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Chapter 3

Acute inhibition of hepatic β-oxidation in APOE*3Leiden mice does not affect hepatic VLDL secretion or insulin sensitivity

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Abstract

Hepatic very low density lipoprotein (VLDL) and glucose production are enhanced in type 2 diabetes and are associated with hepatic steatosis. Whether the derangements in hepatic metabolism are attributable to steatosis or to the increased availability of fatty acid (FA) metabolites is unknown. We used methyl palmoxirate (MP), an inhibitor of carnitine palmitoyl transferase I (CPTI), to acutely inhibit hepatic FA oxidation, and investigated whether the FA were rerouted into VLDL secretion and whether this would affect hepatic glucose production. After an overnight fast, male APOE3*Leiden transgenic mice received an oral dose of 10 mg/kg MP. Administration of MP led to an 83% reduction in plasma β-hydroxybutyrate (keton body) levels compared with vehicle-treated mice (0.47 ± 0.07 vs. 2.81 ± 0.16 mmol/l, respectively, $P < 0.01$), indicative of impaired ketogenesis. Plasma free FA levels were increased by 32% and cholesterol and insulin levels were decreased by 17% and 50%, respectively, in MP-treated mice compared with controls. MP treatment led to a 30% increase in liver triglyceride (TG) content. Surprisingly, no effect on hepatic VLDL-TG production was observed between the groups at 8 h after MP administration. In addition, the capacity of insulin to suppress endogenous glucose production was unaffected in MP-treated mice compared with controls. In conclusion, acute inhibition of FA oxidation increases hepatic lipid content but does not stimulate hepatic VLDL secretion or reduce insulin sensitivity.

Introduction

Hepatic very low density lipoprotein (VLDL) - triglyceride (TG) secretion is an important determinant of plasma lipid levels. The rate of VLDL secretion is generally believed to be substrate-driven (*i.e.*, the hepatic content of TG and fatty acids (FA) determines VLDL assembly and the rate of VLDL secretion)[1-3]. Indeed, addition of FA to hepatocytes *in vitro* leads to increased hepatic TG and eventually to enhanced VLDL secretion[4]. However, the hypothesis that VLDL secretion is substrate-driven *in vivo* has not been proven.

Hepatic TG content is the result of uptake and synthesis on the one hand and β-oxidation and VLDL-TG secretion on the other. The importance of β-oxidation is illustrated by the severe phenotype of humans and mice with impaired β-oxidation. Deficiency in hepatic β-oxidation enzymes results in severe plasma hypoketosis and fatty liver[5,6].

In β-oxidation the rate-limiting enzyme is carnitine palmitoyl transferase I (CPTI), which couples long-chain FA to carnitine for transportation into the mitochondria[7,8]. Methyl palmoxirate (MP) is a specific and irreversible inhibitor of CPTI and thereby inhibits β-oxidation[9,10].

In this study, we tested the effect of acute inhibition of β-oxidation *in vivo* by MP on hepatic VLDL production in fasted hyperlipidemic APOE3*Leiden transgenic mice. APOE3*Leiden mice are characterized by a human-like lipoprotein profile[11,12]. We and

others have shown that these mice provide a suitable model to study hepatic VLDL metabolism, since these mice have decreased VLDL-TG production and fatty liver comparable to human conditions[12-14].

We observed that acute inhibition of FA β-oxidation in the liver leads to increased hepatic TG content but neither increases hepatic VLDL secretion nor induces hepatic insulin resistance.

Materials and methods

Animals and diet

Male 3 to 4 month old APOE3*Leiden transgenic mice were fed a Western-type diet containing 15% cacao butter and 0.25% cholesterol (Hope Farms, Woerden, The Netherlands) for 8 weeks before the experiments. The animals were allowed free access to food and water and were kept on a normal diurnal rhythm under standard conditions. After an overnight fast, the animals were matched for body weight and received a dose of 10 mg/kg MP (McN-3716: methyl 2-tetradecyloxiranecarboxylate; kindly provided by Dr. Hegardt, Barcelona, Spain) by gavage in 0.05% methyl cellulose solution or methyl cellulose solution alone (vehicle) as described earlier[9]. Blood samples were taken by tail-tip bleeding every 2 h after MP or vehicle administration. After 8 h, the animals were sacrificed and liver samples were taken and snap-frozen in liquid nitrogen for lipid content and mRNA expression analysis. Parallel groups of mice were used either to study VLDL-TG production or to perform hyperinsulinemic clamp analyses (see below). The animal care committee of TNO Quality of Life (Leiden, The Netherlands) approved all experiments.

