

Hydrolyzed Casein Reduces Diet-Induced Obesity in Male C57BL/6J Mice^{1–3}

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Abstract

The digestion rate of dietary protein is a regulating factor for postprandial metabolism both in humans and animal models. However, few data exist about the habitual consumption of proteins with different digestion rates with regard to the development of body mass and diet-induced obesity. Here, we used a factorial ANOVA design to investigate the effects of protein form (intact vs. hydrolyzed casein) and protein level (16 vs. 32 energy percent protein) on body mass gain and adiposity in obesity-prone male C57BL/6J mice fed Western diets with 35 energy percent fat. Mice fed the hydrolyzed casein diets had higher spontaneous locomotor activity than mice fed intact casein. During the light phase, mice fed hydrolyzed casein tended ($P = 0.08$) to have a lower respiratory exchange ratio, indicating lower utilization of carbohydrates as energy substrate relative to those fed intact casein. In further support of less carbohydrate oxidation, plasma concentrations of glucose and those of the glucose metabolite lactate were lower in fed mice that consumed the hydrolyzed compared with the intact casein diet. Concomitantly, the plasma insulin concentration was strongly reduced in fed mice given hydrolyzed casein relative to those given intact casein. The mice fed hydrolyzed casein had greater ex vivo inguinal white adipose tissue non- CO_2 β -oxidation capacity along with induced expression of genes involved in mitochondrial fatty acid oxidation and mitochondrial uncoupling. The physiological changes induced by hydrolyzed casein ingestion translated into decreased body and adipose tissue masses. We conclude that chronic consumption of extensively hydrolyzed casein reduces body mass gain and diet-induced obesity in male C57BL/6J mice. J. Nutr. 143: 1367–1375, 2013.

Introduction

The worldwide increment in body weight is of an alarming character. Between 1980 and 2008, the mean BMI worldwide was estimated to have increased by 0.4 and 0.5 kg/m^2 per decade for men and women, respectively (1). The increase in BMI is caused by a positive energy balance over time. Therefore, elucidating dietary impact on energy metabolism is crucial. Dietary protein has been shown to increase postprandial thermogenesis, i.e., convert more of the ingested food energy to heat, relative to carbohydrates in humans (2). The reduced energy efficiency, or the reduced ability to use ingested energy for growth, by intake of dietary proteins has been verified in humans in a cross-

sectional epidemiological study (3), in a controlled intervention study (4), in studies with caloric restriction (5–7), in studies with overeating participants (8,9), and in a weight-maintenance study following weight reduction (10). Therefore, intake of dietary protein might be beneficial to prevent or reduce body weight gain.

Data on the ability of different protein sources to reduce the metabolic efficiency is limited. However, postprandial digestion rate is an independent factor modulating protein retention (11–13). Intake of soluble milk proteins with a high intestinal absorption rate increases the deamination of the ingested amino acids, leading to a higher urinary urea excretion compared with micellar casein or total milk proteins (14). Thus, ingestion of dietary proteins with a rapid amino acid delivery might induce a lower net energy accretion compared with expenditure in the postprandial period (14). Over time, a reduced energy accretion might translate into reduced body weight gain.

In addition to varying intestinal kinetics of amino acid delivery in intact milk proteins (11,12,14), ingestion of extensively hydrolyzed casein induces increased gastric emptying, higher amino acid absorption rates, and elevated plasma glucose-dependent insulinotropic polypeptide compared with ingestion of an equal amount

¹ Supported by the National Institute of Nutrition and Seafood Research and by grants from The Danish Dairy Research Foundation and The Danish Council for Strategic Research, grant no. 2101-08-0053. This work was also supported by the EU FP7 project DIABAT, HEALTH-F2-2011-278373.

² Author disclosures: H. H. Lillefosse, H. S. Tastesen, Z.-Y. Du, D. B. Ditlev, F. A. Thorsen, L. Madsen, K. Kristiansen, and B. Liase, no conflicts of interest.

³ Supplemental Tables 1 and 2 and Supplemental Figure 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

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of intact casein (15). Moreover, in a study with young adults receiving a semi-synthetic meal with 23 g fat, 96 g carbohydrates, and 28.5 g protein either as casein or hydrolyzed casein, the hydrolyzed casein meal favored a higher splanchnic nitrogen utilization but a lower peripheral amino acid anabolism (16). In a crossover design study with elderly men, a single bolus of 35 g hydrolyzed casein induced accelerated protein digestion and absorption from the gut, a transient increase in plasma amino acid concentrations, and a tendency to increase the incorporation rate of amino acids into skeletal muscle protein relative to ingestion of casein (17). Thus, relative to intact casein, ingestion of hydrolyzed casein implies a different nitrogen utilization in human beings that possibly could modulate body weight gain.

All of the above-mentioned studies with casein (11–17) were performed as single meal tests. To the best of our knowledge, no published data exist on longitudinal, controlled intervention studies with ingestion of extensively hydrolyzed casein in adult human participants. However, extensively hydrolyzed casein is used in infant formulas for the prevention of allergy and food intolerance. In fact, 3 independent studies report decreased BMI due to lower body weight gain in the period of exclusive formula feeding with hydrolyzed casein-based formulas relative to those given intact casein-based formulas (18–20). Importantly, there was no decrease in length growth in those studies (18–20). Hence, studies with infants indicate that habitual intake of extensively hydrolyzed casein could be associated with a reduced body mass gain.

Based on the findings that ingestion of extensively hydrolyzed casein alters postprandial metabolism in single meal test studies with adults (15–17) and in feeding studies with rats (21) and pigs (22), as well as the findings that habitual intake of extensively hydrolyzed casein was associated with reduced body mass in infants (18–20), we hypothesized that intake of diets with extensively hydrolyzed casein would prevent or decrease body mass gain. To test this hypothesis, we fed mice diets with intact or extensively hydrolyzed casein.

Materials and Methods

Ethical statement. The animal experiments were approved by the National Animal Health Authorities (Norwegian approval identification: 1062, 1840, and 2500). Adverse events were not observed.

Mice. Data in this paper are based on the results from 4 animal studies (Expts. 1–4). In Expts. 1–3, one group of mice was fed a low-fat diet (D12450B, Research Diets) (Supplemental Table 1). These mice were used as a reference only and data from these mice are not included in the statistical analyses.

In all studies, male C57BL/6JBomTac mice (Taconic) 8 wk of age were assigned by weight to the experimental diets with hydrolyzed (H) or intact (I) casein at 16 energy percent protein to mimic a contemporary Western diet (23), with 16, 49, and 35 energy percent from protein, carbohydrate, and fat, respectively, or with 32 energy percent protein and 33 and 35 energy percent from carbohydrate and fat, respectively. The experimental diets were designated 16 energy percent protein, intact casein (I-16)⁹, 16 energy percent protein, hydrolyzed casein (H-16), 32 energy percent protein, intact casein (I-32), or 32 energy percent protein, hydrolyzed casein (H-32) (Supplemental Table 1). The amino acid content in the diets was balanced. The mice were

individually housed and maintained on a 12-h-light/dark cycle at thermo-neutrality, $28 \pm 1^\circ\text{C}$. To ensure equal energy intake, the mice were pair-fed. The mice were fed daily and body weight was recorded twice per week. At the end of the studies, mice were anesthetized with isoflurane (Isoba Vet) and killed by cardiac puncture.

