Interactions between hepatic glucose and fat metabolism in animal models of insulin resistance
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Chapter 3

Dietary fat-induced hepatic insulin resistance with regard to VLDL production in rats

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Submitted

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ABSTRACT

Insulin resistance and type 2 diabetes are associated with changes in plasma lipoprotein levels. Long-term high-fat feeding induces insulin resistance in rodents but short-term effects of high-fat feeding are poorly defined. After three weeks on low- or high-fat diets, we examined insulin sensitivity, hepatic VLDL production and hepatic insulin signaling during hyperinsulinemia in Wistar rats. Plasma insulin levels were increased by 75% in rats fed the high-fat diet and the insulin response to an intravenous glucose infusion was impaired compared to rats fed the low-fat diet. Rats fed the high-fat diet developed massive liver steatosis. VLDL-apoB100 content (+74%), hepatic VLDL-triglyceride production (+62%), apoB100/B48 ratio (+39%) and VLDL particle size (+42%) were increased in isolated VLDL fractions from rats fed the high-fat diet. Importantly, insulin showed a decreased ability to suppress VLDL-triglyceride production and apoB100/B48 ratio during a hyperinsulinemic clamp in rats fed the high-fat diet. High-fat feeding decreased hepatic insulin receptor gene expression and protein levels. Furthermore, hepatic protein kinase B phosphorylation during acute hyperinsulinemia was decreased suggesting impaired hepatic insulin signaling. Short-term high-fat feeding leads to glucose intolerance and hepatic steatosis in rats. These metabolic changes are associated with increased VLDL-triglyceride and -apoB100 production and a decreased ability of insulin to suppress hepatic VLDL production rate.

Keywords. Diet, liver, VLDL, insulin resistance, rats.
INTRODUCTION

Type 2 diabetes mellitus (DM2) in humans is associated with the development of an atherogenic lipoprotein profile with hypertriglyceridemia, low HDL and a preponderance of small, dense LDL particles (1). This characteristic plasma lipid profile has been attributed to increased production of VLDL-triglyceride (TG) and -apolipoprotein B (apoB) by the liver (2-5). Substrate availability, in particular NEFA supply, is thought to be an important factor in control of hepatic VLDL production (6). Lowering plasma NEFA concentration by a hyperinsulinemic clamp leads to a reduction of VLDL production in healthy volunteers (7-10), but not in DM2 patients (11) and obese individuals (7). Insulin acutely suppresses VLDL-apoB production rate in humans, mainly by reducing the production of large, TG-rich VLDL1 particles without affecting the production of smaller VLDL2 particles (9,10). The ability of insulin to suppress VLDL1 production is impaired in human insulin resistant states (11). Processing of large, TG-rich VLDL1 particles in plasma results in the formation of small, dense LDL particles, also called pattern B LDL (1,12). These particles are more susceptible to oxidation and, therefore, constitute an increased risk for development of cardiovascular disease (13-15). Hepatic steatosis is also a common feature in DM2 patients (16) and may be causally linked to the development of hyperlipidemia. However, the relationship between the disturbed VLDL formation, insulin resistance, and hepatic steatosis in DM2 patients is unclear.

The phosphatidylinositol 3-kinase (PI3K) pathway is thought to be the major pathway involved in insulin-mediated control on glucose homeostasis as well as on VLDL production (17,18). Animal models of diabetes, like the obese ob/ob mouse (19,20), db/db mouse (21) and ZDF rat (22), all show a decreased insulin signaling at the level of the insulin receptor substrate (IRS) and/or PI3K. The serine/threonine protein kinases B and C (PKB or Akt and PKC) are both activated downstream of PI3K and might modulate the insulin signal transduction and thereby influence VLDL secretion. PKB has recently been shown to stabilize IRS activity by phosphorylation of specific serine residues, thereby prolonging insulin-signaling (23). Different PKC isoforms have been associated with impaired insulin signaling (24,25). In a fatty liver, therefore, over-activation of PKC and/or inhibition of PKB signaling may contribute to impaired insulin signaling (23,26) and hence to insulin resistance.

