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Impaired ketogenesis is a major mechanism for disturbed hepatic fatty acid metabolism in rats with long-term cholestasis and after relief of biliary obstruction

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***Background/Aims*: Rats with long-term cholestasis have reduced ketosis of unknown origin.**

***Methods*: Fatty acid metabolism was studied in starved rats with biliary obstruction for 4 weeks (bile duct ligated rats = BDL rats), and 3, 7, 14, 28 and 84 days after reversal of biliary obstruction by Roux-en-Y anastomosis (RY rats), and in sham-operated control rats.**

***Results*: BDL rats had reduced β-hydroxybutyrate concentrations in plasma (0.25 ± 0.10 vs. 0.75 ± 0.20 mmol/l) and liver (2.57 ± 0.20 vs. 4.63 ± 0.61 μmol/g) which increased after restoring bile flow. Hepatic expression and activity of carnitine palmitoyltransferase I (CPT I) or CPT II were unaffected or decreased in BDL rats, respectively, and increased after restoring bile flow. Oxidative metabolism of different substrates by isolated liver mitochondria and activation of palmitate were reduced in BDL rats and recovered 7–14 days after restoring bile flow. Ketogenesis was decreased in mitochondria from BDL rats and recovered 3 months after restoring bile flow. Both mRNA and protein expression of hydroxymethylglutaryl-coenzyme A synthase (HMG-CoA synthase), the rate-limiting enzyme of ketogenesis, was reduced in livers of BDL rats and increased after reversing biliary obstruction.**

***Conclusions*: In BDL rats, impairment of hepatic fatty acid metabolism is multifactorial. After reversing biliary obstruction, reduced activity of HMG-CoA synthase is the major factor.**

***Keywords*: Mitochondria; Respiratory chain; β-Oxidation; Hydroxymethylglutaryl-coenzyme A pathway (ketogenesis); Hydroxymethylglutaryl-coenzyme A synthase**

1. Introduction

Long-term cholestasis in the rat is associated with alterations in hepatic energy metabolism such as impaired glycogen storage [1] and mitochondrial fatty acid metabolism [2,3]. Rats with long-term bile duct ligation have decreased ketosis during starvation, compatible with reduced production of ketone bodies [2]. Similar findings were obtained in perfused livers from bile duct ligated (BDL) rats, which showed reduced production of ketone bodies when long- or short-chain fatty acids were used as substrates [4,5]. We have conducted several studies on the mechanisms causing impaired ketogenesis in BDL rats. In initial studies, we could show that liver mitochondria from BDL rats have a reduced activity of enzyme complexes of the respiratory chain and of enzymes of the mitochondrial β-oxidation which could both explain these findings [4]. Further studies revealed, however, that after reversing bile duct ligation, ketogenesis remained impaired, whereas the function of

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Abbreviations: AST, aspartate aminotransferase; BDL rats, bile duct ligated rats; CoASH, coenzyme A, reduced form; CPT, carnitine palmitoyltransferase; HMG-CoA cycle, hydroxymethylglutaryl-CoA cycle; PCS, palmitoyl-CoA synthase; TMPD, *N*,*N*,*N*′*N*′-tetramethyl-*p*-phenylenediamine.

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complexes I and III of the respiratory chain, which are both essential for hepatic fatty acid metabolism, normalized quickly [6].

Beside complexes I and III of the respiratory chain, formation of ketone bodies can be impaired at many other sites, among them activation and transport of long-chain fatty acids, β-oxidation and the hydroxymethylglutaryl-CoA (HMG-CoA) cycle (ketogenesis). Regarding activation and transport of fatty acids, the activity of carnitine palmitoyltransferase I (CPT I) is considered to be rate-limiting [7,8]. The activity of the HMG-CoA cycle, which produces ketone bodies from acetyl-CoA, the end product of β-oxidation, is controlled by HMG-CoA synthase, an enzyme located in the mitochondrial matrix [9]. Short-term control of the activity of HMG-CoA synthase is achieved by succinylation [10], whereas gene transcription controls long-term activity [9].

In recent studies, we were able to show that rats with short-term cholestasis have also impaired ketogenesis and that this finding can be explained entirely by a reduced activity of HMG-CoA synthase [11]. This mechanism may therefore contribute to impaired ketogenesis also in rats with long-term cholestasis and may explain the observation that ketogenesis remains decreased after reversing biliary obstruction. In order to find an answer to this question, we decided to study hepatic fatty acid metabolism in rats with bile duct ligation for 4 weeks and up to 3 months after reversal of bile duct ligation by biliodigestive anastomosis. The specific questions asked were: (i) which mechanisms contribute to impaired hepatic fatty acid metabolism in rats with long-term cholestasis, (ii) are these changes reversible after biliodigestive anastomosis for 3 months, and (iii) which are the causes, if hepatic fatty acid metabolism does not recover.

