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Regulation of cholesterol and bile acid homoeostasis in bile-obstructed rats

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We examined how total blockage of biliary excretion, the major pathway through which cholesterol and bile acids are removed from the body, affects liver function, cholesterol and bile acid metabolism and homoeostasis. After 4 weeks of bile-duct ligation, rats showed impaired liver function, as documented by elevations in serum bilirubin and alkaline phosphatase activity. Moreover, bile-duct ligation decreased by about 30 % both the amount of microsomal cytochrome *P*-450 in the liver and the elimination of aminopyrine *in vivo*, a reliable index *in vivo* of microsomal mixed-function oxidase activity. Cholesterol and bile acid contents in livers of bile-duct-ligated rats were doubled compared with sham-operated controls. Despite the increase in the contents of cholesterol and bile acids in liver, activities of the respective rate-limiting enzymes, 3-hydroxy-3-methylglutaryl-CoA reductase and cholesterol 7α-hydroxylase, were doubled. Serum concentrations of bile acids and free cholesterol increased 25- and 4-fold respectively. The large increase in serum bile acids was associated with a 380-fold increase in the urinary excretion of bile acids. Although there is a general decrease in cytochrome *P*-450 content and drug metabolism involving cytochrome *P*-450-containing hydroxylases, the activity of cholesterol 7α-hydroxylase, also a cytochrome *P*-450-containing enzyme, is actually increased. These data show that complete obstruction of the bile duct results in the selective impairment of microsomal cytochrome *P*-450. Increased activity of 7α-hydroxylase, bile acid synthesis and urinary excretion provides an alternative excretory pathway that helps to maintain cholesterol homoeostasis when the biliary excretory pathway is eliminated.

INTRODUCTION

The liver is the primary organ responsible for the clearance of lipoproteins from plasma [1]. Cholesterol taken up as lipoprotein particles is metabolized by the liver to bile acids [2–4]. Biliary excretion of cholesterol/phospholipid/bile acid micelles is quantitatively the major pathway responsible for the elimination of cholesterol from the body [2]. In many different experimental systems, bile acid synthesis varies in parallel with increases in hepatic cholesterol content caused by increased synthesis [5], receptor-mediated uptake of lipoproteins [6,7] and cholesterol-rich diets [8,9]. These data are consistent with the proposal that compensatory changes in bile acid synthesis in response to hepatic cholesterol availability may be an important mechanism regulating cholesterol homoeostasis [2–9].

Mechanical obstruction of the bile duct leads to complete loss of the major route responsible for the excretion of both cholesterol and bile acids. In hamsters, as a result of bile-duct ligation urinary excretion of bile acids is dramatically increased [10]. Bile-duct ligation also results in the accumulation of bile acids in the liver [11]. The detergent properties of bile acids have been proposed to play an important role in causing hepatic dysfunction following bile-duct obstruction [12]. One of the several liver functions impaired by bile-duct obstruction is the cytochrome *P*-450 mixed-function oxidase system. Both total cytochrome *P*-450 content and function are severely decreased [12,13]. In short-term (3 days) studies, Danielsson [14] showed that bile-duct-ligated rats displayed a significant increase in the activity of 7α-hydroxylase, the cytochrome *P*-450 enzyme regulating bile acid synthesis [15]. The functional significance of this finding had not been elucidated. The major goal of this research was to examine what happens to cholesterol and bile acid metabolism and homoeostasis when the major excretory route is completely blocked by long-term bile-duct obstruction. In addition, we examined how long-term bile-duct obstruction, which clearly leads to hepatic dysfunction, altered morphology and decreased cytochrome *P*-450 content and enzymic activities, affects the rate-limiting steps in cholesterol synthesis (HMG-CoA reductase; EC 1.1.1.32) and bile acid synthesis (cholesterol 7α-hydroxylase), and what the physiological consequence might be. The results show that in long-term bile-duct-ligated rats the activities of both HMG-CoA reductase and 7α-hydroxylase are increased, resulting in increased cholesterol and bile acid content in liver, increased secretion into plasma and removal of bile acids from the body via increased excretion in urine. Since the major excretory route of cholesterol is lost in bile-duct ligation, and because cholesterol is not excreted via the kidneys in significant amounts, conversion of cholesterol and subsequently urinary excretion of bile acids may provide an alternative pathway to biliary excretion, helping to maintain cholesterol homoeostasis in bile-duct obstruction.

