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C G Dietrich, A Geier, H E Wasmuth, S Matern, C Gartung, D R de Waart and R P J O Elferink

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Background and aims: The liver is the central organ for the detoxification of numerous xenobiotics, including carcinogens. We studied the influence of cholestasis and biliary cirrhosis on the detoxification, elimination, and tissue distribution of a model compound and food derived carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP).

Methods: Wistar rats were injected with $^{14}$C-PhIP into the portal vein one or six weeks after common bile duct ligation (CBDL). Bile flow was reconstituted, bile and urine were collected over 120 minutes, and metabolites were analysed using high performance liquid chromatography. Total tissue radioactivity levels in several organs as well as tissue bound (ethanol insoluble tissue fraction) radioactivity levels were determined.

Results: Significant downregulation of the transport proteins multidrug resistance associated protein 2 and breast cancer resistance protein was observed in biliary cirrhosis. Biliary excretion of radioactivity was significantly reduced in cholestasis and biliary cirrhosis compared with controls ($15 (2.9)\%$ and $3.2 (1)\%$ of the dose $v$ $36.5 (2)\%$, respectively). Phase II metabolism was severely reduced in cirrhotic rats, resulting in a twofold increase in tissue radioactivity levels in the liver, kidney, and colon. Biliary cirrhosis increased tissue binding of reactive metabolites, as expressed in cpm/100 mg tissue in the liver and the colon ($3267 (1218) v$ $1191 (429)$ in the liver, $3044 (1913) v$ $453 (253)$ in the colon).

Conclusions: Biliary cirrhosis induced by CBDL causes impaired metabolism and elimination of PhIP, and leads to higher tissue levels of potentially genotoxic metabolites in the liver and colon of rats. These data may explain the increased incidence of hepatic and extrahepatic cancers in cholestasis and liver cirrhosis.

Epidemiological studies have shown that cirrhosis is associated with an increased risk of hepatocellular carcinoma. The effect of cirrhosis on the incidence of other cancers is unclear but studies suggest an increased risk of cancers in the respiratory and gastrointestinal tracts, as well as urinary organs. Primary sclerosing cholangitis (PSC), a chronic cholestatic disease, is known to be a risk factor for hepatic and extrahepatic malignancies, including colon carcinoma in patients with ulcerative colitis.

Chronic cholestasis leads to downregulation of multidrug resistance associated protein 2 (Mrp2) and the behavioural profile of breast cancer resistance protein (Bcrp) is unknown. Protein levels of cytochrome P450 (CYP) isoforms are differentially regulated in biliary cholestasis and cholestasis is unknown. Protein levels of cytochrome P450 (CYP) isoforms are differentially regulated in biliary cholestasis and cholestasis is unknown. Protein levels of cytochrome P450 (CYP) isoforms are differentially regulated in biliary cholestasis and cholestasis is unknown. Protein levels of cytochrome P450 (CYP) isoforms are differentially regulated in biliary cholestasis and cholestasis is unknown. Protein levels of cytochrome P450 (CYP) isoforms are differentially regulated in biliary cholestasis and cholestasis is unknown. Therefore, the presence of cirrhosis is likely to lead to impaired metabolism and excretion of substrates of CYP isoforms and of the transporter Mrp2. However, protein levels may not always be representative of enzyme activity, and reduced protein levels may still maintain metabolic fluxes, as in the case of Mrp2. Further, in vitro assays measuring isolated activity do not always correctly predict the in vivo behaviour of drugs or toxins. Many xenobiotic compounds are substrates for several different steps in metabolism and detoxification, and some substrates can be metabolised by different isoforms of the same enzyme complex. We have previously shown that the absence of a whole class of metabolising enzymes (in this case, all UGT1A isoforms) does not necessarily lead to impaired metabolism of substrates as there may be upregulation of compensatory mechanisms of redundant enzyme systems such as UGT2 isoforms and sulphotransferases.

Therefore, it is difficult to predict how liver cirrhosis or cholestasis may affect the overall metabolism and detoxification of toxic compounds.

To determine the effect of cholestasis and cirrhosis on metabolism, detoxification, and elimination of a food derived carcinogen, we studied the fate and distribution of a model substrate and further characterised the molecular alterations in biliary cirrhosis after six weeks of common bile duct ligation (CBDL). To determine primarily changes in liver metabolism and to exclude alterations via intestinal uptake, we administered the model substrate 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) intravenously into the portal vein.

