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The Effects of Long- or Medium-Chain Fat Diets on Glucose Tolerance and Myocellular Content of Lipid Intermediates in Rats

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Accumulation of triacylglycerols (TAGs) and acylcarnitines in skeletal muscle upon high-fat (HF) feeding is the resultant of fatty acid uptake and oxidation and is associated with insulin resistance. As medium-chain fatty acids (MCFAs) are preferentially β-oxidized over long-chain fatty acids, we examined the effects of medium-chain TAGs (MCTs) and long-chain TAGs (LCTs) on muscle lipid storage and whole-body glucose tolerance. Rats fed a low-fat (LF), HFLCT, or an isocaloric HFMCT diet displayed a similar body weight gain over 8 weeks of treatment. Only HFLCT increased myocellular TAG (42.3 ± 4.9, 71.9 ± 6.7, and 48.5 ± 6.5 µmol/g for LF, HFLCT, and HFMCT, respectively, $P < 0.05$) and long-chain acylcarnitine content ($P < 0.05$). Neither HF diet increased myocellular diacylglycerol (DAG) content. Intraperitoneal (IP) glucose tolerance tests (1.5 g/kg) revealed a significantly decreased glucose tolerance in the HFMCT compared to the HFLCT-fed rats (802 ± 40, 772 ± 18, and 886 ± 18 area under the curve for LF, HFLCT, and HFMCT, respectively, $P < 0.05$). Finally, no differences in myocellular insulin signaling after bolus insulin injection (10 U/kg) were observed between LF, HFLCT, or HFMCT-fed rats. These results show that accumulation of TAGs and acylcarnitines in skeletal muscle in the absence of body weight gain do not impede myocellular insulin signaling or whole-body glucose intolerance.

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INTRODUCTION

Insulin resistance is an early and key defect in the development of type 2 diabetes, and is characterized by an impairment of insulin to regulate glucose homeostasis. Dietary factors play an important role in the development of insulin resistance. Numerous animal studies have demonstrated that feeding a high-fat (HF) diet (up to 75% kcal fat) impairs insulin action in the liver and skeletal muscle, the major sites of insulin-mediated glucose secretion and disposal, respectively (1–6). Most of these studies used diets rich in lard, palm oil, safflower oil, or corn oil, all of which consist of a different mixture of saturated and unsaturated long-chain triacylglycerols (TAGs) (LCTs) (1–7). These TAGs from plant or animal origin are composed of fatty acids with 12 or more carbon atoms and are the main contributors to the fat intake of the typical western diet (8).

Dairy products or coconut oils are foods with the highest content of medium-chain TAGs (MCTs), which consist of saturated fatty acids with a chain length of 6–12 carbon atoms. After hydrolysis of the MCT in the stomach and proximal small intestine, liberated medium-chain fatty acids (MCFAs) are immediately absorbed in the small intestine. Due to their shorter chain length these fatty acids are better water-soluble and less efficiently incorporated into chylomicrons compared to long-chain fatty acids. Furthermore, the majority enter the portal vein directly and thus bypass the lymphatic system (9). The metabolism of MCFAs is also different compared to long-chain fatty acids, as their uptake into mitochondria is not dependent on the activity of carnitine palmitoyltransferase-1. As the latter is considered to be the rate-limiting enzyme of β-oxidation, medium-chain fatty acids are preferentially oxidized when compared to long-chain fatty acids, which has been shown in acute and short-term diet-intervention studies in humans (10–13). Furthermore, similar findings were found in experiments feeding fatty acids with different chain lengths to rats (14).

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Ectopic fat storage is associated with insulin resistance (15). More specific, accumulation of lipid intermediates such as fatty-acyl CoAs, diacylglycerols (DAGs), and ceramides have been shown to be elevated in insulin-resistant states (16) and to inhibit insulin signaling directly (17). Recently, acylcarnitines were added to the list of lipid intermediates that may interfere with proper insulin signaling in skeletal muscle. Acylcarnitines are formed primarily in mitochondria from their respective fatty acyl-CoAs by carnitine acyltransferases (18) and it has been suggested that acylcarnitine levels increase when fatty acids are incompletely oxidized inside the mitochondria (19). Because MCFAs are preferentially oxidized and do not need the carnitine shuttle system to be taken up into mitochondria, we studied the hypothesis that an HF diet rich in MCT does not lead to accumulation of lipid intermediates in muscle and liver when compared to a diet with a similar fat content in the form of LCT, and therefore does not affect insulin sensitivity.

METHODS AND PROCEDURES

Animals and diets

Five-week-old male Wistar rats (Charles River) were housed individually in a room with controlled temperature (20–22 °C) with lights on from 0700 to 1900 hours. Animals were acclimatized to the housing conditions for 1 week before the start of the experiment. During the experiment, the animals had free access to food and acidified tap water.

