

Impaired glucose tolerance in rats fed low-carbohydrate, high-fat diets

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¹Endocrine Research Unit, Medizinische Klinik und Poliklinik IV, Klinikum der Ludwig-Maximilians-Universität (LMU), Munich, Germany; ²Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio; ³Metabolic Diseases Institute, College of Medicine, University of Cincinnati, Cincinnati, Ohio; ⁴Center for Clinical Veterinary Medicine, Institute of Veterinary Pathology, LMU, Munich, Germany; ⁵Nephrologisches Zentrum, Medizinische Klinik und Poliklinik IV, Klinikum der LMU, Munich, Germany; and ⁶Institute for Diabetes and Obesity, Helmholtz Centre for Health and Environment and Technical University, Munich, Germany

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Bielohuby M, Sisley S, Sandoval D, Herbach N, Zengin A, Fischereider M, Menhofer D, Stoehr BJ, Stemmer K, Wanke R, Tschöp MH, Seeley RJ, Bidlingmaier M. Impaired glucose tolerance in rats fed low-carbohydrate, high-fat diets. *Am J Physiol Endocrinol Metab* 305: E1059–E1070, 2013. First published August 22, 2013; doi:10.1152/ajpendo.00208.2013.—Moderate low-carbohydrate/high-fat (LC-HF) diets are widely used to induce weight loss in overweight subjects, whereas extreme ketogenic LC-HF diets are used to treat neurological disorders like pediatric epilepsy. Usage of LC-HF diets for improvement of glucose metabolism is highly controversial; some studies suggest that LC-HF diets ameliorate glucose tolerance, whereas other investigations could not identify positive effects of these diets or reported impaired insulin sensitivity. Here, we investigate the effects of LC-HF diets on glucose and insulin metabolism in a well-characterized animal model. Male rats were fed isoenergetic or hypocaloric amounts of standard control diet, a high-protein “Atkins-style” LC-HF diet, or a low-protein, ketogenic, LC-HF diet. Both LC-HF diets induced lower fasting glucose and insulin levels associated with lower pancreatic β -cell volumes. However, dynamic challenge tests (oral and intraperitoneal glucose tolerance tests, insulin-tolerance tests, and hyperinsulinemic euglycemic clamps) revealed that LC-HF pair-fed rats exhibited impaired glucose tolerance and impaired hepatic and peripheral tissue insulin sensitivity, the latter potentially being mediated by elevated intramyocellular lipids. Adjusting visceral fat mass in LC-HF groups to that of controls by reducing the intake of LC-HF diets to 80% of the pair-fed groups did not prevent glucose intolerance. Taken together, these data show that lack of dietary carbohydrates leads to glucose intolerance and insulin resistance in rats despite causing a reduction in fasting glucose and insulin concentrations. Our results argue against a beneficial effect of LC-HF diets on glucose and insulin metabolism, at least under physiological conditions. Therefore, use of LC-HF diets for weight loss or other therapeutic purposes should be balanced against potentially harmful metabolic side effects.

dietary intervention; macronutrients; Atkins-style and ketogenic diet; hyperinsulinemic euglycemic clamps; insulin resistance

LOW-CARBOHYDRATE, HIGH-FAT (LC-HF) diets have been suggested to induce weight loss by triggering satiety via a mechanism involving reduced postprandial insulin secretion following meals with a low glycemic index (39). Thus, in addition to their use for the reduction of body weight in obese patients (19, 22, 44, 49), LC-HF diets may have beneficial effects on

glucose and insulin metabolism. Weight loss and improvements in body composition may themselves improve insulin sensitivity and reduce the risk of developing type 2 diabetes in overweight subjects. However, some evidence has suggested that consumption of LC-HF diets could decrease the risk of type 2 diabetes independent of weight loss effects, and recent studies have shown reductions of fasting glucose and insulin levels in subjects consuming LC-HF diets (21, 31, 52). Furthermore, a greater relative improvement in insulin sensitivity has been observed in obese patients following a LC-HF diet than in those on a calorie- and fat-restricted diet with normal carbohydrate content (44). In patients with type 2 diabetes, low-carbohydrate diets have been shown to improve glycemic control (24, 26, 27, 42) and are suggested to reduce the likelihood of exacerbating existing hyperinsulinemia (38). It has also been suggested that LC-HF diets could protect against the development of type 2 diabetes mellitus (1, 29). Nevertheless, the beneficial effects of LC-HF diets on glucose and insulin metabolism have been questioned by some authors (10, 37), and a recent study suggested they could even have negative effects on glucose tolerance in healthy lean men (40).

Ketogenic LC-HF diets are characterized by much lower protein contents compared with “Atkins-style” LC-HF diets. These diets are used to treat epilepsy in children but are currently also under intense research as a potential treatment option for brain (48) and prostate cancer (23) and neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (50). In addition, it has been suggested that ketogenic diets may offer a new therapeutic opportunity for controlling pain and peripheral inflammation (42). Animal studies allow investigation of diet effects in a more controlled and standardized environment than can be achieved in humans. Typically, LC-HF diets contain a very high amount of fat, even greater than standard “high-fat” diets (HFD), which are often used in experimental rodent studies. Therefore, LC-HF diets are very unique, and thus experimental results cannot be compared directly with studies using HFD, which comprise both a high fat content but also a high content of carbohydrates. To date, only a few rodent studies have investigated the effects of LC-HF diets on glucose tolerance. Badman et al. (3) reported that circulating glucose and insulin concentrations were reduced and insulin sensitivity improved in obese mice fed a ketogenic LC-HF diet, with no adverse effects on glucose metabolism in lean, wild-type mice fed the same diet. However, Jornayvaz et al. (33) reported opposing findings, with

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hyperinsulinemic euglycemic clamps showing that ad libitum consumption of a ketogenic LC-HF diet induces hepatic insulin resistance in lean mice despite diet-induced reduction of body weight. In a third study, Garbow et al. (25) reported development of glucose intolerance with no effect on systemic insulin sensitivity in lean C57BL/6 mice fed a ketogenic LC-HF diet for 12 wk. Thus, it remains unclear in either human or animal studies whether LC-HF diets are beneficial for glucose and insulin metabolism. Furthermore, two important aspects have not been studied. First, it is not clear whether LC-HF diets per se affect glucose tolerance or whether the relative abundance of protein and fat in the diets plays a role. This is especially relevant if, like in the aforementioned studies in mice, ketogenic LC-HF diets are used. However, with respect to body weight loss, the majority of subjects typically consume Atkins-style diets, i.e., LC-HF diets that comprise much higher protein contents than ketogenic LC-HF diets. The second aspect that has not been studied until today is the role of energy intake with LC-HF diets. All of the previously cited rodent studies employed ad libitum settings, and mice were obese when fed the ketogenic LC-HF diet. Therefore, it is a matter of debate whether the energetic intake of a LC-HF diet and the resulting effects on body composition are of importance or not. Furthermore, we hypothesized that the lack of carbohydrates in LC-HF diets could affect pancreatic β -cell mass. To close these knowledge gaps, we have studied the effects of LC-HF independent of the energy intake by using an isoenergetic pair-feeding setting. We have shown previously that the isoenergetic pair-feeding setting of the LC-HF diets results in visceral fat accumulation (7, 9, 16). To exclude that the factor fat accumulation exerts significant effects on glucose and insulin metabolism, we also investigated LC-HF diets in a hypocaloric setting, where we restricted the access of the rats to only 80% of the regular pair-fed groups, which prevented fat accumulation and resulted in a comparable fat mass between LC-HF groups and rats fed the control diet.

