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ORIGINAL ARTICLE

Dietary L-leucine and L-alanine supplementation have similar acute effects in the prevention of high-fat diet-induced obesity

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Abstract High-protein diets have been shown to alleviate detrimental effects of high-fat diets and this effect can be partially mimicked by dietary L-leucine supplementation. Here, we aimed to elucidate the early mechanisms and the specificity of leucine effects. We performed a 1-week trial with male C57BL/6 mice fed ad libitum with semisynthetic high-fat diets containing an adequate (10 % w/w, AP) or high (50 % w/w, HP) amount of whey protein, or supplemented with L-leucine corresponding to the leucine content within the HP diet (Leu) or supplemented with equimolar L-alanine (Ala). Food and water intake were monitored continuously using a computer-controlled monitor system and body composition changes were assessed using quantitative NMR. HP completely prevented the AP-induced accumulation of body fat. Leu and Ala resulted in a similar reduction of body fat accumulation which was intermediate between AP and HP. There were no significant effects on plasma glucose or insulin. Triacylglycerol content and gene expression of lipogenesis enzymes in liver as well as plasma cholesterol were reduced by HP compared to AP with Leu and Ala again showing intermediate effects. Body fat gain and liver triacylglycerols were strongly correlated with total energy intake. Water intake was rapidly increased by HP feeding and total water intake correlated strongly with total amino nitrogen intake. We concluded that the positive effects of high-protein diets on metabolic syndrome associated traits are acutely due to effects on satiety possibly linked to amino nitrogen intake and on the subsequent suppression of liver lipogenesis without evidence for a specific leucine effect.

Keywords High-protein diet · L-Leucine supplementation · L-Alanine supplementation · Satiety · Energy intake · Water intake · Metabolic syndrome · Diet-induced fatty liver

Introduction

The epidemic dimension of obesity prevalence and related disorders represents an increasing problem in both developed and developing countries. Therefore, it is important to find effective treatments and preventive strategies to curtail the expansion of overweight. One dietary approach is the consumption of a high-protein diet which can promote weight loss and weight maintenance in animals and humans (for review see Halton and Hu 2004; Westerterp-Plantenga et al. 2009). We could demonstrate that increasing the protein to carbohydrate ratio in a high-fat diet led to a delayed development of obesity and improved glucose tolerance in mice fed ad libitum (Klaus 2005). The underlying mechanisms could involve increased energy expenditure and increased satiety (Hochstenbach-Waelen et al. 2009). Animal as well as human studies showed a decrease in food intake following dietary high-protein intake (Potier et al. 2009; Westerterp-Plantenga et al. 2009; Zhou et al. 2011). Furthermore, it has been suggested that the metabolic improvements related to high-protein intakes could be mediated by specific amino acids such as the

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indispensable branched chain amino acid leucine. Several studies have shown beneficial effects of L-leucine supplementations on obesity development and/or glucose homeostasis using different mouse models of obesity and diabetes mellitus (Arakawa et al. 2011; Guo et al. 2010; Macotela et al. 2011; Zhang et al. 2007).

In a mouse model of diet-induced obesity we have previously compared the metabolic effects of high-fat diets containing either a high-protein content (50 %) or supplemented with L-leucine corresponding to the leucine content of the high-protein diet (Freudenberg et al. 2012). We found that a 20-week lasting high-protein exposure decreased food intake, body fat, and liver triacylglycerols (TG) while increasing insulin sensitivity compared with adequate protein diet fed controls. Gene expression analysis pointed to a decreased liver lipogenesis and increased adipose tissue lipolysis. Most importantly, L-leucine supplementations showed similar effects although less pronounced (Freudenberg et al. 2012). Moreover, leucine has been suggested to activate skeletal muscle protein synthesis by activation of the mammalian target of rapamycin (mTOR) pathway (Drummond and Rasmussen 2008; Murgas Torrazza et al. 2010; Suryawan et al. 2011). Although we found skeletal muscle protein synthesis to be up-regulated after 20 weeks of high-protein diet exposure or L-leucine supplementation, we did not detect any activation of the mTOR pathway which suggests that other mechanisms can be involved (Freudenberg et al. 2012).

However, the observed effects on gene expression could have been secondary to body weight changes in this long-term study rather than directly caused by high-protein or L-leucine feeding. Furthermore, we did not control for the increased amino nitrogen intake caused by L-leucine supplementation, which might also have metabolic effects. Therefore, the aim of the present study was twofold: (a) to assess early, primary effects of high-protein and L-leucine supplementation on energy metabolism, and (b) to assess the specificity of leucine effects by including an additional control supplemented with another amino acid. We also assume that a higher dietary amino nitrogen intake should lead to an increased water intake resulting from the necessity of higher rates of urinary nitrogen elimination as urea and the rise in glomerular filtration rate (Bankir et al. 1996; Seney et al. 1987). Because there is evidence that water intake might affect body weight in humans (Dennis et al. 2009), we also monitored water intake in addition to food intake.

