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 VLDL and glucose production is enhanced in type 2 diabetes and associated with hepatic steatosis. Whether the derangements in hepatic metabolism are attributable to steatosis or to the increased availability of FA metabolites is not known. We used methyl palmoxirate (MP), an inhibitor of carnitine palmitoyl transferase I, to acutely inhibit hepatic FA oxidation and investigated whether the FAs 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 (ketone 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 FFA 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.—Duivenvoorden, I., B. Teusink, P. C. N. Rensen, F. Kuipers, J. A. Romijn, L. M. Havekes, and P. J. Voshol. **Acute inhibition of hepatic β-oxidation in APOE*3Leiden mice does not affect hepatic VLDL secretion or insulin sensitivity. *J. Lipid Res.* 2005.** 46: **988–993.**

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Hepatic 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 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 1 (CPT1), which couples long-chain FAs to carnitine for transportation into the mitochondria (7, 8). Methyl palmoxirate (MP) is a specific and irreversible inhibitor of CPT1 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.

Abbreviations: *acc1*, acetyl-coenzyme A carboxylase 1; apoB, apolipoprotein B; CPT1, carnitine palmitoyl transferase 1; *dgat*, acyl:diacylglycerol transferase; *fas*, fatty acid synthase; β-HB, β-hydroxybutyrate; *hmgs*, 3-hydroxy-3-methylglutaryl-coenzyme A synthase; *mcad*, medium-chain acyl coenzyme A; MP, methyl palmoxirate; MTP, microsomal triglyceride transfer protein; *mttp*, microsomal triglyceride transfer protein; *pparα*, peroxisome proliferator-activated receptor α; *srebp1c*, sterol regulatory element binding protein 1c; TG, triglyceride.

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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 in which to study hepatic VLDL metabolism, because they have decreased VLDL-TG production and fatty liver comparable to human conditions (12–14). We observed that acute inhibition of fatty acid 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 previously (9). Blood samples were taken by tail-tip bleeding every 2 h after MP or vehicle administration. After 8 h, the animals were killed 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 Netherlands Oganization for Applied Scientific Research (TNO) 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 (Roche Diagnostics GmbH, Mannheim, Germany), TG (without free glycerol; Triglyceride GPO-Trinder, Sigma Diagnostics, Inc., St. Louis, MO), glucose (glucose trinder 500, Sigma Diagnostics), β-hydroxybutyrate (β-HB) (Sigma Diagnostics), and FFA levels (NEFA-C, Wako Chemicals GmbH, Neuss, Germany) were determined using standard commercial kits, according to the manufacturer's instructions. Plasma insulin was measured by a radioimmunoassay using rat insulin standards, which show 100% cross-reaction with mouse insulin (Sensitive Rat Insulin RIA Kit; Linco Research, Inc., St. Charles, MO).

Liver lipid levels

Liver samples taken from mice 8 h after administration of MP or vehicle were homogenized in phosphate-buffered saline (10% wet weight per volume), 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 on silica gel plates as described previously (18) and subsequent analysis by TINA2.09 software (19) (Raytest Isotopen Meßgeräte GmbH, Straubenhardt, Germany).

Hepatic VLDL-TG production

Hepatic VLDL-TG production, de novo apolipoprotein B (apoB) 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 [0.5 ml/kg Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and 12.5 mg/g midazolam (Roche, Mijdrecht, The Netherlands)] and injected intravenously with 0.1 ml of phosphate-buffered saline containing 100 μCi of Tran^{35}S-label™ (ICN Biomedicals, Inc., Irvine, CA) 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 (20). 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 killed 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 (21). 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 GmbH). ^{35}S-labeled total apoB content was measured in the VLDL fraction after precipitation with isopropanol as described previously (22, 23).

Hyperinsulinemic clamp analysis

Insulin sensitivity was determined in a group of APOE3* Leiden mice, fed a Western-type diet, that 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 (24, 25).