METHODS

Sprague–Dawley male rats were fed on ordinary laboratory chow and kept on a 12 h-light/12 h-dark cycle. Rats (320–370 g) were subjected to bile-duct ligation or sham operation under pentobarbitone (50 mg/kg body wt.) anaesthesia as previously described [16]. In summary, the bile duct was isolated and ligated by a double ligature. A 1 cm section of the bile duct was then excised and the abdominal incision closed. The sham-operated controls underwent laparotomy and manipulation of the small intestine before the incision was closed. The animals were killed after 4 weeks at mid-dark of the 12 h-light/dark cycle. Liver homogenates and microsomes were prepared in 40 mM-Tris/HCl/1 mM-EDTA/5 mM-dithiothreitol/50 mM-KCl/

Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LDL, low-density lipoprotein.

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50 mM-KF/300 mM-sucrose, pH 7.4, as previously described [9]. The microsomes were resuspended in the same buffer without sucrose.

Aminopyrine breath test was measured as expiration of $^{14}CO_2$ after intraperitoneal injection of di[^{14}C]methylaminoantipyrine [16]. The amount of $^{14}CO_2$ released has previously been shown to be an index of hepatic demethylation capacity [17]. Alkaline phosphatase activity, alanine aminotransferase, bilirubin and phospholipids were measured as previously described [18]. Protein was determined by a modification of the Lowry method [19]. Serum bile acids were quantified by a commercial available radioimmunoassay kit (Beckton–Dickinson). Hepatic bile acids were determined by capillary g.l.c.–m.s. after purification on reverse-phase liquid chromatography (Sep-Pak C_{18}) [20]. The bile acids eluted from the column were subjected to solvolysis, enzymic deconjugation and methylation and preparation of the trimethylsilyl ether derivative as described [20]. Urinary bile acids were determined by the 3-hydroxysteroid dehydrogenase method after solvolysis, hydrolysis and concentration on Sep-Pak columns [21]. Cholesterol and esterified cholesterol were separated on silica Sep-Pak columns. Cholesterol esters were hydrolysed in methanolic 0.5 M-NaOH. Cholesterol was then quantified by g.l.c. as described [9]. The total amount of microsomal cytochrome *P*-450 content was determined by a spectrophotometric method as described by Omura & Sato [22]. NADPH–cytochrome *c* reductase activity was determined at 37 °C by reduction of cytochrome *c* (30 μM) measured at 550 nm after addition of 0.13 mM-NADPH in 0.2 M-sodium phosphate buffer, pH 7.4, containing 32 μM-KCN [23]. Cholesterol 7α-hydroxylase activity was determined by a g.l.c.–m.s. isotope-dilution method with [7β-^{2}H]-7α-hydroxycholesterol as internal standard as described by Straka *et al.* [9]. In summary, 0.5 mg of microsomal protein was incubated with a NADPH-generating system for 20–30 min at 37 °C. The incubation was terminated with chloroform/methanol (2:1, v/v) and 1–2 μg of [7β-^{2}H]-7α-hydroxycholesterol was added. The samples were derivatized with trimethylchlorosilane and injected into a g.l.c.–m.s. apparatus. The mass ions 457 (^{2}H-labelled) and 456 (endogenous) of 7α-hydroxycholesterol were determined by selected ion monitoring. The amount of 7α-hydroxycholesterol formed was calculated from the ratio of mass ion 457/456. HMG-CoA reductase activity was determined by the conversion of [2-^{14}C]HMG-CoA into [^{14}C]mevalonic acid, with [^{3}H]mevalonic acid as internal standard as described [24]; 30 μg of microsomal protein was preincubated at 37 °C for 20 min in an imidazole/$MgCl_2$-containing buffer [24]. [2-^{14}C]HMG-CoA was then added together with a NADPH-generating system and incubated for 20 min at 37 °C. The reaction was terminated with 6 M-HCl, and HMG-CoA and mevalonic acid were separated by t.l.c. The band corresponding to mevalonic acid was scraped into counting vials.