Previously, we have introduced a nutritional rat model in which the experimental diets are precisely defined and controlled (7). In contrast to previous rodent studies on this topic, not only are the experimental diets used in our study well characterized in terms of digestibility and macronutrient composition (6), but also the sources of macronutrients are kept constant in every diet. Therefore, any effects observed in this study are only attributable to differences in the relative abundances of fat, protein, and carbohydrates. It is important to mention that we did not intend to study LC-HF diets in the context of rats already exhibiting type 2 diabetes but rather investigate how the macronutrient composition in LC-HF diets affects glucose tolerance and how healthy organisms adapt to the lack of dietary carbohydrates. In the current investigation, we explored the effects of two different LC-HF diets compared with a standard control chow (CH) diet on glucose and insulin metabolism in rats. The first LC-HF diet (LC-HF-1) is a LC-HF diet with very low carbohydrate content, a high fat content, and a "normal" protein content, which is matched to the CH diet. This diet mirrors a LC-HF diet, which is promoted for induction of weight loss in humans (Atkins-style diet). The second LC-HF diet (LC-HF-2) contains a very low amount of carbohydrates and a comparably low quantity of protein but a very high amount of fat and is thus clearly ketogenic (7). Glucose and insulin metabolism were investigated by dynamic

challenge tests [glucose tolerance tests (GTT), insulin tolerance tests (ITT), and hyperinsulinemic euglycemic clamps] after 3 wk on the respective diets in a strictly controlled experimental setting. On a mechanistic level, we hypothesized that LC-HF diets impair glucose metabolism by affecting cellular glucose uptake in the periphery and that consumption of these diets leads to β -cell loss in the pancreas.

MATERIALS AND METHODS

Animal Husbandry

Most animal experiments were conducted at the Medizinische Klinik und Poliklinik IV in Munich, Germany. However, ITTs and hyperinsulinemic euglycemic clamps were performed at the University of Cincinnati due to the already established methods at this location. All procedures (and experimental diets) described below were identical at both locations and housing, and acclimation conditions at the University of Cincinnati were adapted to those at the Medizinische Klinik und Poliklinik IV in Munich. Male Wistar rats (10 wk old at the time of acquisition; Charles River Laboratories, Sulzfeld, Germany; and Harlan, Cincinnati, OH) were housed individually in type 3 makrolon cages (artificial light, $22 \pm 1^\circ\text{C}$; humidity, $60 \pm 15\%$) with standard bedding and maintained on a 12-h light-dark cycle. Rats were allowed to acclimate to the new environment for 2 wk following delivery. Throughout the acclimation period, rats had free access to a standard rodent diet (Ssniff, Soest, Germany) and tap water. Body weight and 24-h food intake were measured daily to the nearest 0.1 g (Sartorius Competence CP2201, Goettingen, Germany) 1 h before the onset of the dark period. At the end of the acclimation period, rats had reached the age of 12 wk and were divided into diet groups ($n = 10$ –12/diet group for groups matched for body weight after the acclimation period; $n = 7$ /diet group for the experiments conducted in the US). All procedures were approved by the Upper Bavarian Government's ethics committee for animal experiments and by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Diet Composition and Dietary Groups

All diets were custom made and purchased from Klifa Nafag (Kaiseraugst, Switzerland). Diet composition expressed as a percentage of metabolizable energy (ME) was as follows: control diet (referred to as "chow" in the following): 16.7% fat, 19% protein, and 64.3% carbohydrates (ME = 3.8 kcal/g); LC-HF-1: 78.7% fat, 19.1% protein, and 2.2% carbohydrates (ME = 6.2 kcal/g); LC-HF-2: 92.8% fat, 5.5% protein, and 1.7% carbohydrates (ME = 7.2 kcal/g). The macronutrient composition of each diet was independently controlled after production by Weende analysis (AGROLAB group/LUFA ITL, Kiel, Germany). Diets were semipurified, and only a single, identical source was used for each macronutrient (protein source: sodium-casein; fat source: beef tallow; carbohydrate source: starch). The LC-HF diets contained a minimum amount of carbohydrates (1.7 or 2.2% of ME) necessary to deliver minerals and vitamins. The CH diet corresponded to the standard rodent diet used by the American Institute of Nutrition (AIN-93G diet). The amounts of micronutrients (minerals and vitamins) added were based on recommendations from the AIN-93G reference diet and have been adapted to the respective ME content of each diet. This assured equal (and adequate) supply of minerals and vitamins with all experimental diets also in a pair-feeding setting. Rats were pair-fed daily the CH diet on an isoenergetic basis for 4 wk, as described previously (7). For the caloric restriction experiments aiming to eliminate differences in fat mass ($n = 5$ /diet group), rats fed LC-HF-1 and LC-HF-2 diets received only 80% of the food given to rats fed in the regular pair-feeding setting ($n = 5$ /diet group), as a reduction of 20% food intake of LC-HF diets was estimated to approximate fat mass between the different experimental groups.

Dynamic Tests to Assess Glucose Tolerance in Pair-Fed Rats

After 3 wk on the respective diets, rats were divided into several groups for oral glucose tolerance tests (OGTT), intraperitoneal glucose tolerance tests (IPGTT), and ITT. For each dynamic test, a new group of rats was used (5–6 rats/diet group and dynamic test). For OGTT and IPGTT, rats were weighed after a 16- to 18-h fast. Subsequently, a 2 g/kg body wt freshly prepared 50% glucose solution was administered orally through a gavage needle or injected intraperitoneally (ip). Blood samples were obtained from the tail vein at baseline and 15, 30, 60, 120, and 180 min after the glucose challenge (additionally after 5 min for IPGTTs). For ITTs, rats were fasted for 6 h and injected (ip) with 0.75 U insulin/kg body wt.

Hyperinsulinemic Euglycemic Clamp Experiments

A detailed description of experimental procedures has been published elsewhere (45). In brief, overnight-fasted rats were conscious and unrestrained during the experimental period. The morning of the study, rats were weighed and the exteriorized catheters extended for ease of access. To measure glucose kinetics, a primed (26 μCi) constant (0.26 $\mu\text{Ci}/\text{min}$) infusion of high-performance liquid chromatography-purified [$3\text{-}^3\text{H}$]glucose (Perkin-Elmer Life Sciences, Boston, MA) was administered via a precalibrated infusion pump (Harvard Apparatus, South Natick, MA) at 0–120 min. At 120–240 min, the infusion of [$3\text{-}^3\text{H}$]glucose was increased to 0.53 $\mu\text{Ci}/\text{min}$ to maintain constant specific activity. From 155–240 min, a primed (56 $\text{pmol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) continuous (28 $\text{pmol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) infusion of insulin (Eli Lilly, Indianapolis, IN) was administered via a precalibrated infusion pump (Harvard Apparatus). A variable rate of 50% dextrose infusion maintained blood glucose at ~ 7.7 mmol/l. During the experimental period, blood was drawn every 5–10 min for measurements of blood glucose, every 10 min during the experimental period for [$3\text{-}^3\text{H}$]glucose, and at times 140 and 240 min for plasma insulin levels.

Dissection of Rats

After 4 wk on the respective diets, rats were given access to food for 1 h after lights out and then fasted for 6 h (to standardize gastrointestinal filling) before decapitation under short isoflurane anaesthesia. Trunk blood was collected for further analysis, and centrifuged EDTA plasma samples were stored at -80°C until analysis. Rats were dissected, and livers, epididymal, inguinal, and perirenal fat pads (only one side of each fat pad), and pancreas were excised, carefully freed from adherent tissues, and weighed to the nearest 0.1 mg (Scaltec Instruments, Goettingen, Germany). Tissue samples of *M. gastrocnemius* were taken and immediately stored on dry ice (Oil Red O staining) or immediately fixed in 4% paraformaldehyde (for GLUT4 expression). Finally, all remaining organs were removed, and the skin was prepared free from the carcass (i.e., lean mass). The carcass was then weighed. Relative weights of liver and fat pads were calculated by dividing organ weights by the respective body weights of the rats. Relative weights of controls were set to 100%, and organ weights of LC-HF groups are expressed as a percentage of chow controls.

Measurement of Blood Glucose, Lipids, and Circulation Metabolic Hormones

All blood samples were handled, processed, and stored as recommended previously (8). Fasting blood glucose and glucose during dynamic tests were measured using the glucose oxidase method (EcoSolo; Care Diagnostics, Voerde, Germany). Plasma triglycerides and total and HDL cholesterol were measured by an automated system (Cobas Integra 800; Roche Diagnostics, Mannheim, Germany). Plasma Insulin, C-peptide (Alpco, Salem, NH), resistin (Bertin Pharma, Montigny le Bretonneux, France), and glucagon (Wako Chemicals, Neuss, Germany) were analyzed using commercially available kits (specific for rats) according to the manufacturer's instructions. Total plasma

glucose-dependent insulinotropic polypeptide (GIP; Merck Millipore, Billerica, MA) was measured in samples stabilized with a protease inhibitor cocktail (Complete; Roche, Mannheim, Germany) and a specific dipeptidyl peptidase IV inhibitor (Merck Millipore).