2. Materials and methods

2.1. Reagents

[1-^{14}C]-palmitic acid was obtained from Amersham Pharmacia Biotech (Dübendorf, Switzerland). Phosphotransacetylase was obtained from Boehringer Mannheim GmbH (Rotkreuz, Switzerland). All other chemicals were of the highest purity available obtained from different providers.

2.2. Animals

Male Sprague–Dawley rats (BRL, Füllinsdorf, Switzerland) were used throughout the experiments. Rats were housed in wire-bottom cages on a 12 h dark and light cycle. All animals were fed a standard rat chow and tap water ad libitum. The studies were performed with the rats starved for 24 h before investigation. All animal experiments had been approved by the Animal Ethics Board of the State of Berne and were performed according to these guidelines.

2.3. Surgical procedure

Bile duct ligation (BDL) and sham operation were performed as reported previously [6]. The reversal of biliary obstruction was achieved by Roux-en-Y anastomosis as described earlier [6]. In sham-operated animals, laparotomy was performed and the abdominal cavity exposed without any surgical procedure.

2.4. Study design

Seven different groups were investigated with all animals starved for 24 h. Rats of group 1 were bile duct ligated for 4 weeks (BDL, $n = 9$). In rats of groups 2–6, bile duct ligation was reversed by Roux-en-Y anastomosis for 3 (group 2, $n = 4$), 7 (group 3, $n = 6$), 14 (group 4, $n = 6$), 28 (group 5, $n = 6$) or 84 days (group 6, $n = 6$). Group 7 represented sham-operated control rats ($n = 8$). Control rats were studied at the same time point as BDL rats.

2.5. Characterization of the animals

The animals were characterized by their body, liver and spleen weights, activities of alkaline phosphatase and aspartate aminotransferase (AST) in plasma and by their plasma concentrations of glucose, free fatty acids, β-hydroxybutyrate, bilirubin and bile acids. Alkaline phosphatase, AST, glucose, free fatty acids and bilirubin were analyzed on a COBAS analyzer (Hoffmann-La Roche Diagnostics, Basel, Switzerland) with commercially available kits. Bile acids were determined with a radioimmunoassay (Becton and Dickinson, Orangeburg, SC, USA). β-Hydroxybutyrate was determined fluorimetrically as described by Olsen [12].

2.6. Isolation of liver mitochondria and mitochondrial oxidative metabolism

For the isolation of mitochondria, rats were decapitated and a mixed venous/arterial blood sample was obtained into heparinized tubes. A freeze clamped liver sample was obtained quickly and stored at −70°C. Mitochondria were isolated from the remainder of the liver by differential centrifugation as described by Hoppel et al. [13]. As shown previously, this method yields mitochondria of good quality with only minor contamination of peroxisomes or lysosomes also in rats with bile duct ligation [3]. Mitochondrial protein was determined by the Biuret method with bovine serum albumin as a standard [14]. Oxygen consumption by intact mitochondria was measured in a chamber equipped with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) at 30°C as described previously [15].

2.7. In vitro mitochondrial β-oxidation and ketogenesis

β-Oxidation of [1-^{14}C]-palmitic acid by intact liver mitochondria was assessed as described by Fréneaux et al. [16], with some modifications as described before [11]. β-Oxidation was estimated by the formation of acid soluble products from [1-^{14}C]-palmitate [17].

Ketone body formation by disrupted liver mitochondria was determined according to Chapman et al. [18], with some modifications described before [11]. Acetoacetate was analyzed fluorimetrically as described by Olsen [12].

2.8. Ketone bodies in liver homogenate

Frozen liver (100 mg) was homogenized at 0°C with 500 μl 3% PCA and then transferred into a graduated 2 ml Eppendorf tube. The homogenizer was washed twice with 500 μl 3% perchloric acid. The homogenate (1500 μl) was centrifuged for 3 min at full speed. The supernatant was transferred into a new graduated 2 ml Eppendorf tube, the volume was adjusted to 2.0 ml by adding 3% PCA and the supernatant was analyzed for β-hydroxybutyrate [12].

