Interactions between hepatic glucose and fat metabolism in animal models of insulin resistance
Wiegman, Cornelis Harm

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Hepatic VLDL production in ob/ob mice is not stimulated by massive *de novo* lipogenesis and is less sensitive to the suppressive effects of insulin

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Submitted

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ABSTRACT

Type 2 diabetes mellitus in humans is associated with increased de novo lipogenesis (DNL), increased fatty acid (FA)-flux from peripheral tissues, decreased FA oxidation and hepatic steatosis. In type 2 diabetes mellitus, production of VLDL is increased and resistant to the suppressive effects of insulin. The relationships between FA metabolism, hepatic steatosis and VLDL production are incompletely understood. We investigated VLDL formation in relation to DNL and insulin sensitivity in female ob/ob mice, a model of type 2 diabetes with hepatic steatosis. Hepatic triglyceride (5-fold) and cholesteryl ester (15-fold) contents were increased in ob/ob mice compared to lean controls. Hepatic DNL was increased ~10-fold in ob/ob mice whereas hepatic cholesterol synthesis was not affected. Basal rates of hepatic VLDL-triglyceride and -apoB100 production were also similar between the groups. Hyperinsulinemic clamping reduced VLDL-triglyceride and -apoB100 production rate by ~60% and ~75%, respectively, in lean mice but only by ~20% and ~20%, respectively, in ob/ob mice. No difference in hepatic expression of genes encoding apolipoprotein B, microsomal triglyceride transfer protein and diacylglycerolacyltransferase-1 were found. Hepatic gene expression and protein phosphorylation of the insulin receptor and insulin receptor substrate-isoforms were reduced in ob/ob mice. Thus, strongly induced hepatic DNL is not associated with increased basal VLDL production in ob/ob mice, which might be related to differential hepatic zonation of apoB synthesis (periportal) and lipid accumulation (perivenous) and/or relatively low rates of cholesterogenesis. Insulin is unable to effectively suppress VLDL-triglyceride production in ob/ob mice, presumably due to impaired insulin signaling.
INTRODUCTION

Type 2 diabetes mellitus (DM2) is associated with increased de novo lipogenesis (DNL), decreased plasma fatty acid (FA) oxidation and an increased FA-flux from peripheral tissues to the liver (1). These factors may all contribute to hepatic steatosis and increased hepatic VLDL production, two characteristic hallmarks of type 2 diabetes (2), and are probably related to insulin resistance. The relative contribution of the various pathways in hepatic lipid metabolism to the development of a fatty liver and disturbances in VLDL production is unknown but may, at least in part, be related to the localization of these processes within the liver. Fatty acid synthesis and TG accumulation occur predominantly in the perivenous areas (zone 3) of the liver whereas FA oxidation is more associated with the periportal areas (zone 1) of the liver (3,4). VLDL secretion has not been restricted to a specific hepatic zone: zonation of apoB gene expression and/or synthesis has not been reported.

Leptin-deficient ob/ob mice develop a fatty liver, insulin resistance and hyperlipidemia (5-7). The contribution of hepatic lipoprotein production to the development of hyperlipidemia in these mice is not clear. Hyperglycemia together with an increased glycolytic activity in ob/ob mice may lead to an increased availability of acetylCoA residues for DNL (8). The high glycolytic rate in these mice is reflected by increased expression levels and activities of enzymes involved in this metabolic pathway, i.e., glucokinase (9,10), phosphofructokinase (8) and pyruvate kinase (8,9). Furthermore, elevated plasma FFA levels (10) together with increased hepatic expression of fatty acid translocase (FAT or CD36) and plasma membrane-fatty acid binding protein (pmFABP) have been reported in this model (11). Increased endoplasmic reticulum (ER)-associated acetylCoA synthase (ACS) activity may increase the FA availability for esterification rather than for oxidation (11), which could contribute in increased TG and cholesteryl ester deposition and/or incorporation into VLDL.