Quantitative Stereological and Morphometric Analysis of the Pancreas

Immediately after excision, the whole pancreas was weighed and fixed overnight in 4% paraformaldehyde. The pancreas volume was calculated by dividing the pancreas weight by the specific weight of rat pancreas (1.08). For quantitative stereological analyses, pancreas tissues were routinely processed and embedded in paraffin, and paraffin sections were stained for insulin, as described previously (28). Briefly, the pancreas was serially cut perpendicular to the longitudinal axis into $\sim 1.7 \pm 0.1$ -mm thick slices. The slices were embedded in paraffin with the right cut surface facing downward. On average, 186 ± 47 mm² of pancreas tissue and between 100 and 300 islet profiles per rat were evaluated. The volume density of β -cells in the pancreas [$V_V(\beta\text{-cells}/\text{pan})$] was calculated by dividing the insulin-positive cross-sectional area by the pancreas area. The total β -cell volume in the pancreas [$V(\beta\text{-cells}/\text{pan})$] was calculated, multiplying $V_V(\beta\text{-cells}/\text{pan})$ and the pancreas volume. The number of apoptotic and replicating cells per insulin-positive area (N_A) was determined using sections immunohistochemically stained for cleaved caspase 3 (no. 9661, 1:200 in TBS; Cell Signaling Technology) and PCNA (ab29, 1:6,400 in TBS; Abcam), respectively. Briefly, the primary antibodies were incubated overnight at room temperature, a biotinylated goat anti-rabbit antibody (cleaved caspase 3) and a peroxidase-conjugated rabbit anti-mouse antibody (PCNA) served as secondary antibody (1:100 in TBS, 1 h at room temperature), and diaminobenzidine was used as chromogen. The number of immunohistochemically stained nuclear profiles was counted and divided by the insulin-positive cross-sectional area.

Detection of Intramyocellular Lipids, Muscle Triglyceride Content, and Skeletal GLUT4 Expression by Immunohistochemistry

Skeletal muscle samples obtained from the gastrocnemius muscle were stained with Oil Red O to detect intramyocellular lipids, as described previously (36). Extraction and isolation of triglycerides from skeletal muscle have been described previously (41). In brief, ~ 0.1 g of gastrocnemius ($n = 4$ /dietary group) was homogenized in 2:1 chloroform-methanol, and total lipids were extracted. Nonpolar lipids were isolated by solid-phase extraction, and triglyceride content was then determined spectrophotometrically at 500 nm by using an enzymatic colorimetric solution, Infinity Triglycerides (Thermo Scientific). Triglyceride concentrations of samples were quantified against a standard curve of Matrix Plus Chemistry Reference Kit (Verichem Laboratories). GLUT4 expression was determined as explained in detail previously (5). In brief, representative blocks of paraffin-embedded muscles were cut according to routine procedures. To detect the presence of GLUT4 by immunofluorescence staining, sections were incubated overnight at 4°C with an anti-rat GLUT4 antibody (1:100; FabGennix, Shreveport, LA), followed by incubation for 1 h at room temperature with the secondary Cy3-labeled antibody (red staining, 1:1,500; Dianova, Hamburg, Germany). At last, coverslips were mounted with a DAPI-containing medium (blue staining, Vectashield; Vector Laboratories, Burlingame, CA) to stain cell nuclei. Pictures were obtained using a fluorescence microscope with a $\times 40$ lens (Leica DM 2500; Leica Microsystems, Wetzlar, Germany) that was directly connected to a digital camera (Leica DFC340 FX; Leica Microsystems). The final magnification of the pictures was $\times 400$.

Analysis of p-p70 S6 Kinase Expression

At least 40 μg of protein (extracted from livers and *M. quadriceps* samples) was resolved by SDS-PAGE and then transferred on to a

PVDF membrane that had been pretreated with methanol for 1 min. Membranes were blocked in a solution of TBST (Tris-buffered saline and 0.1% Tween-20) containing 3% dry milk for 60 min with constant agitation. After blocking, the PVDF membrane was incubated with the primary rabbit antibody directed against p-p70 S6 kinase (Thr³⁸⁹; New England Biolabs, Frankfurt am Main, Germany) (1:1,000 dilution in TBST) overnight at 4°C. Membranes were washed in 10–20 ml TBST (3 times for 5 min) and incubated for 1 h with anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody at a 1:2,000 dilution at room temperature with constant agitation before enhanced chemiluminescence (Thermo, Schwerte, Germany) and exposure to film.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (Version 5; GraphPad Software, La Jolla, CA). For the statistical comparison between the three dietary groups, nonparametric ANOVA analysis by Kruskal Wallis was performed with subsequent Dunn multiple comparison tests. *P* values <0.05 were considered significant. All data are presented as means ± SE.

RESULTS

Body Weights, Organ Weights, Plasma Lipids, and Circulating Hormones of Rats Pair-Fed Isoenergetic Amounts of the Diets

Table 1 shows the results for body weights, organ weights, and hormone concentrations in rats fed chow or LC-HF diets for 4 wk. Rats fed either chow or LC-HF-1 consistently gained body weight and were significantly heavier after 4 wk on the

Table 1. Body weight, organ weights, circulating fasting blood lipids, and glucose as well as plasma hormone concentrations in rats fed isoenergetic amounts of the 3 different diets

	Chow	LC-HF-1	LC-HF-2
Body weight after 4 wk on the diets, g	396.8 ± 6.7	390.4 ± 3.8##	366.4 ± 4.2***
Lean mass, g	224.5 ± 2.2	213.0 ± 2.0*	206.6 ± 1.5***
Perirenal fat, g	3.1 ± 0.1	3.8 ± 0.2*	4.1 ± 0.2***
Relative perirenal fat, %	100 ± 3.3	133 ± 7.5***	147 ± 6.5***
Epididymal fat, g	3.7 ± 0.1	4.2 ± 0.2*	4.4 ± 0.1**
Relative epididymal fat, %	100 ± 3.4	118 ± 4.4**	128 ± 3.9***
Inguinal fat, g	3.3 ± 0.1	3.3 ± 0.1#	3.8 ± 0.1*
Relative inguinal fat, %	100 ± 3.5	103 ± 3.3#	119 ± 4.0***
Liver weights, g	12.3 ± 0.3	12.9 ± 0.2#	11.9 ± 0.2
Pancreas wet weights, g	0.86 ± 0.04	0.68 ± 0.04*	0.59 ± 0.03***
Total cholesterol, mg/dl	83.9 ± 4.9	86.8 ± 5.8	78.1 ± 3.3
Triglycerides, mg/dl	113.5 ± 12.7	135.8 ± 24.7#	263 ± 57.1**
HDL, mg/dl	60 ± 3.6	57.6 ± 5.3#	37 ± 4.4**
Fasting glucose, mg/dl	103.8 ± 2.8	89.2 ± 3.5*	81.6 ± 2.1***
Fasting insulin, ng/ml	0.69 ± 0.06	0.50 ± 0.04*	0.41 ± 0.05***
Fasting C-peptide, pM	569 ± 46	498 ± 44	382 ± 38*
Fasting total GIP, pg/ml	64.6 ± 15.4	264.5 ± 80*	234.2 ± 86.2*
Fasting resistin, ng/ml	1.19 ± 0.05	0.94 ± 0.09*	0.95 ± 0.08
Fasting glucagon, pg/ml	503 ± 165	498 ± 45	544 ± 118

Data are presented as means ± SE. LC-HF-1, 1st low-carbohydrate, high-fat diet; LC-HF-2, 2nd low-carbohydrate, high-fat diet; GIP, gastric inhibitory peptide. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, significant differences compared with controls fed with chow. #*P* < 0.05 and ##*P* < 0.01, significant differences between LC-HF-1 and LC-HF-2.

diets compared with the starting weight. Body weight gain in rats fed LC-HF-2 was markedly lower than that of rats fed chow or LC-HF-1. At the end of the feeding period, rats fed LC-HF-2 weighed almost 10% less compared with chow (*P* < 0.001) or LC-HF-1 (*P* < 0.01). Both LC-HF diets led to significantly lower lean body mass than with chow (CH vs. LC-HF-1: *P* < 0.05; CH vs. LC-HF-2: *P* < 0.001; LC-HF-1 vs. LC-HF-2: not significant). In contrast, absolute and normalized weights of perirenal and epididymal fat pads were significantly higher in both LC-HF diet groups compared with chow. Weights of inguinal fat pads (absolute and normalized to body weight) were significantly higher with LC-HF-2 compared with chow and LC-HF-1 (*P* < 0.05). Significant differences in liver weights were found when comparing LC-HF-1 and LC-HF-2 (*P* < 0.05). Pancreas weights were significantly lower in both LC-HF groups vs. rats fed chow.

