Liver International ISSN 1478-3223

CLINICAL STUDIES

Expression of bile acid synthesis and detoxification enzymes and the alternative bile acid efflux pump MRP4 in patients with primary biliary cirrhosis

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Keywords
bile acids – cholestasis – hepatocyte-enriched transcription factor – nuclear receptors – transport

Abbreviations
CAR (NR1I3), constitutive androstane receptor; Cyp, cytochrome P450 enzyme; CYP7A1, cholesterol 7α-hydroxylase; CYP27A1, sterol 27-hydroxylase; CYP8B1, sterol 12α hydroxylase; FGF, fibroblast growth factor; FXR (NR1H4), farnesoid X receptor/bile acid receptor; HNF1α (*TCF1*), hepatocyte nuclear factor 1α; HNF4α (NR2A1), hepatocyte nuclear factor 4α; MRP3/4 (*ABC3/4*), multidrug resistance-associated proteins; OST, organic solute transporter; PBC, primary biliary cirrhosis; PXR (NR1I2), pregnane X receptor; RXRα (NR2B1), retinoid X receptor α; SHP (NR0B2), short heterodimer partner; SULT2A1, dehydroepiandrosterone sulphotransferase; UDCA, ursodeoxycholic acid; UGT, UDP-glucuronosyl transferase.

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Received 8 January 2007
accepted 17 March 2007

DOI:10.1111/j.1478-3231.2007.01506.x

Abstract

Background: Bile acid synthesis, transport and metabolism are markedly altered in experimental cholestasis. Whether such coordinated regulation exists in human cholestatic diseases is unclear. We therefore investigated expression of genes for bile acid synthesis, detoxification and alternative basolateral export and regulatory nuclear factors in primary biliary cirrhosis (PBC). *Material/Methods:* Hepatic CYP7A1, CYP27A1, CYP8B1 (bile acid synthesis), CYP3A4 (hydroxylation), SULT2A1 (sulphation), UGT2B4/2B7 (glucuronidation), MRP4 (basolateral export), farnesoid X receptor (FXR), retinoid X receptor (RXR), short heterodimer partner (SHP), hepatocyte nuclear factor 1α (HNF1α) and HNF4α expression was determined in 11 patients with late-stage PBC and this was compared with non-cholestatic controls. *Results:* CYP7A1 mRNA was repressed in PBC to 10–20% of controls, while CYP27 and CYP8B1 mRNA remained unchanged. SULT2A1, UGT2B4/2B7 and CYP3A4 mRNA levels were unaltered or only mildly reduced in PBC. MRP4 protein levels were induced three-fold in PBC, whereas mRNA levels remained unchanged. Expression levels of FXR, RXR, SHP, PXR, CAR, HNF1α and HNF4α were moderately reduced in PBC without reaching statistical significance. *Summary/Conclusions:* Repression of bile acid synthesis and induction of basolateral bile acid export may represent adaptive mechanisms to limit bile acid burden in chronic cholestasis. As these changes do not sufficiently counteract cholestatic liver damage, future therapeutic strategies should aim at stimulation of bile acid detoxification pathways.

Bile acid synthesis, transport and metabolism are markedly altered in cholestasis (1, 2). While some of these alterations may result in or may maintain cholestasis, most changes are believed to be secondary phenomena in response to accumulating bile acids. Many of these alterations are considered compensatory 'anti-cholestatic' defence mechanisms aimed at limiting bile acid-induced liver injury. Mechanisms

implicated in the hepatic defence against bile acid overload are repression of hepatic basolateral bile acid uptake, *de novo* bile acid synthesis and induction of alternative basolateral bile acid excretion (1–4). In addition, increased bile acid metabolism (i.e., phase I bile acid hydroxylation and phase II conjugation with sulphate and glucuronate) renders bile acids more hydrophilic, less toxic and more amenable for urinary excretion (5–8). Most of our knowledge on these adaptive mechanisms has been derived from experimental animal models. We and others have previously demonstrated alterations in hepatobiliary transporter expression in various cholestatic liver diseases in humans including repression of bile acid uptake and induction of alternative basolateral bile acid export systems (9–14). However, little is known about the regulation of bile acid synthesis and detoxification in human cholestatic diseases. The appearance of hydroxylated, sulphated and glucuronidated bile acids in the urine of patients with cholestatic liver diseases indicates that these adaptive mechanisms may also play an important role in humans (5, 6, 8, 15–21).