Table 1
Characterization of the rats[a]

	CON	BDL	RY3	RY7	RY14	RY28	RY84
Body weight (end of study) (g)	358 ± 72	322 ± 27	337 ± 33	321 ± 46	349 ± 65	343 ± 27	482 ± 81[b,c]
Liver weight (g)	2.63 ± 0.56	5.90 ± 0.71[b]	5.09 ± 1.33[b]	3.86 ± 0.62[b,c]	4.01 ± 0.92[b,c]	3.47 ± 0.58[c]	2.80 ± 0.58[c]
Spleen weight (g)	0.20 ± 0.05	0.63 ± 0.13[b]	0.62 ± 0.23[b]	0.56 ± 0.19[b]	0.44 ± 0.18[b]	0.44 ± 0.11[b]	0.36 ± 0.23[c]
Aspartate aminotransferase (U/l)	90 ± 30	480 ± 200[b]	120 ± 20[c]	130 ± 20[c]	140 ± 30[c]	110 ± 20[c]	140 ± 30[c]
Alkaline phosphatase (U/l)	170 ± 60	400 ± 90[b]	140 ± 70[c]	160 ± 50[c]	160 ± 60[c]	200 ± 80[c]	120 ± 30[c]
Bilirubin (μmol/l)	1 ± 1	120 ± 40[b]	11 ± 4[b,c]	4 ± 3[c]	3 ± 3[c]	2 ± 1[c]	2 ± 1[c]
Bile acids (μmol/l)	2 ± 1	130 ± 5[b]	2 ± 1[c]	7 ± 5[c]	5 ± 3[c]	5 ± 4[c]	6 ± 4[c]
Glucose (mmol/l)	7.7 ± 0.9	6.7 ± 0.6	7.1 ± 0.2	7.5 ± 0.9	6.7 ± 0.6	6.9 ± 0.9	7.8 ± 1.4

[a] Rats studied were bile duct ligated for 4 weeks (BDL, $n = 9$), or 3 (RY3, $n = 4$), 7 (RY7, $n = 6$), 14 (RY14, $n = 6$), 28 (RY28, $n = 6$) or 84 days (RY84, $n = 6$) after relief of biliary obstruction by Roux-en-Y anastomosis. Control rats (CON, $n = 8$) were sham-operated and studied at the same time point as the BDL rats. Enzyme activities and metabolite concentrations were determined in heparinized plasma as described in Section 2. Results are given as mean ± sd.
[b] $P < 0.05$ vs. control.
[c] $P < 0.05$ vs. BDL.

2.9. RNA isolation, DNase digestion, reverse transcription and real-time quantitative polymerase chain reaction (PCR) analysis of HMG-CoA synthase

Total RNA was extracted from rat liver according to the general protocol of Sambrook et al. [19]. Quality control of the isolated RNA, DNase digestion and reverse transcription were performed as described recently [11]. Real-time quantitative PCR analysis of HMG-CoA synthase was performed with a PE Applied Biosystems 7700 Sequence Detector (PE Biosystems) as described previously [11].

2.10. Preparation of antibodies and Western blot analysis of HMG-CoA synthase

Preparation of antibodies against mitochondrial HMG-CoA synthase and immunoblotting of HMG-CoA synthase were carried out as described by Serra et al. [20]. The autoradiograms were quantified using a Luminescent Image Analyser LAS-1000 with Image Reader LAS-1000 for Windows® software (Raytest, Urdorf, Switzerland).

2.11. Activities of palmitoyl-CoA synthase (PCS) and CPT, and protein expression of CPT I and II

Activity of PCS was determined in isolated liver mitochondria according to Hesler et al. [21] using [1-^{14}C]-palmitic acid as a substrate. CPT activities were determined in liver homogenate using the CPT assay-1 ('forward direction': palmitoyl-CoA + carnitine → palmitoylcarnitine + CoASH) and the CPT assay-2 ('backward reaction': palmitoylcarnitine + CoASH → palmitoyl-CoA + carnitine) as described by Hoppel and Tomec [22]. Antibodies against rat CPT I and II were a gift from Dr V.A. Zammit (Hannah Research Institute, Ayr, Scotland) [23]. Immunoblotting of CPT I and II was performed as described by Fraser and Zammit [23].

2.12. Data presentation and statistical analysis

Data are presented as mean ± sd unless otherwise specified. Data were analyzed by analysis of variance (ANOVA) followed by a *t*-test with Bonferroni correction to localize the differences in case of a significant ANOVA. A $P < 0.05$ was considered to be statistically significant.