To this end we performed a 1-week trial, feeding male C57BL/6 mice ad libitum with high-fat diets containing either an adequate or a high amount of whey protein or supplemented with L-leucine corresponding to the leucine content within the high-protein diet. An additional group was supplemented with L-alanine equimolar to L-leucine. Alanine was chosen because it is a dispensable amino acid showing high plasma levels largely independent of dietary protein exposures (Holecek and Kovarik 2011; Petzke et al. 2000; Rémésy et al. 1978).

Materials and methods

Experimental design, animals and diets

The experiments were performed in accordance with the guidelines on animal experiments approved by the ethics committee of the Ministry for environment, health, and consumer protection (State Brandenburg, Germany, Permission No. 23-2347-18-2010). 10-week-old male C57BL/6 mice (Charles River, Sulzfeld, Germany) were housed individually at 22 °C with a 12-h light/dark cycle. This mouse strain was shown to be a suitable model for studies of diet-induced obesity (Surwit et al. 1995). Before the experiment mice received a standard pelletized rodent chow diet (ssniff Spezialdiäten GmbH, Soest, Germany) ad libitum. Mice were randomly distributed into four experimental groups ($n = 7$–8 per group), assigned to the different semisynthetic, isoenergetic high-fat diets (Table 1) and fed for 1 week ad libitum. The experimental diets contained either 10 % (adequate protein, AP) or 50 % (high-protein, HP) whey protein. A third group was exposed to an AP diet supplemented with L-leucine (Leu) corresponding to the leucine content of the HP diet. A fourth group received an AP diet supplemented with L-alanine (Ala) equimolar to the amino nitrogen content of L-leucine supplemented in the Leu diet. The amount of L-leucine added to the diets was calculated based on the measured amino acid composition of whey protein (Petzke et al. 2005). The AP diet was defined as the control diet. Before feeding and drinking monitoring started, mice were food deprived for 8 h (during light phase). Dietary switch to different experimental diets was performed at 1500 hours, 3 h before onset of dark phase. Amino acid concentrations in different diets were determined by HPLC as described previously (Petzke et al. 2005). Concentrations of all detected amino acids were correspondingly higher in our HP diet compared to control as well as amino acid supplemented diets with the exception of L-leucine and L-alanine. The amounts of L-leucine in Leu diet as well as L-alanine in Ala diet correspond to the concentrations in HP diet (Table 2). Water was provided ad libitum. Energy intake (kJ/7 days) was calculated by multiplication of consumed food (g/7 days) with metabolizable energy content of used diets. Metabolizable energy was calculated according to following macronutrient energy contents (kJ/g): protein/amino acids 15.7, carbohydrate 16.0, fat 38.0. Nitrogen content of each diet was calculated

according to nitrogen content of whey protein (15.67 g nitrogen/100 g whey protein), L-alanine (15.72 g nitrogen/100 g L-alanine) and L-leucine (10.68 g nitrogen/100 g L-leucine). After 1 week of feeding mice were killed between 0900 and 1000 hours, 2 h after food withdrawal. Li-heparinized plasma was obtained and stored at −80 °C until analyses. Liver was excised, weighed and shock frozen in liquid nitrogen.

Food and water intake

Food and water intake was measured during the whole feeding period using a computer-controlled drinking and feeding monitor system (TSE Systems, Bad Homburg, Germany) which automatically records liquid and food consumption of mice housed in standard animal keeping cages (home cages, macrolon type III). Mice were adapted

Table 1 Composition of semisynthetic diets with an adequate (AP), a high (HP) or an adequate protein concentration supplemented with L-leucine (Leu) or equimolar L-alanine (Ala)

Components	AP	Ala	Leu	HP
Whey protein[a]	100	100	100	500
Wheat starch[b]	480	435	420	80
Saccharose[c]	50	50	50	50
Coconut oil[d]	90	90	90	90
Lard[e]	90	90	90	90
Soybean oil[f]	10	10	10	10
Safflower oil[g]	10	10	10	10
Cellulose[h]	100	100	100	100
L-Leucine[i]	–	–	60	–
L-Alanine[i]	–	45	–	–
Mineral mixture[j]	50	50	50	50
Vitamin mixture[j]	20	20	20	20
Nitrogen content[k]	15.7	22.8	22.1	78.4
Metabolizable energy (kJ/g)[l]	17.7	17.6	17.6	17.5
Protein (energy %)[l]	8.9	12.9	14.2	44.8
Carbohydrates (energy %)[l]	48.0	44.0	42.6	11.9
Fat (energy %)[l]	43.1	43.1	43.1	43.4
L-Alanine content (mole)[m]	0.063	0.572	0.063	0.314
L-Leucine content (mole)[m]	0.114	0.114	0.572	0.572