To study the effect of the fatty acid chain length on ectopic lipid accumulation and muscle oxidation capacity, three groups of rats were fed a semi-purified low-fat (LF), HF long-chain TAG (HFLCT), or isocaloric HF medium-chain TAG (HFMCT) diet for 8 weeks. Rats were randomly assigned to the three different groups. In a second experiment, whole-body glucose tolerance and insulin signaling were studied under identical conditions as the first study.

The macronutrient and fatty acid composition of the diets is shown in Table 1. Diets (LF: 4068.10; HFLCT: 4031.17, and HFMCT 4031.16) were purchased from Hope Farms (Woerden, The Netherlands; see **Supplementary Table S1** online). The experimental protocol was approved by the Institutional Animal Care and Use Committee of Maastricht University and complied with the principles of laboratory animal care.

Net-energy absorption

Food intake and body mass were recorded weekly. During the last week of dietary intervention, feces were collected from the cage and separated from the bedding material with a sieve. After each collection, the fecal samples were frozen at −20 °C until further experiments. Both feces and random samples of the diet were lyophilized and analyzed simultaneously for energy content using adiabatic bomb calorimetry (Ika-calorimeter system C4000, Heitersheim, Germany). Net-energy absorption by the animal was calculated by subtraction of the energy content of the feces from the gross energy intakes.

Isolation and respiration of muscle mitochondria

After 8 weeks of diet intervention, nonfasted rats were sedated between 9 and 10 AM with a mixture of CO_2/O_2 (70/30%) and killed by cervical dislocation. Gastrocnemius muscle was dissected and immediately minced in ice-cold mitochondrial isolation medium with a pair of scissors and mitochondria were isolated as described before (20). State-3 and -4 respiration of the mitochondria were determined as described in detail previously (20). Gastrocnemius muscle from the contralateral leg and liver were frozen in liquid nitrogen-cooled isopentane and stored at −80 °C until further analyses.

Table 1 Composition of the diets

	LF	HFLCT	HFMCT
Protein (energy %)	18	18	18
Carbohydrate (energy %)	72	35	35
Fat (energy %)	10	47	47
Percentage of FAs in the diets			
C8:0	—	—	37
C10:0	—	—	55
C14:0	1	1	—
C16:0	53	75	1
C18:0	4	6	—
C18:1	16	13	2
C18:2	20	4	4
C18:3	6	1	1

FA, fatty acid; LF, low fat; HFLCT, high-fat long-chain triacylglycerol; HFMCT, high-fat medium-chain triacylglycerol.

Glucose tolerance test and insulin bolus

An intraperitoneal (IP) glucose tolerance test (1.5 g/kg body weight) was performed at week 8 after a 6-h fasting period. Blood samples were obtained *via* vena saphena puncture and collected in EDTA pretreated tubes (BD microtainer K2E tubes; Becton Dickinson, Franklin Lakes, NJ) at time intervals indicated and stored directly on ice. After the last collection, all samples were centrifuged at 3000 r.p.m. for 10 min to isolate the plasma. Glucose levels were measured directly using a hand-held glucose meter (Freestyle Freedom Lite; Abbott, Amersfoort, The Netherlands). After the glucose tolerance test, the rats were allowed to recover for 3 days. At 1400 hours after another 6-h fast, the rats were injected IP with an insulin bolus (10 U/kg body weight) and 9 min later, rats were sedated with a mixture of CO_2/O_2 (70/30%) and killed by cervical dislocation. A mixed blood sample was taken using a heart puncture, followed by immediate dissection of muscle and liver that were frozen and stored. In addition, gonadal and retroperitoneal adipose tissue depots were dissected, weighed, and discarded.

Plasma analysis

Insulin concentrations were determined in plasma samples using an ultrasensitive ELISA kit (Ultrasensitive Rat Insulin ELISA; Mercodia, Uppsala, Sweden) according to the manufacturer's protocol.

Histological analysis of intramyocellular and intrahepatic lipids

Cryosections (5 µm) from the liver and the mid-belly region of the gastrocnemius muscle were stained for neutral lipids with Oil red O (21) and quantified as previously described (20).

Skeletal muscle TAG and DAG analysis

Muscle tissue was freeze-dried and dissected from fat and vascular tissue. Lipids were extracted from the muscle fibers using MeOH:CHCl3 (1:2). Extracted lipids were separated by thin-layer chromatography and the TAG and DAG spots were analyzed using an analytical gas chromatograph with ion-flame detection. Results of the TAG and DAG measurements are presented as the sum of all fatty acids detected and expressed in µmol/g dry weight muscle sample.