Long-term high-fat feeding (>15 weeks) in rodents results in insulin resistance, hepatic steatosis, hyperglycemia, hyperinsulinemia and hyperlipidemia, all characteristics of a diabetic phenotype (27,28). Short-term high-fat feeding may already affect hepatic insulin sensitivity and hepatic glucose metabolism, but effects on hepatic lipid metabolism and VLDL formation are still largely unknown. We studied the effects of short-term high-fat feeding (3 weeks) on the insulin sensitivity of the VLDL production process in male Wistar rats.

MATERIAL AND METHODS

Animals. Male Wistar rats weighing between 300-350 gram (Harlan Laboratories, Zeist, The Netherlands) were fed either the high-fat diet or the control, low-fat diet. After two weeks on diet, rats were equipped with a permanent catheter in the right jugular vein to allow infusion and blood collection in unrestrained conditions (29). After surgery rats recovered for a period of 7 days in individual cages in a light- (8am-8pm), and temperature- (20 °C) controlled room with diet and water available at libitum. Food intake returned to preoperative levels within two days. All experimental procedures were approved by the Ethical Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen.
Diet. Low- and high-fat semi-purified diets were developed by Hope Farms (Woerden, The Netherlands). Total fat and carbohydrate content was 8 wt% and 60 wt%, respectively in the low-fat diet (4148.01), whereas the high-fat diet (4148.02) contained 25 wt% fat and 25 wt% carbohydrate. The high-fat diet contained more palmitate (C16:0, 29.13 g/kg vs. 9.12 g/kg) and oleate (C18:1, 32.08 g/kg vs. 100.24 g/kg) compared to the low-fat diet. Other nutritional components were comparable between the two diets. The protein content was slightly lower in the low-fat diet, but due to a higher daily intake (20±1 vs. 17±1 g/day in low-fat and high-fat diet, respectively), total protein intake was similar between the groups.

Intravenous glucose tolerance test. To establish dietary effects on insulin sensitivity, rats underwent an intravenous glucose tolerance test (IVGTT). After a fasting period of 9 hours a blood sample (~150 µl) was taken to determine basal levels of the metabolites of interest. The IVGTT started with a glucose infusion for 20 minutes (10% D-glucose, Merck, Darmstadt, Germany; infusion rate 3 ml/hr). During the infusion, blood samples (~80 µl) were taken after 1, 3, 5, 10, 15 and 20 minutes. After 20 minutes the infusion was stopped but blood sampling continued for another 20 minutes (blood samples after 25, 30, 35 and 40 minutes). Samples were placed on ice in heparin-containing tubes and centrifuged at 5,000 rpm for 10 minutes at 4 ºC. Plasma was stored at -20 ºC until analysis.

Hyperinsulinemic clamp. To study the effects of insulin on lipoprotein metabolism, rats underwent a hyperinsulinemic clamp. The infusion contained insulin (18 mU.kg⁻¹.min⁻¹; Novo Nordisk, Bagsvaerd, Denmark), somatostatin (1.5 µg.kg⁻¹.min⁻¹; UCB, Breda, The Netherlands) to suppress the endogenous insulin production, and glucose (25 mg.kg⁻¹.hr⁻¹; Merck, Darmstadt, Germany) to maintain normal plasma glucose concentrations. Based on euglycemic insulin clamps performed in rats and mice by Hawkins and Rossetti et al. (30,31) fixed at ~25 ng/ml we used a single infusion containing all constituents, aimed to result in hyperinsulinemia and euglycemia. The procedure was tested in pilot experiments. All infusates were freshly prepared in saline containing 1.5% BSA (Sigma, St. Louis, MO, US). The total infusion time was 6 hours. After 3 hours of infusion, Triton WR1339 (Tyloxapol; Sigma, St. Louis, MO, US), 12% w/w in saline, 5 ml/kg BW, was injected. Triton blocks lipolysis of TG contained within secreted lipoprotein particles and, therefore, accumulation of plasma TG in time allows calculation of TG production rates (32). Blood samples were taken at 30, 60, 120 and 180 minutes after the Triton injection and were treated as described above. After the experiment, plasma was collected to isolate VLDL/LDL particles using a solution of 15.3% NaCl (Merck, Darmstadt, Germany) and 35.4% KBr (Merck, Darmstadt, Germany), final concentration 0.65% and 1.52%, respectively, in saline with a density <1.019 g/ml. Plasma (0.5 ml) was mixed with 0.5 ml of a NaCl-KBr solution and centrifuged for 100 minutes at 120.000 rpm and 4 ºC. Tubes were sliced at 1.5 cm and the VLDL-fraction was collected and frozen at -80 ºC for composition analysis and apoB100 quantification (see below). VLDL particle size was determined in isolated VLDL fractions using a Submicron Particle Sizer, (Autodilute, model 370; Nicomp, Santa Barbara, CA, US).