Values are in g/kg diet

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

[b] Kröner, Ibbenbüren/Westfalen, Germany

[c] Nordzucker, Uelzen, Germany

[d] Ostthüringer Nahrungsmittel, Gera, Germany

[e] LARU Langensiepen & Ruckebier, Bottrop, Germany

[f] Kunella Feinkost, Cottbus, Germany

[g] EUCO, Hamburg, Germany

[h] JRS Pharma, Weissenborn, Germany

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

[j] Altromin, Lage, Germany. Mineral mixture (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). Vitamin mixture (per 100 g diet): vitamin A (1500 IE), cholecalciferol (50 IE), vitamin E (15 mg), menadione (1 mg), thiamine (2 mg), riboflavin (2 mg), vitamin B-6 (1.5 mg), vitamin 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), vitamin C (1.95 mg)

[k] Contained in whey protein and amino acid components of each diet

[l] Calculated according to following macronutrient energy contents (kJ/g): whey protein 15.7, carbohydrate 16.0, fat 38.0. L-leucine and L-alanine supplementations were included as protein equivalents

[m] These theoretical concentrations are based on amino acid composition analysis of whey protein (Petzke et al. 2005). The measured concentrations of individual amino acids of each experimental diet are presented in Table 2

Table 2 Amino acid composition of semisynthetic diets with an adequate (AP), a high (HP) or an adequate protein concentration supplemented with L-leucine (Leu) or equimolar L-alanine (Ala)

	AP	Ala	Leu	HP
Aspartic acid + asparagine	1.31	1.46	1.57	10.67
Threonine	0.94	0.97	1.02	6.41
Serine	0.44	0.49	0.52	3.55
Glutamic acid + glutamine	2.10	2.35	2.51	17.16
Glycine	0.21	0.22	0.24	1.62
Alanine	0.62	5.87	0.76	4.96
Valine	0.58	0.64	0.68	4.63
Isoleucine	0.58	0.62	0.64	4.86
Leucine	1.57	1.85	13.77	13.67
Tyrosine	0.29	0.35	0.35	2.39
Phenylalanine	0.38	0.40	0.40	3.19
Histidine	0.21	0.23	0.23	1.70
Lysine	1.12	1.22	1.25	10.02
Arginine	0.26	0.27	0.27	2.12

Values are in g/100 g diet

For diet composition see Table 1. The amino acid content was determined by HPLC essentially as described (Petzke et al. 2005)

to the cages, food baskets and water bottles of the system for 3 days before the diets were changed.

Body weight and composition analysis

Body weight (BW) and composition of mice were measured at the beginning and at the end of the experiment. Body composition was determined non-invasively as described previously (Katterle et al. 2008; Klaus et al. 2005) using a quantitative magnetic resonance (QMR) method. Lean body mass (LBM) was calculated by subtracting body fat mass obtained by QMR from BW.

Biochemical measurements

Free amino acid and urea concentrations in plasma were determined by HPLC according to published protocols (Petzke et al. 2005). Plasma glucose, free fatty acids (NEFA), total cholesterol and TG as well as liver TG were analysed in triplicates on 96-well plates using commercial colorimetric and enzymatic standard assays as described (Noatsch et al. 2011).

Gene expression

Gene expression analysis was performed by quantitative real-time PCR on the 7900 HT Fast Real-Time PCR System (Applied Biosystems) as described in detail before (Freudenberg et al. 2012). Real-time PCR data were analysed by the comparative threshold cycle (C_T) method subtracting the C_T values of genes of interest from C_T values of internal controls (ΔC_T) using 18S rRNA as reference gene. The results were expressed relative to the AP group normalized to a value of 1.

Statistical analysis

Statistical analyses were performed using GraphPad Prism® (vers. 4.03, GraphPad Software, Inc. La Jolla, CA 92037, USA). Data are reported as mean ± SEM. Statistical significance was assessed by one-way ANOVA followed by comparison using the Newman–Keuls multiple range test. Pearson correlation coefficients were calculated to determine the relationship between selected parameters. Differences with $P < 0.05$ were considered statistically significant.

Results

High-protein feeding and amino acid supplementation resulted in decreased energy intake and increased nitrogen intake

The feeding of the HP diet resulted in a significant decrease in food and thus energy intake compared to all other groups (Fig. 1a), an effect which was apparent already immediately after the switch to the experimental diets. The reduced food intake of HP mice compared to controls was significant from 4 h after the dietary switch onwards (Fig. 1b). After 7 days, cumulative food intake was significantly decreased by 33, 12, and 9 % in mice fed the HP ($P < 0.0001$), Ala, and Leu diets ($P < 0.05$), respectively, compared with the control group (Table 3). The two amino acid supplemented groups Ala and Leu had a similar energy intake which was intermediate between HP- and AP-fed mice ($P < 0.05$). Total nitrogen intake was significantly increased in mice exposed to HP ($P < 0.0001$) compared with controls. L-Alanine- and L-leucine-supplemented mice consumed significantly less nitrogen than HP mice and more than AP mice but did not differ among each other.