3. Results

Hepatic fatty acid metabolism was investigated in rats with long-term bile duct ligation and after reversal of biliary obstruction. Body weights were not different between the different groups of rats investigated with the exception of an increased body weight in RY rats observed for 3 months, which is explained by the long observation period (Table 1). Similarly as in other studies, liver and spleen weights were increased in BDL rats and had reached control values 1 and 3 months after reversing bile duct ligation, respectively [6,24]. In comparison to control rats, BDL rats had higher AST and alkaline phosphatase activities, as well as higher bilirubin and bile acid concentrations, which had all normalized 3 or 7 days after reversing biliary obstruction.

BDL rats had decreased plasma and liver β-hydroxybutyrate concentrations after starvation for 24 h in comparison to control rats (Table 2). Over 3 months after reversal of bile duct ligation, the plasma β-hydroxybutyrate concentration gradually increased, but did not reach control values, whereas the liver β-hydroxybutyrate concentration had normalized after 3 months. The plasma free fatty acid concentrations were decreased by 20–30% in BDL rats and reached control values 4 weeks after restoring bile flow. Since the K_m value of palmitoyl-CoA synthase is in the range of 100 μmol/l [25], this decrease is not likely to impair hepatic fatty acid metabolism in BDL rats.

The activity of palmitoyl-CoA synthase was decreased in liver mitochondria from BDL rats and had recovered 14 days after relief of biliary obstruction (Table 3). CPT activity was determined in both the forward and backward directions, which predominantly reflect the activity of CPT I and CPT II, respectively [22]. The activity in the forward reaction was not different between BDL and control rats and showed a significant increase 14 days after reversal of biliary obstruction. In contrast, the activity in the backward reaction was decreased in BDL and partially recovered over 14 days after restoring bile flow. Hepatic expression of CPT I paralleled CPT activity in the forward direction with a significant increase in RY rats as compared to BDL or control rats. Similarly, hepatic expression of CPT II paralleled CPT activity in the backward direction with a decrease

Table 2
Fatty acid metabolism[a]

	CON	BDL	RY3	RY7	RY14	RY28	RY84
Plasma free fatty acids (mmol/l)	1.24 ± 0.30	0.93 ± 0.13[b]	0.71 ± 0.11[b]	0.88 ± 0.16[b]	0.75 ± 0.26[b]	0.79 ± 0.09[b]	1.01 ± 0.19
β-Hydroxybutyrate in plasma (mmol/l)	0.75 ± 0.20	0.25 ± 0.10[b]	0.39 ± 0.10[b]	0.51 ± 0.20[b,c]	0.42 ± 0.15[b,c]	0.45 ± 0.17[b,c]	0.47 ± 0.12[b,c]
β-Hydroxybutyrate in liver (μmol/g liver)	4.63 ± 0.61	2.57 ± 0.20[b]	2.74 ± 0.26[b]	3.08 ± 0.23[b]	3.64 ± 0.30[b,c]	3.48 ± 0.68[b,c]	3.64 ± 0.68[b,c]

[a] Rats studied were bile duct ligated for 4 weeks (BDL, $n = 9$), or 3 (RY3, $n = 4$), 7 (RY7, $n = 6$), 14 (RY14, $n = 6$), 28 (RY28, $n = 6$) or 84 days (RY84, $n = 6$) after relief of biliary obstruction by Roux-en-Y anastomosis. Control rats (CON, $n = 8$) were sham-operated. β-Hydroxybutyrate in plasma and liver, and plasma fatty acids were determined as described in Section 2. Results are given as mean ± sd.
[b] $P < 0.05$ vs. control.
[c] $P < 0.05$ vs. BDL.

in BDL as compared to control rats and a partial recovery after relief of biliary obstruction.

Mitochondrial fatty acid metabolism was studied further by assessing oxidative metabolism of fatty acid derivatives in intact, freshly isolated mitochondria (Table 4). In mitochondria from BDL rats, oxidation of palmitate, palmitoyl-CoA and palmitoylcarnitine was decreased by 20–30% as compared to control mitochondria, compatible with a defect in mitochondrial fatty acid metabolism distal to activation and CPT I. After restoring bile flow, the impairment of mitochondrial fatty acid metabolism was rapidly reversible, reaching normal activities after 7–14 days.

As shown previously, oxidative metabolism of L-glutamate and succinate was impaired in mitochondria from BDL rats, but not oxidation of ascorbate, compatible with reduced activities of complexes I and II (and possibly also III) of the electron transport chain [3,6]. Mitochondrial metabolism of L-glutamate had normalized 7 days and metabolism of succinate 28 days after restoring bile flow. Since oxidative metabolism of L-glutamate, which involves complexes I, III and IV of the electron transport chain, had normalized 7 days after restoring bile flow, impaired activity of the electron transport chain could not explain reduced hepatic fatty acid metabolism 7–28 days after reversing biliary obstruction.