Expt. 1 consisted of 38 mice ($n = 8/\text{group}$, except low-fat diet reference, $n = 6$). After 3 wk of feeding, the mice spent 48 h in wire-bottom cages for feces collection. After 8 wk of feeding, mice were killed during feed access, blood was collected for plasma separation, and tissues were harvested and weighed. A part of the liver, inguinal white adipose tissue (iWAT), soleus, and interscapular brown adipose tissue (iBAT) tissues were homogenized for non- CO_2 β -oxidation capacity measurements. Samples were taken from the epididymal white adipose tissue (eWAT) and iWAT tissues and fixed for histology. The rest of the liver, eWAT, iWAT, soleus, tibialis anterior, and iBAT tissues were freeze-clamped in liquid nitrogen and stored at -80°C . Samples from this study were used for plasma measurements and tissue qPCR analyses from fed mice.

Expt. 2 consisted of 35 mice ($n = 7/\text{group}$). After 7 wk of feeding, one representative mouse from each group was subjected to MRI.

Expt. 3 consisted of 49 mice ($n = 9\text{--}10/\text{group}$). After 7 wk of feeding, a glucose tolerance test was performed. After 8 wk of feeding, the mice were killed. Blood was collected from mice feed deprived overnight and plasma separated. Plasma from this study was used to analyze plasma metabolites and hormones from feed-deprived mice.

Expt. 4 consisted of 16 mice ($n = 4/\text{group}$). Mice were fed for 4 wk, body composition was analyzed at the start and at the end of the feeding period, and indirect calorimetric measurements were started after 3 wk of feeding.

Body composition by quantitative magnetic resonance (QMR). In Expt. 4, whole-body fat and lean tissue masses were determined using the EchoMRI quantitative magnetic resonance whole-body composition analyzer (Echo Medical Systems).

Indirect calorimetry and spontaneous locomotor activity. In Expt. 4, O_2 consumption, CO_2 production and spontaneous locomotor activity of the mice were measured in the PhenoMaster open-circuit indirect calorimetry system (TSE Systems) using CaloCages fitted with infrared light-beam frames (ActiMot2). Indirect calorimetric calculations and spontaneous locomotor activity counts were made based on 3 consecutive light periods (0730–1900 h) and 3 dark periods (1930–0700 h). Energy expenditure [$\text{kJ}/(\text{h} \cdot \text{kg body weight})$] was calculated from the equation; $16.3 \text{ L O}_2 \text{ used} + 4.6 \text{ L CO}_2 \text{ produced}$, as described in details elsewhere (24).

Gross energy in diets. The energy contents were determined in a bomb calorimeter following the manufacturer's instruction (Parr Instruments).

MRI. In Expt. 2, one representative mouse from each group was anesthetized with isoflurane (Isoba Vet). The adipose tissues in the abdominal region of the mice were visualized on a 7-Tesla Bruker Pharmascan small animal Magnetic Resonance scanner (Bruker Biospin MRI). The MRI was performed at the Molecular Imaging Center, Department of Biomedicine, University of Bergen. Axial and coronal T1 weighted, whole-body scans were acquired with the following scan parameters: repetition time, 1300 ms; echo time, 7.5 ms, 6 acquisitions, 256×256 scan matrix; field of view, 4.0 cm; slice thickness, 1.0 mm; interslice distance, 1.0 mm, 12 slices; scan time, 6 min 14 s.

Histology. In Expt. 1, sections of adipose tissue were fixed in 4% formaldehyde in 0.1 mol/L phosphate buffer overnight at 4°C , washed in 0.1 mol/L phosphate buffer, dehydrated in ethanol, and embedded in paraffin after clearing with xylene. Then 5- μm -thick sections of the embedded tissue were stained with eosin and hematoxylin.

Plasma measurements. In Expts. 1 and 3, heparin plasma was prepared from blood collected at killing and stored at -80°C prior to analysis. Leptin (BioVendor) and insulin (DRG Diagnostics) concentrations were analyzed by ELISA kits. Glucagon (Millipore) and adiponectin (LINCO Research) were measured by RIA kits.

⁹ Abbreviations used: eWAT, epididymal white adipose tissue; H-16, 16 energy percent protein, hydrolyzed casein; H-32, 32 energy percent protein, hydrolyzed casein; I-16, 16 energy percent protein, intact casein; I-32, 32 energy percent protein, intact casein; iBAT, interscapular brown adipose tissue; iWAT, inguinal white adipose tissue; OH-butyrate, β -hydroxybutyrate; RER, respiratory exchange ratio; WAT, white adipose tissue.

Plasma β -hydroxybutyrate (OH-butyrate; Randox), TG, FFA, glucose (all DIALAB), and lactate (Sentinel Diagnostics) concentrations were analyzed by conventional enzymatic kits using a MaxMat PL II (MAXMAT).

Non- CO_2 β -oxidation capacity measurements. In Expt. 1, one part of tissue from iWAT and iBAT was homogenized in 5 parts (wt:v) of glycyl-glycine homogenization buffer (25). One part of liver tissue was homogenized in 9 parts (wt:v) homogenization buffer (200 mmol/L mannitol, 50 mmol/L sucrose, 10 mmol/L HEPES-KOH, 1 mmol/L EDTA, 0.1% fatty acid-free BSA, pH 7.4) and one part muscle (soleus) was homogenized in 19 parts (wt:v) homogenization buffer (250 mmol/L sucrose, 10 mmol/L Tris-HCl, 2 mmol/L EDTA, 2 mmol/L taurine, 50 U/mL heparin, pH 7.4). Non- CO_2 palmitate oxidation capacity was measured in the aqueous fraction (iWAT, after centrifugation at $1000 \times g$ for 5 min) or whole homogenates (liver, iBAT, and muscle) by the acid-soluble product method (26). In brief, $[1\text{-}^{14}\text{C}]$ palmitate oxidation by tissue homogenates was performed at 37°C using a medium containing a final concentrations of 13.3 mmol/L HEPES, 83.3 mmol/L KCl, 16.7 mmol/L MgCl_2 , 0.7 mmol/L EDTA, 1.0 mmol/L L-carnitine, 0.4 mmol/L CoASH, 2.0 mmol/L ATP, 0.5 mmol/L L-malate, and 25 mmol/L cytochrome c. Then 40 μL of tissue homogenate was added to each vial containing 0.31 mL reaction medium. After 2 min of preincubation, the reaction was initiated by the addition of 0.15 mL $[1\text{-}^{14}\text{C}]$ palmitate (1.5 Ci/mol, at 100 $\mu\text{mol/L}$ of final concentration) as potassium salt bound to BSA in a 2:1 molar ratio. After incubation for 15 min, the reaction was stopped by the addition of 1.5 mL of 10% perchloric acid. Non- CO_2 palmitate oxidation capacity was normalized to the total protein content in the homogenates, measured using the BCA protein Assay Reagent kit (Pierce).