Overall water consumption strongly correlated with total amino nitrogen intake

The decreased food intake of the HP group was associated with an increased water intake which was significantly higher than in the AP group already 7 h after the dietary switch (Fig. 1c). Total water consumption after 7 days was 1.9-fold higher in HP mice ($P < 0.0001$) compared with AP controls, whereas supplementation of L-alanine or L-leucine did not significantly influence water intake

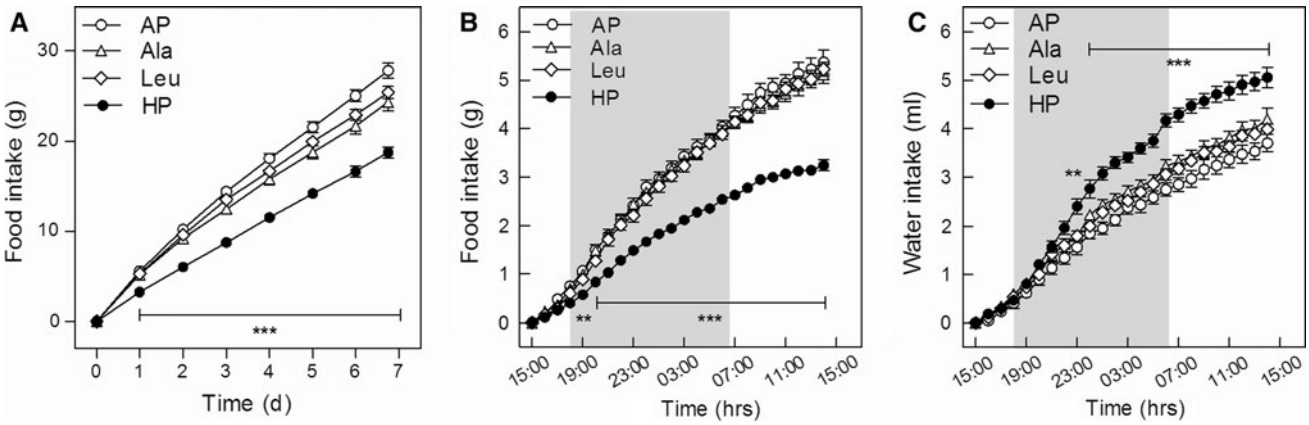

Fig. 1 Food and water intake. Ten-week-old male C57BL/6 mice were fed high-fat semi-synthetic diets with adequate (*AP*) or high (*HP*) protein content or supplemented with L-leucine (*Leu*) or equimolar L-alanine (*Ala*) for 7 days. **a** Cumulative food intake during the whole feeding trial. Food (**b**) and water (**c**) intake on the first day of dietary intervention. Values are mean ± SEM. The *shaded area* refers to night (light off) period. *Asterisks* indicate significant differences from AP (**$P < 0.01$, ***$P < 0.0001$)

Table 3 Body composition, food and water intake, and organ weights of 10-week-old male C57BL/6 mice fed high-fat diets containing an adequate (AP), a high (HP) or an adequate protein concentration supplemented with L-leucine (Leu) or equimolar L-alanine (Ala)for 1 week

	AP	Ala	Leu	HP	ANOVA
Body weight start (*g*)	25.8 ± 0.5	25.9 ± 0.4	26.0 ± 0.3	25.4 ± 0.1	NS
Body weight day 7 (g)	28.7 ± 0.6^{a}	27.0 ± 0.7^{b}	27.6 ± 0.5^{b}	25.3 ± 0.3^{c}	<0.0001
Body weight change (g)	2.94 ± 0.35^{a}	1.06 ± 0.34^{b}	1.60 ± 0.43^{b}	−0.10 ± 0.28^{c}	<0.0001
Fat mass day 7 (g)	8.39 ± 0.74	6.45 ± 1.02	7.77 ± 0.78	6.01 ± 0.55	NS
Fat mass gain (g)	3.08 ± 0.27^{a}	1.15 ± 0.29^{b}	1.92 ± 0.43^{b}	0.06 ± 0.27^{c}	<0.0001
Lean mass day 7 (g)	20.1 ± 0.4	20.3 ± 0.6	19.6 ± 0.5	19.2 ± 0.4	NS
Lean mass change (g)	−0.09 ± 0.28	−0.12 ± 0.24	−0.18 ± 0.23	−0.10 ± 0.23	NS
Cumulative food intake (g/7 days)	27.8 ± 0.12^{a}	24.4 ± 1.0^{b}	25.4 ± 0.6^{b}	18.7 ± 0.6^{c}	<0.0001
Cumulative energy intake (kJ/7 days)	491 ± 15^{a}	429 ± 17^{b}	448 ± 11^{b}	328 ± 10^{c}	<0.0001
Cumulative water intake (ml/7 days)	17.2 ± 0.4^{a}	20.1 ± 1.0^{a}	19.3 ± 0.6^{a}	32.9 ± 1.3^{b}	0.0001
Cumulative nitrogen intake (g/7 days)	0.44 ± 0.01^{a}	0.56 ± 0.02^{b}	0.56 ± 0.01^{b}	1.47 ± 0.05^{c}	<0.0001
eWAT weight (g)	0.73 ± 0.05^{a}	0.51 ± 0.05^{b}	0.63 ± 0.07^{ab}	0.43 ± 0.05^{b}	<0.01
Liver weight (g)	1.29 ± 0.06	1.15 ± 0.03	1.12 ± 0.05	1.22 ± 0.03	NS
Liver TG (mg/liver)	22.7 ± 0.9^{a}	18.7 ± 2.9^{a}	17.5 ± 3.2^{a}	8.4 ± 1.2^{b}	<0.01