The results are given as means ± S.D.; differences between bile-duct-ligated rats and sham-operated controls were compared by unpaired Student's *t* test. Values of $P < 0.05$ were considered to be significant (n.s., not significant).

RESULTS

Bile-duct ligation impairs hepatic metabolism of aminopyrine and decreases hepatic cytochrome *P*-450 content and function

After 4 weeks of complete obstruction of the bile-duct, there was no difference in body weights: control, 480 ± 19 g ($n = 4$), and bile-duct-ligated, 444 ± 27 g ($n = 5$) (n.s.). However, in the bile-duct-ligated group, both liver (control 17.3 ± 0.4 g; bile-duct-ligated 27.1 ± 2.4 g) and spleen (control 1.1 ± 0.2 g; bile-duct-ligated 2.4 ± 0.7 g) weights were significantly increased ($P < 0.05$). Bile-duct-ligated rats were clearly jaundiced, as evidenced by increased serum bilirubin (Table 1). Furthermore, both alkaline phosphatase and alanine transferase activities were elevated in the bile-duct-ligated rats (Table 1).

To quantify the effect of bile-duct ligation on cytochrome *P*-450, the metabolism of aminopyrine *in vivo* was determined by using a breath test. This test has been shown to be a sensitive measurement *in vivo* of aminopyrine *N*-demethylase, a hepatic microsomal cytochrome *P*-450-dependent enzyme [17]. Bile-duct ligation significantly decreased the rate of metabolism of aminopyrine by 28 % (Table 1).

We determined whether the decrease in the metabolism of aminopyrine is associated with decreases in heptic microsomal cytochrome *P*-450 content and activity of NADPH–cytochrome *c* reductase, a component of the microsomal mixed-function oxidase system. There was equal recovery and purity of microsomes isolated form the livers of both groups of rats (relative enrichment of NADPH–cytochrome *c* reductase was 7.0 ± 1.1 for control and 6.2 ± 1.4 for bile-duct-ligated; n.s.). Bile-duct ligation decreased the content of cytochrome *P*-450 in the liver (−27 %; Table 2) to the same extent as the decrease in metabolism of aminopyrine (Table 1). Furthermore, there was a similar decrease in the activity of NADPH–cytochrome *c* reductase (−37 %; Table 2). These data suggest that bile-duct

Table 1. Liver function tests in sham-operated and bile-duct-ligated rats

Data are given as means ± S.D. for five bile-duct-ligated rats and four sham-operated control rats: [a]$P < 0.001$; [b]not significant.

	Rats	
	Sham-operated	Bile-duct-ligated
Serum bilirubin (μM)	not detected	138 ± 32[a]
Serum alkaline phosphatase (units/l)	213 ± 51	450 ± 56[a]
Serum alanine transferase (units/l)	35 ± 3	61 ± 24[b]
Aminopyrene breath-test clearance (h^{-1})	1.16 ± 0.10	0.60 ± 0.11[a]
Serum bile acids (μM)	2.1 ± 0.3	55.8 ± 17.7[a]

Table 2. Hepatic microsomal enzyme activities and cytochrome *P*-450 content in sham-operated and bile-duct-ligated rats

Data are given as means ± S.D. for five bile-duct-ligated and four sham-operated control rats: [a]$P < 0.025$; [b]$P < 0.005$; [c]$P < 0.001$; [d]$P < 0.05$.

	Rats	
	Sham-operated	Bile-duct-ligated
NADPH–cytochrome *c* reductase (μmol/h per mg of protein)	5.6 ± 1.4	3.5 ± 0.7[a]
HMG-CoA reductase (nmol/h per mg of protein)	5.1 ± 0.7	13.7 ± 4.1[b]
7α-Hydroxylase (pmol/min per mg of protein)	18.3 ± 2.5	40.7 ± 7.8[c]
Cytochrome *P*-450 content (nmol/mg of microsomal protein)	0.45 ± 0.08	0.33 ± 0.08[d]

Table 3. Hepatic bile acid content in sham-operated and bile-duct-ligated rats

Data are given as means ± S.D. for six bile-duct-ligated and six sham-operated rats: [a]$P < 0.05$; [b]not significant; [c]$P < 0.005$.