Acylcarnitine profile analysis

Mass spectrometry-based acylcarnitine profiling was performed on gastrocnemius muscle and liver tissue as described previously by van Vlies *et al.* (22). Powdered muscle (3–10 mg) and liver (4–17 mg) were extracted using acetonitrile/water (80/20%) containing internal standards (16,250 pmol d3-C0-, 125 pmol d3-C3-, 50 pmol d3-C8-, and

50 pmol d3-C16-carnitine). After sonication (twice at 8 W output, 40 J, on ice) and centrifugation, the supernatant was collected and dried at 45 °C under N_2, followed by propylation for 15 min at 60 °C using 1-propanol/acetylchloride (4:1), drying at 45 °C under N_2, and dissolving the samples in acetonitrile. Quantitative determination of the formed acylcarnitines was performed using a Quattro II triple-quadrupole mass spectrometer (Micromass, Manchester, UK) and MassLynx 4.0 software (Micromass).

Western blot analysis

Muscle and liver were homogenized as described previously (23). Protein content in the samples was measured using a protein quantification kit (Protein assay; Bio-Rad Laboratories, Hercules, CA). Gels were loaded with the same amount of protein (~25 µg). After sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting, we incubated with Akt en Ser-473 P-Akt antibodies (cat. no. 9271 and cat. no. 9272, respectively; Cell Signaling Technology, Bioké, Leiden, The Netherlands) and with the appropriate secondary antibodies. Specific protein bands were visualized by chemiluminescence and analyzed by Chemidoc XRS system (Bio-Rad, Veenendaal, The Netherlands). OXPHOS protein expression was determined with an antibody cocktail against several subunits of the mitochondrial respiratory chain as described before (24).

Enzyme activity measurements

Muscle and liver β-hydroxyacyl-CoA dehydrogenase (β-HAD), citrate synthase (CS), and cytochrome C oxidase (COX) enzyme activities were determined spectrophotometrically. In short, 30 (20 µm) sections of muscle or liver were added to 100 µl sucrose–EDTA–Tris buffer (250 mmol/l sucrose, 2 mmol/l EDTA, and 10 mmol/l Tris (pH 7.4)) and sonicated (Branson 2210; Branson Ultrasonics, Danbury, CT) for 25 s. Samples were centrifuged (10 min, 10,000*g*, 4 °C), and the supernatants were analyzed spectrophotometrically (Multiskan Spectrum; Thermo Labsystems, Breda, The Netherlands) to determine β-HAD (25) and CS (26) enzyme activities. COX activity was determined after digitonin treatment of the supernatant in a 50 mmol/l potassium phosphate buffer by measuring the rate of oxidation of reduced cytochrome C as reflected by a change in absorbance at 550 nm. Protein concentrations were measured in the same supernatant using a Dc-kit (Bio-Rad, Berkeley, CA) according to the manufacturers instructions. Activity of the proteins is expressed in U/g protein.

Liver cryosections were incubated with D-glucose-6-phosphate to demonstrate D-glucose-6-phosphatase (G6P) activity as described (27). In short, 5-µm thick cryosections were incubated at 37 °C temperature with incubation medium for 20 min. The reaction was stopped by washing the sections in demineralized water, followed by incubation with 1% ammonium sulfide for 2 min. Sections were studied at a ×200 magnification and quantified using a Leica DMRD microscope (Wetzlar, Germany). G6P activity was calculated from the change in absorbance. The system was calibrated by assigning 0% absorbance to a section without the substrate incubation and 100% to a black image.

Statistical analysis

Results are presented as mean ± s.e.m. Statistical analysis was performed using SPSS for Mac, version 16.0 (SPSS, Chicago, IL). Differences between groups were evaluated by one-way ANOVA test. When significant differences were found, a Bonferroni adjusted *post hoc* test was used to determine the exact location of the difference. Outcomes were regarded statistically significant if $P < 0.05$.

RESULTS

Body mass and net-energy uptake

During the 8-week intervention period no differences in body weight gain were observed between groups (see **Supplementary Figure S1** online). Gross energy intake in the HFLCT group was 1.2-fold higher ($P < 0.001$) than in the LF group. Energy intake in the HFMCT was not significantly different compared to the LF or HFLCT groups. However, fecal energy output was 2.5-fold higher in the HFLCT compared to both other groups ($P < 0.001$), resulting in a similar net-energy intake (see **Supplementary Figure S2** online) and energy absorption in all groups (see **Supplementary Table S2** online). Corresponding with the similar body weights, wet weight of the gonadal, and retroperitoneal fat depots was not different between groups (see **Supplementary Table S2** online).

Lipid accumulation in skeletal muscle

Using Oil red O staining, we observed that intracellular fat content in skeletal muscle was significantly increased by eight-fold in the HFLCT compared to the LF group ($P < 0.05$). The HFMCT intracellular lipid content was not different compared to the control group (**Figure 1**). Lipid levels were also quantified in skeletal muscle using thin-layer chromatography and subsequent gas chromatographic analysis. This analysis confirmed higher total myocellular TAG content in the HFLCT compared to LF and no difference between the HFMCT and the LF groups (**Table 2**). In contrast, total intramyocellular levels of DAG were not significantly different between groups (**Table 2**).