Values are mean ± SEM, $n = 7$–8. Within a row, values without a common superscript differ, $P < 0.05$. For diet composition see Table 1

NS not significant, *eWAT* epididymal white adipose tissue, *TG* triacylglycerol

compared to AP mice (Table 3). However, on an individual basis, total water consumption showed a very strong positive correlation with total nitrogen intake ($r^2 = 0.90$, $P < 0.0001$, data not shown).

Fat accumulation is mainly explained by energy intake

Seven days of AP feeding resulted in a significant increase in body weight (Table 3) which was exclusively due to an increase in fat mass ($P < 0.0001$). This increase was completely inhibited by HP feeding. Both, body weight and fat mass gain of the Ala and Leu groups were similar and significantly reduced compared with AP-fed mice. They were significantly higher than observed in the HP group and thus intermediate between AP and HP. LBM and its changes were not different between groups. Liver weight was not affected by the diets but total liver TG content was significantly reduced in the HP group compared with all other groups ($P < 0.01$; Table 3).

Fat mass gain was highly correlated with metabolizable energy intake (Fig. 2a). Energy intake thus explained 77 % of the variation in body fat increase. Liver triacylglycerol content was also significantly correlated with energy intake ($r^2 = 0.65$, $P < 0.0001$; Fig. 2b). Energy expenditure was

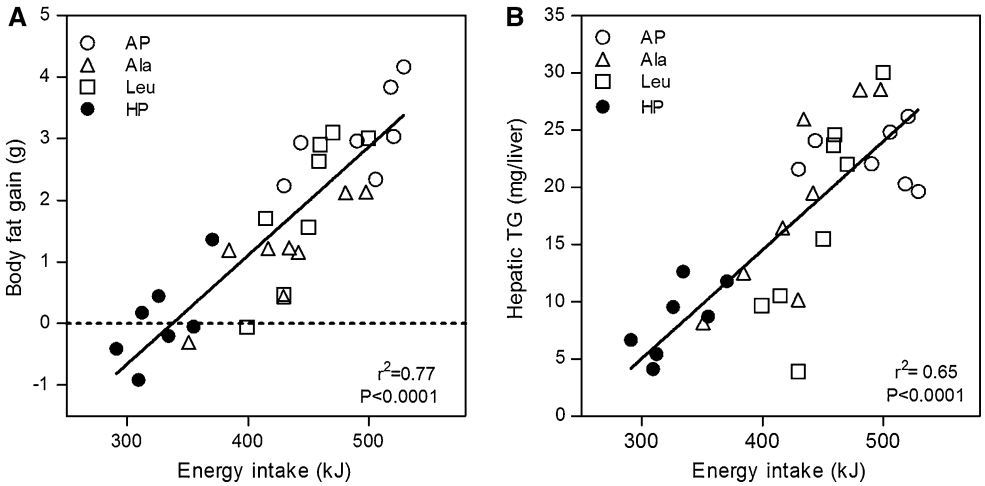

Fig. 2 Correlations of body fat gain and liver triacylglycerol content with energy intake. Ten-week-old male C57BL/6 mice were fed high-fat semi-synthetic diets with adequate (*AP*) or high (*HP*) protein content or supplemented with L-leucine (*Leu*) or equimolar L-alanine (*Ala*) for 7 days. **a** Individual correlation of body fat gain with total energy intake, **b** individual correlation of liver triacylglycerols content with total energy intake

not directly determined in this experiment, but measurements of locomotor activity using infrared motion detectors did not show any differences between the groups (data not shown). The regression line (Fig. 2a) shows an *x*-intercept of 337 kJ at zero fat gain. This means that an energy intake of 335 kJ/week (48 kJ/day) can be considered the maintenance energy expenditure because it leads to neither increase nor decrease of body fat stores. This fits very well to data from our previous long-term study (Freudenberg et al. 2012) where by indirect calorimetry, we determined a mean daily maintenance energy expenditure of 49 kJ in mice of a similar body weight.

Effects of high-protein feeding and amino acid supplementation on plasma metabolites

All plasma metabolites were determined at the end of the experiment after 2 h of food withdrawal. Plasma leucine concentrations were significantly higher in mice exposed to Leu and HP diets compared with AP, but did not differ between AP- and Ala-fed mice (Table 4). Concentrations of other branched chain amino acids (valine and isoleucine) remained unchanged in plasma of L-leucine- and L-alanine-supplemented mice but increased significantly in HP-fed animals (Table 4). The alanine plasma level did not differ between groups. Urea concentrations were significantly increased in HP-fed mice compared with all other groups ($P < 0.0001$; Table 4). Overall plasma urea concentrations were positively correlated with nitrogen intake ($r^2 = 0.76$, $P < 0.0001$, data not shown).