Plasma parameters

Blood samples were taken from the tail vein into chilled paraoxon-coated capillaries to prevent *ex vivo* lipolysis[15]. Plasma was collected via centrifugation and plasma cholesterol, TG (without free glycerol), glucose, β-hydroxybutyrate (β-HB) and FFA levels were determined using standard commercial kits, according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany; Triglyceride GPO-Trinder, glucose Trinder 500 and β-hydroxybutyrate, Sigma Diagnostics, St. Louis, MO, USA; NEFA-C, Wako chemicals, Neuss, Germany, respectively). Plasma insulin was measured by a radioimmunoassay using rat insulin standards, which shows 100% cross-reaction with mouse insulin (sensitive rat insulin RIA kit, Linco research, St. Charles, MO, USA).

Liver lipid levels

Liver samples taken from mice 8 h after administration of MP or vehicle were homogenized in phosphate buffered saline (PBS) (10% wet wt/vol), and samples were taken to measure protein content by the Lowry assay[16]. Lipid content was determined by extraction of lipids using the Bligh and Dyer method[17], followed by lipid separation using high performance thin layer chromatography (HPTLC) on silica gel plates as described

previously[18] and subsequent analysis by TINA2.09 software[19] (Raytest Isotopen Meβgeräte, Straubenhardt, Germany)[20].

Hepatic VLDL-TG Production

Hepatic VLDL-TG production, *de novo* apolipoprotein (apo) B secretion, and VLDL composition were determined in overnight-fasted APOE3*Leiden mice given a gavage of MP (10 mg/kg) or vehicle. After 6 h of MP or vehicle administration, the animals were anesthetized with 0.5 ml/kg Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and 12.5 mg/g Midazolam (Roche, Mijdrecht, The Netherlands) and were injected intravenously with 0.1 ml PBS containing 100 µCi Tran^{35}S-label™ (ICN Biomedicals, Irvine, CA, USA) to measure *de novo* total apoB synthesis. After 30 min, the animals received a 15% (by volume) Triton WR1339 injection (500 mg/kg body weight; Tyloxapol, Sigma Chemicals, Steinheim, Germany) to prevent systemic lipolysis of newly secreted hepatic VLDL-TG[21]. Blood samples were drawn at 0, 15, 30, 60, and 90 min after Triton WR1339 injection, and TG concentrations were determined in the plasma as described above. At 90 min, the animals were sacrificed and blood was collected by cardiac puncture for isolation of VLDL.

VLDL composition analyses

VLDL particles (d < 1.019) were separated from other lipoproteins in plasma by density gradient ultracentrifugation as described[22]. Protein content of the VLDL fraction was determined by the Lowry assay[16]. TG and total cholesterol were measured as in plasma (see above). Phospholipids and free cholesterol were determined using standard commercial kits according to the manufacturer's instructions (Wako Chemicals). ^{35}S-labelled total apoB content was measured in the VLDL fraction after precipitation with isopropanol as described previously[23,24].

Hyperinsulinemic clamp analysis

Insulin sensitivity was determined in a group of APOE3*Leiden mice, fed a Western-type diet, which were fasted overnight, body weight-matched and given either MP or vehicle. Basal and insulin-mediated suppression of endogenous (hepatic) glucose production was studied by hyperinsulinemic euglycemic clamp analysis using [^{3}H]D-glucose as a tracer. The clamp analysis and calculations were performed as described previously[25,26].