Hepatic insulin resistance seen in DM2 is associated with increased VLDL production (2). Acute hyperinsulinemia reduces VLDL production in healthy volunteers (12-14) but not in DM2 patients (2) and obese individuals (13). Despite the insulin resistant condition and an increased hepatic TG content in ob/ob mice, a decreased VLDL-TG production rate under basal fasted conditions has been reported in this model (15,16). However, increased VLDL-TG secretion in ob/ob mice associated with enhanced expression and activity of the microsomal triglyceride transfer protein (MTP) has also been reported (17). The reason for these discrepant observations is unknown. The impact of insulin on VLDL production in the ob/ob mouse model has not been reported previously. Therefore, we quantified hepatic DNL and cholesterol synthesis using mass isotopomer distribution analysis (MIDA) and related the synthesis rates to VLDL-TG and -apoB100 production rates determined under basal conditions and during hyperinsulinemic clamp conditions in ob/ob mice and in lean littermates. Hepatic insulin signaling and expression levels of genes encoding transcription factors and important enzymes involved in fatty acid and cholesterol metabolism, VLDL formation and insulin signaling, were studied to provide a mechanistic basis for our findings.

MATERIAL AND METHODS

Animals. Female ob/ob and lean littermates were purchased from Harlan (Zeist, The Netherlands) and housed in a light- and temperature controlled facility. Experimental protocols were approved by the local Experimental Ethical Committee for Animal Experiments.
**Analytical kits.** Plasma and hepatic TG, cholesterol and glucose levels were determined by commercially available kits (Roche, Mannheim, Germany). Plasma and hepatic phospholipid concentrations and plasma FFA concentrations were determined with Phospholipid-kit and NEFA-C kit, respectively (Wako Chemical GmbH, Neuss, Germany). Plasma insulin was determined by a radio-immunoassay (RIA) RI-13K (Linco Research, Inc., St. Charles).

**Experimental procedures.** Female ob/ob (n=5) and lean mice (n=5), weighing between 51-63 gram and 24-28 gram respectively, were fed normal chow diet (RMH-B 2181, Hope Farms BV, Woerden, The Netherlands) enriched with 2% [1-13C]-acetate (Isotec, Miamisburg, OH), which was given *ad libitum*. After 11 days mice were fasted for a period of 4 hours. Mice were anaesthetized with halotane and the liver was excised. A portion of abdominal fat was also collected. Liver and fat tissue were immediately frozen in liquid nitrogen and stored at -80°C. Blood was isolated by heart puncture and immediately placed on ice in EDTA containing tubes and centrifuged 10 minutes at 5,000 rpm at 4°C. Plasma was stored at -20°C until analysis.

**Hyperinsulinemic clamp.** To study the effects of insulin on lipoprotein metabolism a second groups of lean and ob/ob mice received a hyperinsulinemic clamp or a saline infusion (n=4/group) under anesthesia. Based on euglycemic insulin clamps performed in rats and mice by Hawkins and Rossetti *et al.* (18,19) where insulin concentrations were 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. The infusion contained insulin (18 mU/kg/min; Novo Nordisk, Bagsvaerd, Denmark), somatostatin (1.5 µg/kg/min; UCB, Breda, The Netherlands) to suppress the endogenous insulin production, and glucose (25 mg/kg/hr; Merck, Darmstadt, Germany) to maintain normal plasma glucose concentration. All components were freshly prepared in saline containing 1.5% BSA (Sigma, St. Louis, MO). Plasma glucose concentration was determined with a GlucoTouch-glucose analyzer (LifeScan, Beerse, Belgium). The total infusion time was 2 hours. After 1 hour mice received a Triton WR1339 infusion (Tyloxpol; Sigma, St. Louis, MO) 12% w/w solution dissolved in saline, dose 5 ml/kg lean BW. Triton blocks the lipolysis of secreted lipoprotein particles, therefore, the accumulation of these lipoprotein particles in time allows us to calculate production rates (20). After injection blood samples were taken after 30 and 60 minutes. At the end of the experiment a 200 µl plasma sample was obtained by heart puncture to isolate VLDL particles. VLDL production rates were calculated from the slope of the linear-TG-accumulation-curves in time. Particles were isolated using a solution of 15.3% NaCl and 35.4% KBr (final concentration 0.65% and 1.52%, respectively) in saline with a density <1.019 g/ml. Plasma (0.2 ml) was used with 0.8 ml of the NaCl-KBr solution and centrifuged for 100 minutes at 120,000 rpm and 4°C. Tubes were sliced at 1.5 cm from top and the top-fraction, containing the VLDL-particles, was collected and frozen at -80°C until composition analysis and apoB quantification. VLDL particle size was determined in isolated VLDL fractions using a Submicron Particle Sizer, (Autodilute, model 370; Nicomp, Santa Barbara, CA, US).