Analysis. Plasma and hepatic TG, total cholesterol, and free cholesterol levels were determined by commercially available kits (Roche, Mannheim, Germany). Plasma and hepatic phospholipid and plasma NEFA concentrations were determined with Phospholipid-kit and NEFA-C kit, respectively (Wako Chemical GmbH, Neuss, Germany). Plasma insulin was determined by a radioimmunoassay (RIA) RI-13K (Linco Research, Inc., St. Charles, MO, US). Plasma glucose concentration was determined with a GlucoTouch-glucose analyzer (LifeScan, Beerse, Belgium). Liver lipids were extracted following a modified Bligh & Dyer
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method (33) and determined by commercially available kits. Total protein of tissue homogenates was determined using the method described by Lowry et al. (34).

**Histology.** To visualize fat deposition in the liver an "Oil-red-O" staining was performed on 4 µm liver slices.

**ApoB quantification.** ApoB100 concentrations were quantified with a reference to a human IDL apoB-standard isolated from healthy human subjects (35). Since human liver does not produce apoB48-containing VLDL particles, we were not able to accurately quantify apoB48 levels with our human apoB-standard. The apoB100/B48 ratio, as a measure for the secretion particle type, was therefore calculated from the ratio between Western blot band intensities of apoB100 and apoB48. Isolated VLDL samples (10 µl) were delipidated with methanol and diethylether and dried under nitrogen. Apolipoproteins were separated by SDS-PAGE using 4-15% gradient gels (Ready gels, Biorad, Hercules, CA, US). Delipidated lipoprotein samples were reduced in SDS sample buffer (8 mol/l urea, 10 mmol/l Tris base, 2% SDS, 10% glycerol, 5% β-mercaptoethanol). Samples (15 µl) were applied on gels and run at 100 V for 30 minutes and at 120 V for 120 minutes. Proteins were visualized and quantified by silver-staining procedure. For Western blot analysis proteins were transferred on nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) by blotting for 90 minutes at 250 mA. Blots were put on skimmed milk overnight and stained with a primary polyclonal antibody against human apolipoprotein B, raised in sheep (dilution 1:100,000; Roche, Mannheim, Germany) and secondary IgG, anti-sheep antibody conjugated with horseradish-peroxidase activity (dilution 1:10,000; Calbiochem, San Diego, CA, US). ECL activation of the peroxidase was detected on film.