PhIP is a heterocyclic amine and as such is the most abundant food derived carcinogen in prepared meat. Its metabolism has been extensively studied in several species, and we and others have previously shown that PhIP and its major metabolites are substrates of the efflux pump MRP2 in rats and humans. Additionally, it has been shown recently that the parent compound is transported very efficiently by the breast cancer resistance protein (Bcrp). Both of these transport proteins are present in the liver and colon. Activation of PhIP to N-OH-PhIP, a genotoxic compound and precursor for further activation, occurs via CYP1A1 and CYP1A2. This enzymatic reaction can also yield 4'-OH-PhIP, which is the first detoxified metabolite of PhIP. Both hydroxylation products can be further metabolised by glucuronidation or sulphation. Detoxification of PhIP may

Abbreviations: Bcrp, breast cancer resistance protein; CBDL, common bile duct ligation; CYP, cytochrome P450; Mrp, multidrug resistance associated protein; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; UGT, UDP-glucuronosyltransferase; PSC, primary sclerosing cholangitis; HPLC, high performance liquid chromatography
also occur via direct glucuronidation of the parent compound, a pathway which has only limited importance in rodents but which may account for approximately 20% of metabolites in humans. Data from animal studies have shown that PhIP is involved in rat colon carcinogenesis but data regarding the contribution of PhIP to human colon carcinogenesis are controversial (discussed recently by Berlau and colleagues), which may be due in part to methodological problems. In the light of reports on the association between colon cancer and liver cirrhosis, we studied the metabolism and excretion of PhIP as a carcinogenic model compound in cholestatic (one week of bile duct ligation) and cirrhotic (six weeks of bile duct ligation) animal models.

MATERIALS AND METHODS

Chemicals

[14C]PhIP (specific activity 10 mCi/mmol) was obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). All solvents used for high performance liquid chromatography (HPLC) separation (HPLC or analysis grade) were from Merck (Darmstadt, Germany).

Animals

Female Wistar rats (Janvier, Le Genest-St Isle, France), 200–250 g each, were used for all experiments. Rats were housed in cages with a 12 hour light/dark cycle and given access to food and water ad libitum. CBDL was executed as previously described. Briefly, after subcutaneous injection of ketamine (80 mg/kg body weight) and xylazine (4 mg/kg body weight), the abdomen was opened through a midline incision. The bile duct was identified, isolated, and double ligated in the proximal part. In sham operated rats, the bile duct was isolated and touched but not ligated. After one and six weeks, respectively, all rats again received ketamine/xylazine, the abdomen was opened, and the fate and distribution of PhIP was studied, as previously described. Briefly, the bile duct was cannulated, in CBDL rats proximal to the ligation (to restore bile flow). The urine bladder was also cannulated. Rats received 2 μCi of [14C]PhIP, dissolved in 500 μl of 10 mM tauroursodeoxycholate, intravenously into the portal vein. Bile and urine were collected, rats were sacrificed after two hours by bleeding from the aorta, and the liver, colon, small intestine, pancreas, kidneys, heart, and lungs were harvested. Faeces were separated from the intestine before storage at −80°C. All animal experiments were approved by the local animal care committee.

Analysis of bile and urine samples

Scintillation fluid was added to aliquots from bile and urine samples and total radioactivity of each sample was measured; 20 μl from each bile and urine sample were directly injected onto a HPLC system, as previously described. Briefly, a 0.1% diethylamine/methanol gradient was used at pH 6.0 to separate the metabolites on a Gynkotek-HPLC-system (Germering, Germany). Radioactivity was monitored with a Packard 515TR scintillation analyser (Meriden, Connecticut, USA). HPLC chromatograms were used for quantification of each metabolite using identification data from previous experiments.

Organs

Aliquots (~100 mg) of each organ (from at least two different sites) were dissolved in Soluene 350 (Packard; Meriden) at 40°C, decolourised with 10% (v/v) H2O2, scintillation fluid was added, and the samples were counted. Other aliquots were used for ethanol extraction of metabolites (for analysis on HPLC), as previously described. Differences in radioactivity content before and after ethanol extraction were used to estimate the covalent binding of reactive PhIP metabolites to tissue components.