Hepatic mRNA expression

Livers were immediately removed from the mice and snap-frozen in liquid nitrogen. Total RNA was isolated as described by Chomczynski and Sacchi[27] by use of RNA-Bee™ (Campro Scientific, Berlin, Germany). cDNA synthesis was done according to Bloks et al.[28]. Real-time quantitative PCR[29] was performed using an Applied Biosystems 7700 Sequence Detector according to the manufacturer's instructions. Primers were obtained from Invitrogen (Paisley, UK), and fluorogenic probes, labeled with 6-carboxyfluorescein (6-FAM), and 6-carboxytetramethylrhodamine (6-TAMRA), were supplied by Eurogentec

(Seraing, Belgium). Primers and probes used in this experiment were described previously[30-32]. All expression data were subsequently standardized for cyclophylin RNA, which was analyzed in a separate run.

Statistical analysis

The Mann-Whitney U test was used to determine differences between MP- and vehicle-treated mice. The level of significance was set at $P < 0.05$. All data presented as mean ± SD. Analyses were performed using SPSS11.0 (SPSS, Chicago, IL, USA).

Results

MP decreases β-HB and increases FFA levels in plasma

Male APOE3*Leiden mice, fed a Western-type diet for 8 weeks, were fasted overnight and treated with 10 mg/kg MP or vehicle. Plasma β-HB (one of the keton bodies produced by the liver) was decreased by 83% 8 h after MP administration, confirming inhibition of the β-oxidation. A strong decrease in plasma β-HB was already apparent after 2 h, and levels remained constant up to at least 8 h, whereas the solvent had no effect (**Figure 1**). **Table 1** summarizes plasma parameters measured 8 h after MP or vehicle administration. Plasma FFA levels were increased by 32% upon MP treatment. Glucose and TG did not show significant differences, whereas plasma insulin and cholesterol levels were both decreased significantly, by 50% and 17%, respectively, in the MP-treated group versus controls.

Inhibition of β-oxidation increases lipid storage in liver without affecting VLDL production

Liver lipid analysis showed that MP-treated mice had 30% increased TG contents in liver compared with vehicle-treated animals (**Figure 2A**). To analyze whether the increased hepatic TG content was associated with altered hepatic VLDL-TG production, mice were injected with Triton WR1339 at 6 h after MP or vehicle gavage, and the accumulation of endogenous VLDL-TG in plasma was determined over time. Plasma β-HB levels in this subset of mice were decreased similarly, as shown in **Figure 1**. As evident from **Figure 2B**, the VLDL-TG production rate, as determined from the slope of the curve, was unchanged in MP-treated animals compared with controls (2.12 ± 0.58 vs. 2.51 ± 0.53 mmol/l TG/h, respectively). Furthermore, the composition of the VLDL particles (**Table 2**) as well as the rate of *de novo* total apoB production of newly synthesized VLDL particles did not differ between MP- and vehicle- treated mice (35 ± 3 vs. 36 ± 4 x 10^4 dpm/ml plasma/mg protein, respectively).

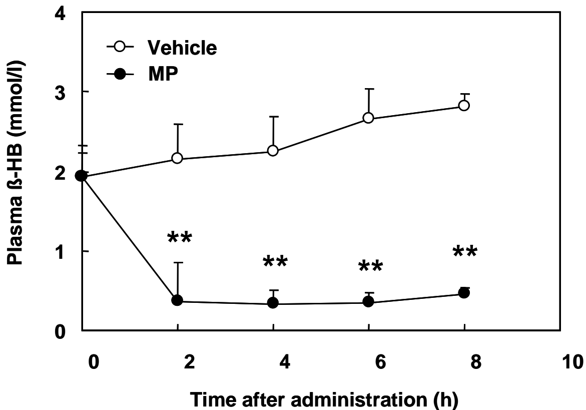

Figure 1. Time course effect of MP on β-HB levels
Plasma samples from overnight-fasted APOE*3Leiden mice, treated with MP (closed circles) or vehicle (open circles), were taken every 2 h to determine plasma β-HB levels. Values represent mean ± SD of 5 mice per group, **P < 0.01. MP methyl palmoxirate, β-HB β-hydroxybutyrate

Table 1. Plasma parameters determined in overnight-fasted APOE*3Leiden mice at 8 h after MP administration