	Content (μg/g of liver)	
	Sham-operated	Bile-duct-ligated
Chenodeoxycholic acid	1.0 ± 0.6	2.4 ± 0.8[a]
Cholic acid	27.7 ± 10.4	21.0 ± 8.4[b]
α-Muricholic acid	5.2 ± 2.6	4.2 ± 2.5[b]
β-Muricholic acid	5.1 ± 4.8	66.6 ± 32.8[c]
Total liver bile acids	40.1 ± 19.3	88.2 ± 31.9[a]

Table 4. Liver cholesterol content in sham-operated control and bile-duct-ligated rats

Data are given as means ± S.D. for five bile-duct-ligated and four sham-operated control rats: [a]$P < 0.01$; [b]$P < 0.001$; [c]$P < 0.005$.

	Rats	
	Sham-operated	Bile-duct-ligated
Liver free cholesterol (mg/g of liver)	1.4 ± 0.3	2.0 ± 0.2[a]
Liver cholesterol esters (mg/g of liver)	0.18 ± 0.03	0.09 ± 0.02[b]
Microsomal free cholesterol (μg/mg of protein)	4.33 ± 0.75	9.94 ± 2.13[c]

Table 5. Plasma cholesterol concentrations in sham-operated control and bile-duct-ligated rats

Data are given as means ± S.D. for five bile-duct-ligated and four sham-operated control rats: [a]$P < 0.005$; [b]not significant; [c]$P < 0.05$; [d]$P < 0.001$.

	Rats	
	Sham-operated	Bile-duct-ligated
Free cholesterol (mM)	0.25 ± 0.06	1.02 ± 0.32[a]
Esterified cholesterol (mM)	1.55 ± 0.25	1.60 ± 0.56[b]
Total cholesterol (mM)	1.80 ± 0.30	2.57 ± 0.85[c]
Esterified cholesterol (% of total)	86 ± 2	60 ± 6[d]

ligation causes a general impairment of the cytochrome *P*-450 mixed-function oxidase system, consistent with the results of others [12,13,16].

Bile-duct ligation increases the accumulation of bile acids in the liver

The major goal of this study was to determine what would happen to hepatic cholesterol metabolism when the major excretory route via the bile was blocked by bile-duct ligation. As expected, the concentration of bile acids in liver and serum was increased 2- ($P < 0.05$) and 25- ($P < 0.01$) fold respectively (Tables 3 and 1). The major bile acid in liver of sham-operated animals was cholic acid, accounting for about 70 % of the liver bile acids (Table 3). Chenodeoxycholic acid was a minor hepatic form (less than 3 % of the total hepatic bile acid pool). Bile-duct ligation caused a dramatic change in the composition of the hepatic bile acid pool. There was a 2- ($P < 0.05$) and 10- ($P < 0.005$) fold increase in chenodeoxycholic acid and β-muricholic acid respectively (Table 3). In bile-duct-ligated rats, β-muricholic acid became the predominant bile acid, accounting for about 70 % of total hepatic bile acid pool. Similar changes in the hepatic bile acid pool composition have been reported to occur in short-term (3 days) studies of bile-duct-ligated rats [11,25].

Cholesterol 7α-hydroxylase, the cytochrome *P*-450 enzyme regulating bile acid synthesis, is increased by bile-duct ligation

In contrast with the marked decrease in cytochrome *P*-450 in the livers of bile-duct-ligated rats, there was a greater than 2-fold increase in the activity of 7α-hydroxylase (Table 2). These data clearly show that the marked accumulation of bile acids in the livers of bile-duct-ligated rats (Table 3) is not associated with inhibition of 7α-hydroxylase. Similar changes in 7α-hydroxylase activity has been reported in short-term (3 days) bile-duct-ligation studies [14].

Bile-duct ligation increases hepatic HMG-CoA reductase activity, cholesterol content and plasma cholesterol concentrations

Livers from bile-duct-ligated rats accumulated 40 % more total cholesterol compared with sham-operated rats. The increase in total hepatic cholesterol was due to a 46 % increase in free cholesterol, whereas the cholesterol ester concentration was actually decreased by 50 % (Table 4). The accumulation of cholesterol was also evident in microsomes. Microsomes from the livers of bile-duct-ligated rats contained twice as much cholesterol as did those from sham-operated controls (Table 4).