**Liver lipid analysis.** Liver lipids were extracted following a modified Bligh & Dyer method (21) and determined by commercially available kits. Total protein of tissue homogenates was determined using the method described by Lowry *et al.* (22).

**Histology.** To study the localization of hepatic TG accumulation and VLDL production, liver morphology, neutral lipids and apolipoprotein B (apoB) mRNA were visualized as indicators.
for TG deposition and VLDL formation, respectively. Hepatic morphology was visualized by standard Hematoxilin Eosin (HE)-staining and neutral lipids were visualized by "Oil-Red-O" (ORO) staining. ApoB in situ hybridization technique was described previously for apoE (23). The pGEM-3z vector in which apoB cDNA was placed was a gift from dr. H.M. Prinsen (TNO, Leiden).

**Mass isotopomer distribution analysis.** The MIDA technique allows measuring the biosynthesis of polymers in vivo and is described in great detail elsewhere (24,25). Briefly, the relative abundance of different mass isotopomers during feeding of a [1-13C]-acetate-enriched diet was determined. The enrichment of the pool of acetylCoA precursor units (p) that has entered newly synthesized cholesterol and palmitate can be calculated by comparing it with a theoretical table generated using binomial expansion and known isotope frequencies of the atomic isotopes. When the enrichment of the acetylCoA pool is known it becomes possible to calculate the fraction (f) of newly synthesized cholesterol and palmitate molecules in liver and adipose tissue homogenates. To determine the absolute amount of newly synthesized hepatic cholesterol and palmitate we multiplied f by the total amount of hepatic free cholesterol and palmitate, respectively.

**Gas chromatography/mass spectrometry (GC/MS) analysis.** Plasma cholesterol was extracted and derivatized as described elsewhere (26). Palmitate from plasma and liver samples were trans-methylated according to Lepage *et al.* (27). Cholesterol and fatty acid derivates were analyzed on a magnetic sector mass spectrometer (70-250S; VG, Manchester, U.K.) using a Chrompack CP-Sil 19 column (Middelburg, the Netherlands) for assessment of isotopomer distribution patterns. For cholesterol samples, the oven temperature increased from 120 to 260ºC at a rate of 20ºC/min, from 260 to 280ºC at 2.5ºC/min and finally from 280 to 300ºC at 20ºC/min. The ions at m/z 368 to 371 were measured under selected ion recording. For FA samples the oven temperature increased from 100 to 300ºC at a rate of 12.5ºC/min. The ions of the palmitate derivative were measured at m/z 270 to 272 under selected ion recording. FA methyl esters were separated and quantified by gas liquid chromatography as earlier described (28) using heptadecanoic acid (17:0) as internal standard.