**Insulin signaling.** IR protein levels were determined in a total membrane fraction of liver homogenates. Liver parts (~250 mg) were homogenized in 2 ml PBS with protein inhibitors (Roche, Mannheim, Germany). After centrifugation (1 hour, 45,000 rpm, 4 °C) the pellet was resuspended in 0.5 ml PBS. Protein content of the isolated total membrane fraction was determined by the method of Lowry et al. (34). Proteins were separated by SDS-PAGE and blotted on nitrocellulose membrane as described above. Western blot analysis was performed with the primary polyclonal antibody against the IRβ-chain (C-19), raised in rabbit (dilution 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, US) and secondary anti-rabbit antibody conjugated with horseradish-peroxidase activity (dilution 1:10,000; Calbiochem, San Diego, CA, US). For analysis of PKB and PKCζ phosphorylation, liver parts were homogenized in buffer (10 mmol/l Tris.Cl pH 7.5, 150 mmol/l NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% NP40, 10 mmol/l NaF and 1 mmol/l Na₃VO₄) containing protease and phosphatase inhibitors. Extracts were cleared by centrifugation and protein content was measured using a BCA kit (Pierce, Rockford, IL, US). Proteins (40 µg) were separated by SDS-PAGE and blotted on PVDF-membrane (Millipore, Bedford, MA, US). Western blot analysis was performed with the primary phosphospecific polyclonal antibody against PKB-Ser473 and PKCζ/λ (Thr410/403), raised in rabbit (dilution 1:1,000, Cell Signaling Technologies, Beverly, MA, US) and a secondary anti-rabbit antibody conjugated with horseradish-peroxidase activity (dilution 1:10,000, Calbiochem, San Diego, CA, US).

**RNA analysis, cDNA synthesis, and RT-PCR.** Liver samples of approximately 30 mg were used for total RNA isolation with the Trizol method (GIBCO, Grand Island, NY, US) followed by the SV Total Isolation System (Promega, Madison, WI, US) according to the protocols provided by the manufacturer. Single stranded cDNA was made using materials from Roche according to manufacturers instructions. Samples were incubated at 25 °C for 10
minutes, 45 °C for 60 minutes and, 95 °C for 5 minutes. RT-PCR was done in 50 µl preparations using 2 µl cDNA, 0.25 taq polymerase, 5 µl tenfold buffer, 0.75 µl dNTP-mix (10 mmol/l) (all from Roche, Mannheim, Germany), 2 µl DMSO (Merck, Darmstadt, Germany), 1 µl of each primer (50 pmol, GIBCO, Grand Island, NY), and 38 µl water. The following primers sets were used: β-Actin, sense primer AAC CCA GCC ATG TAC G and anti-sense primer AGT TCA CGC ACG ATT TCC C (Gen-Bank no. V01217), microsomal triglyceride transference protein (Mttp) sense primer ATC TGA TGT GGA CGA CGT TGT GT, and anti-sense primer CCT CTA TCT TGT AGG TAG TG (Gen-Bank no. L47970), Fatty acid synthase (Fas) sense primer ATG CCA TAC TGG AGA ACC AG, and anti-sense primer TCT CGG ATG CCT AGG ATG TG (Gen-Bank no. X13135), acetylCoA carboxylase (Acc) sense primer GGG ACT TCA TGA AGT TGC TAG G, and anti-sense primer GCT ATT ACC ATC TTC ATT ACC TCA ATC TC (Gen-Bank no. J03808), HMG-CoA synthase (Hmgs) sense primer TAC TAT GGT GTA GAT GG, and anti-sense primer AGT TCT TCT GTG CTT TTC ACT CAC (Gen-Bank no. X52625), HMG-CoA reductase (Hmgr) sense primer GAC ACT TAC AAT CTG TAT GAT G, and anti-sense primer CTT GGA GTA AAA CTG CCA (Gen-Bank no. M29249), apolipoprotein B (Apob) sense primer GAC ATG GTG AAT GGA AAT, and anti-sense primer TGA AGA CTC CAG ATG AGG AC (Gen-Bank no. M14952), apolipoprotein E (Apoe) sense primer ATG TCT CCT GCT CTC TCT ACA, and anti-sense primer CAG AGC TCA ACA GCC AGT (developed by dr. G. Hooiveld, unpublished data), sterol regulatory element binding protein-1a (Srebp-1a) sense primer GCC GAG ATG TGC GAA CTG GAC A, and anti-sense primer GGC CTG AGC TCA ACA GTG GAG (developed by dr. G. Hooiveld, unpublished data), sterol regulatory element binding protein-1c (Srebp-1c) sense primer GAC GGA GCC ATG GAT TGC ACA TT, and anti-sense primer GGC CTG AGC TCA ACA GTG GAG G (Gen-Bank no. L16995).

