followed by comparison using the Newman–Keuls multiple-range test. Differences with $P<.05$ were considered statistically significant.

3. Results

3.1. BW, food intake, body composition and organ weight

Mice fed HP or AP+L diets gained significantly less weight than AP control animals ($P<.01$, Table 1). Differences became manifest after 4 or 10 weeks of dietary intervention for HP and AP+L groups, respectively (Fig. 1). The fat mass of HP-fed mice was significantly reduced ($P<.001$) compared to controls from week 2 on. AP+L mice had significantly less fat than the control but more than HP mice. The LBM was significantly higher in HP mice compared to AP mice. Feeding AP+L did not result in significant changes of LBM compared to control (Fig. 1). Food intake was significantly lower in HP (3.01±0.03 g/d, $P<.001$) and also in AP+L mice (3.37±0.10 g/d, $P<.05$) compared to AP controls (3.61±0.04 g/d). Feed energy efficiency was significantly lower for HP and AP+L than for AP diet, with intermediate values for AP+L (Table 1).

Final organ weights are shown in Table 1. The relative epididymal white fat pad (eWAT) weights were similar in controls and AP+L fed mice, whereas HP mice showed significantly reduced relative eWAT mass. Moreover, relative weights of *M. quadriceps* were 15% and 28% increased ($P<.05$), respectively, in mice fed AP+L and HP compared to those of control animals (Table 1). Relative liver weights were 16% lower in AP+L and 15% higher in HP mice than those of control mice ($P<.05$; Table 1). Kidney weights of mice fed HP were significantly increased compared to AP and AP+L mice.

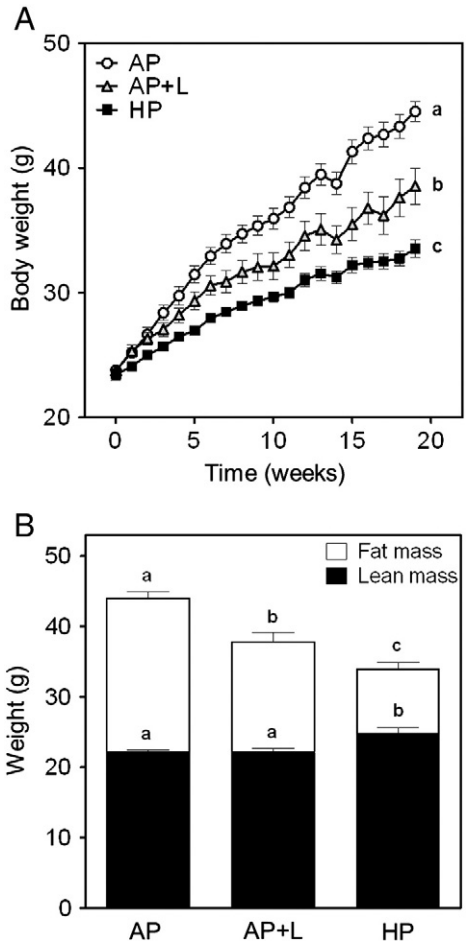

Fig. 1. Body weight development during 20 weeks (A) of mice fed experimental high-fat diets containing different protein and leucine concentrations. Body composition (B) of mice after 20 weeks on experimental diets. Data are means±S.E.M., $n=9–10$, $^{a,b}P<.05$.

3.2. Plasma and tissue parameters

Plasma leucine concentrations were significantly different between the three groups. HP- and AP+L-fed mice had increased plasma leucine levels compared to control mice, with a significant difference between HP and AP+L (Table 1). Plasma concentrations of the two other BCAAs were significantly increased in HP-fed mice compared to AP-fed ones, whereas both isoleucine and valine levels did not differ between leucine-supplemented mice and control animals (Table 1). Total plasma cholesterol levels were decreased in HP-fed mice (−39.4%) compared to other groups (Table 1). Plasma NEFA concentrations were not significantly different between groups. Total plasma TG levels showed no differences between AP+L and AP control mice but were significantly increased in mice fed HP (Table 1). Hepatic TG content was attenuated by approximately 36% and 60% in mice fed AP+L and HP diet, respectively, compared to AP controls (Fig. 2). Analysis of glycogen contents in liver and skeletal muscle showed no significant differences between groups (Table 1).