qRT-PCR. In Expt. 1, total RNA was purified from samples with TRIzol Reagent (Invitrogen) and the quality was assessed with the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies) and the Agilent 2100 Bioanalyzer (Agilent Technologies). A 50- μL RT (0.5 μg total RNA) reaction was performed at 48°C for 60 min utilizing a GeneAmp PCR 9700 machine (Applied Biosystems). Individual RT reactions contained $1 \times$ TaqMan RT buffer ($10\times$), 5.5 mmol/L MgCl_2 , 500 $\mu\text{mol/L}$ dNTP (of each), oligo(dT) primers (2.5 $\mu\text{mol/L}$), 0.4 U/ μL RNase inhibitor, 1.25 U/ μL Multiscribe Reverse Transcriptase (N808-0234, Applied Biosystems), and RNase-free water. qRT-PCR was run in 10- μL reactions on a LightCycler 480 Real-Time PCR System (Roche Applied Sciences) containing 2.0 μL cDNA and using SYBR Green Master Mix (Light Cyler 480 SYBR Green master mix kit, Roche Applied Sciences) and gene-specific primers designed using Primer Express 2.0 (Applied Biosystems) (Supplemental Table 2). GeNorm was used to determine the most suitable normalization gene for each tissue [TATA box binding protein (*Tbp*): eWAT, liver, and soleus; and β -actin (*Actb*): iBAT and iWAT].

Glucose tolerance test. In Expt. 3, after 7 wk of receiving the experimental diets, mice were feed deprived for 12 h before intraperitoneal injection of 2 g glucose/kg body weight. Blood was collected from the saphenous vein and blood glucose was measured with a Bayer Contour glucometer (Bayer) before and 15, 30, 60, and 120 min after glucose injection.

Statistical analyses. All data represent means $\pm$ SEs. All data sets were tested for homogeneity of variances using the Levene's test. Data for growth curves were analyzed with repeated-measures ANOVA with body mass in weeks 0–8 as the dependent variable and protein level and protein form as categorical predictors. Repeated measures (within-subject effects) were defined as body mass at the 9 different times of weighing (weeks 0–8). The rest of the data were analyzed using a factorial ANOVA test with protein level and protein form as categorical predictors. In the case of an interaction effect between protein level and protein form, a post-hoc Fisher least significant difference test was performed. Data with heterogeneous variances were log-transformed before statistical analyses. $P < 0.05$ was considered significant. Statistics were performed with STATISTICA 9.0 (StatSoft).

Results

Reduced body mass gain with hydrolyzed casein ingestion (Expt. 1). After 8 wk of feeding, the body mass gain of mice fed hydrolyzed casein was significantly lower than that of the mice fed intact casein (Fig. 1A,B). Because there was no difference in the accumulated energy intake between experimental groups (3.28 ± 0.06 MJ/8 wk), the mice fed intact casein had significantly higher energy efficiency (body weight gain per energy intake) than the mice fed hydrolyzed casein. Yet, no significant difference in tissue weight was found for liver or skeletal muscles, soleus, and tibialis anterior (Table 1). Moreover, no significant difference was found in fecal fat concentration, even though mice fed hydrolyzed casein (42 ± 4 mg fat/g feces) tended ($P = 0.09$) to have a greater fecal fat concentration compared with those fed intact casein (34 ± 2 mg fat/g feces). Thus, we cannot exclude the possibility that a somewhat higher fecal fat excretion, particularly in the H-32-fed mice, might have contributed to their reduced body mass and lean phenotype.

Increased spontaneous locomotor activity and altered body composition with hydrolyzed casein ingestion. Another set of mice was fed the experimental diets for 4 wk (Expt. 4). Again, at equal energy intake, hydrolyzed casein feeding resulted in less body mass gain relative to intact casein feeding. The body mass gain was less in the mice fed 32 energy percent protein compared with those fed 16 energy percent protein irrespective of casein type (Fig. 1C). The reduction in body mass gain by hydrolyzed casein feeding was due to both reduced fat and lean mass (Fig. 1D). During this shorter feeding trial (4 wk), the H-32 fed mice actually lost body mass, which was caused by a loss of lean mass (Fig. 1C,D). In the previous 8-wk feeding trial (Expt. 1), the mice did not experience reduced body mass loss (Fig. 1A,B) or lower lean tissue masses (Table 1); the negative body mass gain was therefore likely to be a short-term effect. Interestingly, the hydrolyzed casein-fed mice had higher spontaneous locomotor activity (Expt. 4) during both the light and dark periods (Fig. 1E). Despite the lower body mass gain, the altered body composition, and the greater spontaneous locomotor activity induced by hydrolyzed casein ingestion, no difference was found in energy expenditure during either the light [42.6 ± 0.5 kJ/(h $\cdot$ kg body weight)] or dark periods [61.0 ± 0.7 kJ/(h $\cdot$ kg body weight)]. However, the respiratory exchange ratio (RER; CO_2 exhaled/ O_2 inhaled) tended ($P = 0.08$) to be lower during the light periods in mice fed hydrolyzed casein compared with those fed intact casein. In the dark periods, ingestion of the higher amount of protein caused reduced RER (Fig. 1F). The plasma concentrations of glucose and the glucose metabolite lactate were lower in fed mice (Expt. 1) that consumed hydrolyzed compared with intact casein (Table 2). The plasma concentration of OH-butyrate was higher in fed mice (Expt. 1) given hydrolyzed compared with intact casein (Table 2). No significant difference between dietary treatments (Expt. 1) was found for the plasma concentration of TG or FFAs in fed mice (Table 2). Moreover, no difference in plasma concentrations in any of these variables were found in plasma from mice (Expt. 3) deprived of feed overnight (Table 2).