The formation of acid soluble products by isolated mitochondria, mainly reflecting formation of ketone bodies and ketone body precursors, was decreased in BDL rats but normalized quickly after reversing biliary obstruction (Table 5). These findings indicate that mitochondrial β-oxidation is decreased in BDL rats, but this decrease cannot explain impaired ketogenesis in RY rats observed for longer than 7 days.

The formation of acetoacetate from acetyl-CoA (activity of the HMG-CoA cycle) was decreased in mitochondria from BDL rats in comparison to control rats, and recovered only 3 months after restoring bile flow (Table 5). Since HMG-CoA synthase is rate-limiting for the activity of the whole cycle [26], we assessed mRNA and protein expression of this enzyme. Hepatic expression of HMG-CoA synthase was decreased on both the protein and the mRNA level in BDL as compared to control rats (see Fig. 1 for protein expression of HMG-CoA synthase). Recovery of HMG-CoA expression after reversing biliary obstruction was only partial both for protein and mRNA. The hepatic HMG-CoA synthase mRNA content showed a close linear correlation with the hepatic concentration of β-hydroxybutyrate (Fig. 2), indicating that impaired expression of this key enzyme is the major factor in explaining reduced hepatic fatty acid metabolism in rats with chronic biliary obstruction.

Table 3
Activities of palmitoyl-CoA synthase and carnitine palmitoyltransferase (CPT I and II) and expression of CPT I and II[a]

	CON	BDL	RY7	RY14
Enzyme activities				
Palmitoyl-CoA synthase (nmol/mg mitochondrial protein/min)	49 ± 6	21 ± 9[b]	28 ± 7[b]	40 ± 8[c]
CPT 'forward reaction' (μmol/min/g liver wet weight)	1.01 ± 0.16	1.10 ± 0.27	1.39 ± 0.20	1.56 ± 0.15[b,c]
CPT 'backward reaction' (μmol/min/g liver wet weight)	11.2 ± 1.0	5.42 ± 1.44[b]	6.05 ± 2.05[b]	7.83 ± 0.79[b,c]
Protein expression				
CPT I	100 ± 25	105 ± 21	142 ± 16[b,c]	182 ± 14[b,c]
CPT II	100 ± 6	60 ± 1[b]	62 ± 7[b]	77 ± 4[b,c]

[a] Rats studied were bile duct ligated for 4 weeks (BDL, $n = 9$), or 7 (RY7, $n = 8$), or 14 days (RY14, $n = 6$) after relief of biliary obstruction by Roux-en-Y anastomosis. Control rats (CON, $n = 8$) were sham-operated. Results are presented as mean ± sd. Protein expression was studied in $n = 3$ samples per group, and units are arbitrary (control values were set at 100).
[b] $P < 0.05$ vs. control.
[c] $P < 0.05$ vs. BDL.

Table 4
State 3 oxidation rates by isolated rat liver mitochondria[a]

Activity	CON	BDL	RY7	RY14	RY28	RY84
Hydrophilic substrates						
L-Glutamate (20 mmol/l)	82 ± 9	66 ± 6[b]	78 ± 31	73 ± 10	78 ± 20	109 ± 28[b,c]
Succinate (20 mmol/l)	140 ± 29	90 ± 25[b]	111 ± 38	102 ± 30[b]	111 ± 44	152 ± 26[c]
Ascorbate (7.2 mmol/l)	257 ± 68	235 ± 33	259 ± 33	220 ± 15	271 ± 26	215 ± 26
Fatty acids and derivatives						
Palmitate (80 μmol/l)	68 ± 19	33 ± 8[b]	43 ± 18[b]	54 ± 9[c]	53 ± 20[c]	59 ± 9[c]
Palmitoyl-CoA (20 μmol/l)	88 ± 14	67 ± 12[b]	75 ± 13	75 ± 16	72 ± 20	98 ± 8[c]
Palmitoyl-L-carnitine (40 μmol/l)	96 ± 12	77 ± 12[b]	86 ± 21	89 ± 8	92 ± 22	109 ± 17[c]

[a] Rats studied were bile duct ligated for 4 weeks (BDL, $n = 9$), or 7 (RY7, $n = 6$), 14 (RY14, $n = 6$), 28 (RY28, $n = 6$) or 84 days (RY84, $n = 6$) after relief of biliary obstruction by Roux-en-Y anastomosis. Control rats (CON, $n = 8$) were sham-operated. Mitochondria were isolated by differential centrifugation and state 3 oxidation rates were determined using a Clark-type oxygen electrode as described in Section 2. Units are natoms/min/mg mitochondrial protein. Results are presented as mean ± sd.
[b] $P < 0.05$ vs. control.
[c] $P < 0.05$ vs. BDL.