In addition to intramyocellular TAG and DAG levels, tandem mass spectrometry/mass spectrometry analysis was used to study the accumulation of acylcarnitines. Acetylcarnitine was lower in the HFLCT and higher in the HFMCT compared to

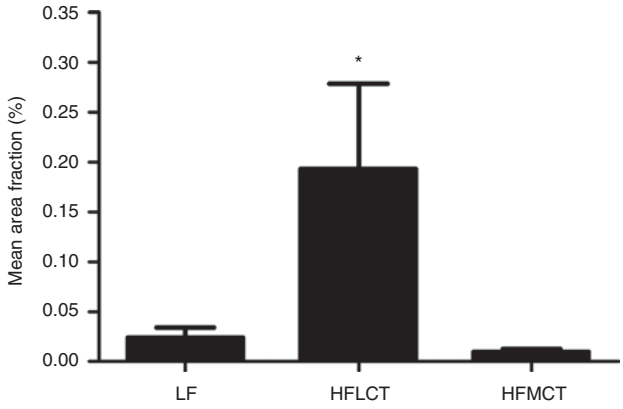

Figure 1 Effects of an 8-week intervention with an LF, HFLCT, or HFMCT diet on intramyocellular lipid content of the gastrocnemius muscle in adult male rats. Area fraction represents the percentage of the total muscle area covered by lipid droplets ($n = 9$, $^*P < 0.05$ vs. LF and HFMCT).

Table 2 Effects of an 8-week intervention with an LF, HFLCT, or HFMCT diet on gastrocnemius muscle triacylglycerol and diacylglycerol content ($n = 6–9$)

	LF	HFLCT	HFMCT
TAG (µmol/g dry weight)	42.3 ± 4.9	71.9 ± 6.7*	48.5 ± 6.5
DAG (µmol/g dry weight)	2.5 ± 1.1	3.3 ± 1	1.9 ± 0.6

LF, low fat; HFLCT, high-fat long-chain triacylglycerol; HFMCT, high-fat medium-chain triacylglycerol.
*$P < 0.05$ vs. LF and HFMCT.

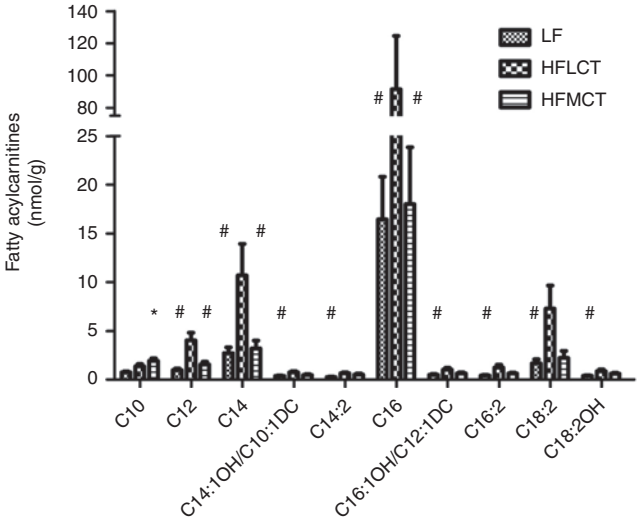

Figure 2 Effects of an 8-week intervention with an LF, HFLCT, or HFMCT diet on skeletal muscle acylcarnitine profiles in adult male rats ($n = 9$, $^{*}P < 0.05$ vs. LF, $^{\#}P < 0.05$ vs. HFLCT).

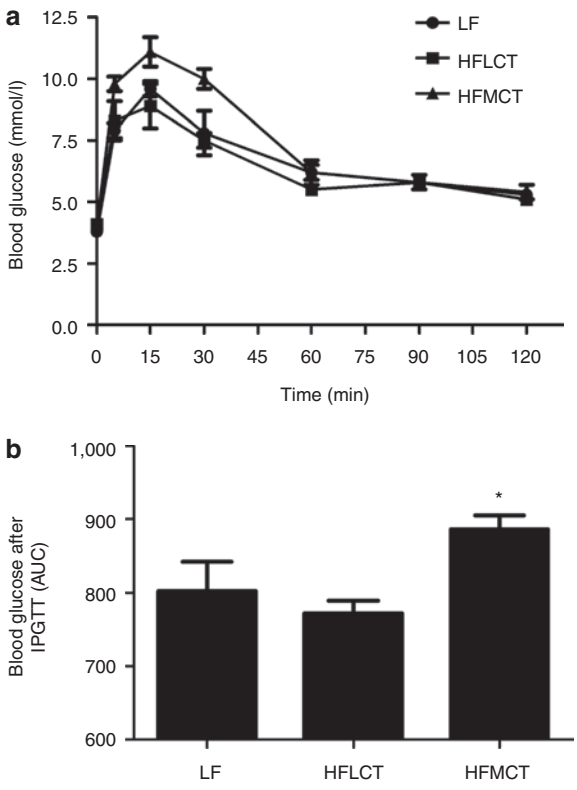

Figure 3 Effects of an 8-week intervention with an LF, HFLCT, or HFMCT diet on whole-body glucose tolerance of adult male rats. (**a**) Glucose clearance after IP glucose bolus (1.5 g/kg) and (**b**) area under the curve of the IPGTT ($n = 5$–6, $^{*}P < 0.05$ vs. HFLCT).