Total serum cholesterol was not significantly different among the three groups. In contrast, circulating triglycerides were significantly higher with LC-HF-2 compared with chow and LC-HF-1. Serum HDL was significantly lower with LC-HF-2 compared with chow and LC-HF-1. After 6 h of fasting, glucose and insulin levels were significantly lower in both LC-HF-fed rats compared with rats fed with chow. Concentrations of C-peptide were lower with both LC-HF diets, but the difference reached statistical significance comparing only rats fed chow and LC-HF-2. Total GIP was significantly higher with LC-HF-1 and LC-HF-2 compared with controls. Circulating levels of resistin were significantly higher in LC-HF-1 compared with LC-HF-2. No significant differences were detected in circulating concentrations of fasting glucagon.

Dynamic Challenge Tests

OGTTs and IPGTTs. Rats fed LC-HF diets showed a more pronounced increase in glucose levels during OGTT (Fig. 1A), with a significantly higher area under the curve (AUC) in both LC-HF groups vs. the chow group (Fig. 1B). No significant differences were observed between rats fed LC-HF-1 and LC-HF-2. As expected, plasma insulin levels during OGTT peaked 15 min after the oral glucose load (Fig. 1C). AUC for insulin tended to be lower in LC-HF groups vs. chow, but this difference did not reach statistical significance (Fig. 1D). As in OGTT, IPGTT revealed impaired glucose tolerance in rats fed LC-HF diets (Fig. 1, E and F). Insulin secretion during IPGTT was more pronounced in LC-HF diet groups compared with chow controls (Fig. 1G), resulting in a significantly higher AUC for insulin during the IPGTT (*P* < 0.05; Fig. 1H).

ITTs. Circulating glucose levels declined in all diet groups 2 h after the ITT was started (Fig. 1I). However, compared with chow-fed rats, the decline in glucose during ITT was delayed significantly in rats fed either LC-HF diet. In addition, the insulin challenge led to significantly lower glucose levels 120 min after insulin application to chow-fed rats (chow, 42.7 ± 1.7 mg/dl; LC-HF-1, 75 ± 3.6 mg/dl; LC-HF-2, 93.1 ± 6.5 mg/dl). There was no significant difference between LC-HF-1 and LC-HF-2 groups. As expected, circulating glucose levels increased again in all groups 6 h after the exogenous insulin load.

Hyperinsulinemic euglycemic clamps. A steady glucose concentration was achieved in all rats during the last 30 min of the clamp experiments (Fig. 2A). As expected, circulating insulin

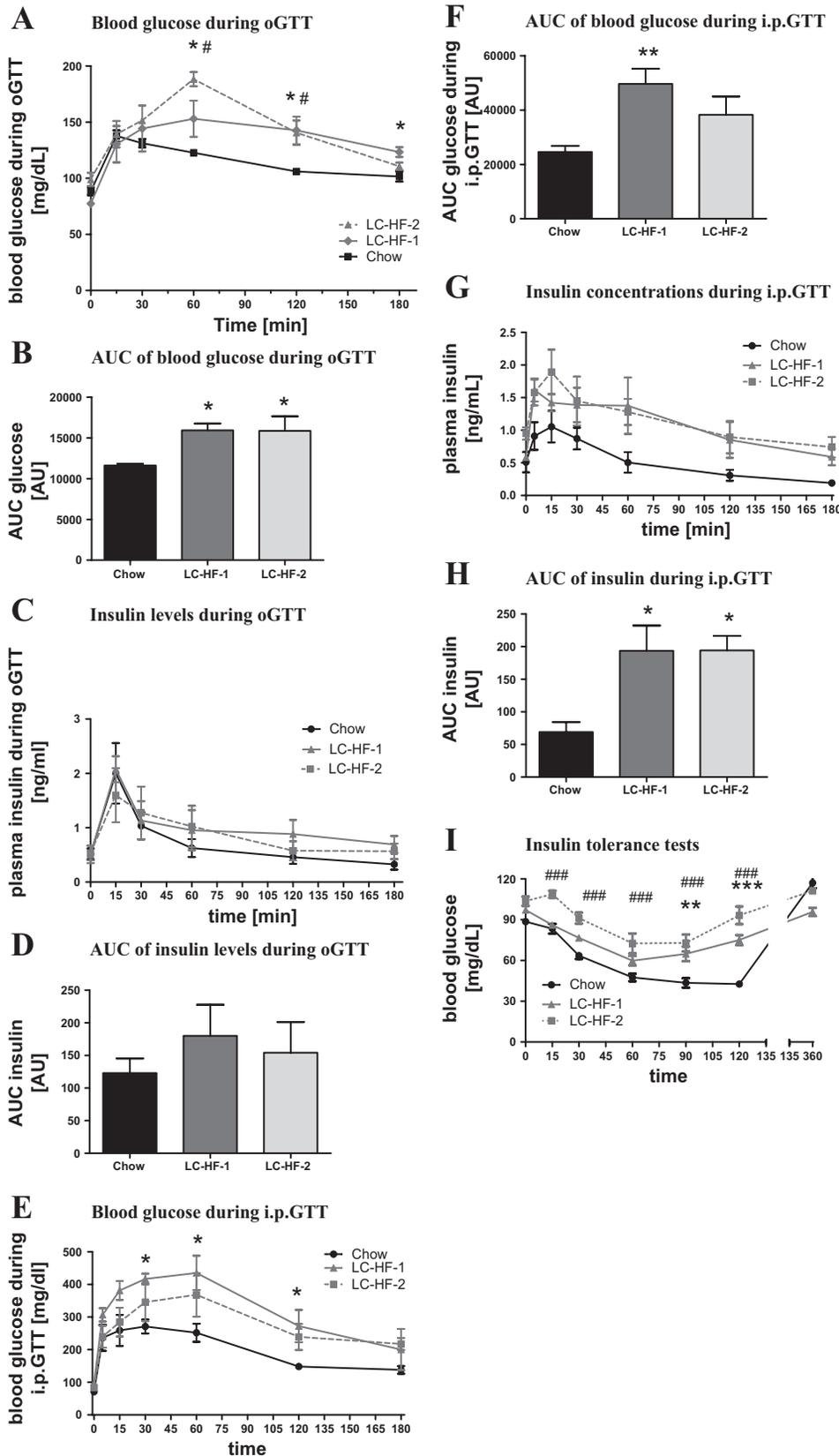
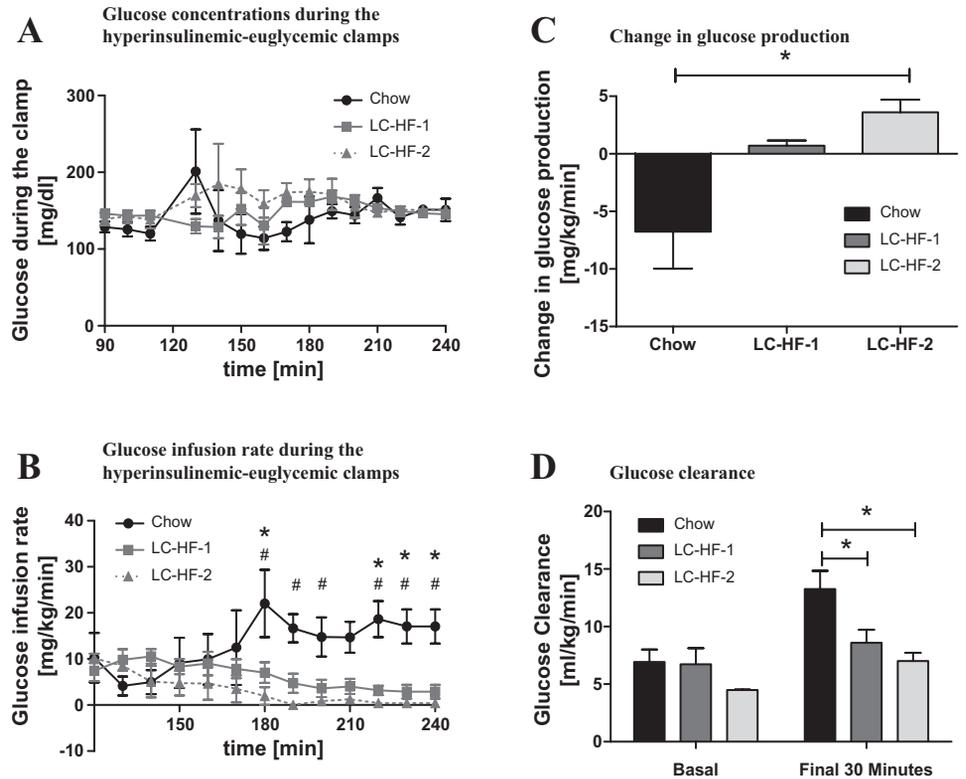