cDNA levels of the insulin receptor, insulin receptor substrate-1 and -2, were measured by real time polymerase chain reaction using ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, US). For the PCR-amplification studies, an amount of cDNA corresponding to 10 ng of total RNA was amplified using the qPCR core kit (Eurogentec, Seraing, Belgium) essentially according to manufacturer’s protocol and optimised for amplification of the particular gene using the appropriate forward and reverse primers (GIBCO, Paisley, UK) and a template specific 3’-TAMRA, 5’-6-FAM labeled Double Dye Oligonucleotide probe (Eurogentec, Seraing, Belgium). In the same experiments, calibration curves were run on serial dilutions of a 8x concentrated cDNA solution as used in the assay, resulting in a series containing 8x, 4x, 2x, 1x, 0.5x, 0.125x, 0.062x, and 0.031x of the cDNA present in the assay incubation. Both assay and calibration incubations were done simultaneously. During the amplification, the breakdown of the probe releases the fluorescent 6-FAM-dye, resulting in an increase in fluorescence. The fluorescence data obtained were processed using the software program ABI Sequence Detector v1.6.3 (System Applied Biosystems, Foster City, CA, US). All quantified expression levels were within the linear part of the calibration curves and calculated using these curves. The following primer sequences were used: β-Actin sense primer AGC CAT GTA CGT AGC CAT CCA, anti-sense primer TCT CGG GAG TCC ATC ACA ATG and probe TGT CCC TGT ATG CCT GTC GTA CCA C (Gen-Bank no. NM_031144), Ir sense primer TGA GTC AGC CAG TCT TCG AGA A, anti-sense primer GCC ATC AGT TCC ATC ACT ACC A and probe CCA CGT GAT GAC AGG TGA AGC CCT TC (Gen-Bank no. NM_017071), Irs-1 sense primer AGC ACC TGG TGG TCC TCT ACA, anti-sense primer CAG CTG CAG AAG AGC CTG GAT and probe CTC GCT ATC CGC CGC AAT GCC (Gen-Bank no. NM_012969), Irs-2 sense
primer CAG TCC CAC ATC AGG CTT GAG, anti-sense primer GGT CTG CAC GGA TGA CCT TAG and probe CCT TCA AGT CAG CCA GCC CCC TG (Gen-Bank no. AF083418).

Statistical analysis. All values reported are mean±s.d. Statistical significance is considered when p<0.05 and is determined non-parametrically with the Mann-Whitney U test.

### RESULTS

Animal characteristics. Mean body weight did not differ between the groups of rats, i.e., 326±21 gram before the experiment and 399±14 gram vs. 398±20 gram after three weeks on low-fat and high-fat diet, respectively. The energy content of the high-fat diet was somewhat higher compared to the low-fat diet but the rats compensated for this by decreasing their total daily food intake (20±1 vs. 17±1 g/day in rats fed the low-fat and high-fat diet, respectively). This resulted in a similar daily caloric intake (81±5 vs. 77±6 kcal/day in rats fed the low-fat and high-fat diet, respectively).

After three weeks on the diets, plasma insulin concentrations were significantly higher and, somewhat paradoxically, plasma TG levels were significantly lower in rats fed the high-fat diet (Table 1). Plasma glucose, cholesterol, and NEFA concentrations were not different between the groups. Rats developed massive hepatic steatosis after three weeks on the high-fat diet (Table 1). Hepatic TG content was increased more than four-fold in this group compared to rats fed the low-fat diet. Hepatic cholesteryl ester levels tended to be elevated but this difference did not reach statistical significance. Neutral fat accumulated predominantly in periportal areas (zone 1) of the liver in rats fed the high-fat diet, as revealed by Oil-red-O (ORO) staining (Figure 1).
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Intravenous glucose tolerance test. Rats were subjected to an intravenous glucose tolerance test (IVGTT) after three weeks on diet (n=4/diet). Plasma glucose and insulin responses are cumulatively displayed as the area under the curve (AUC) in Figure 2. Rats fed the high-fat diet showed elevated plasma glucose concentrations during the infusion compared to rats fed the low-fat diet, indicating a fat-induced decrease in the capacity to dispose glucose (Figure 2A). This was, at least in part, related to a smaller rise in plasma insulin levels during the infusion in rats fed the high-fat diet (Figure 2B), indicating an impaired insulin response.