There were no diet effects on plasma glucose, insulin, NEFA, and TG. Plasma cholesterol was significantly decreased in HP mice compared with other groups (Table 4). Plasma cholesterol concentrations also showed a significant correlation with overall energy intake ($r^2 = 0.62$, $P < 0.0001$, data not shown).

Table 4 Postabsorptive plasma parameters of 10-week-old male C57BL/6 mice fed semi-synthetic diets with an adequate (AP), a high (HP) or an adequate protein concentration supplemented with L-leucine (Leu) or equimolar L-alanine (Ala) for 1 week

	AP	Ala	Leu	HP	ANOVA
Alanine (μmol/l)	363 ± 28	336 ± 16	357 ± 24	327 ± 15	NS
Leucine (μmol/l)	118 ± 7[a]	116 ± 6[a]	144 ± 6[b]	156 ± 13[b]	<0.01
Isoleucine (μmol/l)	63.1 ± 3.7[a]	61.5 ± 3.7[a]	72.0 ± 4.2[a]	87.0 ± 7.3[b]	<0.01
Valine (μmol/l)	147 ± 6[a]	141 ± 7[a]	157 ± 11[a]	194 ± 13[b]	<0.05
Urea (mmol/l)	5.04 ± 0.36[a]	5.32 ± 0.37[a]	5.35 ± 0.21[a]	8.34 ± 0.46[b]	<0.0001
Glucose (mmol/l)	8.69 ± 0.47	8.34 ± 0.57	8.31 ± 0.46	7.70 ± 0.24	NS
TG (mmol/l)	0.37 ± 0.05	0.40 ± 0.04	0.44 ± 0.04	0.42 ± 0.04	NS
NEFA (mmol/l)	0.74 ± 0.15	0.81 ± 0.15	0.65 ± 0.15	0.64 ± 0.16	NS
Insulin (pg/ml)	1.20 ± 0.36	0.89 ± 0.13	1.03 ± 0.20	0.75 ± 021	NS
Cholesterol (mg/dl)	153 ± 5[a]	133 ± 3[a]	130 ± 10[a]	94 ± 2[b]	<0.001

Values are mean ± SEM, $n = 7$–8. Within a row, values without a common superscript differ, $P < 0.05$. For diet composition see Table 1

NS not significant, *TG* triacylglycerol, *NEFA* non-esterified fatty acid

Table 5 Gene expression in liver and epididymal white adipose tissue of 10-week-old male C57BL/6 mice fed semi-synthetic diets with an adequate (AP), a high (HP) or an adequate protein concentration supplemented with L-leucine (Leu) or equimolar L-alanine (Ala) for 1 week

	AP	Ala	Leu	HP	
Liver					
ACCα	1.00 ± 0.09[a]	0.78 ± 0.12[a]	0.67 ± 0.09[a]	0.35 ± 0.03[b]	<0.01
CD36	1.00 ± 0.08	0.73 ± 0.10	0.99 ± 0.10	1.04 ± 0.16	NS
FAS	1.00 ± 0.26[a]	0.55 ± 0.10[b]	0.37 ± 0.05[b]	0.18 ± 0.03[b]	<0.05
L-FABP	1.00 ± 0.14[a]	0.77 ± 0.05[b]	0.59 ± 0.05[bc]	0.48 ± 0.03[c]	<0.05
PK	1.00 ± 0.09[a]	0.79 ± 0.07[ab]	0.52 ± 0.10[bc]	0.24 ± 0.04[bc]	<0.001
Epdidymal white fat					
CD36	1.00 ± 0.04	1.10 ± 0.10	0.90 ± 0.16	1.21 ± 0.08	NS
FAS	1.00 ± 0.18	0.93 ± 0.12	1.09 ± 0.14	0.56 ± 0.09	NS
SCD1	1.00 ± 0.15	0.88 ± 0.16	1.07 ± 0.10	0.82 ± 0.17	NS
GLUT4	1.00 ± 0.09	1.15 ± 0.07	1.09 ± 0.14	1.31 ± 0.09	NS
ATGL	1.00 ± 0.07[a]	1.28 ± 0.15[a]	1.38 ± 0.13[a]	1.86 ± 0.25[b]	<0.01
HSL	1.00 ± 0.05	1.10 ± 0.10	0.89 ± 0.15	1.21 ± 0.08	NS

Values are mean ± SEM, $n = 6$–8. Within a row, values without a common superscript differ, $P < 0.05$. For diet composition see Table 3

NS not significant, *ACCα* acetyl CoA carboxylase, *CD36* fatty acid translocase, *FAS* fatty acid synthase, *L-FABP* liver-type fatty acid binding protein, *ATGL* adipose triglyceride lipase, *HSL* hormone sensitive lipase, *SCD1* stearoyl-CoA desaturase, *PK* pyruvate kinase