	Vehicle	MP
β-Hydroxybutyrate (mmol/l)	2.81 ± 0.16	0.47 ± 0.07**
Triglycerides (mmol/l)	0.77 ± 0.19	0.85 ± 0.06
Total cholesterol (mmol/l)	5.00 ± 0.61	4.14 ± 0.82*
Free fatty acids (mmol/l)	1.17 ± 0.20	1.55 ± 0.26*
Glucose (mmol/l)	4.71 ± 1.14	4.13 ± 0.71
Insulin (pmol/l)	103.0 ± 37.9	50.7 ± 11.1*

Overnight-fasted APOE*3Leiden mice were administered 10 mg/kg body weight methyl palmoxirate (MP), 8 h later plasma was obtained from the mice via tail-tip incision. β-hydroxybutyrate, triglycerides, total cholesterol, free fatty acids, glucose, and insulin levels were determined in plasma. Values represent mean ± SD of 5 mice per group. *P < 0.05, **P < 0.01

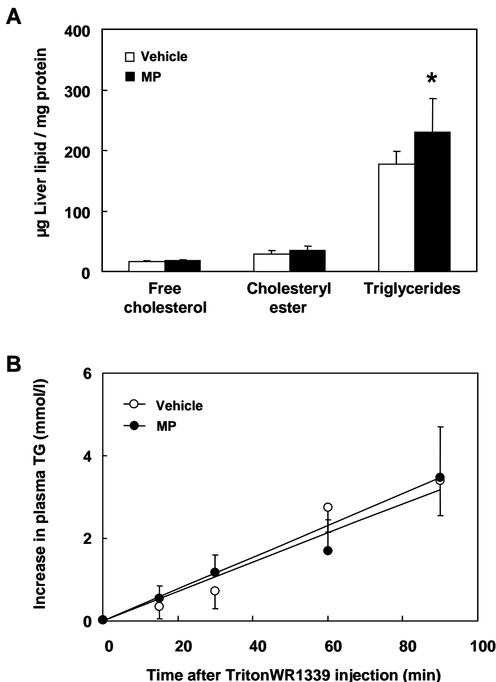

Figure 2. Effect of MP on hepatic lipid levels (A) and VLDL production (B)
Liver samples were taken 8 h after MP or vehicle treatment. Lipids were extracted from liver homogenates and subsequently separated on thin-layer silica gel plates as described. Liver free cholesterol, cholesteryl ester, and TG levels are depicted for MP-treated (closed bars) and vehicle-treated (open bars) mice (A). The increase in plasma TG levels in MP-treated (closed circles) and vehicle-treated (open circles) mice was measured in time after Triton WR1339 injection. (B). Values represent mean ± SD for 5 mice per group, $*P < 0.05$

Table 2. Composition of VLDL obtained from Triton WR1339-injected mice at 8 h after treatment with MP or vehicle

Parameter	Vehicle	MP
Triglycerides (% of total)	72.8 ± 2.1	69.0 ± 3.7
Free cholesterol (% of total)	2.4 ± 0.2	2.6 ± 0.3
Cholesteryl esters (% of total)	12.2 ± 2.3	14.1 ± 3.3
Phospholipids (% of total)	9.0 ± 1.3	10.3 ± 1.2
Protein (% of total)	3.7 ± 0.7	4.1 ± 0.8

VLDL was isolated using ultracentrifugation and triglycerides, free cholesterol, cholesteryl esters, phospholipids and protein contents were determined and expressed as percentages of total. Values represent mean ± SD for 5 mice per group

Acute inhibition of β-oxidation does not lead to hepatic insulin resistance.

Increased hepatic TG content is negatively associated with insulin sensitivity[33]. Since plasma insulin levels decreased in the MP-treated mice, we analyzed the insulin-mediated suppression of endogenous (hepatic) glucose production during a hyperinsulinemic clamp procedure. Although hepatic TG content was increased in the MP-treated mice, endogenous (hepatic) glucose production was equally suppressed by insulin in both groups. The glucose production decreased from 32.5 ± 6.5 to 19.1 ± 10.1 µmol/min/kg body weight (-40%) in MP-treated mice and from 28.7 ± 7.1 to 14.7 ± 8.8 µmol/min/kg body weight (-46%) in vehicle-treated mice. These results show that the livers of MP-treated mice displayed normal hepatic insulin sensitivity.