Nuclear receptors (NRs) and hepatocyte-enriched transcription factors play a key role in the transcriptional regulation of hepatobiliary transport systems and enzymes involved in bile acid metabolism and detoxification. Bile acids and other compounds (e.g., hormones, drugs) bind to and activate specific NRs facilitating feedback regulation of their metabolism and transport (4, 22–25). The importance of NRs in orchestrating the adaptive response to toxic bile acids has been demonstrated in various NR knockout models (2). As such, mice lacking the classical bile acid receptor FXR or the pregnane X receptor PXR are more susceptible to bile acid-induced liver injury than their wild-type littermates (26–32). However, the potential alterations of NRs have not been studied in human cholestatic liver diseases.

Whether bile acid synthesis, detoxification and alternative export are coordinately regulated in humans is still unclear. We therefore assessed gene expression levels of enzymes involved in bile acid synthesis, metabolism and transport and their regulatory NRs in patients with late-stage primary biliary cirrhosis (PBC), serving as a prototypic chronic cholestatic liver disease. We herein demonstrate that only some defence mechanisms known from experimental studies are activated at a transcriptional level in long-standing cholestasis in humans, suggesting that additional pharmacological stimulation may be required to reduce liver damage efficiently.

Table 1. Patient characteristics

	Total number (males/ females)	Age (years)	ALT (IU/l)	ALP (IU/l)	Bilirubin (mg/dl)
Controls	7 (2/5)	58 ± 7	14 ± 4	95 ± 17	1 ± 0.1
PBC III	6 (1/5)	51 ± 3	88 ± 43*	897 ± 307*	6 ± 2*
PBC IV	5 (1/4)	56 ± 3	69 ± 6*	263 ± 6*	8 ± 2*

Values are means ± SEM.
*$P < 0.001$ vs. Controls
PBC, primary biliary cirrhosis.

Material and methods

Tissue specimens and patients characteristics

Eighteen liver specimens comprising samples from patients with PBC ($n = 11$) and controls without liver disease ($n = 7$) were analysed. All PBC specimens were obtained during liver transplantation. The tissue samples have been used in a previous study (10). The diagnosis of PBC was based on standard criteria (33, 34). Six patients had PBC stage III (PBC III) and five stage IV (PBC IV) according to Ludwig et al. (35). Most PBC patients (four/six PBC III; two/five PBC IV) received standard ursodeoxycholic acid (UDCA) treatment (33). Control liver samples were acquired by liver biopsy for exclusion of liver disease or staging of haematologic disorders ($n = 3$); in addition, normal liver tissue was obtained from patients undergoing resection of liver metastases ($n = 4$). Biochemical characteristics are listed in Table 1. Liver tissue was immediately snap frozen and stored in liquid nitrogen until analysis. Patients had given their informed consent for this study, and the experimental protocol was approved by the local Ethics Committees in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted according to Krieg et al. (36). Steady-state mRNA levels of transporters and 28S rRNA were assessed by TaqMan® RT-PCR or using internal RNA standards as described (9, 37, 38). The primers and probes used in this study are given in Table 2.

Preparation of liver membranes and Western blot analysis

Liver membranes were prepared as described (10, 39) and protein concentrations were determined using a Bradford kit (BioRad, Richmond, CA, USA). Similar amounts of protein (20 μg) were loaded onto a 7.5%

Table 2. Human primer and probe sets

Gene	Forward primer	Reverse primer	Probe
CAR	gctggaagctgtgaagtcag	ccgacagtatcatgtctttcc	*
CYP27A1	agaggagattccacgtctaggac	ccctaagtaggacatccacattg	tgcgcttcttctttcagctgttcgttca
CYP3A4	gatgaagaatggaagagattacgat	cctcagatttctcaccaacaca	atggtccctatcattgcccagtatgga
CYP7A1	tttgatttgggggattgctata	gaccatgtttcctttgatttgctc	tggttcacccgtttgccttctcct
CYP8B1	tgctacaggcaggagagttattca	gggagtagacaaaccttgggaaa	catggagttccgcaagtttgaccttctt
FXR	ctcattgaacattcccatttacctac	ggacctgccacttgttctgtta	*
HNF1α	ggccttgttctgtcaccaat	cctggggtcacctctttctt	†
HNF4α	ggtgtccatacgcatccttga	tggctttgaggtaggcatactca	ccttccaggagctgcagatcgatgac
MRP4	caagatgctgcccgtgta	attgagccaccagaagaaca	ccaggaggtgaagcccaaccc
SHP	gcttcaatgctgtctggagtc	cttggaggcctggcacatc	*
SULT2A1	taatattgacttaccccaaatcagga	ctgagtgctgtatacccaatct	ccaagtggatccaatctgtgcccatctg
PXR	tccccaaatctgccgtgtat	agcccttgcatccttcacat	acaaggccactggctatcacttcaatgtca
RXR	ccggccgggcatgagttagtcg	tgtcgcggcaggtgtaggtcaggt	†
UGT2B4	ttcaatttcctcacccactctta	aaactcttccatttccttcggta	actccactgcaaacctgccaaaccc
UGT2B7	catccactcttaccaaatgttga	gtcatgttactgaccattgacc	ctgcaaacctgccaaacccctgcct