Gene expression analysis in liver and white adipose tissue (WAT)

Analysis of gene expression in liver and epididymal white adipose tissue (eWAT) showed an HP diet-induced decrease in gene expression of acetyl CoA carboxylase alpha (ACCα), fatty acid synthase (FAS), liver-type fatty acid binding protein (L-FABP), and pyruvate kinase (PK) compared with the AP control, consistent with a decrease in hepatic lipogenesis (Table 5). L-Alanine- and L-leucine-supplemented groups showed intermediate values between AP and HP which were significantly different from AP-fed controls for FAS, L-FABP, and PK. Gene expression in eWAT was little affected with the exception of adipose triglyceride lipase (ATGL) which was up-regulated by HP. Hormone sensitive lipase (HSL), on the other hand, was not affected. L-Alanine and L-leucine supplementation had no significant effects on eWAT gene expression levels.

Discussion

Previously, we have shown that dietary L-leucine supplementation could partially mimic the beneficial effects of high-protein feeding on metabolic syndrome associated traits in a long-term high fat feeding study (Freudenberg et al. 2012). Here, we have shown that most of these effects are already evident after just 1 week of intervention. Mice fed a high-fat containing HP diet had a decreased food intake, body weight and fat mass as well as decreased liver lipogenesis compared to AP controls already after 1 week. Dietary L-leucine supplementation was able to mimic the HP effects although the effects were less pronounced. An unexpected and novel finding was that supplementation of L-alanine had the same effects as L-leucine in all parameters measured. This puts into question the specificity of L-leucine effects on obesity and glucose homeostasis reported by us and others (Freudenberg et al. 2012; Macotela et al. 2011; Zhang et al. 2007).

In our study, mice showed a decrease in body weight already after 1 week feeding of the high fat containing HP diet. This was due to a complete prevention of the high-fat diet-induced body fat accumulation. A reduced body weight gain following long-term high-protein ingestion was already observed in humans and animal studies and mainly attributed to an increase in satiety (Cota et al. 2006; Hochstenbach-Waelen et al. 2009; Klaus 2005; Potier et al. 2009; Westerterp-Plantenga et al. 2009; Zhou et al. 2011). This was confirmed in the present study which shows that the intake of a high-fat diet was acutely affected when simultaneously containing a high-protein content with significant effects apparent as early as 5 h after the dietary switch. Interestingly, supplementation of L-leucine as well as L-alanine also led to a significant reduction in energy intake, although to a much lesser extent than HP. This reduction in energy intake subsequently led to a reduction in body fat gain which was completely abolished in the HP group. Taking all data together, it becomes evident that energy intake alone was responsible for about 77 % of the variation in body fat accumulation. This is in accordance with a previous study in which we have shown that after longer term feeding of diets with different macronutrient ratios, about 84 % of the variation in final body weight was determined by energy intake (Klaus 2005). It is possible

that the remaining 23 % of variation in fat accumulation are due to differences in energy expenditure. However, physical activity (measured by infrared motion detectors) was not different between groups. Changes in resting energy expenditure due to changes in muscle mass are also unlikely because LBM did not change after 1 week of dietary intervention. In two previous studies, we did not find any significant effects of high-protein diets or L-leucine supplementation on total energy expenditure compared with controls measured by indirect calorimetry after several weeks of exposure (Freudenberg et al. 2012; Noatsch et al. 2011). Together, these data suggest only a minor impact of energy expenditure on changes in body fat accumulation due to HP or amino acid supplementation. Interestingly, hepatic fat accumulation was also highly correlated with energy intake which explained 65 % of the variation in liver fat.

Conceivable mechanisms for the satiating effect of dietary protein are among others a delayed gastric emptying and changes in the secretion of intestinal anorexigenic and orexigenic hormones. An increase in plasma amino acids and leucine, in particular, has been suggested to play a role in hypothalamic regulation of food intake by acting as satiety signals (Cota 2009; Cota et al. 2006; Potier et al. 2009). However in our study, increased circulating leucine levels were only observed in the HP and Leu groups. Therefore, elevated circulating leucine levels cannot explain the L-alanine effect on satiety and fat accumulation. Because L-alanine supplementation did not affect plasma amino acid levels compared with the control (AP), it is not likely that circulating amino acid levels contributed to the satiety effect observed in our study.