**ApoB quantification.** ApoB100 concentrations were quantified with a reference to an IDL apoB100 standard isolated from healthy human subjects (29). Since human liver does not produce ApoB48 containing lipoprotein particles we were not able to quantify apoB48 levels. Isolated VLDL samples (10 µl) were delipidated with methanol and diethylether and dried under nitrogen. Delipidated lipoproteins were reduced in SDS sample buffer (8 M urea, 10 mM Tris base, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) and separated by SDS-PAGE using 4-15% gradient gels (Ready gels, Bioready, Hercules, CA) at 100 V for 30 minutes followed by 120 V for 120 minutes. Gels were either stained with a silver-staining procedure (29) or were prepared for Western blot analysis. Proteins were transferred on nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) by Western blotting for 120 minutes at 250 mA. Blots were put on skimmed milk over night and stained with the 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). ECL activation of the peroxidase was detected on film. ApoB100 levels were quantified with a reference to an IDL apoB100 standard isolated from healthy human subjects.
Hepatic gene expression. Hepatic gene expression levels were quantified by real time polymerase chain reaction (PCR) analysis during basal conditions and hyperinsulinemia. Total RNA was isolated from approximately 30 mg of liver tissue using Trizol-method (GIBCO, Paisley, UK) followed by the SV Total RNA Isolation System (Promega, Madison, WI) according to the protocols provided by the manufacturer. Isolated total RNA was converted to single stranded cDNA by a reverse transcription procedure with M-Mulv-RT (Boehringer Mannheim, Mannheim, Germany) according to manufacturer’s protocol and optimised for amplification of the particular gene using the appropriate forward and reverse primers. The expression levels were measured by real-time PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). 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). Procedures were 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). All quantified expression levels were within the linear part of the calibration curves and calculated using these curves. The primers and probe sets used are listed in Table 1. The relative intensity of the product bands was determined by a CCD video camera of the ImageMaster VDS system (Pharmacia, Upsalla, Sweden) and quantified by Image software.

**Hepatic insulin signaling.** For analysis of IRβ and IRS-1,2,3 phosphorylation, liver parts were homogenized in RIPA-buffer (30 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and protease inhibitors (Complete: Boehringer Mannheim)) using a Ultraturrax mixer. Lysates were cleared by centrifugation (14 krpm; 15 min; 4°C). Protein content of supernatant were determined using BCA-kit (Pierce, Rockford, IL). A total of 25 μg of protein was analysed by immunoblotting for expression of IRβ subunit (Transduction Laboratories), IRS-1 (30), IRS-2 (31) and IRS-3. Anti- IRS3 antibody was obtained by immunized rabbits with a recombinant His-tagged IRS3 fusion protein produced from pET16B-IRS3 (aa198-494 of rat IRS3) as described by Ouwens et al. 1994 (30).

### Table 2. Plasma and hepatic parameters after a 4 hr fast in lean and ob/ob mice (n=5/group), *p<0.05, Mann-Whitney U.

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<tr>
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<th>lean</th>
<th>ob/ob</th>
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<tr>
<td><strong>Plasma</strong></td>
<td></td>
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<tr>
<td>Glucose (mM)</td>
<td>8.6±3.0</td>
<td>16.9±4.6*</td>
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<tr>
<td>Insulin (ng/ml)</td>
<td>1.3±0.9</td>
<td>6.9±0.6*</td>
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<tr>
<td>Triglycerides (mM)</td>
<td>0.3±0.03</td>
<td>0.7±0.1*</td>
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<tr>
<td>Cholesterol (mM)</td>
<td>1.7±0.4</td>
<td>4.1±0.3*</td>
</tr>
<tr>
<td>Free fatty acids (mM)</td>
<td>0.7±0.1</td>
<td>1.3±0.1*</td>
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<tr>
<td><strong>Liver</strong></td>
<td></td>
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<tr>
<td>Liver weight (gram)</td>
<td>1.3±0.1</td>
<td>2.7±0.3*</td>
</tr>
<tr>
<td>Triglycerides (μmol/liver)</td>
<td>1.5±0.5</td>
<td>7.5±1.1*</td>
</tr>
<tr>
<td>Total cholesterol (μmol/liver)</td>
<td>1.7±0.6</td>
<td>4.4±1.1*</td>
</tr>
<tr>
<td>Free cholesterol (μmol/liver)</td>
<td>1.7±0.1</td>
<td>2.7±0.5*</td>
</tr>
<tr>
<td>Cholesteryl esters (μmol/liver)</td>
<td>0.1±0.01</td>
<td>1.5±0.8*</td>
</tr>
<tr>
<td>Phospholipids (μmol/liver)</td>
<td>0.4±0.1</td>
<td>0.6±0.3</td>
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<tr>
<td>Glucose-6-phosphate (μmol/liver)</td>
<td>0.2±0.4</td>
<td>0.3±0.1</td>
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<tr>
<td>Glycogen (μmol/liver)</td>
<td>272±27</td>
<td>501±54*</td>
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RESULTS