Fluorogenic probes for TaqMan® real-time polymerase chain reaction (RT-PCR) are 5′ FAM- and 3′ TAMRA-labelled.
*RT-PCR using the Sybr Green assay was performed.
†Competitive RT-PCR was performed as previously described (9).

sodium dodecyl sulphate-polyacrylamide gel and subjected to electrophoresis (40, 41). Equal protein loading was confirmed by Coomassie blue staining of gels and Ponceau S staining of membranes. After electro transfer to nitrocellulose membranes (BioRad), blots were blocked with Tris-buffered saline containing 0.1% Tween and 5% dry milk for 1 h at room temperature and incubated overnight at 4 °C with polyclonal antibodies against MRP4 dilution, 1:1.500; kindly provided by Dr. Dietrich Keppler (German Cancer Research Center, Heidelberg, Germany). Blots were reprobed with an antibody to β-actin (dilution, 1:5000; Sigma, Steinheim, Germany) to confirm the specificity of changes in transporter protein levels. Detection of immuno-complexes and semiquantification of band intensities was performed as described (10, 39).

Statistical analysis

Data are expressed as mean values ± standard error of mean (SEM). Differences among patient groups were analysed by multivariate analysis of variance with Bonferroni post-testing using the SIGMASTAT® statistic programme (Jandel Scientific, San Rafael, CA, USA). A *P*-value of < 0.05 was considered to be significant.

Results

Expression of bile acid-synthesizing enzymes in PBC

Bile acids inhibit their own synthesis in a negative feedback fashion (42). Whether repression of bile acid synthesis also occurs at a molecular level in human cholestasis is controversial (43). In PBC III and IV, mRNA levels of CYP7A1 (mediating the rate-limiting step in the classical bile acid synthesis pathway) were markedly reduced to 10 ± 9% and 17 ± 13% of controls respectively (both $P < 0.001$) (Fig. 1A). Expression of CYP27A1 (the key enzyme in the alternative bile acid synthesis pathway) was unchanged in PBC III and moderately reduced in PBC IV to 64 ± 3% of controls (NS) (Fig. 1B). CYP8B1 expression (determining the ratio of cholic acid to chenodeoxycholic acid) was not altered in PBC III and IV (Fig. 1C).

Expression of phase I and II bile acid detoxification systems in PBC

Increased bile acid phase I and II detoxification may represent another strategy of hepatocytes to overcome bile acid toxicity as shown under the experimental conditions in rodents (44, 45). In PBC III, mRNA expression of CYP3A4 (mediating phase I bile acid hydroxylation) was unchanged and a trend towards lower levels was observed in PBC IV (59 ± 14% of controls, NS) (Fig. 2A). SULT2A1 (mediating phase II conjugation with sulphate) and UGT2B4 mRNA levels (catalysing conjugation with glucuronate) were unaltered in PBC III and IV (Fig. 2B and C). UGT2B7 mRNA levels (bile acid conjugation with glucuronate) were moderately reduced to 69 ± 5% and 71 ± 11% of controls in PBC III and IV respectively (both NS) (Fig. 2D). Neither serum bilirubin levels nor the presence or absence of UDCA therapy correlated with mRNA