Fig. 1. Glucose and insulin tolerance tests. Blood glucose and plasma insulin levels [and the respective area under the curve (AUC)] in rats fed isoenergetic amounts of the respective diets for 3 wk during oral glucose tolerance test (OGTT; A–D) and intraperitoneal glucose tolerance test (IPGTT; E–H). I: glucose concentrations in rats fed isoenergetic amounts of the respective diets for 3 wk during an insulin tolerance test (0.75 U insulin/kg body wt). Black, control chow (CH) diet; gray and solid line, “Atkins-style” low-carbohydrate/high-fat (LC-HF) diet (LC-HF-1); gray and dotted line, ketogenic LC-HF-2 diet. Data are presented as means ± SE. Different symbols indicate significant differences compared with controls fed with chow at individual time points (CH vs. LC-HF-1: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001; CH vs. LC-HF-2: #*P* < 0.05 and ###*P* < 0.001).

Fig. 2. Hyperinsulinemic euglycemic clamps in rats fed isoenergetic amounts of the respective diets for 3 wk. **A**: blood glucose concentrations during the hyperinsulinemic euglycemic clamps. **B**: glucose infusion rate during the hyperinsulinemic euglycemic clamps. **C**: change in glucose production. **D**: glucose clearance. Black, CH diet; gray on solid lines, Atkins-style LC-HF-1 diet; gray on dotted lines, ketogenic LC-HF-2 diet. Data are presented as means \pm SE. * and #Significant differences compared with controls fed chow. * $P < 0.05$. In **B**, CH vs. LC-HF-1: * $P < 0.05$, CH vs. LC-HF-2; # $P < 0.05$.



concentrations were increased more than twofold in all dietary groups at the end of the clamp compared with baseline (before insulin infusion; data not shown). The glucose infusion rate was approximately threefold higher in chow-fed rats compared with both LC-HF diet groups, indicating whole body insulin resistance in LC-HF fed groups ($P < 0.05$; Fig. 2B). Whereas endogenous glucose production was decreased in rats fed with chow, endogenous glucose production increased in both LC-HF groups, reaching statistical significance in chow vs. LC-HF-2 fed rats (Fig. 2C). Glucose clearance was also significantly reduced in both LC-HF diet groups (Fig. 2D). Together, these data show insulin resistance in LC-HF diets at both the hepatic and muscle levels compared with chow-fed animals.

Caloric Restriction With LC-HF Diets

As expected, the reduction of daily energy intake of LC-HF diets to 80% of the amount consumed by the pair-fed groups resulted in lower body weight compared with the chow- and pair-fed groups. Moreover, with caloric restriction to 80%, fat mass in both LC-HF diet groups did not differ from that of the respective chow-fed control rats (Table 2). Fasting glucose and insulin concentrations were further decreased in the LC-HF groups with 80% caloric restriction compared with the 100% pair-fed LC-HF groups (Table 2). No significant differences in body and organ weights or in fasting glucose and insulin were detected between the calorically restricted LC-HF-1 and LC-HF-2 groups. During OGTT, both calorically restricted LC-HF diet groups showed a greater increase in maximal glucose concentrations compared with chow. The difference was especially pronounced in rats fed with LC-HF-2, which also resulted in a significantly higher AUC for glucose during the

OGTT [chow, $12,986 \pm 801$; LC-HF-1 (80%), $15,662 \pm 1,111$; LC-HF-2 (80%), $23,809 \pm 1,485$; CH vs. LC-HF-1 (80%), $P = 0.087$; CH vs. LC-HF-2 (80%), $P < 0.01$]. Insulin secretion during OGTT tended to be higher in both calorically restricted LC-HF diet groups compared with unrestricted chow, but the difference did not reach statistical significance between the three diet groups [AUC: chow, 152.7 ± 51.1 (100%); LC-HF-1 (80%), 213 ± 16 ; LC-HF-2 (80%), 271.7 ± 38.3 ; Fig. 3, A and B].

Pancreas Morphology and Proliferative/Apoptotic Index

Total pancreas volume was lower in LC-HF-1 (-21% , $P < 0.01$) and LC-HF-2 groups (-31% , $P < 0.001$) compared with chow-fed controls (Fig. 4A). Quantitative stereological analyses revealed that feeding of either LC-HF diet led to significantly ($P < 0.001$) lower volume density and total volume of

Table 2. Body weights, lean body mass, fat pad weights, and circulating fasting blood glucose and plasma insulin in rats fed only 80% of the amount given to the pair-fed LC-HF groups (chow ad libitum)

	Chow	LC-HF-1 (80%)	LC-HF-2 (80%)
Body weight after 4 wk, g	356.4 ± 7.0	332.9 ± 8.3	$316.1 \pm 3.2^{**}$
Lean mass, g	184.7 ± 3.9	176.6 ± 4.1	$164.3 \pm 7.0^*$
Inguinal fat, g	2.1 ± 0.1	2.2 ± 0.3	2.2 ± 0.3
Perirenal fat, g	3.0 ± 0.3	2.9 ± 0.2	3.3 ± 0.1
Epididymal fat, g	3.1 ± 0.3	3.3 ± 0.4	3.4 ± 0.4
Fasting glucose, mg/dl	99.3 ± 3.2	87.8 ± 4.6	$53.7 \pm 3.6^{**}$
Fasting insulin, ng/ml	0.82 ± 0.22	0.52 ± 0.11	$0.23 \pm 0.06^*$

Data are presented as means \pm SE. * $P < 0.05$ and ** $P < 0.01$, significant differences in LC-HF groups compared with controls fed with chow.