Satiety effects and body weight regulation could also be related to water ingestion as suggested by human studies (Davy et al. 2008; Popkin et al. 2006; Stookey et al. 2008). In the present study, we observed a significant increase in water intake of mice fed the HP diet, which was almost doubled compared with the AP group. Most interestingly, overall water intake was very strongly correlated with dietary nitrogen intake. Metabolic adaptation to dietary high protein and thus to high amino nitrogen intake involves an increase in amino acid oxidation since amino acids supplied above metabolic requirement cannot be stored (Jean et al. 2001; Petzke et al. 2000). The resulting excessive amino nitrogen has to be excreted via urine. Excess nitrogen is mainly excreted in terms of urea, which is mainly produced by the liver in the urea cycle. Although it has been suggested that the rate of urea synthesis is relatively constant over a wide range of protein intake (Jackson 1998), other studies have shown a parallel change of urea production and secretion with the level of ingested protein in healthy adults (Forslund et al. 1998; Young et al. 2000). This is in line with our study. Plasma urea concentrations were significantly increased in mice fed HP diets and further positively correlated with amino nitrogen intake. For the urinary excretion and filtration of urea, water is needed (Bankir et al. 1996; Seney et al. 1987). It was shown that an increased protein intake in pigs is accompanied by increased water consumption and a corresponding higher volume of excreted urine (Pfeiffer et al. 1995). In our study, a high water intake was associated with a low energy intake suggesting that an increased obligatory water intake due to increased amino nitrogen intake could be linked to the satiety effect of high-protein diets. However, our data provide correlations only which are no proof of a causal relationship. This would need to be addressed by further studies.

The role of essential amino acids, particularly BCAA in the development of insulin resistance especially in the context of a high-fat diet is currently discussed quite controversially (Adams 2011). In our short-term feeding experiment, we did not observe significant changes in plasma insulin or glucose levels. In a previous long term feeding study we also did not observe any detrimental effects of HP or leucine supplementation on glucose tolerance and insulin resistance which is in line with the conclusion by Adams (2011) that there is overall little evidence that protein-rich diets negatively affect glucose homeostasis.

Several recent studies focussed on the effects of high-protein diets on the preservation of LBM exploring the role of branched chain amino acids in stimulating muscle protein synthesis (Drummond and Rasmussen 2008; Suryawan et al. 2011; Wilson et al. 2011). Here, we could not detect any differences in lean mass as well as weights of *m. quadriceps* (data not shown) between the different groups probably due to the short intervention period of 1 week. However, in our previous long-term study, we found an increased skeletal muscle protein synthesis as assessed by ^{15}N-lysine incorporation after 20 weeks of feeding a high-fat diet with either high-protein content or supplemented with L-leucine compared with controls (Freudenberg et al. 2012). In the same study we observed pronounced effects of the high-protein diet on gene expression of lipogenesis-related enzymes in liver as well as lipases in WAT indicative of decreased hepatic lipogenesis and increased WAT lipolysis. Our present study shows that already after 1 week there was a strong down-regulation of liver lipogenesis genes by HP and to a lesser extent by L-leucine and L-alanine supplementation, suggesting that this is the primary physiological mechanism limiting liver as well as body fat accretion. On the other hand, gene expression in white fat was only little affected after 1 week. There were no changes in lipogenic genes and the only significant effect was an up-regulation of adipose triglyceride lipase (ATGL) by HP feeding. ATGL

is the first, rate limiting enzyme in TG lipolysis and it is known to be inhibited by insulin and induced by fasting in adipose tissue (Ahmadian et al. 2010). This is in line with the strongly reduced energy and also carbohydrate intake of the HP-fed mice compared with the AP group.

It was unexpected that an equimolar substitution of L-leucine by L-alanine had the same effect as L-leucine on all measured parameters as shown in the present study. Alanine and leucine have very different functions in metabolism (Wu 2009). Alanine is an important substrate for hepatic gluconeogenesis and one of the main amino acids released by skeletal muscle. Dietary alanine is largely extracted by the splanchnic bed (Felig 1973). The catabolism of dietary branched chain amino acids, on the other hand, requires previous transamination in skeletal muscle following the oxidation of the resulting ketoacids. During fasting, plasma levels of alanine are decreased, whereas those of leucine are increased (Nair et al. 1983). Consistent with the different metabolic functions, we observed a similar increase in circulating leucine concentrations by HP feeding and L-leucine supplementation, whereas circulating alanine concentrations were not affected by any of the diets. Our results suggest that changes in energy and substrate metabolism induced by high-protein diets are not related to specific effects of any one amino acid such as leucine, but rather a general result of an increased protein, i.e. amino nitrogen consumption.

In conclusion, the present study shows that acute metabolic effects of high-protein as well as amino acid supplemented diets are strongly related to their effects on satiation and on the suppression of liver lipogenesis. Interestingly, energy intake was linked to amino nitrogen intake which in turn strongly correlated with water intake. The latter observation could be an interesting starting point for mechanistic explanations of the effectiveness of high-protein diets in weight loss and weight maintenance since they automatically increase amino nitrogen intake. Furthermore, we found no evidence for a specific effect of the branched chain amino acid leucine because dietary L-leucine as well as equimolar L-alanine supplementation had the same effects which we suggest to result from the increase in amino nitrogen content of both diets. However, additional long-term studies using different types and amounts of dietary amino acid supplementations are needed for confirmation. Our data also imply that future studies investigating specific metabolic effects of a single amino acid in vivo should include additional isonitrogenous controls in order to delineate molecular mechanisms which are specific for one particular amino acid.

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