**Figure 1.** Hepatic neutral lipid "Oil-Red-O" staining in rats fed the low-fat diet (A) and the high-fat diet (B), p = portal area (zone 1), c = central area (zone 3).

**Figure 2.** Glucose (A) and insulin (B) response during an IVGTT (n=4/group) in rats fed a high-fat diet (closed circles) and a low-fat diet (open circles), *p<0.05, Mann-Whitney U test.
**Hyperinsulinemic clamp.** To determine if the capacity of insulin to inhibit the VLDL-TG production is affected by high-fat feeding, rats received a hyperinsulinemic clamp after three weeks on diet (n=4/group). Insulin levels increased 10-15 times during the clamp and reached stable levels at 27±5 vs. 2±1 ng/ml in insulin and saline-infused rats, respectively (Figure 3A). Plasma glucose levels showed slight differences during the clamp between the two groups, probably reflecting different glucose disposal rates (Figure 3B). During the insulin clamp, plasma NEFA level decreased by ~75% in both groups (Figure 3C).

![Figure 3](image-url)

**Figure 3.** Plasma insulin (A), glucose (B) and NEFA (C), concentrations (n=4/group) during a hyperinsulinemic clamp in high-fat (closed squares) and low-fat diet fed rats (closed circles) and saline infusion in high-fat (open squares) and low-fat diet fed rats (open circles), *p<0.05 from basal, †p<0.05 insulin effect, ‡p<0.05 diet effect, Mann-Whitney U test.

Rats received a Triton WR1339 injection at 90 minutes after onset of the hyperinsulinemic clamp to block VLDL lipolysis. VLDL-TG production rate was ~60% higher in rats fed the high-fat diet compared to the low-fat diet fed rats during saline infusion, i.e., 250±7 vs. 154±29 µmol.kg⁻¹.hr⁻¹, respectively (Figure 4A). Insulin inhibited the VLDL-TG production in high-fat fed rats by 32% to 169±19 µmol.kg⁻¹.hr⁻¹. This inhibition of VLDL-TG production was less pronounced compared to low-fat fed rats, in which insulin inhibited the VLDL-TG production rate by 64% to 55±3 µmol.kg⁻¹.hr⁻¹. Net inhibition of VLDL-TG production rate was less in rats fed the high-fat diet compared to rats fed the low-fat diet (-74 vs. -101
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\[ \mu \text{mol.kg}^{-1}.\text{hr}^{-1}, \text{respectively, } p<0.05. \]

ApoB100 content in isolated VLDL fractions was increased in rats fed the high-fat diet (Figure 4B and 4D). Acute hyperinsulinemia resulted in a decreased apoB100 content in isolated VLDL fractions. The apoB100/B48 ratio reflects the distribution of particle sizes that is secreted from the liver; the higher the ratio, the larger the average particles size. High-fat feeding resulted in an increased apoB100/B48 ratio (Figure 4C), indicating increased production of larger, apoB100-containing particles. Indeed, high-fat feeding increased VLDL particle size by \(~40\%\), i.e., \(52.5\pm10.0\) vs. \(74.3\pm10.0\) nm (\(p<0.05\)) in rats fed the low-fat and high-fat diet, respectively. Acute hyperinsulinemia resulted in a decreased apoB100/B48 ratio in low-fat but not in high-fat fed rats (Figure 4C).