3.3. Energy metabolism

TEE was not different between groups, but weight-specific TEE was significantly higher in mice fed HP compared to control animals. Weight-specific REE was significantly increased in HP- and AP+L-fed mice compared to controls. The mean RQ was significantly reduced in HP, whereas AP+L mice showed no differences in mean RQ compared to controls (Table 1).

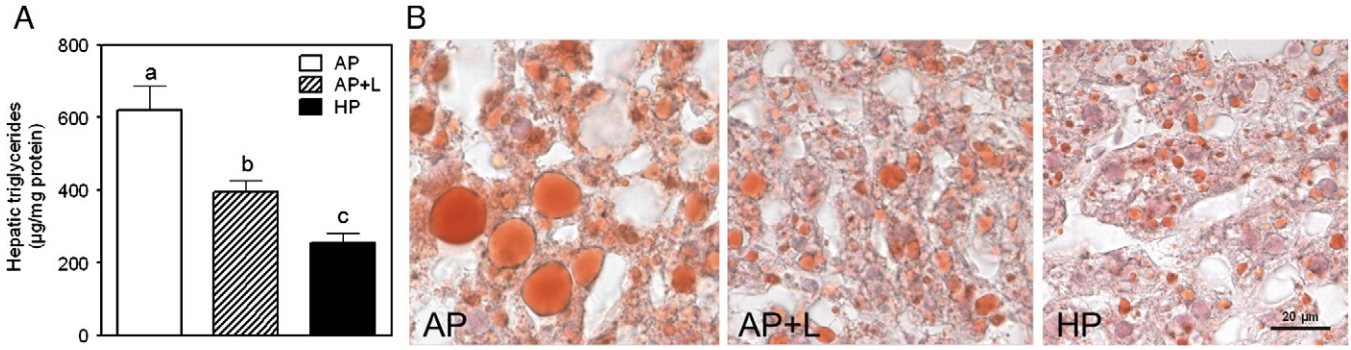

Fig. 2. Hepatic triglyceride concentrations of mice after 20 weeks of feeding different diets (A). Oil red O staining of livers from mice fed high-fat diets containing different protein and leucine concentrations for 20 weeks (B). Data are means±S.E.M., n=9–10, [a,b]P<.05.

In order to further elucidate the effect of HP and leucine supplementation on energy metabolism at the molecular level, we examined the mRNA and protein levels of the UCP isoforms in brown adipose tissue (UCP1), skeletal muscle (UCP3) and liver (UCP2). We could not detect any significant differences in relative mRNA or protein expression levels of UCP1, UCP2 or UCP3 (Table 2).

3.4. Glucose homeostasis

In week 7, postabsorptive plasma insulin levels were significantly decreased in mice fed HP compared to AP control mice, whereas blood glucose levels were similar in all three groups (Table 1). The OGTT in week 14 revealed significantly reduced fasting plasma insulin concentrations in the AP+L and HP group compared to the control group (154±26, 43±13 and 55±9 pmol/L for AP, AP+L and HP, respectively). The rise of plasma insulin levels was significantly reduced within 30 min following oral glucose application in AP+L and HP compared to control mice, whereas blood glucose responses were not significantly different between the three groups (Fig. 3). Additionally, mRNA expression levels for insulin-dependent glucose transporter 4 (GLUT4) in eWAT were 1.6- and 2.8-fold higher, respectively, for AP+L and HP than those of AP controls (Table 3).

3.5. Lipid metabolism

We measured mRNA expression levels of several genes and transcription factors involved in lipolytic and lipogenic pathways in liver and white adipose tissue. In liver, HP feeding led to a reduction in gene expression of lipogenesis-related genes such as acetyl-CoA-carboxylase-alpha (ACCα), fatty acid binding protein 1 (L-FABP) and peroxisome proliferator-activated receptor gamma (PPARγ), whereas AP+L had no effect. There were no significant differences in relative mRNA expression of CD36, PPARα, fatty acid synthase (FAS), cholesterol-7-alpha-hydroxylase (CYP7A1) and HMG-CoA-reductase (HMG-CoA-R) in liver between the groups (Table 3). However, CD36 protein levels were about 68% and 55% reduced in mice fed HP and AP+L, respectively, compared to AP mice (Fig. 4). In white fat, HP but not AP+L feeding led to increased gene expression of the lipases HSL (hormone sensitive lipase) and ATGL (adipose triglyceridelipase) and to decreased leptin gene expression (Table 3).