Reduced adipose tissue masses in mice fed hydrolyzed casein. In agreement with the reduced body fat mass measured after 4 wk of hydrolyzed casein feeding (Fig. 1D), MRI in another set of mice (Expt. 2) revealed that the reduced adiposity by hydrolyzed casein feeding was still present after 7 wk (Fig. 2A). At termination after 8 wk of feeding (Expt. 1), the masses of

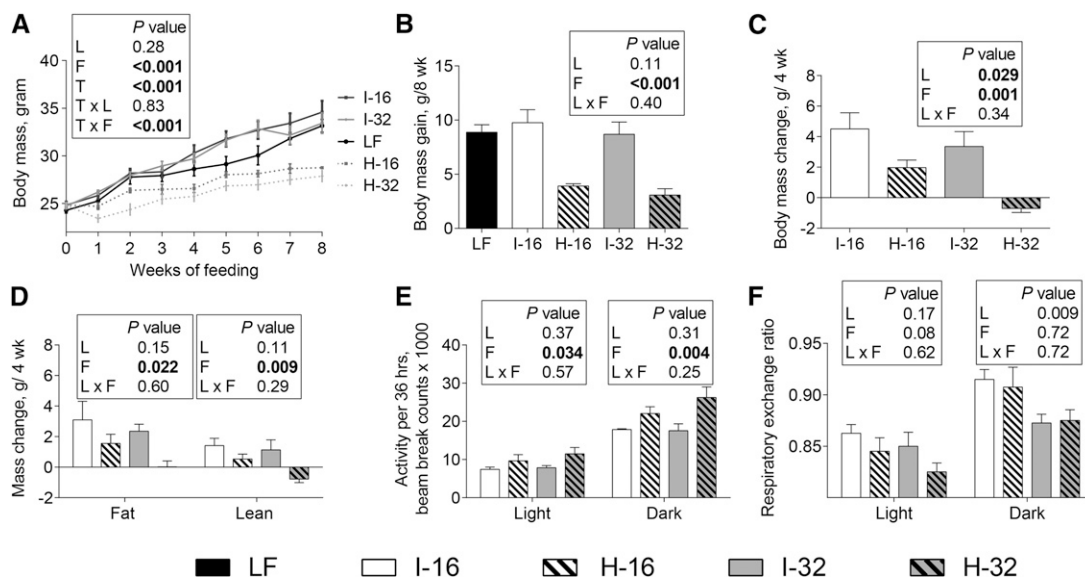


FIGURE 1 Body mass (A, Expt. 1), changes in body mass gain (B, Expt. 1; C, Expt. 4), and change in fat and lean mass (D, Expt. 4), activity (E, Expt. 4), and the RER (F, Expt. 4) in mice fed a low-fat diet or diets containing 16% or 32% of energy as intact or hydrolyzed casein for 8 wk (A,B) or 4 wk (C-F). Values are means $\pm$ SEs, $n = 6-8$ (A,B) or 4 (C-F). Statistics in B performed on log-transformed data. F, protein form; H-16, 16 energy percent protein, hydrolyzed casein; H-32, 32 energy percent protein, hydrolyzed casein; I-16, 16 energy percent protein, intact casein; I-32, 32 energy percent protein, intact casein; L, protein level; LF, low-fat diet; RER, respiratory exchange ratio; T, time.

eWAT and iWAT (Table 3) and also that of iBAT (Table 2) were all significantly reduced in the mice fed hydrolyzed casein compared with the mice fed intact casein. Lean tissues such as liver and skeletal muscles (soleus and tibialis anterior) did not differ in weight after 8 wk (Expt. 1) of feeding (Table 1). Thus, the small reduction in body lean mass measured after 4 wk (Expt. 4) of hydrolyzed casein feeding (Fig. 1D) was not present in lean tissue masses after 8 wk (Expt. 1) of feeding. Histology of eWAT and iWAT samples (Expt. 1) showed that the mice fed hydrolyzed casein had smaller adipocytes, which was particularly evident in iWAT (Fig. 2B). As expected, both plasma leptin concentrations and white adipose tissue (WAT) leptin (*Lep*) mRNA levels reflected the WAT masses, with lower levels in the mice fed hydrolyzed casein (Table 3). The plasma adiponectin concentration and WAT adiponectin (*Adipoq*) mRNA levels, on the other hand, were not affected by WAT accumulation, but the plasma adiponectin concentration was lower in the mice receiving 32 energy percent protein compared with those fed 16 energy percent protein irrespective of the type of casein (Table 3).

Increased iWAT non- CO_2 β -oxidation capacity in mice fed hydrolyzed casein (Expt. 1). The liver and soleus ex vivo non- CO_2 β -oxidation capacity did not differ between dietary treatments (Table 1). Moreover, the hepatic expression of genes involved in fatty acid oxidation, i.e., the mitochondrial carnitine palmitoyltransferase 1a (*Cpt1a*) and medium-chain acyl-CoA dehydrogenase (*Mcad*) or the peroxisomal acyl-CoA oxidase 1 (*Acox1*), did not differ between treatment groups (Table 1). In soleus, the expression of genes encoding mitochondrial fatty acid oxidation enzymes was reduced (*Cpt1b*), tended ($P = 0.053$) to be lower (*Mcad*), or was unchanged [cytochrome c oxidase, subunit VIIIb (*Cox8b*)] in hydrolyzed casein-fed mice (Table 1). In iBAT, both ex vivo non- CO_2 β -oxidation capacity and uncoupling protein 1 (*Ucp1*) mRNA level were downregulated by hydrolyzed casein feeding relative to intact casein feeding (Table 1). Moreover, mice that consumed H-32 or I-32 diets had lower iBAT *Ucp1* mRNA levels than mice that consumed H-16 or I-16 diets (Table 1). Taken together, our data do not support

that hydrolyzed casein feeding induced higher fatty acid oxidation capacity in liver, soleus muscle, or iBAT.

In contrast, mice fed the H-16 and H-32 diets had significantly higher ex vivo non- CO_2 β -oxidation capacity in iWAT (Fig. 3A) and also significantly increased mRNA levels of *Ucp1*, *Cox8b*, and myelin protein zero-like 2 (*Mpzl2*), a gene marker of brown adipose tissue that is also known as Eva, in this tissue (Fig. 3B-D). In addition, gene expression of the thyroid activating enzyme deiodinase, iodothyronine, type II (*Dio2*) tended ($P = 0.06$) to be induced by hydrolyzed casein feeding (Fig. 3E). We also measured the FFA concentration in the iWAT homogenates that were used to measure ex vivo non- CO_2 β -oxidation capacity and found that it was unaltered in homogenates from mice fed the different experimental diets (Fig. 3F). Thus, our data suggest that mice fed hydrolyzed casein had induced fatty acid oxidation capacity and uncoupling in iWAT.

Reduced plasma insulin concentrations in mice fed hydrolyzed casein. Mice fed hydrolyzed casein tended ($P = 0.08$) to exhibit lower RER during light periods (Expt. 4), indicating less use of carbohydrates as an energy source (Fig. 1F). Concomitantly, plasma glucose and lactate concentrations (Expt. 1) were lower in mice fed hydrolyzed casein diets (Table 2). In agreement with the lower plasma glucose, the plasma insulin concentration (Expt. 1) was also reduced in mice fed hydrolyzed casein diets (Table 2). The plasma concentration of glucagon, a hormone that contributes to raise blood glucose, was also lower (Expt. 1) in fed mice hydrolyzed casein diets (Table 2). Despite the low plasma insulin concentration in mice fed hydrolyzed casein diets, these mice showed no signs of reduced glucose tolerance (Expt. 3) (Supplemental Fig. 1).