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 (26) by use of RNA-Bee™ (Campro Scientific, Berlin, Germany). cDNA synthesis was done according to Bloks et al. (27). Real-time quantitative PCR (28) 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 and 6-carboxytetramethylrhodamine, were supplied by Eurogentec (Seraing, Belgium). Primers and probes used in this experiment were described previously (29–31). All expression data were subsequently standardized for cyclophilin 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 are presented as means ± SD. Analyses were performed using SPSS11.0 (SPSS, Inc., Chicago, IL).

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 ketone bodies produced by the liver) was decreased by 83% at 8 h after

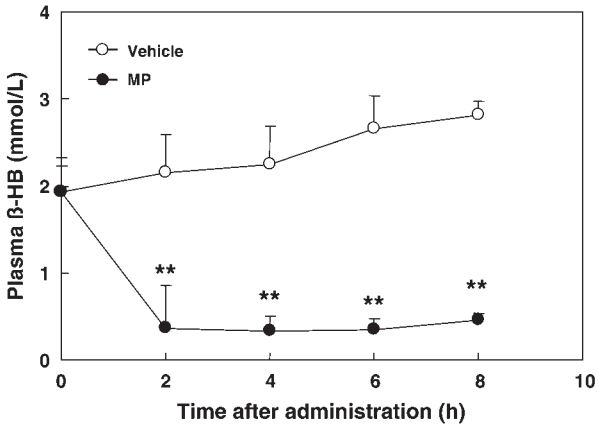

Fig. 1. Time course effect of methyl palmoxirate (MP) on β-hydroxybutyrate (β-HB) levels. Plasma samples from overnight-fasted APOE*3 Leiden mice, treated with MP (closed circles) or vehicle (open circles), were taken every 2 h to determine plasma β-HB levels. Values represent means ± SD of five mice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

MP administration, confirming the inhibition of 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 (**Fig. 1**). **Table 1** summarizes plasma parameters measured at 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 content in liver compared with vehicle-treated animals (**Fig. 2A**). To analyze whether the increased hepatic TG content was associated with altered hepatic VLDL-TG production, mice were injected with Triton

TABLE 1. Plasma parameters determined in overnight-fasted APOE*3Leiden mice at 8 h after MP administration (10 mg/kg body weight)

Parameter	Vehicle	MP
β-HB (mmol/l)	2.81 ± 0.16	0.47 ± 0.07[a]
TG (mmol/l)	0.77 ± 0.19	0.85 ± 0.06
TC (mmol/l)	5.00 ± 0.61	4.14 ± 0.82[b]
FFA (mmol/l)	1.17 ± 0.20	1.55 ± 0.26[b]
Glucose (mmol/l)	4.71 ± 1.14	4.13 ± 0.71
Insulin (pmol/l)	103.0 ± 37.9	50.7 ± 11.1[b]

β-HB, β-hydroxybutyrate; MP, methyl palmoxirate; TC, total cholesterol; TG, triglyceride. Overnight-fasted APOE*3Leiden mice were administered 10 mg/kg body weight MP, and 8 h later plasma was obtained from the mice via tail-tip incision. β-HB, TG, TC, FFA, glucose, and insulin levels were determined in plasma. Values represent means ± SD of five mice per group.

[a] $P < 0.01$.

[b] $P < 0.05$.