4. Discussion

The current studies show that impaired hepatic fatty acid metabolism in rats with long-term cholestasis is multifactorial (reduced activation of palmitate, and reduced activities of CPT II, of the mitochondrial β-oxidation, of the electron transport chain and of the HMG-CoA cycle). While most of these alterations are rapidly reversible after restoring bile flow, this is not the case for the HMG-CoA cycle.

Long-chain fatty acids are activated by palmitoyl-CoA synthase, an enzyme located on the outer mitochondrial membrane [21,27], before they are transported into the mitochondrial matrix for β-oxidation (Fig. 3). Similar to rats with short-term cholestasis [11], activation of palmitate was also reduced in rats with long-term cholestasis, suggesting that cholestasis (and not secondary biliary cirrhosis) affects synthesis of palmitoyl-CoA. Since oxidative metabolism of palmitate had reached control values 14 days after reversal of biliary obstruction, impaired activation of palmitate cannot explain reduced formation of ketone bodies in RY rats at later time points.

Palmitoyl-CoA is converted by CPT I to palmitoylcarnitine which is then transported into the mitochondrial matrix. CPT I is located at the contact sites between outer and inner mitochondrial membranes [23] and is regulated by malonyl-CoA (short-term inhibition) and by protein expression [28]. Our results show that both activity and expression of CPT I were not affected by long-term cholestasis. CPT II is located in the inner mitochondrial membrane and catalyzes the formation of palmitoyl-CoA from palmitoylcarnitine [28].

Table 5
Activity of β-oxidation and ketogenesis[a]

	CON	BDL	RY3	RY7	RY14	RY28	RY84
Acid soluble β-oxidation products (nmol/min/mg mitochondrial protein)	2.75 ± 0.88	1.21 ± 0.47[b]	n.d.[d]	2.37 ± 0.66[c]	3.17 ± 0.66[c]	2.46 ± 0.26[c]	2.55 ± 0.68[c]
Acetoacetate from acetyl-CoA (nmol/min/mg mitochondrial protein)	3.8 ± 1.4	0.4 ± 0.1[b]	n.d.[d]	0.7 ± 0.5[b]	1.0 ± 0.5[b]	2.2 ± 0.9[b,c]	4.8 ± 1.8[c]
HMG-CoA synthase							
mRNA expression (unitless)	100 ± 10	17 ± 3[b]	43 ± 12[b,c]	52 ± 12[b,c]	59 ± 24[b,c]	46 ± 15[b,c]	69 ± 12[b,c]
Protein expression (unitless)	100 ± 11	44 ± 8[b]	32 ± 5[b,c]	76 ± 3[b,c]	76 ± 18[b,c]	71 ± 10[b,c]	68 ± 12[b,c]

[a] Rats studied were bile duct ligated for 4 weeks (BDL, $n = 9$), or 3 (RY3, $n = 4$), 7 (RY7, $n = 6$), 14 (RY14, $n = 6$), 28 (RY28, $n = 6$) or 84 days (RY84, $n = 6$) after relief of biliary obstruction by Roux-en-Y anastomosis. Control rats (CON, $n = 8$) were sham-operated. Mitochondria were isolated by differential centrifugation and disrupted by freeze-thawing. β-Oxidation was determined in freshly isolated mitochondria using [1-^{14}C]-palmitate as a substrate as described in Section 2. Production of acetoacetate from acetyl-CoA was determined flourimetrically using disrupted mitochondria. Protein and mRNA expression of HMG-CoA synthase were performed as described in Section 2 using standard methods. These data are unitless and the control values are arbitrarily set at 100%. Results are presented as mean ± sd.
[b] $P < 0.05$ vs. control.
[c] $P < 0.05$ vs. BDL.
[d] n.d., not determined.