Western blotting

Liver microsomal extracts were produced to analyse protein levels of Mrp2 and UGT1A isoforms. Liver tissue samples (~100 mg) were homogenised in 1 ml of homogenisation buffer (0.25 M sucrose, 10 mM HEPES, pH 7.5, 1 mM EDTA, pH 8). After centrifugation (31 000 rpm at 4°C), the pellet was resuspended in 80 μl of storage buffer (0.3 M sucrose, 10 mM Hepes, pH 7.5) and stored at −80°C. Both buffers contained a commercial mixture of protease inhibitors at the recommended concentration (Mini Complete, Roche, Basel, Switzerland), 0.5 μg/ml pepstatin A, and 40 μg/ml phenylmethylsulphonylfluoride. Protein concentrations were determined according to Bradford. After determination of the linear range (25–200 μg), similar amounts of protein (25 μg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis within three days and probed with the antibodies M2II-6 (for rat Mrp2), BXP-53 (for rat Bcrp, kindly provided by Dr Alfred Schinkel), and HEB7 (for rat UGT1A isoforms) after transfer to nitrocellulose membranes. Equal loading was confirmed by Ponceau staining.

Statistical analysis

Significance was assumed at p<0.05. Testing was done with ANOVA and post testing with the Bonferroni method. All data represent means (SD) from four animals per group.

RESULTS

Physiological and anatomical parameters

Biliary and urinary flow were not significantly different between groups (fig 1A). Liver mass in cirrhotic and cholestatic animals was significantly increased (21.7 (4.7) g and 11.2 (0.6) g, respectively, vs 7.9 (0.9) g in sham operated rats) (fig 1B). After six weeks of bile duct ligation, the livers were firm and showed nodule formation. Microscopically, ductular proliferation and cirrhosis were seen mainly in perportal areas.
Protein expression of Mrp2, Bcrp, and UGT1A isoforms in cirrhotic liver after six weeks of bile duct ligation

As the influence of CBDL on Mrp2 expression in rat liver has been investigated only up to 14 days of CBDL, we determined protein levels of Mrp2 in rats with liver cirrhosis after six weeks of CBDL. We confirmed that Mrp2 protein expression decreased further as the duration of bile duct ligation increased, and was ~10% of sham controls in liver tissue from rats with biliary cirrhosis (p<0.001) (fig 2A). Regulation of Bcrp during CBDL has not been studied previously. We observed a 28% decrease in Bcrp protein to 72 (17)% of sham controls (p = 0.05) (fig 2B). We also confirmed that levels of UGT1A isoforms were unchanged in cirrhotic livers (fig 2C).

Biliary and urinary excretion of radioactive metabolites following injection of 14C-PhIP into the portal vein

Total excretion of radioactivity in two hours was significantly reduced in cholestatic (35.6 (2.8)% and cirrhotic (9.5 (1.6)% rats compared with sham operated controls (49 (2.9)%)) (fig 3A). Biliary excretion of the radiolabel is shown in fig 3B and was 3.2 (1)% in cirrhotic, 15 (2.9)% in cholestatic, and 36.5 (2)% in sham operated rats, respectively (see fig 3A). In cholestatic rats, the decrease in biliary excretion was partially compensated for by increased urinary excretion (20.6 (1.9)% v 12.5 (1.7)% in sham operated rats) while in cirrhotic rats urinary excretion was dramatically reduced (6.3 (1)% (fig 3)).

PhIP metabolites in bile, urine and liver

PhIP is metabolised in the body to N-OH- or 4'-OH-PhIP (phase I). Quantitative important phase II metabolites comprise glucuronides of both phase I metabolites (conjugation on the N2-, N3-, or 4'-OH position) as well as sulphation of 4'-OH-PhIP. All of these metabolites were analysed using separation by HPLC.