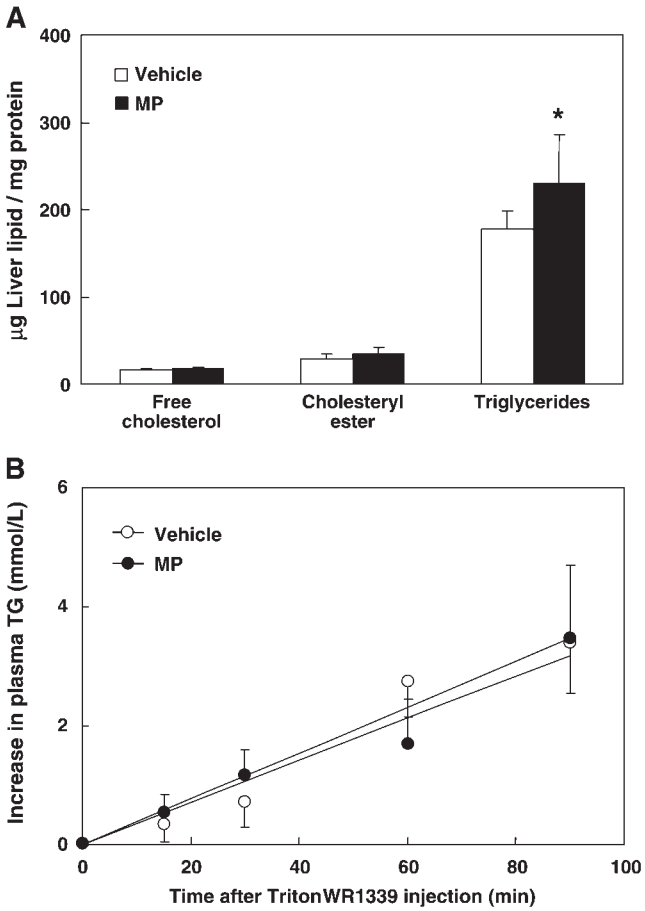

Fig. 2. Effect of MP on hepatic lipid levels and VLDL production. A: Liver samples were taken 8 h after MP or vehicle treatment. Lipids were extracted from liver homogenates and subsequently separated on thin-layer silica gel plates as described. Liver free cholesterol, cholesteryl ester, and triglyceride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

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 Fig. 1. As is evident from Fig. 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 × 10^4 dpm/ml plasma/mg protein, respectively).

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

Increased hepatic TG content is negatively associated with insulin sensitivity (32). Because plasma insulin levels decreased in the MP-treated mice, we analyzed the insulin-

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

Parameter	Vehicle	MP
TG (% 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
Phospholipid (% 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 TG, free cholesterol, cholesteryl esters, phospholipid, and protein contents were determined and expressed as percentages of total. Values represent means ± SD for five mice per group.

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*), and HMG-CoA synthase (*hmgs*)] were all 40–56% higher in MP-treated livers compared with controls, albeit 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 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.

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

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%[a]	↑
cpt1a	100 ± 24%	143 ± 18%[a]	↑
mcad	100 ± 32%	140 ± 9%	NS
hmgs	100 ± 37%	156 ± 18%	NS
TG synthesis/VLDL production			
mttp	100 ± 24%	159 ± 26%[a]	↑
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%[a]	↓
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

acc1, acetyl-coenzyme A carboxylase 1; apoB, apolipoprotein B; *dgat*, acyl:diacylglycerol transferase; *fas*, fatty acid synthase; *hmgs*, 3-hydroxy-3-methylglutaryl-coenzyme A synthase; *mcad*, medium-chain acyl-coenzyme A; *mttp*, microsomal triglyceride transfer protein; *pepck*, phosphoenolpyruvate carboxykinase; *pk*, pyruvate kinase; *pparα*, peroxisome proliferator-activated receptor α; *srebp1c*, sterol regulatory element binding protein 1c. Values represent means ± SD of four MP-treated and five vehicle-treated mice per group.

[a] $P < 0.05$.

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 ketone bodies (i.e., β-HB) decreased and remained low for up to 8 h after gavage (Fig. 1). Because plasma ketone bodies are derived solely from hepatic β-oxidation, we concluded that hepatic β-oxidation of long-chain FAs 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 ketone body and glucose levels in rodents, dogs, and humans (9, 33–36). 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 mitochondria.

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 observed only 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 by 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 ketone bodies was associated with strongly decreased plasma insulin levels (Table 2). In agreement with this, Boden and Chen (37) 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 (Fig. 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 (38). 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 (Fig. 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 triglyceride 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. Because insulin increases hepatic *srebp1c* expression (39), it is possible that the low plasma insulin levels caused this downregulation of liver *srebp1c* expression. However, the 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 (32), 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 (37). 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 still be functional at the applied low dose of MP. It has been shown by Dobbins et al. (40) 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 does not affect either hepatic or 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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