Animal characteristics. Mean body weight was $26 \pm 1$ vs. $58 \pm 5$ gr in the lean and ob/ob mice, respectively ($p<0.05$). Fasting plasma glucose, insulin, TG, cholesterol, and FFA concentrations were elevated in ob/ob mice (Table 2). Excess TG and cholesterol in ob/ob plasma was predominantly found in VLDL-sized fractions upon FPLC separation (data not shown). Liver weight (2-fold), hepatic TG (5-fold), total cholesterol (~2.6-fold), free cholesterol (~1.6-fold), cholesteryl ester (~15-fold) and glycogen levels (~1.8-fold) were all increased in ob/ob mice. No differences in hepatic phospholipid and glucose-6-phosphate (G6P) levels were detected between lean and ob/ob mice (Table 2).

Neutral fat deposition (not shown in figure) in ob/ob mice was clearly associated with the perivenous (zone 3) area of liver lobules resulting in enlarged hepatocytes in these parts of the liver (Figure 1, A vs. B). To check whether localization of fat in ob/ob liver was compatible with that of apolipoprotein B gene expression, Apob mRNA was visualized by in situ hybridization in lean and ob/ob mouse liver (Figure 1, C and D, respectively). Apob mRNA was present in the entire liver lobe but a stronger signal was observed in the periportal zone of the liver both in lean and ob/ob mice, suggesting zonal differentiation between VLDL formation and TG deposition in ob/ob mouse liver.

Figure 1. Hematoxilin Eosin (HE) staining (A,B) and apoB in situ hybridization (C-F) in liver slides from lean and ob/ob mice, respectively, p=portal area (zone 1), c=central area (zone 3).
De novo lipogenesis and cholesterol synthesis. Palmitate and cholesterol synthesis rates in lean and ob/ob mice fed a [1-13C]-acetate-enriched diet are summarized in Table 3. The enrichment of acetylCoA pool and fractional synthesis rate values could not be calculated in adipose tissue of ob/ob mice due to low isotopic enrichments. Enrichments of the hepatic acetylCoA pools for DNL and cholesterogenesis were similar and not different between lean and ob/ob mice. Hepatic fractional de novo lipogenesis (DNL) was increased 1.7-fold in ob/ob mice in comparison with lean controls. The absolute amount of newly synthesized hepatic palmitate was 10-fold higher in livers of ob/ob mice than in those from controls. DNL could not be quantified in adipose tissue from ob/ob mice: values in lean controls indicate that adipocytes may significantly contribute to total DNL.

The fractional hepatic cholesterol synthesis was decreased ~1.4 fold in ob/ob mice compared to lean mice. Values for hepatic free cholesterol contents from Table 2 were used to calculate the total amount of newly synthesized cholesterol present in the liver, which was similar in lean and ob/ob mice (Table 3).