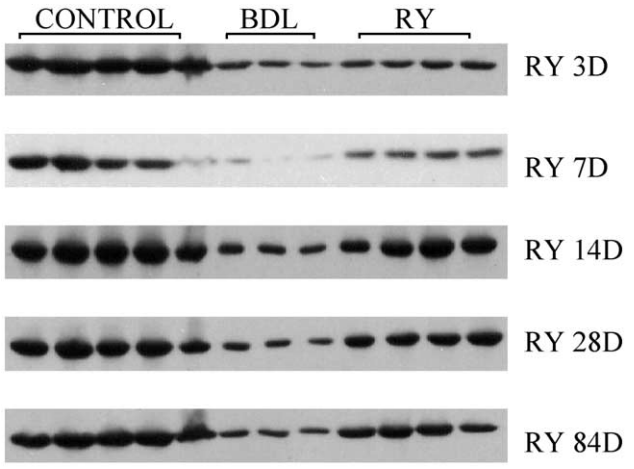

Fig. 1. Hepatic expression of HMG-CoA synthase protein. The Western blots were performed as described in Section 2 and quantified by densitometric analysis of the bands. The numeric results of these blots are given in Table 5 with the control values set at 100%. Note that all blots contain control (CON) and BDL samples for comparison.

In contrast to CPT I, activity and protein expression of CPT II were reduced in BDL rats with an only partial reversibility after restoring bile flow. In comparison, oxidative metabolism of palmitoylcarnitine was decreased in mitochondria from BDL rats, but this decrease was reversible already 7 days after restoring bile flow. The discrepancy between CPT II activity and mitochondrial metabolism of palmitoylcarnitine can be explained by the concept that CPT II is normally not rate-limiting for hepatic fatty acid metabolism [29,30].

An impaired function of the electron transport chain is a well-recognized reason for reduced fatty acid metabolism in patients with mitochondrial cytopathies [31,32]. As shown in the current and in previous studies [3,6], the function of complexes I and III of the electron transport chain of hepatic

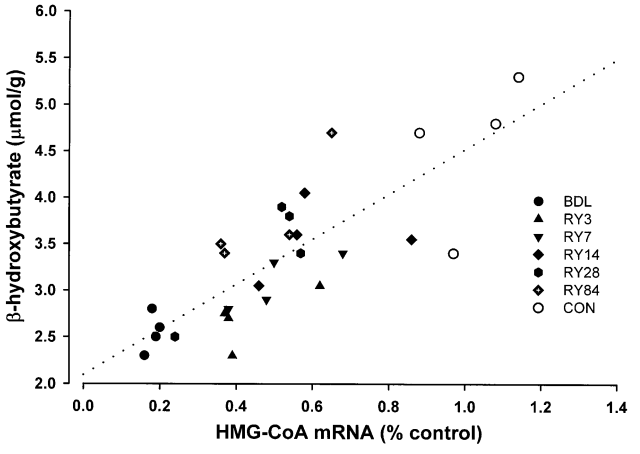

Fig. 2. Relationship between hepatic mRNA expression of HMG-CoA synthase and the hepatic content of β-hydroxybutyrate. The hepatic level of HMG-CoA synthase mRNA was determined by quantitative PCR and the content of β-hydroxybutyrate by a fluorimetric method as described in Section 2. The relation is described by the following equation: $y = 2.1 + 2.4x$ ($r^2 = 0.69$, $P < 0.05$).

mitochondria is decreased in rats with chronic cholestasis but recovers within 7 days after reversal of biliary obstruction. In contrast, the function of complex II (succinate metabolism) had normalized only after 4 weeks. Since complex II is not involved in fatty acid metabolism, a decreased function of the electron transport is not the reason for impaired fatty acid metabolism in RY rats.

An impaired function of enzymes of the β-oxidation cycle is also known to result in decreased hepatic fatty acid metabolism [33–35]. β-Oxidation was assessed using intact mitochondria by the formation of acid soluble products from [1-^{14}C]-palmitate. The formation of acid soluble products was reduced in mitochondria from BDL rats and normalized 7 days after reversal of biliary obstruction. While impaired mitochondrial β-oxidation contributes to reduced hepatic fatty acid metabolism in BDL rats (see also Ref. 3), it offers no satisfactory explanation for the findings in RY rats.

Since impaired function of the HMG-CoA cycle is the reason for reduced formation of ketone bodies in rats with short-term cholestasis [11], we focused on the investigation of key reactions of this cycle. The activity of the entire cycle was decreased in BDL rats and had recovered only 3 months after reversing biliary obstruction, showing a similar pattern as the hepatic content of ketone bodies. Since the assay used reflects the activity of three enzymes (acetoacetyl-CoA thiolase, HMG-CoA synthase and HMG-CoA lyase), each of these enzymes could have been affected. However, HMG-CoA synthase, an enzyme regulated short-term by succinylation and long-term by protein expression, is considered to be rate-limiting for the activity of the whole cycle [26]. Activity and expression of mRNA and protein of HMG-CoA synthase were decreased in BDL rats and slowly increased (mRNA and protein expression) or normalized (activity) after reversal of biliary obstruction. Since HMG-CoA determines the activity of the whole HMG-CoA cycle, these findings offer a satisfactory explanation for impaired ketogenesis and hepatic fatty acid metabolism in BDL rats and also after relief of biliary obstruction. However, our studies do not allow drawing conclusions about the mechanism leading to reduced expression of HMG-CoA synthase in long-term cholestasis. Further studies assessing transcription of the HMG-CoA synthase gene are needed to answer this question.