Hepatic mRNA levels

Hepatic expression of several genes was studied in mice at 8 h after treatment with MP or vehicle using RT-PCR. The expression of genes involved in β-oxidation (peroxisome proliferator-activated receptor α [*pparα*], *cpt1a*, medium-chain acyl-CoA [*mcad*], HMG-CoA synthase [*hmgs*]) were all 40-56% higher in MP-treated livers compared with controls, albeit that statistical significance was achieved for only *pparα* and *cpt1a* (**Table 3**). Regarding genes involved in VLDL production, microsomal TG transfer protein (*mttp*) expression was increased in MP-treated mice, whereas no differences were observed in acyl:diacylglycerol transferase 1 (*dgat1*), *dgat2*, *apob*, and *apoe* expression. Expression of the sterol regulatory element-binding protein 1c (*srebp1c*), which is involved in the activation of genes involved in the uptake and synthesis of FA, TG and cholesterol, was strongly decreased in MP-treated mice. In FA synthesis, gene expression levels of fatty acid synthase (*fas*) and acetyl-CoA carboxylase 1 (*acc1*) were not changed between the two groups. Also no changes were observed in gene expression levels of pyruvate kinase (*pk*) and phosphoenolpyruvate carboxykinase (*pepck*), which are involved in hepatic glucose metabolism.

Table 3. Hepatic mRNA expression levels in control and MP-treated mice as determined by RT-PCR and related to cyclophilin

mRNA source	Vehicle	MP	Change
β-oxidation			
pparα	100 ± 28 %	149 ± 17 %*	↑
cpt1a	100 ± 24 %	143 ± 18 %*	↑
mcad	100 ± 32 %	140 ± 9 %	NS
hmgs	100 ± 37 %	156 ± 18 %	NS
TG synthesis/VLDL production			
mttp	100 ± 24 %	159 ± 26 %*	↑
dgat1	100 ± 33 %	147 ± 11 %	NS
dgat2	100 ± 29 %	119 ± 15 %	NS
apob	100 ± 42 %	95 ± 11 %	NS
apoe	100 ± 21 %	104 ± 18 %	NS
Fatty acid synthesis			
srebp1c	100 ± 48 %	27 ± 13 %*	↓
fas	100 ± 49 %	79 ± 27 %	NS
acc1	100 ± 35 %	103 ± 18 %	NS
Glucose production			
pk	100 ± 42 %	84 ± 16 %	NS
pepck	100 ± 29 %	111 ± 26 %	NS

Values represent mean ± SD of 4 MP treated and 5 vehicle-treated mice per group, *P < 0.05, NS not significant. acc1 acetyl-coenzyme A carboxylase 1, apo apolipoprotein, dgat acyl:diacylglycerol transferase, cpt1a carnitine palmitoyl transferase 1a, fas fatty acid synthase, hmgs 3-hydroxy-3-methylglutaryl-coenzyme A synthase, mcad medium-chain acyl-coenzyme A, mttp microsomal triglyceride transfer protein, NS not significant, pepck phosphoenolpyruvate carboxykinase, pk pyruvate kinase, pparα paroxisome proliferators-activated receptor α, srebp1c sterol regulatory element binding protein 1c

Discussion

Hepatic FA and TG metabolism encompasses a number of interacting and dynamic processes, including uptake of plasma FFA and TG from lipoprotein remnants, storage in the form of TG, β-oxidation, and VLDL-TG formation/secretion. The rate of VLDL-TG secretion has long been thought to be substrate-driven[1-3]. We questioned whether an acute inhibition of hepatic β-oxidation would lead to a redirection of FA toward hepatic TG synthesis specifically directed to VLDL secretion. MP was used to irreversibly inhibit the crucial enzyme in β-oxidation, CPT1A[9,10]. Indeed, within 2 h after oral dosing of MP, plasma keton bodies (*i.e.*, β-HB) decreased and remained low for up to 8 h after gavage (**Figure 1**). Since plasma keton bodies are derived solely from hepatic β-oxidation, we concluded that hepatic β-oxidation of long-chain FA was almost completely inhibited by the applied dose of MP. It is known that, at low doses, MP inhibits mostly CPT1A (present in liver and heart), whereas higher doses also inhibit CPT1B (present in skeletal muscle and heart) in rat[7,9].