**Figure 4.** VLDL-TG production rates (A), VLDL-apoB100 concentration (B), apoB100/B48 ratio (C), and apoB Western Blot levels (D) after saline infusion (white bars) or a hyperinsulinemic clamp (black bars) in high-fat diet (HF) and low-fat diet (LF) fed rats, * \(p<0.05\) insulin effect, † \(p<0.05\) diet effect, Mann-Whitney U test.
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**Gene expression.** High-fat feeding did not change hepatic expression of genes directly involved in VLDL assembly (Figure 5). Expression of genes encoding apolipoprotein B (Apolob), apolipoprotein E (Apoee), microsomal triglyceride transfer protein (Mttp), and diacylglycerol acyltransferase (Dgat) were not affected. In addition, expression of genes involved in cholesterol synthesis, HMG-synthase (Hmgs) and HMG-reductase (Hmgr), were not affected by high-fat feeding (not shown). Fatty acid synthase (Fas) and acylCoA carboxylase (Acc), two important genes involved in fatty acid synthesis, were decreased in livers of rats fed the high-fat diet (Figure 5). The expression levels of the sterol regulatory element binding protein-1a and -1c (Srebp-1a and Srebp-1c), transcription factors involved in control of lipogenesis, were not different between the groups.

![Figure 5](image-url). Hepatic expression levels of genes involved in VLDL formation and fatty acid synthesis in rats fed the low-fat diet (LF) or high-fat diet (HF).

**Insulin signal transduction.** To evaluate the effects of high-fat feeding on insulin signal transduction, we determined gene expression and protein phosphorylation of IR, IRS-1, IRS-2, PKB, and PKCζ in livers of rats fed the low-fat or high-fat diet during a hyperinsulinemic euglycemic clamp. IR protein levels and IR gene expression levels were both decreased by ~40% in rats fed the high-fat diet (Figure 6A and 6B, respectively). IRS-1 and IRS-2 expression levels (-30% and -54%, respectively) were also decreased in rats fed the high-fat diet. However, after 90 minutes of insulin clamping no differences in hepatic IRS-1 and IRS-2 phosphorylation were detected between both diet groups (not shown). As expected, saline-infused rats showed low levels of phosphorylated PKB (Figure 7). Insulin infusion resulted in a ~460% increase in phosphorylated PKB in rats fed the low-fat diet. Rats fed the high-fat diet showed less activation of PKB (~210%), suggesting a high-fat diet induced decrease in insulin signaling (Figure 7). PKCζ activation was not detectable in this experiment.

**DISCUSSION**

Very-Low Density Lipoprotein (VLDL) production is increased and less sensitive to the suppressive actions of insulin in DM2 patients (11). DM2 is frequently associated with hepatic steatosis (16), but the relation between increased VLDL formation, hepatic insulin insensitivity, and steatosis have remained elusive. Our data show that increased VLDL production, hepatic insulin resistance and steatosis are readily induced by short-term high-fat
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feeding in rats. Therefore, the metabolic features observed in DM2 do not necessarily reflect intrinsic abnormalities of DM2 but may be direct consequences of dietary habits.

High-fat feeding resulted in an impaired ability to dispose glucose during an IVGTT due, at least in part, to a decreased insulin response (Figure 2). Basal plasma glucose levels were not differently affected by both diets, but postabsorptive insulin concentrations were clearly increased in rats fed the high-fat diet, indicating that more insulin is needed to maintain normal plasma glucose levels (Table 1). High-fat feeding induced slightly lower triglyceride levels, probably caused by increased lipoprotein lipase (LPL) activity due to prevailing high

Figure 6. Hepatic IR protein levels (A), hepatic gene expression levels of the IR (B), IRS-1 (C), and IRS-2 (D) in rats fed a low-fat diet (LF) and a high-fat diet (HF), *p<0.05, Mann-Whitney U test.
insulin concentrations as was previously described by Kusunoki and Storlien et al. (36), leading to accelerated hydrolysis of VLDL-TG. After three weeks of high-fat feeding, rats developed a massive, periportally localized, TG accumulation in the liver coinciding with a decreased expression of genes involved in FA synthesis (Fas, Acc). Also, rats on the high-fat diet had an increased VLDL-TG production. Insulin acutely inhibited VLDL-TG production rate, but this effect was considerably decreased in high-fat diet fed rats compared to rats fed the low-fat diet (Figure 4A). As a result, VLDL-TG production under insulin-clamped conditions was higher due to hepatic resistance with respect to insulin control of hepatic VLDL production.