Discussion

We tested and substantiated the hypothesis that chronic consumption of extensively hydrolyzed casein given in a Western-type diet would prevent body mass gain and diet-induced obesity in male C57BL/6J mice. Our data from mice are in agreement

TABLE 1 Liver, soleus, tibialis anterior, and iBAT tissue masses, ex vivo non-CO₂ palmitate-oxidation capacities, and relative gene expressions from mice fed diets containing 16% or 32% of energy as intact or hydrolyzed casein for 8 wk (Expt. 1)¹

	Diet				P value		
	I-16	H-16	I-32	H-32	L	F	L x F
Tissue mass							
Liver, ² g	1.42 ± 0.08	1.34 ± 0.03	1.44 ± 0.05	1.42 ± 0.06	0.40	0.50	0.79
Soleus, mg	26 ± 1.1	23 ± 0.7	25 ± 1.3	26 ± 1.0	0.32	0.61	0.052
Tibialis anterior, ² mg	113 ± 6	108 ± 3	113 ± 3	116 ± 4	0.43	0.79	0.42
iBAT, ² mg	99 ± 11	68 ± 3.2	90 ± 7.7	70 ± 4.9	0.62	<0.001	0.50
Liver							
β-oxidation, nmol 1- ¹⁴ C-palmitate carboxyl carbon to ASP/(min · mg protein)	3.0 ± 0.2	3.5 ± 0.2	3.0 ± 0.2	3.3 ± 0.1	0.38	0.46	0.81
Relative mRNA expression							
<i>Cpt1a</i>	1.0 ± 0.2	1.3 ± 0.3	1.3 ± 0.1	1.3 ± 0.1	0.32	0.25	0.91
<i>Mcad</i>	94 ± 11	87 ± 11	113 ± 13	89 ± 8	0.37	0.20	0.46
<i>Acox1</i>	2.3 ± 0.4	2.6 ± 0.4	3.6 ± 0.4	2.7 ± 0.5	0.056	0.59	0.25
Soleus							
β-oxidation, nmol 1- ¹⁴ C-palmitate carboxyl carbon to ASP/(min · mg protein)	2.5 ± 0.4	3.0 ± 0.4	2.6 ± 0.4	2.1 ± 0.3	0.27	0.87	0.16
Relative mRNA expression							
<i>Cpt1b</i>	12 ± 0.8	10 ± 1.0	12 ± 0.6	10 ± 0.5	0.68	0.013	0.87
<i>Mcad</i> ²	165 ± 21	134 ± 6	170 ± 10	147 ± 7	0.31	0.053	0.85
<i>Cox8b</i>	640 ± 82	814 ± 122	774 ± 38	625 ± 86	0.50	0.71	0.85
iBAT							
β-oxidation, nmol 1- ¹⁴ C-palmitate carboxyl carbon to ASP/(min · mg protein)	6.4 ± 0.7	4.5 ± 0.3	5.8 ± 0.4	4.9 ± 0.2	0.84	0.002	0.25
Relative mRNA expression							
<i>Mcad</i>	35 ± 7	30 ± 4	29 ± 3	29 ± 6	0.57	0.69	0.62
<i>Cox8b</i>	219 ± 43	193 ± 38	193 ± 39	147 ± 27	0.35	0.36	0.80
<i>Ucp1</i>	76 ± 10	43 ± 6	50 ± 8	25 ± 5	0.012	0.001	0.49

¹ Values are means ± SEs, *n* = 6–8. ASP, acid-soluble products; F, protein form; H-16, 16 energy percent protein, hydrolyzed casein; H-32, 32 energy percent protein, hydrolyzed casein; I-16, 16 energy percent protein, intact casein; I-32, 32 energy percent protein, intact casein; iBAT, interscapular brown adipose tissue; L, protein level.

² Statistics on log-transformed data.

with the reduced body weight gain that was previously observed in infants consuming extensively hydrolyzed casein-based formulas (18–20). The underlying mechanisms for the infants' reduced body weight gain is not yet fully elucidated, even though some studies indicate that infants fed the hydrolyzed casein-based formula consume less energy due to a greater satiating effect (19,27). In the present study in mice, we observed reduced body weight gain and adiposity at equal energy intake, which is in agreement with data from the infant study reported by Giovannini et al. (18). Therefore, it is likely that another mechanism than satiety could also contribute to the body weight-reducing effect of hydrolyzed casein ingestion.

Mice fed the hydrolyzed casein diets had higher spontaneous locomotor activity after 3 wk of the experimental diets during both the light and dark periods. Despite this, we were unable to detect differences in energy expenditure quantified by indirect calorimetric measurements. However, the mice that consumed hydrolyzed casein diets tended to have a lower RER during the light periods, indicating that these mice used less carbohydrate as an energy substrate. This notion was further supported by lower plasma concentrations of glucose and lactate in mice fed hydrolyzed relative to intact casein diets. During the light periods, the RER approached 0.83 for mice fed the hydrolyzed casein diets. A RER value <1 could be due to higher whole-body long-chain fatty acid oxidation, or it could be due to higher amino acid oxidation, which would drive RER toward 0.8 (24). We did not measure higher non-CO₂ palmitate oxidation

capacity in iBAT, skeletal muscle, or liver. Moreover, plasma from mice fed hydrolyzed casein did not have a higher concentration of FFA. Thus, it is likely that the mice fed hydrolyzed casein used more amino acids as their energy substrate. A higher deamination of rapidly absorbed proteins would be in agreement with previous observations in humans (14). A higher deamination of ketogenic amino acids would also explain the higher plasma concentrations of OH-butyrate in mice fed hydrolyzed relative to intact casein diets.

In the present study, the plasma insulin concentrations were lower in mice fed hydrolyzed compared with intact casein. Dietary modulation of insulin signaling is important in the development of obesity (28,29). Insulin concentrations normally rise after feeding, promoting anabolic processes in the body such as protein and glycogen synthesis and lipid storage (30). Inhibition of insulin signaling in adipose tissue by fat-specific knockout of the insulin receptor protects against obesity in mice (31), whereas overexpression of the insulin receptor substrate (IRS) increases adiposity in mice (32). We previously showed in mice that the obesogenic potential of high-fat diets is related to elevated plasma insulin concentrations (33–35). In humans, weight gain has been observed in diabetic patients when they started on insulin treatment (36). In keeping with insulin's ability to promote body weight gain, it is likely that the reduced plasma insulin concentrations could contribute to the reduced body weight gain and adiposity in mice fed the hydrolyzed casein diets.