Several studies have demonstrated that oxirane carboxylates (such as etomoxir and MP) are effective at decreasing both keton body and glucose levels in rodents, dogs and humans[9,34-37]. However, in our overnight-fasted mice, plasma glucose levels were similar between MP-treated and control mice, suggesting that muscle β-oxidation was not

completely inhibited, in contrast to liver β-oxidation, as judged from the extremely low β-HB levels. The residual level of β-HB in MP-treated animals may be derived from short-chain FA oxidation that does not depend on CPT1A for transport into mitochondia.

We observed that the hepatic expression of genes involved in β-oxidation (*i.e., pparα, cpt1a, mcad, and hmgs)* was higher in MP-treated mice (**Table 3**), albeit statistically significant differences were only observed for *pparα* and *cpt1a*, as a result of the moderate sample sizes in combination with relatively large standard deviations in the control group. This increase of genes involved in β-oxidation may be an attempt of the liver to compensate for the strongly decreased hepatic β-oxidation.

Plasma FFA levels increased significantly at 8 h after MP treatment (**Table 2**). This increase was most likely caused by the decreased hepatic FA oxidation. Interestingly, we observed that acute inhibition of β-oxidation and/or the decrease of plasma keton bodies was associated with strongly decreased plasma insulin levels (**Table 2**). In agreement with this, Boden and Chen[38] showed that in humans there is a positive correlation between plasma β-HB concentrations and insulin secretion capacity.

As expected, inhibition of hepatic β-oxidation led to a significant accumulation of TG within the liver (**Figure 2A**). Hepatic TG accumulation is in agreement with the symptoms seen in patients and animal models with β-oxidation disorders[5,6]. Microscopic analysis of liver slices revealed a mixed micro/macrovesicular accumulation of lipid droplets, also called micro/macrovesicular steatosis (data not shown). This observation of microvesicular steatosis seems to be in agreement with the recent finding with cyclopropane carboxylic acid (CPCA), another β-oxidation inhibitor, in rats[39]. Although one might expect to observe increased liver mRNA levels of TG-synthesizing enzymes such as DGAT1, this was not observed in our experimental setting.

Interestingly, the increased TG accumulation in the liver was not associated with increased hepatic VLDL-TG production and/or changes in VLDL-composition (**Figure 2B, Table 2**). We did observe an increase in gene mRNA expression of *mttp* in the livers of MP-treated mice. We did not analyze microsomal transfer protein (MTP) activity, so we can only conclude that this increased *mttp* expression did not (yet) lead to increased MTP protein levels or activity. We cannot exclude the possibility that chronic, long-term inhibition of hepatic β-oxidation might induce hepatic VLDL-TG production.

Hepatic *srebp1c* expression was strongly decreased in MP-treated mice. Since insulin increases hepatic *srebp1c* expression[40], it is possible that the low plasma insulin levels caused this downregulation of liver *srebp1c* expression. However, this low *srebp1c* expression did not seem to affect RNA expression levels of enzymes involved in *de novo* lipogenesis, such as *fas* or *acc1*, at least at the examined time point.

Because TG accumulation is known to be associated with insulin resistance in the liver[33], we performed hyperinsulinemic clamp procedures to assess the insulin-mediated suppression of endogenous glucose production. Under normal conditions, the major part

of endogenous glucose production is derived from liver[38]. Our results clearly show that there was no significant difference in insulin-mediated suppression of endogenous (hepatic) glucose production between treated and untreated mice. Gene expression of enzymes involved in hepatic glucose production also did not show any differences between the two groups. Whole-body insulin-mediated glucose uptake was not significantly different between MP-treated and control mice (data not shown). This confirms, in part, that muscle FA β-oxidation must be still functional at the applied low dose of MP. It has been shown by Dobbins et al.[41] that chronic suppression of hepatic β-oxidation leads to insulin resistance in both liver and muscle in rats. We clearly show that acute inhibition of FA β-oxidation affects neither hepatic nor muscle insulin sensitivity *in vivo* in mice.

We conclude that acute inhibition of hepatic FA β-oxidation leads to hepatic micro/macrovesicular steatosis, but does not affect either hepatic VLDL secretion or hepatic insulin sensitivity.

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