Table 2
Relative UCP protein expression levels in mice fed AP, HP or AP+L for 20 weeks[1,2]

	AP	AP+L	HP	P value
UCP1 (brown adipose tissue)	1.00±0.19	1.31±0.25	0.86±0.16	NS
UCP2 (liver)	1.00±0.15	0.83±0.12	1.33±0.31	NS
UCP3 (skeletal muscle)	1.00±0.05	1.02±0.11	0.80±0.11	NS

[1] Values are means±S.E.M. n=5. NS, not significant.
[2] For diet composition, see Supplement 1.

3.6. Protein synthesis

The incorporation of ^{15}N-lysine — used as a marker for protein synthesis activity — was significantly increased in skeletal muscle of

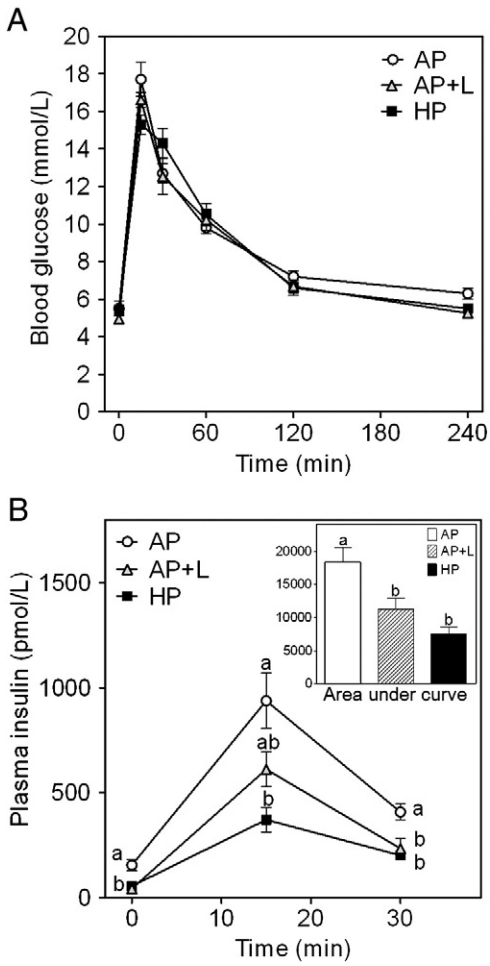

Fig. 3. Glucose tolerance test at week 14 after fasting for 16 h in mice fed experimental diets containing different protein and leucine concentrations. Blood glucose concentrations before (t=0) and after oral glucose application of 2 mg/kg BW (A), and plasma insulin concentrations before (t=0) and after oral glucose application (B). Data are means±S.E.M., n=9–10, [a,b]P<.05.

Table 3
Relative gene expression level in mice fed AP, HP or AP+L for 20 weeks[1]

	AP	AP+L	HP	*P* value
Liver				
FAS	1.00±0.27^{ab}	1.45±0.39^{a}	0.40±0.04^{b}	<.05
HMG-CoA-R	1.00±0.17	1.39±0.32	1.04±0.22	NS
CYP7A1	1.00±0.44	1.96±1.03	0.98±0.26	NS
CD36	1.00±0.40	0.67±0.25	0.86±0.25	NS
ACCalpha	1.00±0.17^{a}	0.88±0.17^{ab}	0.46±0.08^{b}	<.05
L-FABP	1.00±0.12^{a}	1.12±0.12^{a}	0.62±0.12^{b}	<.05
PPARalpha	1.00±0.24	1.12±0.18	0.99±0.17	NS
PPARgamma	1.00±0.15^{a}	0.81±0.13^{ab}	0.48±0.09^{b}	<.05
Epididymal white fat				
HSL	1.00±0.14^{a}	1.21±0.13^{a}	1.70±0.15^{b}	<.05
Perilipin	1.00±0.08	1.03±0.05	1.15±0.09	NS
Leptin	1.00±0.13^{a}	0.81±0.12^{a}	0.47±0.05^{b}	<.05
ATGL	1.00±0.09^{a}	1.05±0.13^{a}	1.95±0.18^{b}	<.001
GLUT4	1.00±0.07^{a}	1.63±0.21^{b}	2.84±0.21^{b}	<.01

[1] Values are means±S.E.M., n=10. Within a row, values without a common superscript differ, P<.05; NS, not significant.
[2] For diet composition, see Supplement 1.

mice exposed to HP and AP+L diets compared to AP controls (Fig. 5). However, protein levels of phosphorylated and unphosphorylated mTOR, as well as p-4E-BP1 and p-rS6 protein, in skeletal muscle were not significantly different between groups (Fig. 5).