To understand the molecular basis of the differences in hepatic lipid synthesis and contents between lean and ob/ob mice, expression levels of relevant genes were determined. Expression levels of key genes involved in FA synthesis, i.e. fatty acid synthase (Fas), and acylCoA carboxylase (Acc) were clearly increased in livers of ob/ob mice. The mRNA levels for the transcription factor sterol regulatory element binding protein-1c (Srebp-1c) was also increased, but gene expression of liver X receptor (Lxr) and carbohydrate responsive element-binding protein (Chrebp), also implicated in control of Fas expression, were significantly decreased. Expression level of the sterol regulatory binding element protein-2 (Srebp-2), the transcription factor mainly involved in control of cholesterol synthesis, was decreased in ob/ob mice liver but this did not result in reduced expression of its target gene HMGCoA reductase (Hmgr). Hepatic expression levels of the transcription factor peroxisomal proliferator activated receptor-α (Ppar-α) was decreased but the expression level of Ppar-γ was increased in ob/ob mouse liver. Genes involved in β-oxidation, that are controlled by PPARα, i.e., mitochondrial HMGCoA-synthase (Hmgs), carnitine palmitoyl transferase-1a (Cpt1a) and medium chain acyl dehydrogenase (Mcad), tended to decrease in ob/ob mice, suggesting a decreased β-oxidation in ob/ob mouse liver (Figure 2B).

Table 3. Acetyl CoA pool enrichment and fractional palmitate synthesis values of adipose tissue and liver (A) and hepatic acetyl CoA pool enrichment and fractional cholesterol synthesis values (B) in [1-13C]-acetate-enriched diet fed lean and ob/ob mice (n=5/5). *p<0.05, Mann-Whitney U test, nd = not detectable.

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<th>de novo lipogenesis</th>
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<td>Liver</td>
<td>lean</td>
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<tr>
<td></td>
<td>Acetyl CoA pool enrichment (%)</td>
<td>6.3 ± 0.3</td>
<td>5.9 ± 0.8</td>
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<td>f liver palmitate (%)</td>
<td>31.0 ± 6.1</td>
<td>53.1 ± 4.7*</td>
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<td>Hepatic palmitate (μmol/liver)</td>
<td>1.1 ± 0.4</td>
<td>6.3 ± 0.9*</td>
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<td>Newly synthesized hepatic palmitate (μmol/liver)</td>
<td>0.3 ± 0.1</td>
<td>3.4 ± 0.6*</td>
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<th>Adipose tissue</th>
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<tr>
<td></td>
<td>Acetyl CoA pool enrichment (%)</td>
<td>1.6 ± 0.5</td>
<td>nd</td>
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<td>f adipose tissue palmitate (%)</td>
<td>21.5 ± 8.7</td>
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<th>Cholesterol synthesis</th>
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<tr>
<td></td>
<td>A</td>
<td>lean</td>
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<tr>
<td></td>
<td>Acetyl CoA pool enrichment (%)</td>
<td>6.3 ± 0.1</td>
<td>5.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>f hepatic free cholesterol (%)</td>
<td>17.8 ± 4.0</td>
<td>12.6 ± 3.4*</td>
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<tr>
<td></td>
<td>Hepatic free cholesterol (μmol/liver)</td>
<td>1.7 ± 0.1</td>
<td>2.7 ± 0.5*</td>
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<tr>
<td></td>
<td>Newly synthesized hepatic cholesterol (μmol/liver)</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.2</td>
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Hepatic VLDL production under basal conditions and hyperinsulinemic clamp. VLDL-TG and apoB100 production was measured in anesthetized lean and ob/ob mice during saline infusion (control) and during a hyperinsulinemic clamp. During the hyperinsulinemic clamp, plasma glucose levels were clamped at basal, fasting (9 hr) plasma concentrations and were reached within 60 minutes. Average plasma glucose levels were 7±1 mM and 15±1 mM for lean and ob/ob mice, respectively (Figure 3A). Plasma insulin increased to stable levels of 34±3 ng/ml and 34±2 ng/ml in lean and ob/ob mice, respectively (Figure 3B). Saline infused mice maintained their fasting insulin level during the clamp (0.7±0.4 ng/ml and 5±2 ng/ml in lean and ob/ob mice, respectively). Plasma FFA levels decreased in the insulin-infused mice only (Figure 3C). Although ob/ob mice have higher basal plasma FFA concentrations, insulin reduced plasma FFA levels to the same concentration within 60 minutes as in lean mice.
After 60 minutes of saline infusion or hyperinsulinemia, Triton WR1339 was injected to determine VLDL-TG and apoB100 production rates. Basal VLDL-TG production rates were similar in lean and ob/ob mice (64±14 and 52±7 µmol/kg/hr, respectively, Figure 4A). Acute hyperinsulinemia reduced VLDL-TG production rate to 27±1 µmol/kg/hr (-58%) in lean mice but only to 41±1 µmol/kg/hr (-21%) in ob/ob mice (Figure 4A). ApoB100 production determined by Western blotting procedure showed a similar pattern as VLDL-TG production rates during the insulin clamp. Insulin suppressed apoB100 production much more pronounced in lean mice than in ob/ob mice (Figure 4B). The apoB100/B48 ratio in nascent VLDL particles, as determined by intensity scanning of Western blots, was much higher in ob/ob mice than in lean controls: this ratio was decreased by insulin infusion.
Expression of genes encoding structural proteins of VLDL particles, i.e., apoB and apoE, was similar in liver of lean and ob/ob mice. In spite of the increased apoB100/B48 ratio in ob/ob VLDL, expression of apobec-1, encoding the Apob mRNA-editing protein, was not different between both groups indicating a posttranscriptional upregulation of editing activity in ob/ob mice. Expression of the genes encoding MTP and DGAT, enzymes essential for VLDL lipidation, did not differ between lean and ob/ob mice (Figure 2C).