TABLE 2 Plasma metabolite and hormone concentrations from mice fed diets containing 16% or 32% of energy as intact or hydrolyzed casein for 8 wk and terminated after overnight feed deprivation (Expt. 3) or during feed access (Expt. 1)¹

	Diet				P value		
	I-16	H-16	I-32	H-32	L	F	L x F
Feed deprivation							
Metabolites, mmol/L							
Glucose	6.3 ± 0.4	8.2 ± 0.8	7.6 ± 0.7	7.7 ± 0.6	0.53	0.14	0.18
Lactate	3.2 ± 0.3	4.0 ± 0.3	4.0 ± 0.2	3.8 ± 0.3	0.50	0.44	0.20
TG ²	0.70 ± 0.11	0.61 ± 0.09	0.57 ± 0.07	0.89 ± 0.15	0.49	0.41	0.12
FFA	0.70 ± 0.05	0.77 ± 0.06	0.75 ± 0.09	0.89 ± 0.07	0.25	0.15	0.57
OH-butyrate	2.1 ± 0.2 ^{ab}	2.0 ± 0.2 ^{ab}	1.6 ± 0.1 ^b	2.4 ± 0.1 ^a	0.85	0.11	0.031
Hormones, pmol/L							
Insulin ²	11 ± 2	23 ± 7	10 ± 2	17 ± 6	0.96	0.12	0.56
Glucagon	51 ± 3	52 ± 5	54 ± 5	45 ± 3	0.76	0.40	0.29
Feed access							
Metabolites, mmol/L							
Glucose	15.4 ± 0.6	15.0 ± 0.5	15.6 ± 0.5	13.6 ± 0.6	0.35	0.047	0.19
Lactate	6.3 ± 0.3	4.9 ± 0.4	5.2 ± 0.4	4.3 ± 0.4	0.057	0.011	0.58
TG	1.0 ± 0.2	0.7 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	0.38	0.15	0.96
FFA	0.32 ± 0.03	0.42 ± 0.06	0.32 ± 0.04	0.40 ± 0.04	0.81	0.10	0.85
OH-butyrate ²	0.11 ± 0.02	0.31 ± 0.08	0.16 ± 0.02	0.32 ± 0.03	0.087	0.005	0.82
Hormones, pmol/L							
Insulin ²	163 ± 39	31 ± 13	101 ± 25	34 ± 10	0.67	0.001	0.21
Glucagon	50 ± 6	36 ± 6	71 ± 7	32 ± 3	0.16	<0.001	0.055

¹ Data are means ± SEs, *n* = 6–10. F, protein form; H-16, 16 energy percent protein, hydrolyzed casein; H-32, 32 energy percent protein, hydrolyzed casein; I-16, 16 energy percent protein, intact casein; I-32, 32 energy percent protein, intact casein; L, protein level; OH-butyrate, β -hydroxybutyrate.

² Statistics on log-transformed data.

Brown adipose tissue is considered the major adipose tissue for dissipation of chemical energy in the form of heat (37), and the presence of brown-like adipocytes has recently been reported in adult humans (38,39). Importantly, cold exposure induces

expression of UCP1 in the brown-like adipocytes (40,41). Also in animal models, the expression of *Ucp1* can be induced in WAT under certain conditions, such as elevated intracellular cAMP (34,42–46). In keeping with the fact that insulin may

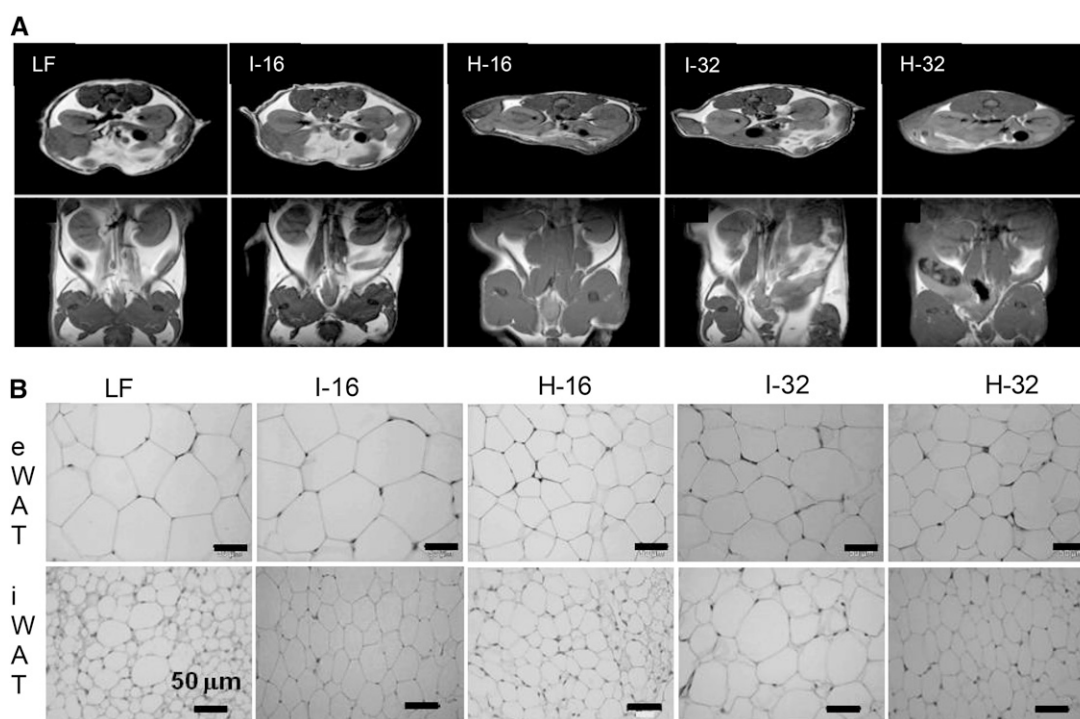


FIGURE 2 Abdominal MRI scan (A, Expt. 2) showing transverse and longitudinal pictures of the abdomen (white areas represent adipose tissue) and histology (B, Expt. 1) of eWAT and iWAT adipose depots from mice fed a low-fat diet or diets containing 16% or 32% of energy as intact or hydrolyzed casein for 7 wk (A) or 8 wk (B). eWAT, epididymal white adipose tissue; H-16, 16 energy percent protein, hydrolyzed casein; H-32, 32 energy percent protein, hydrolyzed casein; iWAT, inguinal white adipose tissue; I-16, 16 energy percent protein, intact casein; I-32, 32 energy percent protein, intact casein; LF, low-fat diet.

TABLE 3 eWAT and iWAT masses, plasma leptin and adiponectin concentrations, and relative gene expressions from fed mice consuming diets containing 16% or 32% of energy as intact or hydrolyzed casein for 8 wk (Expt. 1)¹

	Diet				P value		
	I-16	H-16	I-32	H-32	L	F	L x F
Tissue mass, g							
eWAT ²	1.35 ± 0.20	0.43 ± 0.07	1.09 ± 0.20	0.32 ± 0.04	0.12	<0.001	0.96
iWAT ²	0.49 ± 0.08	0.15 ± 0.01	0.38 ± 0.06	0.13 ± 0.02	0.19	<0.001	0.72
Plasma concentration, $\mu\text{mol/L}$							
Leptin ²	0.51 ± 0.09	0.08 ± 0.04	0.46 ± 0.09	0.013 ± 0.003	0.25	<0.001	0.25
Adiponectin ²	10 ± 0.9	10 ± 0.9	8.3 ± 0.5	8.4 ± 0.4	0.035	0.95	0.85
eWAT, relative mRNA gene expression							
<i>Lep</i> ²	96 ± 16	28 ± 7	72 ± 12	15 ± 4	0.09	<0.001	0.52
<i>Adipoq</i>	665 ± 97	766 ± 57	585 ± 79	639 ± 115	0.25	0.39	0.79
iWAT, relative mRNA gene expression							
<i>Lep</i> ²	0.46 ± 0.11	0.04 ± 0.01	0.25 ± 0.05	0.08 ± 0.02	0.97	<0.001	0.14
<i>Adipoq</i>	80 ± 9	89 ± 27	137 ± 44	214 ± 96	0.15	0.49	0.58

¹ Data are means ± SEs, $n = 5-8$. eWAT, epididymal white adipose tissue; F, protein form; H-16, 16 energy percent protein, hydrolyzed casein; H-32, 32 energy percent protein, hydrolyzed casein; I-16, 16 energy percent protein, intact casein; I-32, 32 energy percent protein, intact casein; iWAT, inguinal white adipose tissue; L, protein level.