4. Discussion

We aimed to examine whether the BCAA leucine is responsible for the metabolic effects of high-protein diets in mice. Here we show that several effects of high dietary protein were at least partially mimicked by leucine supplementation, such as decreased BW and fat gain, increased REE, the prevention of high-fat-diet-induced hepatic steatosis, increased insulin sensitivity and increased postabsorptive muscle protein synthesis. Otherwise, leucine supplementation did not affect LBM, hepatic lipogenesis and white fat lipolysis.

4.1. BW and energy metabolism

The present study shows reduced weight gain in HP- and AP+L-fed mice using high-fat diets to induce obesity, suggesting improvements of the metabolic syndrome due to increased dietary protein exposure and leucine supplementation. This is in line with results showing that high dietary protein intake can induce lower BW gain in humans and animal models, which was mainly explained by increases in satiety and diet-induced thermogenesis (for review, see Refs. [2,26]). Here we found a 29% and 53% reduced BW gain of AP+L and HP mice, respectively, compared to controls. This seems to be partly due to a lower energy intake of −7% and −17% for AP+L and HP groups, respectively. In addition, feed energy efficiency was also reduced by about 27% and 48% when fed AP+L and HP diets, respectively. We cannot exclude that the reduction in food intake of mice in both intervention groups was a result of taste aversion rather than increased satiety. But there is strong evidence that an increase in satiety, and not taste aversion, is responsible for reduced energy intake following high-protein diets [27–30]. Furthermore, a role of leucine in regulating satiety due to an activation of hypothalamic mTOR was previously described [31]. Additionally, our results suggest an influence of dietary high protein and leucine on BW regulation beyond the modulation of food intake. Indeed, both HP feeding and leucine supplementation increased weight-specific REE to the same extent, supporting the suggested role of HP diets and leucine supplementation in increasing EE [2,7] and confirming data by Zhang et al. [15].

In a former study in rats, we have shown that gene expression of UCPs was induced after feeding a high-protein diet for 8 weeks [32], which was suggested to cause higher rates of EE. Here we could not detect any significant changes in UCP1, 2 and 3 expression, which argues against a participation of UCPs in high-protein- or leucine-mediated effects on EE.

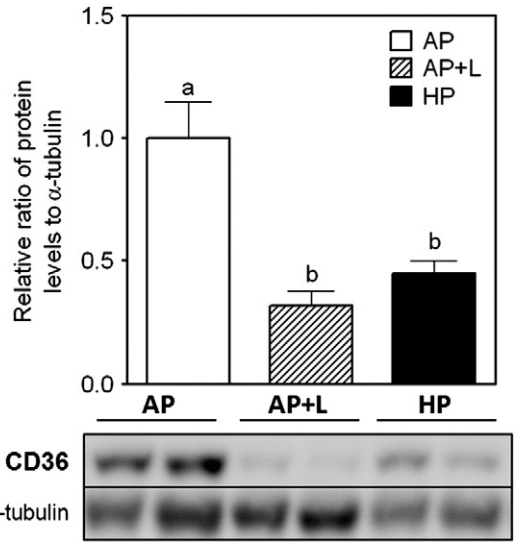

Fig. 4. Relative ratio of hepatic protein levels of CD36 to α-tubulin in mice fed experimental diets containing different protein and leucine concentrations for 20 weeks. All values are expressed as means±S.E.M., $^{a,b}P$<.05.