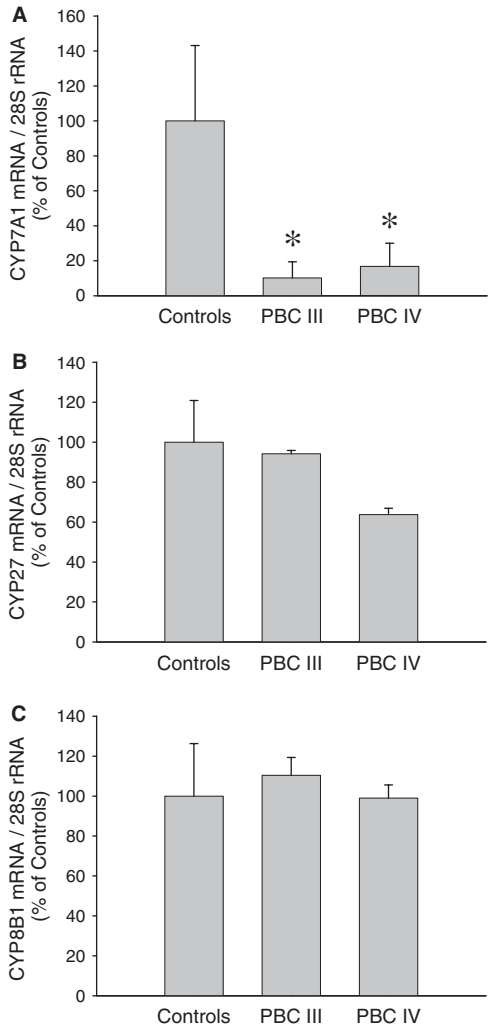

Fig. 1. Expression of bile acid synthetic enzymes in primary biliary cirrhosis (PBC). Total RNA was isolated from control and PBC livers and analysed by TaqMan® reverse transcription polymerase chain reaction as described in 'Material and methods'. (A) Compared with controls, CYP7A1 steady-state mRNA levels are profoundly reduced in PBC III and IV (*$P < 0.01$ vs. controls). (B) Expression of CYP27A1 is unchanged in PBC III and moderately reduced in PBC IV, although this does not reach statistical significance. (C) CYP8B1 mRNA is unchanged in PBC III and IV. Data (means ± SEM) are expressed as percentage of controls. Controls ($n = 7$), PBC stage III ($n = 6$) and PBC stage IV ($n = 5$).

expression levels of phase I or II detoxification enzymes.

Expression of the basolateral alternative export pump MRP4 in PBC

In addition to increased bile acid detoxification, enhanced basolateral excretion of their metabolites reduces intrahepatic bile acid load and is a prerequisite for renal bile acid elimination (46). Recent data suggest that MRP4 rather than MRP3 represents the major hepatic basolateral bile acid export pump (47, 48). In the present study, MRP4 mRNA levels were unchanged in PBC III and IV (Fig. 3A). MRP4 protein levels, however, were markedly induced in PBC III and IV to 345 ± 52% and to 317 ± 26% of controls respectively ($P < 0.001$), suggesting post-transcriptional regulation of MRP4 in cholestasis (Fig. 3B). MRP4 mRNA and protein expression did not differ in patients with or without UDCA therapy (Fig. 3A and B).

Expression of NRs and liver-enriched transcription factors in PBC

In contrast to experimental models of cholestasis and bile acid overload, the expression of detoxification enzymes was hardly altered in PBC. We therefore investigated whether reduced NR expression could explain the lack of transcriptional upregulation of detoxification enzymes. Expression of FXR was unchanged in PBC III and moderately reduced in PBC IV without reaching statistical significance (Table 3). Expression of the nuclear repressor short heterodimer partner (SHP), a downstream target gene of bile acid-activated FXR, was unchanged in PBC III and IV (Table 3) despite elevated intrahepatic bile acid levels (10). mRNA levels of the NRs PXR, CAR, RXR and hepatocyte nuclear factor 1α (HNF4α) were moderately reduced to 40–60% in PBC III and IV, although these changes did not reach statistical significance (Table 3). Expression of the liver-enriched transcription factor HNF1α remained unchanged in PBC III and IV (Table 3). Relatively low levels of these NRs may explain the lack of phase I and II enzyme induction.

Discussion

Most of the changes in hepatobiliary transport and bile acid metabolism encountered in cholestasis are secondary phenomena aimed at reducing liver injury that may at least in part be explained by NR-mediated transcriptional effects. However, most of our knowledge is based on experimental animal models and information in human cholestatic liver disease is still very scarce. As a better understanding of such mechanisms may have major clinical and therapeutic implications, we aimed to explore the hepatocellular defence mechanisms against bile acid accumulation and their regulatory NRs in PBC as prototypic cholestatic liver disease.