**Insulin signaling.** Hepatic gene expression levels of the insulin receptor (Ir) and insulin receptor substrate isoforms (Irs1 and Irs2) were decreased in ob/ob mice (Figure 5A). Also, phosphorylation of IRβ, IRS-1 and IRS-2 proteins was reduced in ob/ob mice liver (Figure 5B), indicating decreased hepatic insulin signaling. IRS-3 phosphorylation was slightly increased in ob/ob mice liver (Figure 5B).
DISCUSSION

The primary defect in the ob/ob mouse model is the absence of leptin, resulting in an obese and diabetic phenotype (5). Ob/ob mice have increased plasma FFA levels, a 10-fold increase in hepatic de novo lipogenesis (DNL) and a severe, perivenous localized, hepatic steatosis. Despite these diabetic characteristics, hepatic VLDL production was not increased, as is the case in humans with insulin resistance. We propose that absence of a simultaneous appropriate upregulation of hepatic cholesterol synthesis, recognized as a crucial factor in control of VLDL production rates (32-35) contributes to this discordant phenotype. We further demonstrated that the VLDL production process in ob/ob mice was insensitive to the suppressive effects of insulin, as previously reported in human (2,13) and rodent (36) insulin resistant conditions. This is likely the result of a decrease in the transduction pathway of insulin: in livers of ob/ob mice IRβ, IRS-1 and IRS-2 gene expression levels and protein phosphorylation were clearly decreased. Similar decreases of hepatic and muscle IRS phosphorylation after insulin stimulation in vivo were observed in other studies (37-40).

We found DNL, suggested to be a regulator of VLDL production, to be 10-fold
increased in ob/ob liver using MIDA approach. The increase in DNL is probably related to dysregulation of transcriptional control of this pathway due to leptin-deficiency. Expression of enzymes involved in the lipogenesis is under control of at least three transcription factors, i.e., SREBP-1c, LXR and CHREBP (41-43). Interestingly, hepatic SREBP-1c expression levels were increased in ob/ob mice, whereas LXR and CHREBP expression levels were decreased, indicating that SREBP-1c is independently able to strongly induce DNL. SREBP-1c expression is influenced by insulin (44,45). Insulin levels are elevated in ob/ob mouse and may continuously induce expression of SREBP-1c and, thereby, of its target genes (41). Thus, insulin resistance may not involve all branches of insulin signaling. Alternatively, leptin has been shown to be able to downregulate SREBP-1c expression and protein levels and expression of its target gene (Fas) in ob/ob adipocytes (46) and in wildtype mouse liver (47). IRS2−/− mice, like ob/ob mice, have increased hepatic SREBP-1c levels, which normalize upon leptin treatment (48). Irrespective of the underlying mechanism, however, our results indicate that upregulated DNL per se is not a regulator of hepatic VLDL production by mouse liver.