the LF-fed rats, although this did not reach statistical significance (915 ± 33, 848 ± 42, and 984 ± 64 nmol/g dry weight muscle for LF, HFLCT, and HFMCT, respectively, $n = 9$, $P = 0.15$). Several long-chain acylcarnitines were significantly elevated in the HFLCT group compared to the LF and HFMCT groups. This increase reached statistical significance for C14:1OH/C10:1DC, C14:2, C16:1OH/C12:1DC, C16:2, and C18:2OH acylcarnitines when compared to the LF group and for C12:0, C14:0, C16:0, C16:2, and C18:2 acylcarnitines when compared to both LF and HFMCT groups (**Figure 2**). Only the C10 medium-chain acylcarnitine was significantly elevated in the HFMCT compared to the LF group, which is consistent with the high level of C10 fatty acid in the diet (**Figure 2**). The other short-, medium- (<C12:0), and long-chain acylcarnitines were not significantly different between groups as shown in **Supplementary Table S3** online.

Muscle oxidative capacity

To study whether the observed lipid contents in skeletal muscle of the LF and HF groups was paralleled by changes in muscle oxidative capacity, we determined muscle mitochondrial function upon carbohydrate or fatty acid substrates. However, ADP-stimulated respiration with pyruvate, palmitoyl-CoA, or octanoyl-CoA as substrates in isolated mitochondria was not different between groups (see **Supplementary Table S4** online). Because these measurements were performed in isolated mitochondria, we next determined the expression of several subunits of the mitochondrial respiratory chain (30-kDa subunit of complex II, core protein 2 subunit of complex III, the α-subunit of complex V, and the ND6 subunit of complex I) and the activity of enzymes involved in fatty acid oxidation and oxidative capacity in whole muscle homogenates. Corroborating our findings, expression of mitochondrial respiration subunits (see **Supplementary Figure S3** online) and activity of the enzymes β-HAD, CS, and COX were not different between groups (**Table 3**).

Whole-body glucose tolerance

To investigate whether HF diets enriched in LCT or MCT affected glucose tolerance, we examined whole-body glucose clearance following an IP glucose tolerance test. Eight weeks of intervention did not result in significant differences in whole-body glucose clearance between the LF and HF-fed rats (**Figure 3a**). However, in contrast to our hypothesis, whole-body glucose tolerance was significantly lower in the HFMCT group compared to the HFLCT group (area under the curve, $P < 0.05$, $n = 5$, **Figure 3b**). Fasting plasma insulin levels (2.4 ± 0.6, 2.0 ± 0.3, and 3.0 ± 0.5 ng/ml in HFLCT, HFMCT, and LF, respectively, $n = 6$, P = not significant) and insulin levels during the glucose tolerance test (202 ± 24, 173 ± 17, and 243 ± 27 area under the curve in HFLCT, HFMCT, and LF, respectively, $n = 3$–4, P = not significant) were not significantly different between groups. Finally, the ratio of the [insulin]/glucose (area under the curve), which illustrates the relation between insulin secretion and glucose clearance revealed a significantly lower ratio in the HFMCT compared to the LF group ($P < 0.05$), but no significant differences between both HF groups ([insulin]/glucose; 0.01 ± 0.001, 0.007 ± 0.001, and 0.006 ± 0.001 in LF, HFLCT, and HFMCT, respectively).

Table 3 Effects of an 8-week intervention with an LF, HFLCT, or HFMCT diet on skeletal muscle and liver enzyme activity of β-HAD, CS, and COX ($n = 9$, $^*P < 0.05$ vs. HFLCT)

Diet	Muscle	Liver
β-HAD (U/g protein)		
LF	23.9 ± 2.1	78.6 ± 9.7
HFLCT	26.9 ± 1.8	66.7 ± 5.1
HFMCT	24.9 ± 1.8	97.9 ± 7.7*
CS (U/g protein)		
LF	19.1 ± 2.3	45.6 ± 1.9
HFLCT	17 ± 2.0	43.7 ± 2.5
HFMCT	19.8 ± 2.2	44.7 ± 1.7
COX (U/g protein)		
LF	62.9 ± 5.7	153.1 ± 14.4
HFLCT	59.6 ± 2.9	143.5 ± 12.7
HFMCT	58.6 ± 4.4	121.9 ± 8.1

β-HAD, β-hydroxyacyl dehydrogenase; COX, cytochrome C oxidase; CS, citrate synthase; LF, low fat; HFLCT, high-fat long-chain triacylglycerol; HFMCT, high-fat medium-chain triacylglycerol.