The key enzyme in the classical bile acid synthesis pathway, CYP7A1, is repressed by bile acids, thus regulating their own synthesis in a negative feedback

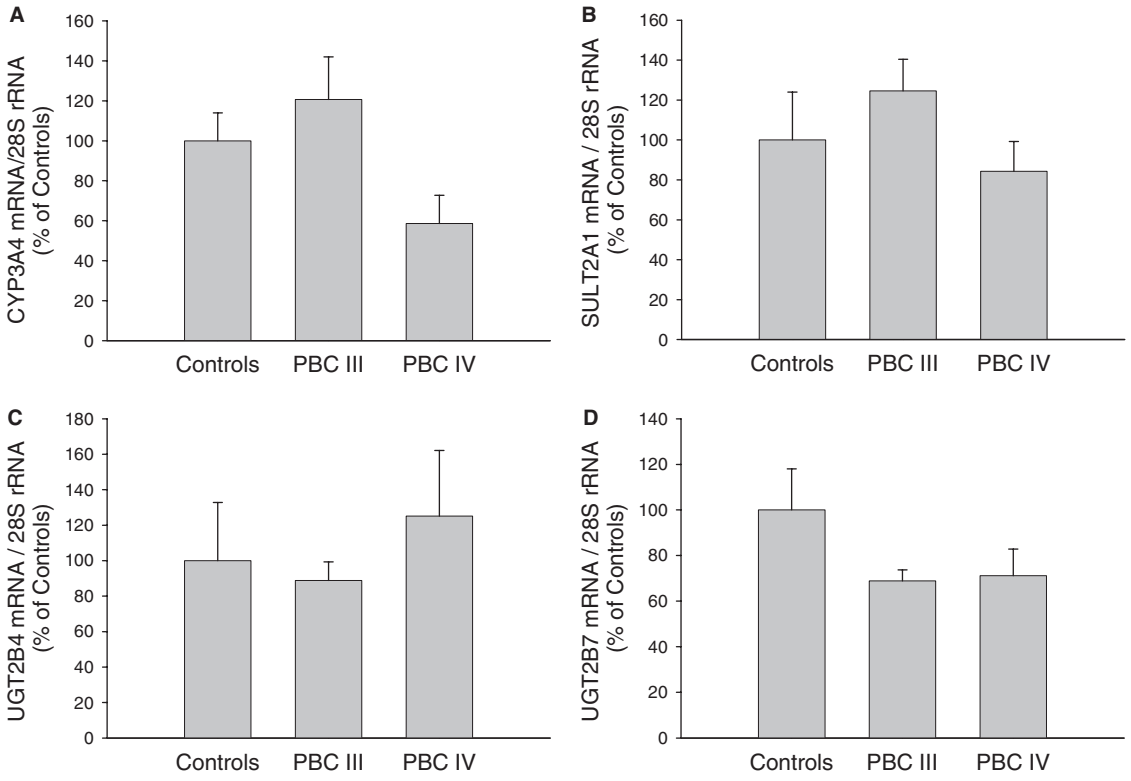

Fig. 2. Expression of phase I and phase II bile acid detoxification enzymes in primary biliary cirrhosis (PBC). Total RNA was isolated from control and PBC livers and analysed by TaqMan® reverse transcription polymerase chain as described in 'Material and methods'. (A) CYP3A4 mRNA (phase I hydroxylation) is unchanged in PBC III and slightly reduced in PBC IV (NS) while (B) expression of SULT2A1 (phase II conjugation with sulphate) and (C) UGT2B4 (phase II conjugation with glucuronidate) is unaltered in PBC. (D) UGT2B7 (also mediating conjugation with glucuronidate) is moderately reduced in PBC III and IV (NS). Data (means ± SEM) are expressed as percentage of controls. Controls ($n = 7$), PBC stage III ($n = 6$), PBC stage IV ($n = 5$).