Biliary excretion of all glucuronidated metabolites was significantly reduced in cholestatic and, more severely, in cirrhotic rats (3.3 (0.2)% and 0.5 (0.2)%, respectively) compared with normal controls (21.1 (1.6)% of injected dose). Biliary excretion of PhIP, the unmetabolised parent compound, was significantly diminished in both animal models to a similar extent. The bile content of PhIP-sulphate was significantly elevated in cholestatic rats (6.8 (1)% v 4.4 (0.3)% in controls) but this compensatory metabolism was absent in cirrhotic rats (0.45 (0.1)%) (fig 4A). In urine, all phase II metabolites were significantly elevated (glucuronides and sulphate) in rats with acute cholestasis compared with controls. This compensation for reduced biliary excretion was again absent in cirrhotic rats (reduced urinary excretion for the N2- and N3-glucuronides and sulphate)
Liver tissue levels of total and bound (ethanol insoluble) radioactivity

In the liver of cirrhotic rats, there was a fourfold accumulation of radioactivity at two hours after intravenous administration compared with control rats (15.9 (1.4)% vs 4.5 (1.4); p<0.001) (fig 5A). However, when corrected for liver mass, these differences were less apparent (7291 (1906) vs 4067 (435) cpm/100 mg tissue; p = 0.02) (fig 5B). A similar effect was observed in cholestatic rats (8.4 (2.6)% radioactivity accumulating in the liver compared with 4.5 (1.4)% in controls; p = 0.02) (fig 5A). However, this difference was not statistically significant when expressed per 100 mg liver mass (4946 (1702) vs 4067 (435) cpm/100 g tissue; p = 0.2).

Tissue levels of total and bound (ethanol insoluble) radioactivity in other organs

There were no significant differences in the weight of the kidney, heart, lung, or intestine between the treatment groups. The following results were obtained (see fig 5A–D): accumulation of radioactivity from injected $^{14}$C-PhIP was significantly increased in the colon in cirrhotic rats but not in rats with acute cholestasis. In contrast, tissue bound radioactivity was significantly elevated in the colon in both cirrhotic and cholestatic rats compared with control rats (3044 (1913) cpm and 1410 (203) cpm, respectively, vs 453 (253) cpm per 100 mg tissue, p = 0.04 and <0.001, respectively). In the kidney, total radioactivity was decreased in cirrhotic compared with cholestatic rats but was significantly elevated in both when compared with control animals (16911 (3987) cpm and 21101 (6248), respectively, vs 9809 (2264), p = 0.014 for both). However, tissue bound radioactivity in the kidney was not significantly elevated in cirrhotic or cholestatic rats compared with controls.

Differences in other organs (small intestine, pancreas, heart, lung) were borderline significant, and are not shown.

DISCUSSION

Our data demonstrate that chronic cholestasis and biliary cirrhosis cause impaired hepatic metabolism and excretion of the heterocyclic amine and food derived carcinogen PhIP. Reduced biliary excretion after one week of bile duct ligation could partially be compensated for by increased urinary elimination of PhIP and its metabolites but biliary cirrhosis led to a marked decrease in both elimination routes. One of the consequences of decreased metabolism and elimination was accumulation of PhIP and its metabolites in the liver, kidney, and colon, with the latter being a potential target organ of the carcinogen PhIP in rats. Tissue binding, defined as ethanol insoluble tissue fraction of radioactivity, was significantly increased in the colon of cirrhotic rats, indicating the formation of adducts of activated PhIP metabolites to tissue components, including DNA.

PhIP is a substrate for CYP1A1 and CYP1A2 isoforms, several different UGT isoforms, sulphotransferases, and the transport proteins Mrp2 and Bcrp. As such, its metabolism, tissue distribution, and elimination are influenced in a complex way by progressive cholestasis and biliary cirrhosis where adaptive changes in protein expression and activity at different levels of metabolism and transport take place. We have recently shown that cholestasis downregulates intestinal Mrp2 expression and increases oral bioavailability of PhIP in rats, indicating that local processes such as cholestasis may influence gene expression in other organs via systemic mediators. The complex metabolism of PhIP with competing pathways and widespread changes in protein activity or expression, not only in the liver, complicates assessment of the present data, even in our experimental setting (portal intravenous administration of the compound, dose higher than in physiological situations to allow quantification of metabolites). This can give rise to misinterpretation of our data and has to be kept in mind when applying results of the current study to different conditions.