² Statistics on log-transformed data.

lower intracellular cAMP (47,48) and that mice fed the hydrolyzed casein diets had lower plasma insulin concentrations in the present study, the gene expression pattern and the increased β -oxidation capacity in iWAT strongly suggest a higher amount of brown-like adipocytes in iWAT. Brown-like adipocytes in WAT is one of the characteristics of obesity-resistant mouse strains such as the A/J mouse (49). Importantly, hyperinsulinemia was recently shown to drive diet-induced obesity in mice, and prevention of hyperinsulinemia reprogrammed WAT to express Ucp1 and increase energy expenditure (50). Thus, in the present study, the reduced fed plasma insulin concentration was most likely one underlying factor of the

alterations observed in iWAT in the hydrolyzed casein-fed mice. Even though the observed differences in iWAT functions are insufficient to explain all the metabolic consequences of intake of hydrolyzed compared with intact casein, it is conceivable that the WAT phenotype contributed to the lower body mass gain in mice consuming hydrolyzed casein.

Whether our data obtained in mice are of relevance for ingestion of hydrolyzed casein in humans still needs to be investigated. However, our data from the mouse study resemble those from studies of infants where those given extensively hydrolyzed casein formula also exhibited reduced body mass gain relative to those given an intact casein formula (18–20).

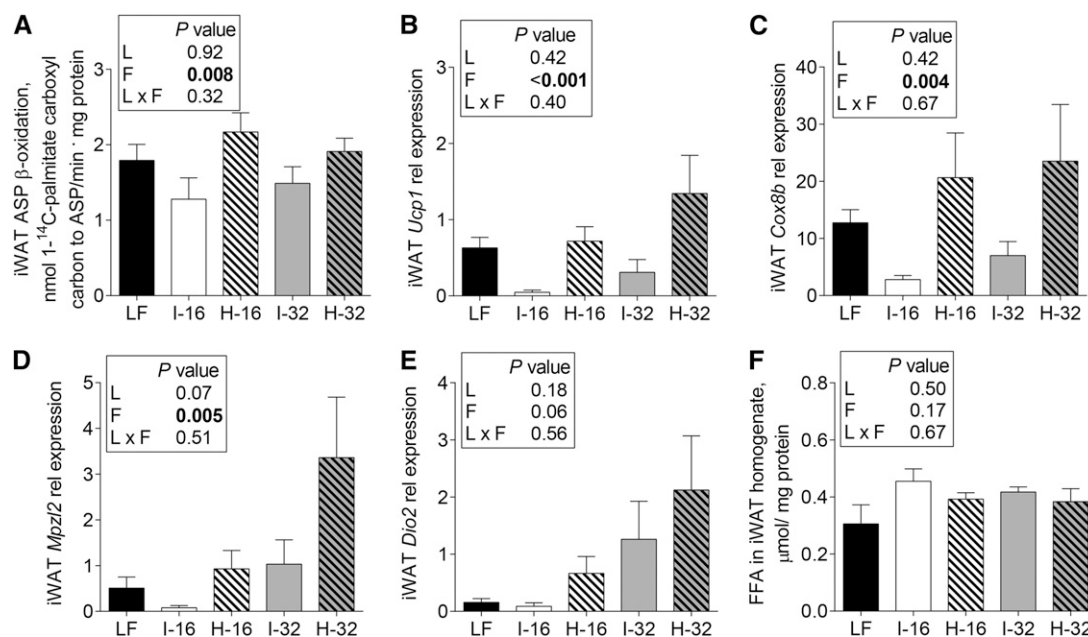


FIGURE 3 iWAT non- CO_2 ex vivo palmitate oxidation capacity (A, Expt. 1), iWAT relative gene expressions (B–E, Expt. 1), and FFAs in iWAT homogenates used for the ex vivo palmitate oxidation capacities (F, Expt. 1) in mice fed a low-fat diet or diets containing 16% or 32% of energy as intact or hydrolyzed casein for 8 wk. Values are means ± SEs, $n = 4-8$. Statistics in B–E performed on log-transformed data. ASP, acid-soluble products; F, protein form; H-16, 16 energy percent protein, hydrolyzed casein; H-32, 32 energy percent protein, hydrolyzed casein; I-16, 16 energy percent protein, intact casein; I-32, 32 energy percent protein, intact casein; iWAT, inguinal white adipose tissue; L, protein level; LF, low-fat diet.

Data on postprandial hormone concentrations from the infant studies are not available from the literature at this time. In healthy adults, single meal tests (given as solutions) containing either hydrolyzed casein alone (15,17) or in a mixture with carbohydrates and fat (16) are reported to either transiently increase postprandial plasma insulin (16,17) or not alter the plasma insulin concentration (15). The transient increase in plasma insulin is thought to be secondary to a transient increase in plasma amino acids, in particular leucine, one amino acid that can stimulate pancreatic insulin release (51–53). Further, in type 2 diabetic subjects, single meal tests with mixtures of carbohydrate and hydrolyzed casein transiently elevate plasma insulin concentration and decrease the plasma glucose concentration (54). The insulinotropic effect of hydrolyzed casein seems to be dose dependent (55).

In both healthy and type 2 diabetic adults, intake of a bolus of ~30–35 g hydrolyzed casein generally results in a postprandial, transient elevation in plasma insulin concentration. This is in contrast to our findings in the present study with mice consuming hydrolyzed casein as the sole protein source for 8 wk. However, all of the reported human studies were performed with single test meals only, which very well may not reflect the situation with repeated/habitual intake of hydrolyzed casein. Therefore, human intervention studies with intake of hydrolyzed casein over time are needed to elucidate whether the insulinotropic effects of hydrolyzed casein intake are maintained beyond single meal tests in humans.

Acknowledgments

The authors are grateful to Aase Heltveit, Øyvind Reinshol, and Jacob Wessels for technical assistance. B.L., K.K., L.M., H.H.L., and H.S.T. designed research; H.H.L., H.S.T., B.L., L.M., Z.-Y.D., D.B.D., and F.A.T. conducted research; K.K., L.M., H.S.T., H.H.L., and B.L. analyzed data and conducted statistical analysis; H.H.L. and B.L. wrote the paper; and K.K., L.M., H.H.L., and B.L. had primary responsibility for the final content. All authors read and approved the final manuscript.