4.2. Body composition and muscle protein synthesis

Several recent studies focused on the effects of high-protein diets on the preservation of LBM, exploring the role for BCAA in stimulating muscle protein synthesis [10,11,33]. We observed a significant increase in total LBM only in HP-fed mice. However, we determined LBM as fat-free BW. Thus, we cannot exclude biased results due to other organ weights beside skeletal muscle. However, the *M. quadriceps* weight was increased in leucine-supplemented as well as HP-fed mice. Furthermore, we found higher postabsorptive ^{15}N-lysine incorporation rates into skeletal muscle of both HP and AP+L groups, indicating an anabolic function of dietary leucine in skeletal muscle protein metabolism. Leucine is generally known as a potent activator of the mTOR pathway, stimulating muscle protein synthesis on translational level [10,33]. However, we could not detect significant differences between groups either in the protein level of mTOR, in the phosphorylated mTOR or in protein expression of mTOR downstream targets such as p4EB-P1 and p-rS6 protein in skeletal muscle after 20 weeks of dietary intervention. These results seem to contradict the suggested role of leucine on the mTOR pathway. Increased protein levels of the mTOR downstream target S6K1 were observed in humans following short-term infusion of amino acids in healthy patients as well as during long-term high-protein diet in obese subjects demonstrating diet-induced changes of the mTOR pathway [34,35]. It should be noted that most investigations supporting a role of leucine supplementation on mTOR-induced muscle protein synthesis were done in humans or animals others than mice (with the exception of mTOR heterozygous mice) [11,36–38]. Furthermore, these investigations were performed under different conditions such as leucine infusion, exercise, neonatal/postweaning, single meal application, etc. [11,36–38]. It thus may well be that there are substantial differences between mice and humans with regard to the molecular mechanisms of HP or leucine activation of protein synthesis. Our findings suggest that, in mice, mechanisms beside the mTOR pathway may contribute to the long term-regulation of skeletal muscle protein synthesis by dietary protein and leucine. Depression of protein degradation by inhibition of the ubiquitin pathway due to

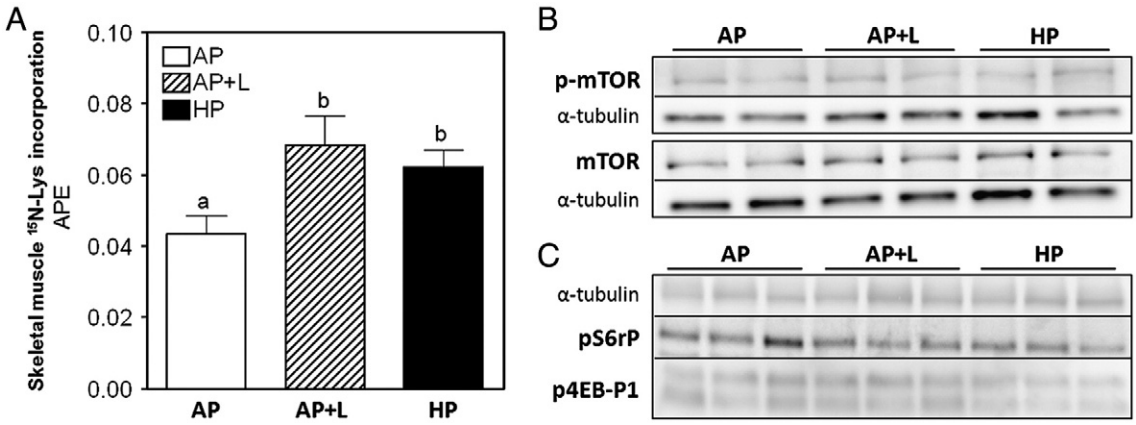

Fig. 5. Skeletal muscle protein synthesis in mice fed high-fat diets containing different protein and leucine concentrations for 20 weeks. Incorporation of ^{15}N-lysine into skeletal muscle expressed in APE relative to plasma lysine concentrations in mmol/L (A). Relative ratios of protein levels of p-mTOR, mTOR (B), pS6rP and p4EB-P1 (C) to α-tubulin in skeletal muscle. Representative bands for each protein are shown. Values are expressed as mean±S.E.M., $^{a,b}P<.05$.

metabolites such as beta-hydroxyl-beta-methylbutyrate derived from leucine and alpha-ketoisocaproate is also conceivable. Sugawara et al. [39] have recently shown that feeding supplementation with 1.5% of leucine prevented muscle protein degradation in rats fed a protein-deficient diet.

In any case, the reduced BW gain of HP-fed and leucine-supplemented mice was predominantly due to a decreased gain of body fat mass. In addition to that, weights of eWAT fat pads were also lower in HP- but not in AP+L-fed mice, indicating differences in fat distribution between groups.