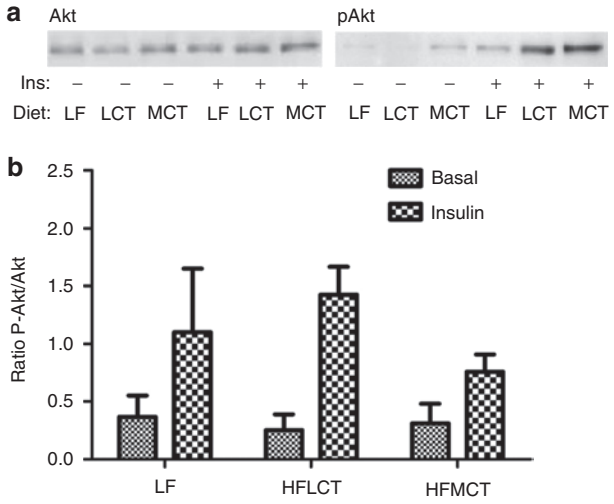

Figure 4 Effects of an 8-week intervention with an LF, HFLCT, or HFMCT diet on rat skeletal muscle (**a**) expression of Akt and P-Akt before and after an IP insulin bolus (Ins) (10 U/kg) and (**b**) the ratio of P-Akt/Akt (basal; $n = 2$–3, insulin stimulated; $n = 5$–6).

Muscle insulin signaling

To determine whether the HF diets modified insulin action in skeletal muscle, we injected rats IP with an insulin bolus and measured Akt Ser-473 phosphorylation. Insulin administration increased the phosphorylation of Akt in all groups (Figure 4a), without significant differences in the ratio of P-Akt/Akt between groups (Figure 4b).

Liver lipids and insulin signaling

Medium-chain fatty acids are efficiently absorbed in the intestines and transported predominantly to the liver *via* the portal vein. Therefore, we also studied the effects of the diets on liver lipid accumulation and liver insulin signaling. Hepatic lipid content was threefold higher in the HFLCT compared to the LF ($P < 0.05$) and not significantly different in the HFMCT compared to the LF group (0.73 ± 0.35, 1.93 ± 0.69, and 0.50 ± 0.10 area fraction (%) in LF, HFLCT, and HFMCT, respectively, $n = 9$). Analysis of the acylcarnitine subtypes revealed a significant elevation of C18 acylcarnitine in the HFLCT compared to the LF group. The HFMCT group had higher levels of the C4-3OH acylcarnitine and several medium-chain acylcarnitine subtypes (C8, C10, and C12:1OH/C8:1DC) compared to the LF controls (see **Supplementary Figure S4** online). Furthermore, no differences in long-chain acylcarnitine levels were observed between groups. Results of all analyzed acylcarnitine subtypes are shown in **Supplementary Table S5** online.

To determine whether the HF diets modified insulin action in liver, we injected rats IP with an insulin bolus and measured Akt Ser-473 phosphorylation. Insulin-stimulated Akt phosphorylation in all groups (see **Supplementary Figure S5A** online) without differences in the P-Akt/Akt ratio between groups (see **Supplementary Figure S5B** online).

Table 4 G6P activity in periportal and pericentral zones from livers of rats after an 8-week diet intervention with LF, HFLCT, or HFMCT ($n = 9$)

	LF	HFLCT	HFMCT
Periportal G6P activity (% absorbance)	59 ± 2	67 ± 4	87 ± 3*
Pericentral G6P activity (% absorbance)	45 ± 2	52 ± 3	56 ± 3#

G6P, glucose-6-phosphatese; LF, low fat; HFLCT, high-fat long-chain triacylglycerol; HFMCT, high-fat medium-chain triacylglycerol.
$^*P < 0.001$ HFMCT compared to LF or HFLCT. $^\#P < 0.05$ HFMCT compared to LF.

Liver enzyme activity

Analysis of the activity of enzymes involved in mitochondrial oxidation and oxidative phosphorylation revealed that the HF diets did not have a significantly different activity of β-HAD, CS, and COX compared to the LF groups. However, β-HAD activity was higher in the HFMCT compared to the HFLCT (Table 3). This effect of MCTs on a marker of fatty acid oxidation was substantiated by a significantly ($P < 0.05$) higher expression of 3-hydroxyacyl Coenzyme A dehydrogenase (Ehhadh) and cytochrome P450, family 4, subfamily a, polypeptide 14 (Cyp4a14), which are also markers of fatty acid metabolism, compared to the LF and HFLCT groups. The fold-change of 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (Hmgcs2), which is involved in ketogenesis, was only significantly higher in the HFMCT compared to the LF group ($P < 0.01$) (see **Supplementary Figure S6** online). Above results of the fatty acid metabolism markers might indicate that fatty acid oxidation is increased, which is associated with a higher liver glucose production (28). We measured the activity of G6P, which is a rate-limiting enzyme for the hepatic release of glucose from the liver. We showed that the G6P activity was higher in the periportal zone compared to the pericentral zone in all livers (see **Supplemental Figure S7A–C** online), which was expected as the contribution of this region is predominantly

associated with gluconeogenesis. More importantly, we observed a significantly upregulated ($P < 0.001$) G6P activity in the periportal zone of the MCT livers compared to the LCT group (**Table 4**), which points to a higher glucose output capacity and may therefore explain the difference in glucose tolerance between the MCT and LCT-fed rats.