mechanism (42). In the present study, mRNA levels of CYP7A1 were markedly repressed in patients with PBC. Repression of CYP7A1 may not only act as a hepatic defence mechanism but may also contribute to hypercholesterolemia in these patients (49, 50), as bile acid synthesis is a major pathway for degrading and eliminating cholesterol (51). CYP7A1 regulation by bile acids involves many redundant pathways (25, 42). Induction of SHP by activated FXR is not the only mediator of Cyp7a1 repression as indicated in mice lacking SHP (52, 53). CYP7A1 repression in the absence of SHP induction in the present study strongly argues for the presence of alternative pathways. Potential pathways contributing to CYP7A1 repression are reduced *CYP7A1* promoter activation by HNF4α (which was moderately reduced in PBC in the present study) (25, 42, 54, 55) and/or increased levels of pro-inflammatory cytokines (56, 57). Repression of CYP7A1 in PBC contrasts unchanged or even increased CYP7A1/Cyp7a1 expression and function observed in patients with obstructive cholestasis (43) and in common bile duct-ligated rodents (37, 58, 59). A potential explanation for this discrepancy has recently been provided by Inagaki et al. (60), describing a bile acid signalling pathway between the gut and liver. An intestinal factor (fibroblast growth factor, Fgf15) is induced in the small intestine by bile acids in an FXR-dependent fashion and in turn signals to the liver to repress murine *Cyp7a1* expression. The absence of bile acids in the intestine because of complete bile duct obstruction causes reduced Fgf15 signalling to the liver consequently leading to *Cyp7a1* overexpression (60). In PBC and most other chronic cholestatic diseases (where complete bile duct obstruction is absent and bile acids still reach the gut), however, this signalling pathway should still be intact, which would explain differences in CYP7A1 expression in obstructive cholestasis and PBC. However, the role of the human orthologue FGF19 in *CYP7A1* repression in cholestasis remains to be determined (61, 62).

Expressions of CYP27A1 (catalysing the first step in the alternative pathway of bile acid synthesis) and

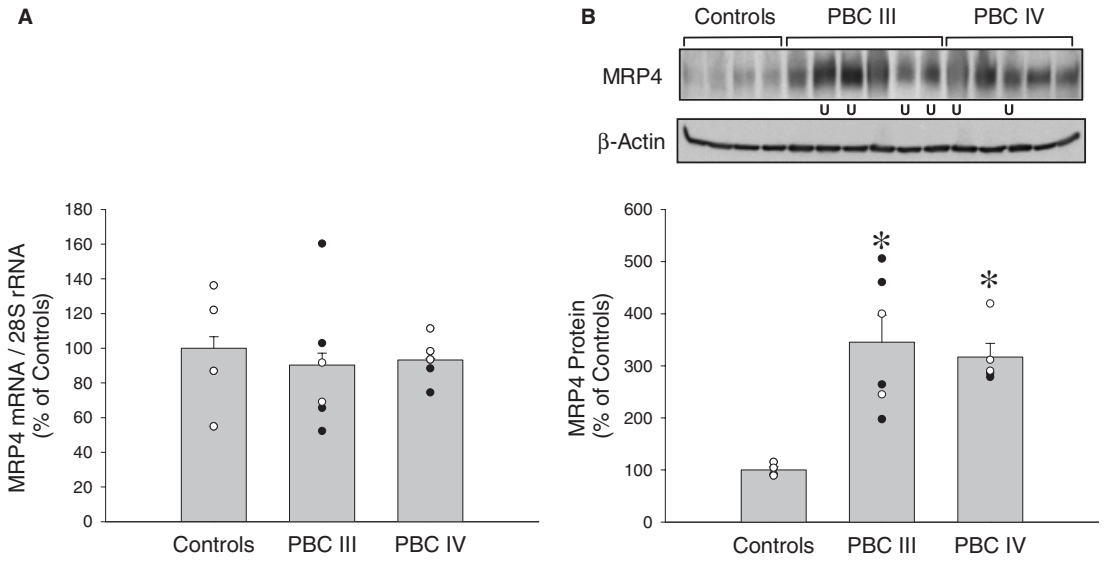

Fig. 3. Expression of the alternative basolateral bile acid efflux pump MRP4 in primary biliary cirrhosis (PBC). Total RNA and liver membranes were isolated from control and PBC livers and analysed by TaqMan® reverse transcription polymerase chain (A) and Western blotting (B) as described in 'Material and methods'. (A) MRP4 mRNA levels are unchanged in PBC, while (B) MRP4 protein levels are markedly increased in PBC III and IV. Patients receiving ursodeoxycholic acid (UDCA) are marked with 'U' beneath the Western blot. MRP4 densitometry data are given in the graph below a representative blot. The specificity of the observed MRP4 induction is confirmed by unchanged β-actin protein expression (B). Data (means ± SEM) are expressed as percentage of controls. $^*P < 0.01$ vs. controls. Each data point represents a single patient (•, on UDCA therapy; ○, without UDCA therapy). Controls ($n = 7$), PBC stage III ($n = 6$), PBC stage IV ($n = 5$).