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Online supporting material

Supplemental Table 1: Experimental diet composition

	LF ¹	I-16 ²	H-16 ²	I-32 ³	I-32 ³
<i>Composition</i>			(g/ kg)		
Casein ⁴	189.6	177.1	-	349.7	-
Hydrolyzed casein ⁵	-	-	177.1	-	349.7
Supplemental AA ⁶	-	4.7	14.4	9.3	28.6
L-Cystine	2.8	2.6	2.6	5.2	5.2
KCl	-	5.1	-	10.2	-
Corn starch	298.6	223.4	219.2	38.8	30.3
Maltodextrin 10	33.2	109.3	109.3	109.3	109.3
Sucrose	331.8	188.9	188.9	188.9	188.9
Cellulose	47.4	54.6	54.6	54.6	54.6
Soybean oil	23.7	27.3	27.3	27.3	27.3
Lard	19	144.3	144.3	144.3	144.3
Mineral mix S10026 ⁷	9.5	10.9	10.9	10.9	10.9
DiCalcium Phosphate	12.3	14.2	14.2	14.2	14.2
Calcium Carbonate	5.2	6.0	6.0	6.0	6.0
Potassium Citrate, 1 H ₂ O	15.6	18.0	18.0	18.0	18.0
Vitamin Mix V10001 ⁸	9.5	10.9	10.9	10.9	10.9
Choline Bitartrate	1.9	2.2	2.2	2.2	2.2
<i>Analyzed</i>			(kJ/g)		
Gross energy	not analyzed	20.5	20.3	21.5	21.2

¹The low fat control diet (LF) was bought commercially: D12450B (Research Diets, USA), and contained 20 % protein, 70 % carbohydrate and 10 % fat (% of energy), gross energy 16.1 kJ/g

²Containing 16 % protein, 49 % carbohydrate and 35 % fat (% of energy)

³Containing 32 % protein, 33 % carbohydrate and 35 % fat (% of energy)

⁴MP Biomedicals prod# 901293

⁵MP Biomedicals prod# 960138

⁶The concentration of individual amino acids was analyzed and supplemental amino acids were added to balance each amino acid in the final diets.

⁷Containing (g/kg mineral mix): sodium chloride, 259; magnesium oxide, 41.9; magnesium sulfate, 7H₂O, 257.6; ammonium molybdate, 4H₂O, 0.3; chromium potassium sulfate, 1.925; copper carbonate, 1.05; ferric citrate, 21; manganese carbonate, H₂O, 12.25; potassium iodate, 0.035; sodium fluoride, 0.2; sodium selenite, 0.035; zinc carbonate, 5.6; sucrose, 399.105

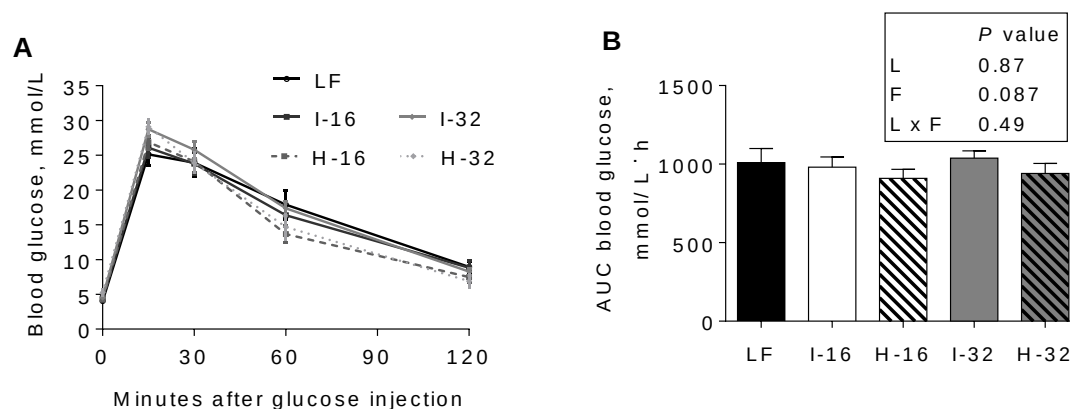
⁸Containing (g/kg vitamin mix): retinyl palmitate, 0.8; cholecalciferol, 1.0; all-rac- α -tocopheryl acetate, 10; menadione sodium bisulfite, 0.08; biotin, 2; cyanocobalamin, 1; folic acid, 0.2; nicotinic acid, 0.2; calcium panthotenate, 1.6; pyroxidine-HCl, 0.7; riboflavin, 0.6; thiamin HCl, 0.6, sucrose, 978.42

Online Supporting Material

Supplemental Table 2: Primer sequences used for real-time qPCR analyses

Abbreviation	Gene name	5'prime	3'prime
Acox1	Acyl-Coenzyme A oxidase 1	GGGTCATGGAACATCATCTTCGA	GAATGAACTCTTGGGTCTTGGG
Adipoq	Adiponectin	GATGGC AGA GATGG ACTCC	CTTGCCAGTGCTGCCGTC AT
Actb	Beta Actin	ATGGGTCAGAAGGACTCCTACG	AGTGGTACGACCAGAGGCATAC
Cox8b	Cytochrome c oxidase, subunit VIIIb	GAACCATGAAGCCAACGACT	GCGAAGTTCACAGTG GTTCC
Cpt1a	Carnitine Palmitoyltransferase 1a	ACAAATTATGTGAGTGACTGG	GATCCCAGAAGACGAATAGG
Cpt1b	Carnitine palmitoyltransferase 1b	CACAATTCACCGGTACTTGG	TAATAAGCACAAATCCATGGC
Dio2	Deiodinase, iodothyronine, typeII Acyl-CoA dehydrogenase, medium- chain	GCC CAG CAA ATG TAG AC	TGG CAA TAA GGA GCT AGA A
Mcad		AGTATGCCCTGGATAGGAAGACAT	CTTGGTGCTCCACTAGCAGCT
Mpzl2 (Eva1)	Myelin protein zero-like 2	GTCCCAACCAGACCATCAAC	CTCCATCTTGCTCTGGAAGC
Lep	Leptin	ATTTACACACGCAGTCGGTAT	AAGCCCAGGAATGAAGTCCA
Tbp	TATA-Box Binding Protein	ACCCTTCACCAATGACTCCTATG	ATGATGACTGCAGCAAATCGC
Ucp1	Uncoupling protein 1	AGCCGGCTTAATGACTGGAG	TCTGTAGGCTGCCCAATGAAC

Online supporting material



Supplemental Figure 1 Blood glucose concentrations during the intraperitoneal glucose tolerance test (A, Expt. 3), calculated blood glucose area under the curve (AUC) (B, Expt. 3) in mice fed a low-fat diet or diets containing 16 or 32 % of energy as intact or hydrolyzed casein for 7 weeks. Values are given as mean + SE, n = 9-10. F, protein form; L, protein level.