Rat liver secretes both apoB100- and apoB48-containing lipoprotein particles. The amount of apoB100-containing lipoprotein particles was increased in isolated VLDL fractions from rats fed the high-fat diet (Figure 4B). Insulin decreased the apoB100/B48 ratio, and therefore probably VLDL particle size, in rats fed the low-fat diet but in not rats fed the high-fat diet (Figure 4C). The observed effect of insulin on the apoB100/B48 ratio is probably attributable to its effects on the activity of Apobec (37), the apoB mRNA editing enzyme.

We performed a hyperinsulinemic clamp study employing fixed infusion rates of insulin and glucose. This resulted in similar plasma insulin levels in low-fat and high-fat diet fed rats. Yet, probably due to differences in insulin sensitivity, plasma glucose levels were slightly different between the two groups. Although we cannot exclude an effect of these differences in glucose levels on VLDL production during hyperinsulinemia, this effect is probably small. The contribution of glucose to VLDL-TG production is related to de novo lipogenesis and this process only contributes 3-5% to total VLDL-TG production (38). In addition, this contribution of glucose is only apparent after many hours of high-dose glucose infusion (39). Most importantly, de novo lipogenesis was reduced in high-fat fed rats
experiencing the highest plasma glucose concentration. Insulin inhibited VLDL-TG production to such a large extent that it is highly unlikely that our results are merely explained by the slight differences in plasma glucose levels.

Increased lipid availability can increase different PKC-isoforms and might therefore contribute to the development of insulin resistance, as has been shown in rats (24,25) and humans (40,41). The hepatic activation of PKCζ could not be detected after 90 minutes of hyperinsulinemia in our experiment. However, this does not imply that PKCζ was not able to attenuate IRS protein and hence insulin signaling. Further studies are required to determine the precise role of PKCζ in diet-induced effects on hepatic insulin signaling and VLDL metabolism.

Protein kinase B (PKB or Akt) is hypothesized to be involved in the feedback regulation of IRS activation. Phosphorylation of specific serine residues lowers the rate of tyrosine dephosphorylation, which stabilizes the IRS protein and maintains insulin signaling (23). Hepatic PKB was still detectable after ninety minutes of hyperinsulinemia. In rats fed the high-fat diet hepatic PKB activation was less pronounced than in rats on the low-fat diet (Figure 7). This indicates either decreased insulin signaling and/or a decreased ability to maintain the insulin signal. Both conditions lead to a decreased insulin functioning and response. We found that hepatic insulin receptor mRNA and protein levels were decreased in rats fed the high-fat diet (Figure 6). These results are in agreement with the observed effects of high-fat feeding on hepatic IR activation. High-fat feeding resulted in decreased insulin-stimulated phosphorylation of the β-subunit of the insulin receptor in rat liver, muscle and adipose tissue (42,43). In accordance, high-fat feeding in our study resulted in impaired hepatic insulin signaling. Insulin accelerates apoB degradation by post-transcriptional modification (44,45), and, thereby, decreases VLDL assembly and production. The insulin signal transduction pathway involved in the inhibitory effects of insulin on VLDL metabolism is the PI3K pathway. Wortmannin and LY 294002, two distinct PI3K inhibitors, prevent insulin-dependent inhibition of apoB secretion (18). It is currently not known how insulin affects apoB degradation. Defective insulin signaling may prevent the insulin effect on apoB degradation and thus allows for ongoing resulting in lesser reduction of VLDL production.

In conclusion, short-term high-fat feeding leads to hepatic steatosis, increased hepatic VLDL production and impaired hepatic insulin function. Hepatic fat accumulation may reflect a primary event related to dietary habits in the cascade that ultimately results in hepatic insulin resistance and the development of an atherogenic plasma lipoprotein profile.

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