DISCUSSION

The accumulation of lipids in insulin sensitive tissues other than adipose tissue, such as skeletal muscle and liver, is considered to play a causative role in the development of insulin resistance (7). This study demonstrates that feeding a high amount of dietary fat in the form of medium-chain TAGs to rats did not lead to accumulation of fat in muscle and liver, in contrast to feeding a similar amount of fat in the form of long-chain TAGs. Unexpectedly, MCT impaired whole-body glucose tolerance compared to LCT and neither HF diet impaired whole-body glucose tolerance compared to the LF group. This illustrates that accumulation of fat in skeletal muscle and liver are not necessarily linked to whole-body glucose intolerance.

Fat accumulation in skeletal muscle has been related to the development of insulin resistance (29). Indeed, lipid infusion in humans leads to the acute induction of insulin resistance, in concert with increased intramuscular lipid accumulation in both humans (30,31) and animals (32). In addition, several genetic animal models, such as the Zucker rats with a genetic form of obesity (33) or transgenic mice that lack white adipose tissue (34) showed an inverse relation between intramyocellular lipid accumulation and insulin resistance. Furthermore, numerous animal studies showed an association between myocellular lipid accumulation and insulin resistance using an HF enriched diet intervention. The HF diets used in these studies consisted mostly of mixtures of saturated and unsaturated fatty acids (1,7,35). We found that changing the fatty acid composition from an HF diet consisting of predominantly LCT to MCT did not result in the accumulation of lipids in the skeletal muscle. In fact, despite the high dietary fat intake in the MCT group, levels of intramyocellular lipids were as low as in the LF group. These results are consistent with our previous observation in humans, in which a combined MCT/LCT lipid infusion resulted in markedly lower muscular fat content compared to a 100% LCT infusion (31).

Based on these results, we hypothesized that the low level of muscle fat content after an MCT diet would result in unchanged or improved myocellular and whole-body insulin sensitivity. Surprisingly, in contrast to our expectation, whole-body glucose tolerance was significantly reduced after MCT compared to the LCT diet. Thus, the impaired whole-body glucose tolerance after HFMCT feeding is not associated with increased myocellular lipid levels.

Although a substantial body of data has implicated increased myocellular lipid levels in the development of insulin resistance, it has been suggested that accumulation of DAG rather than TAG is responsible for the development of insulin resistance (36). Therefore, we also determined muscle DAG levels in this study, but we did not observe an effect of HFMCT or LCT diets on myocellular DAG levels compared to LF diets, consistent with the absence of glucose intolerance in the LCT group.

Other recently suggested lipid intermediates potentially inducing insulin resistance are the acylcarnitines. Koves *et al.* (19,37) proposed that in case of lipid overflow, mitochondrial β-oxidation may exceed the capacity of the electron transport chain, resulting in the accumulation of fatty-acyl CoAs. To keep CoA available for the mitochondrial oxidative processes, the overload of fatty-acyl CoAs are converted into acylcarnitines that can leave the mitochondria (38). Indeed, Koves *et al.* (19) reported that acylcarnitines accumulate in insulin-resistant states. In addition, prevention of the accumulation of acylcarnitines using malonyl-CoA decarboxylase knockout mice resulted in improved glucose tolerance. Furthermore, Adams *et al.* (39) recently showed an association between plasma levels of C10–C14 acylcarnitines and HbA_{1c} in type 2 diabetes patients and healthy controls. They hypothesized that high levels of medium-chain acylcarnitines induce a proinflammatory state by activation of NF-κB, which is implicated in promoting insulin resistance (39). A potential direct effect of acylcarnitines on insulin signaling remains, however, to be investigated. Because MCT are preferentially oxidized and do not need the carnitine shuttle system to enter mitochondrial oxidation, we determined the acylcarnitine ester content in muscle of MCT-fed rats and compared it to LCT-fed rats. We found higher levels of mainly long-chain acylcarnitines in muscles of rats fed the HFLCT diet, as shown previously (19,40). In accordance with lower myocellular TAG levels after MCT, we found that also medium- to long-chain acylcarnitines levels were not elevated after HFMCT diet, when compared to HFLCT diet. In addition, only a small increase in medium-chain acylcarnitines was observed (C10).