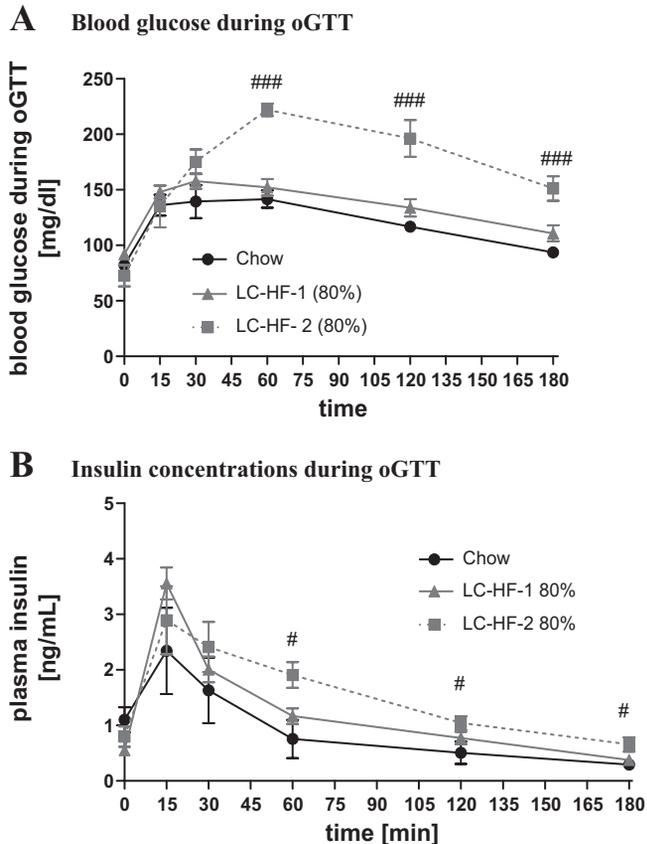


Fig. 3. OGTTs in rats that were fed with 80% of the pair-fed LC-HF diet groups. Blood glucose (A) and plasma insulin (B) concentrations during an OGTT in rats fed the respective LC-HF diets under caloric restriction (80%) for 3 wk (chow fed at 100%). Black, CH diet; gray on solid lines, Atkins-style LC-HF-1 diet; gray on dotted lines, ketogenic LC-HF-2 diet. Data are shown as means \pm SE. # $P < 0.05$ and ### $P < 0.001$, significant differences between LC-HF-2 compared with controls fed with chow at individual time points.

β -cells in the pancreas compared with chow-fed rats (Fig. 4, B–D). Analysis of the number of cleaved caspase-3-positive nuclear profiles as an indicator of apoptosis in pancreatic islets revealed no significant differences between the three diet groups [cleaved caspase-3-positive cells/islet area ($\times 10^{-6}$): chow, 1.84 ± 0.76 ; LC-HF-1, 1 ± 1.23 ; LC-HF-2, 1.18 ± 1.05 ; $n = 5$ /group; not significant]. Similarly, there were no significant differences in the number of PCNA-positive nuclear profiles per β -cell area as a marker of β -cell proliferation (chow, 17.3 ± 12.2 ; LC-HF-1, 11.6 ± 6.2 ; LC-HF-2, 22.6 ± 18.5 ; nos. shown $\times 10^{-6}$; $n = 5$ /group; not significant).

Determination of Intramyocellular Lipids and GLUT4 Expression

Skeletal muscle sections (m. gastrocnemius) stained with Oil Red O from rats pair-fed the LC-HF diets appear to contain more intramyocellular lipids than those from chow-fed rats (Fig. 5A). Lipid droplets appeared to be more numerous and larger in both LC-HF groups. Isolation of triglycerides from skeletal muscle (m. gastrocnemius) by Folch extraction and subsequent quantification revealed higher total triglyceride concentrations in both LC-HF diet groups, supporting the results from the Oil Red O analysis (chow, 15.1 ± 4.0 mg/dl; LC-HF-1, 30.3 ± 2.6 mg/dl; LC-HF-2, 42.8 ± 8.3 mg/dl; chow

vs. LC-HF-1: not significant; chow vs. LC-HF-2: $P < 0.5$). Cell surface expression of GLUT4 in gastrocnemius muscle samples appeared to be higher in rats fed chow compared with both LC-HF diet groups (Fig. 5B).

Expression of p-p70 S6 Kinase in Liver and Muscle

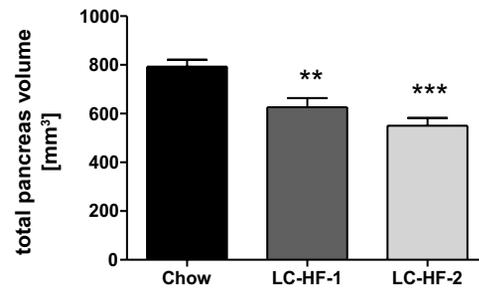
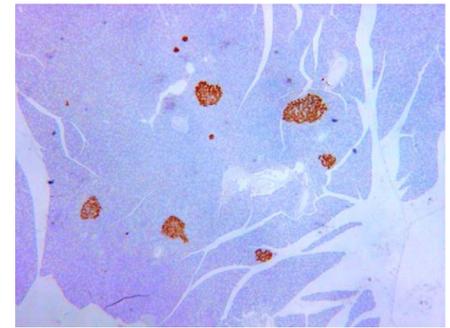
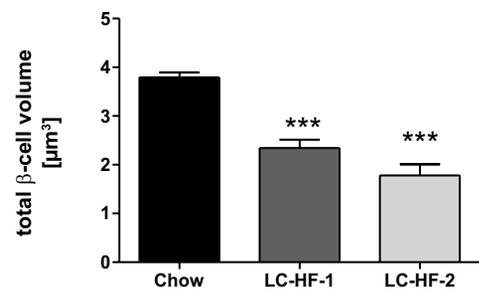
Relative to β -actin, the expression of p-p70 S6 kinase in the liver of rats fed with LC-HF-1 was significantly lower when compared with chow ($P < 0.05$) and with LC-HF-2 ($P < 0.01$). There were no significant differences when comparing chow with the LC-HF-2 group (Fig. 5C). No significant differences between the three groups were detected when the p-p70 S6 kinase in muscle samples was analyzed (data not shown).

DISCUSSION

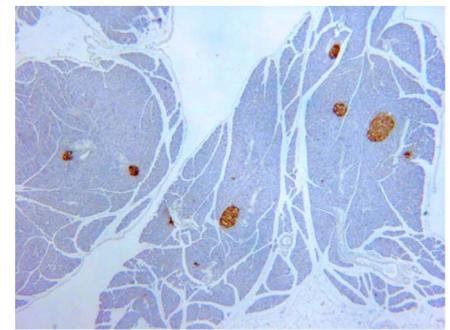
In this study, we have shown that rats fed LC-HF diets exhibit impaired glucose tolerance and insulin resistance despite showing reduced glucose and insulin levels. Glucose intolerance was observed with both LC-HF diets. The use of isoenergetic pair-feeding ensured that the effects were not due to energy overconsumption. In addition, caloric restriction of LC-HF to 80% of the isoenergetic pair-fed groups revealed that the glucose-intolerant phenotype is not dependent upon visceral fat accumulation. Thus, according to our findings, LC-HF diets cannot be recommended for prevention of impaired glucose metabolism, and care should be taken regarding the potential metabolic effects of these diets when used for other therapeutic purposes.

LC-HF diets have generally been shown to reduce circulating glucose and insulin in both humans and animal models. However, despite being used as a surrogate measurement for the risk of type 2 diabetes, it has been unclear whether such reductions correspond to improved glucose tolerance. In our study, reduced glucose tolerance was observed alongside insulin resistance despite reduced circulating concentrations of glucose and insulin in animals fed LC-HF diets. In agreement with our findings, a recent study by Jornayvaz et al. (33) demonstrated that mice fed a ketogenic LC-HF diet displayed hepatic insulin resistance despite lower body weight gain. However, other authors have reported weight loss and improved glucose tolerance during IPGTTs and ITTs in obese but not in lean wild-type mice fed a ketogenic LC-HF diet (3, 34). One similarity between our study and the study by Jornayvaz et al. (33) is the use of hyperinsulinemic euglycemic clamp experiments, which are considered to be the gold standard to investigate insulin sensitivity. In contrast, both Badman et al. (3) and Kennedy et al. (34) analyzed glucose metabolism using only GTTs and ITTs. However, it is not clear whether this can explain the differences in the results obtained.