Table 3. mRNA expression of nuclear receptors and hepatocyte nuclear factor 1α in PBC

	Controls	PBC III	PBC IV
FXR	100 ± 10	88 ± 21	58 ± 16
SHP	100 ± 17	124 ± 52	79 ± 26
PXR	100 ± 31	54 ± 8	47 ± 6
CAR	100 ± 29	59 ± 7	53 ± 5
RXR	100 ± 24	52 ± 10	42 ± 6
HNF4α	100 ± 33	68 ± 12	64 ± 6
HNF1α	100 ± 10	86 ± 22	85 ± 36

Changes were statistically not significant (analysed by multivariate analysis of variance). Values are means ± SEM

CAR, constitutive androstane receptor; FXR, farnesoid X receptor; HNF, hepatocyte nuclear factor; PBC, primary biliary cirrhosis; PXR, pregnane X receptor; RXR, retinoid X receptor; SHP, short heterodimer partner.

CYP8B1 (regulating the ratio of cholic acid to chenodeoxycholic acid) were unchanged in PBC. While differences in the regulation of CYP7A1 and CYP27A1 have been reported in the human liver (63), the lack of coordinated regulation of CYP7A1 and CYP8B1 was unexpected, as both genes harbour similar bile acid response elements in their gene promoters (64–67). This might indicate that other, yet unknown pathways are involved in CYP7A1 and CYP8B1 regulation in long-standing cholestasis. Taken together, our data demonstrate that the key enzyme in bile acid synthesis, CYP7A1, is markedly repressed in PBC, which can be regarded as defence mechanisms to reduce bile acid overload and may account for increased serum cholesterol levels in these patients.

Increased phase I (hydroxylation) and phase II bile acid detoxification may also contribute to the hepatocytes' adaptive response to bile acids. Hydroxylation and conjugation with glucuronate or sulphate reduces the hydrophobicity and toxicity of bile acids (8, 68, 69). Moreover, these water-soluble substances can be eliminated via the kidney into urine. While these bile acid metabolites are absent under normal conditions, they appear in the urine of patients with cholestatic diseases, indicating that such detoxification pathways play an important role in humans (5, 6, 8, 16–21). The enzymes mediating hydroxylation (CYP3A4, the human orthologue of rodent Cyp3a11) (28, 29, 44, 45, 70–72), sulphation (SULT2A1/Sult2a1) (73, 74) and glucuronidation (UGT2B4/2B7) (75, 76) are regulated by bile acids via the action of NRs in animal models and *in vitro*. As these enzymes are induced at a transcriptional level by bile acids in animal studies and *in vitro*, the unchanged or even slightly reduced mRNA levels of CYP3A4, SULT2A1, UGT2B4 and UGT2B7 in PBC in the present study were unexpected. In particular, conjugation with sulphate

is an important mode of bile acid detoxification and elimination, as renal clearance of these conjugates is much higher than that of non-sulphated bile acids (77). In patients with acute cholestatic hepatitis or obstructive jaundice, urinary levels of sulphated bile acids were reported to be as high as 60–80% of the total bile acids (5). These data suggest that sulphation or at least renal excretion of sulphated bile acids is increased in cholestasis despite unchanged *SULT2A1* gene expression in the present study.

Unchanged for moderately reduced expression of phase I and II detoxification enzymes could be because of the relatively low expression levels of FXR, PXR, the constitutive androstane receptor CAR and the retinoid X receptor RXR or could indicate that other, post-transcriptional mechanisms may be involved. It has to be kept in mind that NR mRNA levels cannot directly be extrapolated to NR function, as their activity is a complex result of many factors such as nuclear translocation, the action of repressors and coactivators, mechanisms that cannot all be assessed in limited human material. Another explanation for unchanged levels of phase I and II detoxification enzymes could be disease-stage-dependent alterations. Induction could be present only in the early stages, whereas these genes are downregulated with disease progression, as shown for several other genes using the microarray technique (78). However, maintained expression of phase I and II detoxification enzymes in PBC is apparently sufficient to hydroxylate and conjugate bile acids with sulphate and glucuronate as reflected by the appearance of these metabolites in urine (5, 6, 8, 16–21). Increased alternative basolateral efflux of sulphated, glucuronidated and hydroxylated bile acids during cholestasis may also significantly contribute to their renal elimination.