4.3. Fatty liver and lipid metabolism

Excessive fat accumulation (mostly TG) in the liver is considered as the hepatic feature of the metabolic syndrome. Interestingly, we observed that the decreased hepatic TG accumulation following HP feeding was to a great extent mimicked by leucine supplementation. Possible reasons for the development of a fatty liver phenotype can be increased rates of hepatic *de novo* lipogenesis, reduced lipid oxidation, increased fatty acid uptake and/or defective discharge of lipids. Herein, we measured lower mRNA levels of hepatic ACCα, indicating reduced rates of lipogenesis only after HP feeding, suggesting that leucine does not affect hepatic lipogenesis. Indeed, in contrast to the HP feeding, we found no effects of leucine supplementation on gene expression in liver, suggesting that HP effects on liver lipid metabolism are not due to specific effects of leucine. It should be pointed out that HP-fed mice had an almost fivefold lower total dietary carbohydrate intake (52±1 g) compared to AP-fed control mice (254±3 g), which could lead to decreased hepatic lipogenesis and thus reduced hepatic TG levels. On the other hand, carbohydrate intake of the AP+L-fed mice (210±6 g) was only little affected, while hepatic TG content was over 30% decreased compared to AP. This could be linked to a decreased fatty acid uptake due to decreased levels of lipid transport proteins, such as CD36, which was dramatically reduced by HP as well as AP+L feeding. Zhu et al. [40] showed that an increased fatty acid uptake due to higher expression level of lipid transport proteins such as CD36 and L-FABP is more important for deposition of fat in the liver than changes in *de novo* lipogenesis or fat oxidation. Interestingly, HP feeding led to an increased lipolysis in white fat as indicated by increased gene expression of the main lipases ATGL and HSL. This was not observed in the AP+L group and could again be due to the decreased carbohydrate intake of the HP group. Taken together, our data suggest that HP effects on liver lipogenesis and adipose tissue lipolysis could primarily be due to a decreased dietary carbohydrate intake, whereas effects on liver fatty acid uptake might be due to leucine-specific effects.

4.4. Glucose homeostasis

Obesity and fatty liver are often accompanied by insulin resistance [41,42]. Here we found decreased fasting insulin levels and a reduced insulin response following oral glucose application, indicating increased insulin sensitivity in mice fed HP and AP+L compared to high-fat diet controls probably due to the decreased body fat accumulation. Additionally, mice fed HP as well as AP+L displayed increased GLUT4 mRNA concentrations in eWAT. Decreased GLUT4 expression in adipose tissue accompanied by increased fasting plasma insulin levels is well documented in diabetic mouse models (for review see Ref. [43]). Tissue-specific knockout of the GLUT4 gene results in insulin resistance [44], while overexpression of GLUT4 improves insulin sensitivity [45]. The role of HP diets and amino acids in the pathogenesis of insulin resistance is discussed controversially. High intakes of dietary protein as well as of leucine were described to activate the mTOR pathway, thereby inducing insulin resistance via phosphorylation of IRS-1 [46]. In our study, mTOR was not affected by dietary intervention, which could also be a reason for the fact that there was no induction of insulin resistance by HP diet or leucine supplementation. On the other hand, Macotela et al. [16] observed that glucose homeostasis could be improved in mice fed high-fat diets by doubling the leucine intake via the drinking water despite an activation of the mTOR pathway. The role of mTOR pathway in mediating the development of insulin resistance is thus still unresolved.

In conclusion, our results show that leucine is able to mimic several HP-mediated effects on the prevention of the development of obesity and the metabolic syndrome such as the reduced BW gain, increased skeletal muscle protein synthesis and REE, as well as the prevention of liver steatosis and improvement of insulin sensitivity. Most leucine effects were less pronounced than HP effects, suggesting that additional factors are responsible for the metabolic effects of HP diets. One such factor could be the reduced dietary carbohydrate intake associated with an increased protein intake which could mainly be responsible for the decreased liver lipogenesis and increased adipose tissue lipolysis observed after HP feeding. However, leucine supplementation led only to a minor decrease in dietary carbohydrate intake, suggesting specific effects of leucine on energy balance. The molecular mechanisms are not yet clear but do not seem to involve the mTOR pathway.

Supplementary materials related to this article can be found online at doi:10.1016/j.jnutbio.2011.10.005.

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