In a different study, Garbow et al. (25) observed systemic glucose intolerance but preserved whole body insulin responsiveness in mice fed a ketogenic diet. These authors suggested that the preserved response to insulin was explained by their lower lean mass resulting in a proportionally higher insulin dose in insulin tolerance tests compared with controls (25). In addition to fat mass, body weight and lean body mass significantly affect glucose tolerance and insulin sensitivity. In the current investigation, the restriction of food intake to only 80% of pair-fed rats resulted in a similar fat mass between all

A Total pancreas volume**D** Immunohistochemistry for insulin in rats fed with the different diets**B** Total β -cell volume

LC-HF-1



LC-HF-2

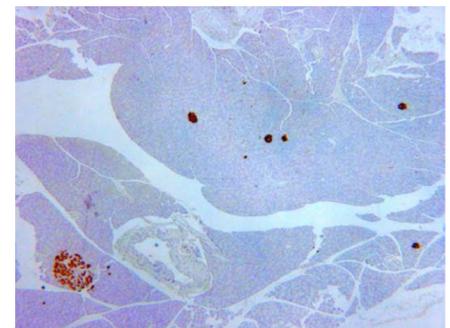
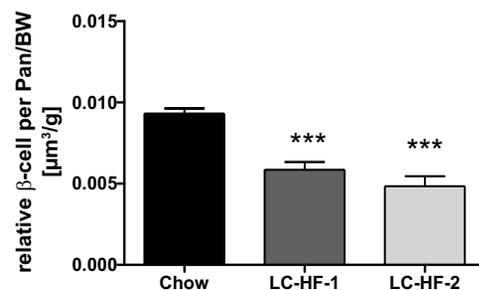
**C** Relative β -cell volume (normalized to pancreas volume and body weight)

Fig. 4. Pancreas and islet morphology. Pancreas volume (A), total β -cell volume (B), and volume density of β -cells in the pancreas (normalized to pancreas volume and body weight; C) determined by quantitative stereological methods in pancreas sections from rats fed isoenergetic amounts of the respective diets for 4 wk. D: lower β -cell mass in rats fed the LC-HF diets (brownish color: insulin). Black bars, CH diet; dark gray bars, Atkins-style LC-HF-1 diet; light gray bars, ketogenic LC-HF-2 diet. ** $P < 0.01$ and *** $P < 0.001$, significant differences compared with controls fed with chow.

groups. However, body weight and lean body mass differed between the groups. Therefore, future studies should also investigate the effect of LC-HF diets on glucose tolerance in rodents controlling for body weight and lean body mass. Although conflicting results have been obtained regarding the effect of LC-HF diets on glucose and insulin (22, 43, 49), most of the studies were designed primarily to assess weight loss, and therefore, it is difficult to distinguish the direct effects of diet from indirect effects due to weight loss and changes in body composition (12). In this investigation, as well as in previous studies (7, 9, 16), isoenergetic feeding of the Atkins-style and the ketogenic LC-HF diet resulted in a significant accumulation of visceral fat mass in rats compared with chow controls. No significant differences in the degree of visceral fat accumulation between the two LC-HF diets were detected, which is in accord with previous studies in which unchanged circulating leptin between LC-HF-1 and LC-HF-2 was also

shown (9). To distinguish the direct effects of LC-HF diets on glucose homeostasis from such indirect effects (i.e., accumulation of visceral fat mass), we calorically restricted rats fed with the LC-HF diets to 80% intake of pair-fed groups. This procedure eliminated the differences in fat mass between chow and LC-HF diet groups, but the LC-HF-2 group especially still showed impaired glucose tolerance. The use of a single source of fat and protein in our study suggests that the observed impairment of glucose tolerance induced by LC-HF diets cannot be attributed to differences in amino and fatty acid profile, which have themselves been suggested to influence glucose metabolism (46). Importantly, only the LC-HF-2 diet, being very low in its protein content, is a true ketogenic diet, whereas the LC-HF-1 diet does not induce permanent ketosis, as shown previously (7). By comparing both LC-HF diets, we found that the relative abundance of fat and protein in LC-HF diets did not significantly affect glucose tolerance, since rats

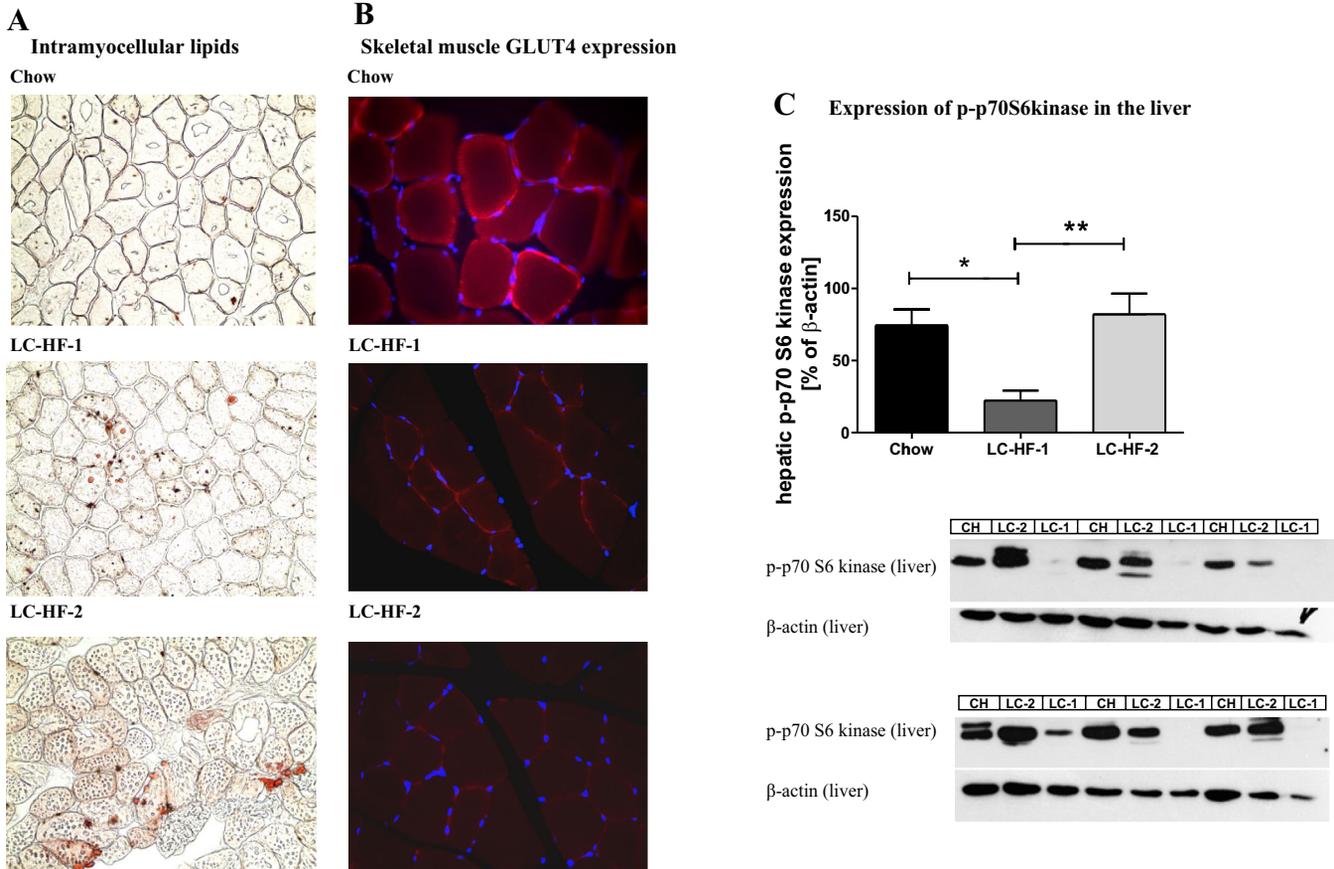


Fig. 5. A–C: determination of intramyocellular lipids, GLUT4 expression in muscle, and hepatic expression of p-p70 S6 kinase. A: muscle sections of rats pair-fed isoenergetic amounts of the 3 different diets stained for intramyocellular lipids (Oil Red O). Intramyocellular lipids appear as red lipid droplets. B: GLUT4 was stained with Cy3 (red), revealing a decreased expression on the plasma membrane and in the cytosolic vesicles of both LC-HF groups compared with the CH group. Cell nuclei appear in blue (DAPI). C: the hepatic expression of phospho-p70 S6 kinase was significantly lower in rats fed with LC-HF-1 (LC-1) compared with CH ($P < 0.05$) and LC-HF-2 (LC-2; $P < 0.01$). Expression of p-p70 S6 was normalized against the expression of β -actin. Data are shown as means \pm SE. * $P < 0.05$; ** $P < 0.01$.