However, the difference in acylcarnitine levels between the LCT and MCT groups was not due to differences in muscle oxidative capacity, as we observed no differences between the groups in the maximal mitochondrial respiration response to oxidize a carbohydrate or lipid substrate. Furthermore, maximal activity of key enzymes involved in the β-oxidation (β-HAD), TCA cycle (CS), and oxidative phosphorylation (COX) in muscle homogenates were not different between the LF and HF groups. Finally, the expression of four distinct proteins involved in mitochondrial oxidative phosphorylation, used here as a marker of mitochondrial content, was also similar between all groups.

This may indicate that the increased long-chain acylcarnitine levels are merely due to an imbalance between the mitochondrial fatty acid supply and the demand for fatty acid oxidation and ATP production in the muscle and not due to a decreased capacity of complete β-oxidation. Moreover, acylcarnitine production in tissues probably represents only a small proportion of the total fatty acid oxidation flux (41). Alternatively, the low level of acylcarnitines in the MCT group could be due to a lower supply of lipids to the muscle, which was not associated with improved whole-body glucose tolerance in this study. If the increased acylcarnitine levels are associated with an increased inflammatory status remains to be investigated.

In this study, we did not determine muscle insulin sensitivity using clamp experiments. We did determine insulin-stimulated Akt phosphorylation, which is an important regulator of insulin-stimulated glucose uptake and an accepted marker for insulin sensitivity. It has been suggested that fat accumulation in skeletal muscle interferes with muscle insulin signaling and thereby leads to insulin resistance. However, in this study we could not detect differences in P-Akt between LF, HFMCT, and HFLCT. This shows that our HF diets did not result in muscle insulin resistance at the level of insulin signaling, and therefore this pathway is most likely not responsible for the reduced whole-body glucose tolerance after MCT. However, it also shows that marked differences in muscle TAG and acylcarnitine content do not necessarily result in differences in muscle insulin signaling.

One of the metabolic characteristics of MCTs is their efficient intestinal absorption (42) and fast transport directly to the liver *via* the portal vein (43,44). Therefore, we also studied liver lipid accumulation and effects of the diets on liver insulin signaling. We observed no lipid accumulation in the liver after feeding the HFMCT diet, whereas the rats fed the HFLCT diet had higher lipid levels compared to the LF-diet-fed rats. Furthermore, liver acylcarnitine levels showed only small differences between the three groups, which suggest a distinct regulation between accumulation of acylcarnitine species in the liver and the skeletal muscle. Again, no differences in insulin signaling were observed between groups, dissociating liver lipid accumulation and impaired insulin signaling. In addition, upon HFMCT feeding we observed, compared to the HFLCT, an increased activity of β-HAD, an important enzyme involved in mitochondrial β-oxidation, and a high expression of Ehhadh and Cyp4a14, both markers of fatty acid oxidation. It is known that fatty acid oxidation is associated with hepatic glucose production (28). Therefore, a high rate of fatty acid oxidation in the HFMCT group may not only result in a higher glucose production but may also result in a higher glucose output compared to the HFLCT. We stained all liver sections to determine G6P activity, which is one of the rate-controlling enzymes involved in glucose release from the liver. Indeed G6Pase activity was strongly induced in the periportal zone, which is the gluconeogenic region (45), of the livers in the HFMCT compared to the HFLCT-fed rats. We therefore speculate that the HFMCT-induced glucose intolerance can be traced to an increased output of glucose from the liver. Animals on an HFMCT diet might thus have a reduced muscle glucose uptake due to the preferential oxidation of MCT over glucose. However, further exploration of these results requires additional studies.

Results from epidemiological and controlled dietary intervention studies suggest that replacing saturated fatty acids for other lipids has beneficial effects on insulin sensitivity (46). In this study, we observed lipid accumulation in the skeletal muscles and livers of the animals fed the HFLCT diet but could not detect a change in glucose tolerance or the development of obesity compared to the LF-fed rats. In contrast, many rodent studies showed detrimental effects of HF diets on glucose tolerance and the development of obesity.

However, comparisons of dietary fat types in other rodent studies also failed to confirm a clear association between fat saturation and insulin sensitivity (4,7). One of the differences between the diets used in literature and the HF long-chain diet used in this study is the degree of saturation. One of the most used HF diets in the literature is D12451 from Research Diets, which has approximately a 50/50% ratio in saturated vs. unsaturated lipids. In addition, this diet contains 17% (kcal) sucrose, which is a rich source of fructose. Dietary fructose is associated with increased levels of obesity and the development of insulin resistance (47,48) and is absent in our diets. It will require additional intervention studies to evaluate if the percentage of dietary saturated fatty acids and/or the presence of dietary fructose are indeed important for the development of obesity and insulin resistance.

In summary, our results suggest that accumulation of TAG and acylcarnitines in skeletal muscle in the absence of gain of body weight not necessarily lead to impaired muscular insulin signaling and whole-body glucose intolerance. This also shows that the effects of different HF sources on insulin sensitivity are not yet clear and require further study.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/oby

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DISCLOSURE

The authors declared no conflict of interest.

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