Similar to rats, cholestasis is also associated with impaired hepatic mitochondrial metabolism in humans [36,37], which may represent a mechanism for secondary biliary cirrhosis and may be a risk factor for an unfavorable outcome [38]. After restoring bile flow, hepatic mitochondrial metabolism rapidly normalizes [36] and also liver fibrosis and cirrhosis may be reversible [38].

In conclusion, multiple factors (impaired activation of long-chain fatty acids, and impaired activities of β-oxidation, electron transport chain and HMG-CoA cycle) lead to reduced hepatic metabolism of fatty acids in rats with long-term cholestasis. After reversing biliary obstruction, hepatic

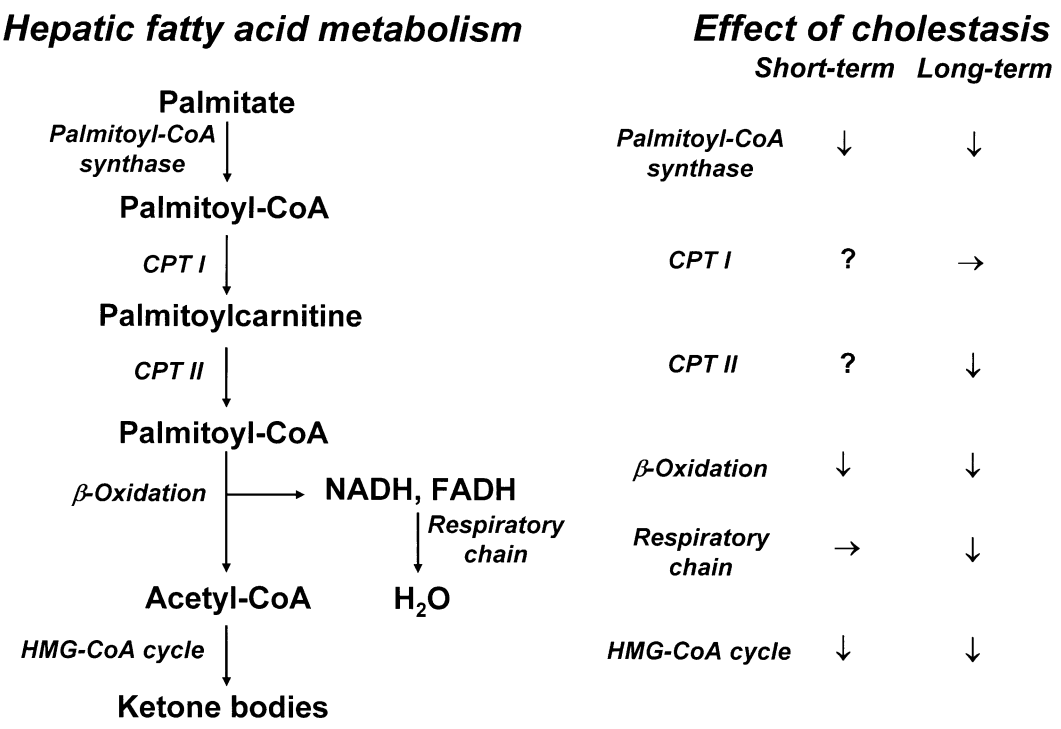

Fig. 3. Effect of short- and long-term cholestasis on hepatic fatty acid metabolism. Long-chain fatty acids are activated by palmitoyl-CoA synthase on the outer mitochondrial membrane, and then converted to the carnitine derivative by carnitine palmitoyltransferase I (CPT I) and transported across the inner mitochondrial membrane by carnitine translocase. In the mitochondrial matrix, they are reconverted to the CoA derivative by CPT II and undergo β-oxidation. NADH and FADH produced by β-oxidation are metabolized by the respiratory chain. Besides NADH and FADH, β-oxidation also produces acetyl-CoA, which can be used for the formation of ketone bodies or is degraded to CO_2 and H_2O by the Krebs cycle. The effect of short-term cholestasis on CPT I or II is not known. See text for more explanations.

fatty acid metabolism recovers only slowly, a finding explained by reduced activity of HMG-CoA synthase, the rate-limiting enzyme of the HMG-CoA cycle.

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