However, a limitation of our study is the lack of enzyme activity and protein data as a result of the limited amount of liver tissue. Nevertheless, it is attractive to speculate that further transcriptional induction of these detoxification mechanism with specific NR ligands [ligands or activators of PXR (rifampicin), CAR (phenobarbital, 6,7-dimethylesculetin–a compound present in Yin Chin, a traditional Chinese herbal decoction)] would be beneficial via decreasing bile acid toxicity (2, 46).

Hepatocellular excretion of bile acids via the basolateral membrane represents an alternative elimination pathway for bile acids during cholestasis. This decreases intrahepatic bile acid load and reduces systemic bile acid accumulation as bile acids escaping clearance by the liver are filtered at the glomerulus from plasma into urine (79). We and others have previously reported increased expression of basolateral MRP3 and the heteromeric organic solute transporter OSTα/OSTβ in human cholestatic diseases (10, 11, 80). Another key candidate basolateral bile acid export system is MRP4 (81–83). We herein report a pronounced induction of MRP4 in PBC, suggesting that this bile acid transporter may also contribute to the adaptive response to cholestasis. Unchanged MRP4 mRNA levels indicate a post-transcriptional type of regulation in PBC. In patients with progressive intrahepatic cholestasis (PFIC) types II and III, both MRP4 mRNA and protein expressions were induced in most patients, suggesting age-dependent and/or disease-specific differences in the molecular regulation of MRP4 expression in PFIC and PBC (14). The relative contributions of OSTα/OSTβ, MRP3 and MRP4 to alternative bile acid export have not yet been fully elucidated. However, some conclusions may be drawn from Mrp3 and Mrp4 knockout mice. Serum bile acid levels in common bile duct-ligated (CBDL) $Mrp4^{-/-}$ were significantly lower than in wild-type animals (suggesting decreased alternative basolateral bile acid secretion), whereas no differences in bile acid levels between CBDL $Mrp3^{-/-}$ and $Mrp3^{+/+}$ could be detected (47, 48, 84). Moreover, $Mrp4^{-/-}$ but not $Mrp3^{-/-}$ displayed more severe liver injury after CBDL than their wild-type littermates and induction of both Mrp3 and Ostα/Ostβ was unable to compensate for loss of Mrp4 (47, 48). These findings suggest that at least in rodents, Mrp4 but not Mrp3 is required to effectively extrude bile acids from the cholestatic liver.

Most patients investigated received standard UDCA treatment, which could possibly influence mRNA expression of the genes investigated (85). There were no significant differences in the expression of genes investigated between PBC patients receiving UDCA or those who did not. The negative effects of UDCA on phase I and II metabolism in the present study are in line with absent effects of UDCA on CYP3A-dependent steroid metabolism and on serum and urinary levels of sulphated and glucuronidated bile acids in PBC patients (86, 87). Despite MRP4 induction, UDCA had no effect on mRNA of levels CYP7A1 and phase I and II detoxification enzymes in otherwise healthy gallstone patients undergoing cholecystectomy (85). Lack of a significant MRP4 induction by UDCA in the present study might be attributed to the small number of patients not receiving UDCA in each PBC group (PBC III two out of six, PBC IV three out of five) in the present study. However, the number of patients investigated is too small to draw definitive conclusions on UDCA's effect on MRP4 expression in PBC.

In summary, we herein demonstrate repression of the key enzyme in bile acid synthesis, CYP7A1, and

induction of the alternative basolateral bile acid export pump MRP4 in late-stage PBC. These alterations represent adaptive responses to cholestasis. However, phase I and II bile acid detoxification enzymes were not upregulated in PBC at the mRNA level, which might be attributed to the relatively low expression of NRs involved in their regulation. As the observed adaptive mechanisms cannot counteract cholestatic liver injury as reflected by the frequent need for liver transplantation in PBC patients, future therapeutic strategies may be in particular targeted at stimulating phase I and II bile acid detoxification pathways.

Acknowledgements

This work was supported by Grant P18613-BO5 from the Austrian Science Foundation (to M. T.), Grant 10266 from the Jubilee Funds of the Austrian National Bank and by a GEN-AU Grant from the Austrian Ministry for Science. The antibody against MRP4 was kindly provided by Dr. Dietrich Keppler (Deutsches Krebsforschungszentrum Heidelberg, Germany).

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