Despite the substantial increase in hepatic cholesterol concentrations, the activity of HMG-CoA reductase in hepatic microsomes was increased 2-fold (Table 2). The elevated HMG-CoA reductase activity displayed by bile-duct-ligated rats was also associated with increased plasma concentrations of both free and total cholesterol (Table 5). However, esterified cholesterol in plasma was unchanged (Table 5).

Urinary excretion of bile acids is increased by bile-duct ligation

In regard to regulation of bile acid synthesis, the combined data show that both substrate (cholesterol) and enzyme (7α-hydroxylase) are increased in the bile-duct-ligated rats. Since the biliary excretion was blocked by ligation, we examined whether bile acids produced in bile-duct-ligated rats were excreted through an alternate route (i.e. via the urine). Urinary excretion of bile acids was increased by more than 380-fold in bile-duct-ligated rats, from 0.05 ± 0.02 to 19.4 ± 11.4 μmol/day. These data suggest that the bile acids produced by the bile-duct-ligated rats are secreted through the sinusoidal membrane (rather than via the canaliculus) and are then subsequently excreted via the urine.

DISCUSSION

An important factor in determining whole-body cholesterol homoeostasis is the elimination of free cholesterol and bile acids via biliary excretion. Extrahepatic cholestasis occurs in several different pathological conditions and often is associated with severe impairment of hepatic function [16]. The mechanism responsible for the loss of hepatic function is complex and may involve injury due to the retention of toxic compounds including bilirubin and bile acids [12]. Long-term mechanical biliary obstruction results in altered hepatocyte morphology and impaired function [16,26,27]. Some of the most prominent morphological findings are decreased amounts of smooth and rough endoplasmic reticulum as well as the Golgi complex,

together with increased numbers of peroxisomes and lysosomes [26,28].

The data in the present study show that some functions are dramatically impaired in long-term bile-duct ligation (aminopyrine breath test, microsomal cytochrome *P*-450 content, NADPH–cytochrome *c* reductase). In marked contrast, other enzymes such as HMG-CoA reductase and 7α-hydroxylase show increased activities in bile-duct-ligated rats. The increased activities of these enzymes are paradoxical, considering that long-term bile-duct ligation results in a 2-fold elevation of intrahepatic concentration of both free cholesterol and bile acids. These results are inconsistent with the hypothesis that the activity of these two enzymes is regulated strictly by the hepatic content of cholesterol (HMG-CoA reductase) and bile acids (7α-hydroxylase). Previous studies using short-term (2 days) bile-duct-ligated rats show variable results. In two studies there was no elevation in heptic cholesterol, and negative-feedback inhibition of cholesterol synthesis occurred [29,30]. However, in another study 2 days after ligating the common bile duct, there was a 22% increase in hepatic free cholesterol [31] and no negative-feedback regulation [32]. A possible explanation for these different results is that it may take longer than 48 h of bile-duct obstruction before the changes necessary for cholesterol accumulation and loss of negative-feedback regulation consistently occur. Clearly, our studies show that after 4 weeks of bile-duct obstruction there is marked 43% increase in hepatic free cholesterol, accompanied by a 2-fold increase in the activity of HMG-CoA reductase, consistent with a loss of negative-feedback regulation.

Recent studies on the molecular mechanism of regulation of HMG-CoA reductase show that cholesterol itself is not regulatory, but rather an oxysterol metabolite is involved [33,34]. One of the most compelling experiments supporting this mechanism is one in which oxysterol production was inhibited by ketoconazole, an inhibitor of cytochrome *P*-450-dependent reactions [34]. When intestinal epithelial cells are incubated in the presence of ketoconazole, LDL cholesterol does not down-regulate HMG-CoA reductase; in marked contrast, adding 25-hydroxycholesterol, which mimics oxysterol metabolites, to ketoconazole-treated cells caused rapid repression of HMG-CoA reductase.