Theoretically, it may be that TG and apoB, required for VLDL assembly, are functionally separated in the ob/ob liver. Using in situ hybridization, we found that Apob mRNA is present in all cells in the liver lobule, but with highest intensity in periportal hepatocytes of control mice. Funahashi et al. (49) reported a uniform distribution of Apob mRNA in rat liver, suggesting the existence of species-differences in this respect. In any case, our results suggest that perivenously localized TG may be less available for VLDL production.

VLDL production in ob/ob mice has been reported to be decreased (15,16) and increased (17) in comparison of that in lean controls. In this study, we found a moderate, not statistically significant decrease in VLDL-TG production in female ob/ob mice under basal conditions (Figure 4A). Although basal production rates for VLDL-TG and -apoB100 were similar in ob/ob and lean mice, acute hyperinsulinemia did not decrease these production rates to the same extent in both genotypes. The insulin-insensitivity in ob/ob mice may lead to a relative overproduction of VLDL particles in postprandial conditions and hence contribute to the hypertriglyceridemia seen in these mice.

In our clamp experiments plasma insulin levels were similar between both groups. However, both groups were clamped at their basal glucose levels resulting in higher glucose levels in ob/ob mice. The question arises to which extent this may have influenced our results with respect to insulin sensitivity of VLDL-TG production. For instance, it may be argued that hyperglycemia in the presence of hyperinsulinemia may stimulate DNL further. This effect might counteract the inhibitory effects of insulin on VLDL-TG secretion. However, in the basal state VLDL production was not increased despite hyperglycemia and a manyfold increase in DNL in ob/ob mice, indicating that this was not a driving force for VLDL secretion. Therefore, it is rather unlikely that hyperglycemia per se the profound insulin resistance with respect to the suppressive effects on VLDL production.

Substrate availability has been proposed to regulate hepatic VLDL output (32,35). Since the availability of plasma FFA, de novo synthesized FA and hepatic TG were all increased in ob/ob mice, it is unlikely that the supply of TG is rate-controlling in this respect. In addition, the availability of newly synthesized cholesterol may influence VLDL formation, as has been shown in rats (32), pigs (33), rabbits (34) and in humans treated with cholesterol synthesis inhibitors (35). Total hepatic cholesterol content in ob/ob mouse liver was increased but the absolute cholesterol synthesis rate, as determined by MIDA, was not different compared to lean mice. The transcription factor that is in control of cholesterol synthesis is the sterol regulatory element binding protein-2 (SREBP-2). Sterol regulation is the primary mechanism that dominates cleavage of intracellular, membrane-bound SREBP-2, allowing entry of the active transcription factor into the nucleus to induce expression levels of genes
involved in cholesterol synthesis and uptake. Increased hepatic cholesterol levels were associated with a decreased expression level of SREBP-2 in ob/ob mice, however, this decrease in SREBP-2 expression did not decrease the expression levels of its target gene HMGCoA reductase (Figure 2) nor the absolute cholesterol synthesis rate (Table 3). We hypothesize that limited availability of newly synthesized cholesterol reduces the ability of the liver to remove excess TG as VLDL.

In conclusion, hepatic cholesterol synthesis is not affected but DNL is clearly increased in ob/ob mice, probably related to increased SREBP-1c expression levels and despite downregulation of LXR and CHREBP expression. Insufficient supply of newly synthesized cholesterol may become rate-controlling for VLDL production in ob/ob mice in a situation in which the supply of FA from plasma and DNL is excessive. Metabolic zonation of TG accumulation and apoB production may contribute in this respect. The inability to induce VLDL-production under these conditions, in combination with impaired hepatic β-oxidation, contributes to development of hepatic steatosis, which in itself may contribute to development of hepatic insulin resistance. Hepatic insulin signaling is clearly impaired in ob/ob mice resulting in a decreased ability of insulin to suppress VLDL production.

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