fed both LC-HF diets showed the same insulin-resistant phenotype. High concentrations of plasma resistin have been linked to obesity and insulin resistance (14, 53). However, in our study, circulating concentrations of resistin were not different between the dietary groups. Fasting plasma levels of GIP were significantly higher in rats fed both LC-HF diets, but this did not result in increased insulin secretion. Interestingly, it has been reported that obese patients with type 2 diabetes also show higher circulating GIP concentrations (18, 47). However, the higher GIP concentrations of rats fed LC-HF diets are most likely explained by the high intake of dietary fats, which have been shown to increase GIP secretion (13, 47). Our observation of reduced basal insulin secretion following short-term feeding with LC-HF diets was correlated with a decrease in β -cell mass. Our finding is in contrast to earlier studies by Al-Khalifa et al. (1, 2), who have shown that feeding of a ketogenic LC-HF diet did not affect the β -cell number in rats. This discrepancy might be explained by differences in histological techniques since we used quantitative stereological methods, whereas Al-Khalifa et al. (1, 2) investigated the β -cell number by staining single slides with Gomori's chrome alum hematoxylin-phloxine stain. We were unable to attribute the decrease in β -cell mass to differences in apoptotic and proliferative indices of pancreatic islets. This may be explained by the

difficulty of observing such changes histologically due to the low rate of proliferation and fast clearance of apoptotic cells in the adult endocrine pancreas. Since it is not currently possible to estimate proliferation and apoptosis of β -cells in vivo, conclusions cannot be drawn regarding when and how remodeling of β -cell mass with LC-HF diets occurs. Furthermore, it remains unclear whether this process is reversible, especially after long-term adherence to a LC-HF diet. During short-term feeding studies, we have shown previously that switching rats from a ketogenic LC-HF diet back to the standard control diet normalizes baseline levels of glucose and insulin within days (17). In agreement with the data reported in the current study, Kinzig et al. (35) showed that feeding a ketogenic LC-HF diet resulted in decreased sensitivity to peripheral insulin and impaired glucose tolerance in rats. It has been shown that the responsiveness to central insulin and the expression of insulin receptor mRNA was increased in rats fed a ketogenic diet. After food was changed to control chow diet again, the negative effects of the ketogenic LC-HF diet on insulin sensitivity and glucose tolerance were rapidly reversed. These results from short-term feeding trials indicate that LC-HF diet induced impairment of glucose tolerance that could be reversible.

Our results suggest that insulin resistance occurs at two sites. First, hepatic insulin resistance was observed most likely

because of diet-induced hepatic fat accumulation (7, 33), the nonalcoholic fatty liver disease-like phenotype (25), and associated impairment of hepatic insulin signaling (33). In addition, hyperinsulinemic euglycemic clamp and ITT experiments provided evidence for peripheral insulin resistance. This could be due to lower lean body mass of rats fed LC-HF diets. However, we also observed greater accumulation of intramyocellular lipids and higher triglyceride concentrations in gastrocnemius muscle samples from rats fed both LC-HF diets, which have been shown to be an early marker for the development of insulin resistance (11). It has recently been shown that the expression of p70 S6 kinase in the liver has an important impact upon development of hepatic steatosis and systemic insulin resistance. Bae et al. (4) have shown that mice exhibiting liver-specific p70 S6 kinase depletion are protected against both pathophysiologies. We investigated the hepatic expression of the phosphorylated form of this protein and found a significantly lower expression in rats fed the protein-matched LC-HF-1 diet but not in rats fed the LC-HF-2 diet. It is possible that the reduction in phosphorylated p70 S6 represents an adaptive mechanism that is partially protective and enables the organism to counteract the development of hepatic steatosis and insulin resistance. However, this mechanism seems to require normal protein ingestion. Indeed, we have shown previously that hepatic steatosis with LC-HF-1 was more severe compared with chow but less pronounced compared with the liver phenotype observed with the ketogenic LC-HF-2 diet (9). Furthermore, whereas both LC-HF diets impaired glucose tolerance during ITT, during hyperinsulinemic euglycemic clamps, and in the caloric restriction experiments, the (higher protein) LC-HF-1 diet showed an attenuated insulin-resistant phenotype compared with rats fed with the ketogenic LC-HF-2 diet. This might also hint toward a protective role of lower p-p70 S6 kinase expression. Another hypothesis to explain the effect of LC-HF diets on glucose and insulin resistance would be that cellular glucose uptake is altered via effects on the expression of glucose transporters (GLUTs). We found reduced cell surface expression of GLUT4 in skeletal muscle samples (m. gastrocnemius) of LC-HF fed rats. This may play a significant role in explaining reduced glucose uptake in the periphery and thus could contribute to the insulin resistant phenotype. Also in muscle, the mTOR/p70 S6 kinase pathway seems to play an important role for the modulation of glucose transport (51). However, we did not detect significant differences in p-p70 S6 kinase expression in muscle quadriceps samples between the dietary groups. Interestingly, LC-HF diets seem not to generally affect muscle GLUT4 expression. It is known that especially the anatomic location of the muscle and the type of muscle fiber (including the myosin heavy chain isoform expression profile) plays an important role for GLUT4 expression (15, 20, 30, 32). Our own preliminary data from Western blot experiments using quadriceps muscle samples indicate that GLUT4 expression was not affected by LC-HF diets in that muscle type (data not shown). Therefore, future studies are required to investigate the impact of LC-HF diets on skeletal muscle expression of GLUTs in more detail, especially if GLUT4 membrane translocation is affected by LC-HF diets and how the GLUT4 expression in different muscle types responds to the absence of dietary carbohydrates.

A limitation of our study certainly is that feeding of the LC-HF diets was initiated while rats were still lean and that we

did not investigate whether LC-HF diets improve glycemic control in rats that displayed a diabetic phenotype before the dietary intervention. However, the aim of this study was not to investigate whether LC-HF diets can improve type 2 diabetes but instead study how the dramatic differences of macronutrient composition in LC-HF diets affect glucose metabolism in the context of normal physiology (nondiabetic rats).

In conclusion, our findings show that lack of dietary carbohydrates with LC-HF diets leads to glucose intolerance and insulin resistance in rats despite causing a reduction in fasting glucose and insulin concentrations. Importantly, these effects were observed under both pair-feeding conditions (to control for the effects of energy intake) and conditions of caloric restriction (to control for the effects of visceral fat accumulation). Furthermore, the relative abundance of fat and protein in the two LC-HF diets did not play a major role in explaining the unfavorable outcome. Thus, despite their beneficial effects on body weight and a reduction of fasting insulin, C-peptide, and glucose levels, our results do not support a beneficial role of LC-HF diets for sustained improvements in glucose metabolism or for the prevention of diabetes mellitus. Furthermore, when using these diets for weight loss and the treatment of other conditions, our data imply that potentially harmful metabolic consequences should be kept in mind.

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DISCLOSURES

All authors declare that they have no conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

M. Bielohuby, D.A.S., R.W., R.J.S., and M. Bidlingmaier contributed to the conception and design of the research; M. Bielohuby, S.S., N.H., A.Z., M.F., D.M., B.J.S., and K.S. performed the experiments; M. Bielohuby, S.S., D.A.S., N.H., D.M., B.J.S., K.S., and M. Bidlingmaier analyzed the data; M. Bielohuby, S.S., D.A.S., N.H., A.Z., B.J.S., R.W., M.H.T., R.J.S., and M. Bidlingmaier interpreted the results of the experiments; M. Bielohuby and S.S. prepared the figures; M. Bielohuby, N.H., and M. Bidlingmaier drafted the manuscript; M. Bielohuby, M.F., M.H.T., R.J.S., and M. Bidlingmaier edited and revised the manuscript; M. Bielohuby, S.S., D.A.S., N.H., A.Z., M.F., D.M., B.J.S., K.S., R.W., M.H.T., R.J.S., and M. Bidlingmaier approved the final version of the manuscript.

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