Further analysis shows that miconazole, a more selective inhibitor of cytochrome *P*-450, does not affect the ability of LDL to down-regulate expression of the LDL receptor [35]. These data suggest that a cytochrome *P*-450 inhibitable by ketoconazole, but insensitive to miconazole, is required. These data suggest that oxysterols, produced by a cytochrome *P*-450-dependent enzyme, regulate HMG-CoA reductase, whereas cholesterol itself does not. A possible explanation for the apparent dissociation of HMG-CoA reductase activity and hepatic cholesterol concentrations may be the inability to form regulatory oxysterols because of decreased cytochrome *P*-450 content and activity (Table 2). A similar discordance between hepatic cholesterol concentrations and HMG-CoA reductase has been observed in rats treated with high concentrations of ethinyloestradiol [36], which also causes intrahepatic cholestasis, owing to change in surface membrane lipid structure and function [37].

Bile acids are thought to be the major regulators of 7α-hydroxylase. However, it is clear that in the livers of bile-duct-ligated rats there are increases in both bile acids and activity of 7α-hydroxylase. Two possible explanations for these data are: (1) the bile acids (chenodeoxycholic and β-muricholic acids) that accumulate in the livers of bile-duct-ligated rats are not the ones that inhibit 7α-hydroxylase; (2) an effector other than the intrahepatic concentration of bile acids regulates the activity of 7α-hydroxylase. In regard to the first possibility, Heuman *et al.* [38] have shown that the hydrophilic bile acid ursodeoxycholic acid does not inhibit 7α-hydroxylase, whereas more hydrophobic bile acids do. These data have been confirmed by others [39]. Our data show that the relatively hydrophilic bile acid β-muricholic acid accumulates in the greatest quantities (10-fold increase) in the livers of bile-duct-ligated rats. The change in bile acid composition to more hydrophilic bile acids is consistent with a lack of negative-feedback regulation. Furthermore, since the bile acids accumulating in bile-duct-ligated rats are to a large degree conjugated with glucuronic acid and sulphate, thus making them more hydrophilic, they may not be efficient inhibitors of 7α-hydroxylase.

The results obtained in this experimental model are consistent with those of other studies showing that bile acid synthesis and cholesterol synthesis vary in parallel in response to several different treatments: bile diversion, cholestyramine feeding, fasting/feeding, diurnal variation and hormone and drug treatments [15]. One exception is cholesterol feeding, which inhibits HMG-CoA reductase and stimulates cholesterol 7α-hydroxylase activity [15]. Furthermore, treatment of liver cells [40] and animals [5] with drugs that block cholesterol synthesis results in a concomitant decrease in bile acid synthesis. In contrast, supplying cholesterol via mevalonolactone or lipoproteins increases bile acid formation in cultured hepatocytes [40,41]. We have proposed that cholesterol regulates bile acid synthesis both by supplying substrate and by increasing the activity (i.e. enzyme units) of 7α-hydroxylase [9]. The finding in the present study of a concomitant increase in microsomal cholesterol and the activity of 7α-hydroxylase is consistent with this proposal.

Jelinek *et al.* [42] have recently shown that cholesterol feeding increases the mRNA levels for cholesterol 7α-hydroxylase. One interpretation of the parallel change in HMG-CoA reductase and 7α-hydroxylase activities is that under some circumstances a common effector regulates the activity of both these enzymes. The increase in both 7α-hydroxylase and HMG-CoA reductase by bile-duct ligation can be explained by induction of both enzymes by a common effector.

The daily synthesis of bile acid in a normal 350 g rat has been estimated to be 5–12 mg [43,44]. Daily urinary excretion of bile acids in bile-duct-ligated rats was 7–8 mg. These data show that urinary excretion of bile acids can account for elimination of a significant amount of bile acids that would normally be secreted in bile. Sulphation and/or glucuronidation of bile acids will render them sufficiently soluble for their excretion in urine. The large capacity to sulphate and/or glucuronidate bile acids and excrete them into urine suggests that, in the pathological situation of bile-duct obstruction, cholesterol homoeostasis is partially maintained by converting cholesterol into hydrophilic bile acids, which are subsequently conjugated and excreted in urine. Conversion of cholesterol into bile acids, and subsequently excretion into urine, might therefore be a mechanism that helps to maintain cholesterol homoeostasis when the principal route of excretion is blocked.

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