Our data show that under the current conditions, phase I metabolism is preserved despite reduced protein levels of the CYP1A isoforms. Glucuronidation is decreased in cholestatic animals using both calculation methods (7.1 (2)% and 3.4 (1)% respectively, vs 1.3 (0.4)% of the total dose, p = 0.005 and 0.008, respectively (fig 5C); 3267 (1218) cpm and 21101 (644) cpm, respectively, vs 1191 (429) cpm in mass related radioactivity per 100 mg tissue, p = 0.02 and 0.04, respectively (fig 5D)).
and, more so, in biliary cirrhosis despite the persistence of normal UGT levels (fig 2). We and others have previously shown that decreased activity of Mrp2 is sufficient to reduce phase II metabolism in liver cells, and this is probably an effect of product inhibition due to reduced biliary elimination. Downregulation of Mrp2 to 10% of its normal expression may have similar effects on glucuronidation activity. In this study, we have shown for the first time that Bcrp, which secretes the parent compound PhIP into bile, is also downregulated during long term CBDL. This modest downregulation may contribute to the high tissue levels of PhIP observed in the livers of rats with biliary cirrhosis (fig 4C).

As the main step of detoxification, a reduction in glucuronidation may seriously intensify the genotoxicity of PhIP. Increased sulphation as a possible compensatory detoxification pathway was only present in cholestatic but not in cirrhotic rats (fig 4A–C), indicating a further deterioration in metabolism in the cirrhotic liver. Instead of the use of alternative detoxification pathways, overall metabolism in cirrhotic livers was reduced, as shown by the elevated PhIP concentrations, and resulted in a shift to microsomal activation (production of N-OH-PhIP, see fig 4C). N-OH-PhIP is already genotoxic per se but it can be metabolised to N-acetoxy- and N-sulfoxy-PhIP, metabolites which both bind highly to DNA and induce genotoxic effects in vitro and in vivo.

The metabolism of compounds outside the liver is difficult to quantify. Differences in biliary and urinary metabolite concentrations indicate preserved, or even compensatory metabolism in extrahepatic tissues such as the duodenum, this mechanism is a prerequisite for UGT mediated detoxification in extrahepatic tissues. However, little is known regarding the influence of liver disease on protein expression levels of extrahepatic metabolising enzymes. It is possible that increased N-OH-PhIP concentrations in the colon not only trigger detoxification via glucuronidation but also provoke further activation by acetylation or sulphation, resulting in increased tissue binding, as observed in our experiments. Clearly, further studies are needed to elucidate these systemic effects in vivo.

Neither biliary nor urinary flow were reduced in cirrhotic rats (fig 1). Maintenance of bile flow after resolution of six weeks of bile duct obstruction is probably due to the high bile salt pool in cirrhotic livers which compensates for the apparent reduction in bile salt independent bile flow via Mrp2. Despite preserved urinary flow, cirrhotic animals were not able to compensate for the loss of biliary excretion by renal elimination (fig 3). In addition, the observed remaining urinary elimination was delayed (fig 3C). In parallel with this result, we saw reduced total tissue concentrations of PhIP and its metabolites in the kidneys of cirrhotic animals compared with cholestatic animals (fig 5). It has previously been shown that liver cirrhosis results in reduced renal plasma flow in rats, which may explain the reduced and delayed urinary excretion. Reduced hepatic basolateral re-secretion into blood appears to play a minor role as blood levels of radioactivity were unchanged two hours after injection and tissue levels in other organs were elevated (colon) or unchanged.

Tissue levels of PhIP and its metabolites in the liver are not easy to interpret in our animal models due to the steady increase in liver mass with increased duration of bile duct ligation. We measured the absolute increase in radioactivity in cirrhotic livers, which may be the consequence of similar tissue content combined with a three fold enlarged organ (fig 5A). Weight dependent calculations however also showed a relative increase in tissue concentrations (fig 5B). Most critical for potential genotoxic effects is the relative tissue load of tissue bound metabolites. Here we found a threefold increase in the liver of cirrhotic animals but in the colon the respective values were elevated sixfold (fig 5D). Given the fact that PhIP is a rat colon carcinogen, this supports the biological significance of hepatic biliary cirrhosis in the development of colon cancers in rats. However, only binding of reactive PhIP metabolites to DNA of colon stem cells determines the extent of possible carcinogenicity by this heterocyclic amine. With our experimental setup, it is not possible to reliably measure the carcinogenic effect of liver cirrhosis on the colon.

Protein expression levels of metabolising enzymes in human liver fibrosis and cirrhosis have not been systematically investigated. In PSC, MRP2 mRNA was reduced to 27% of healthy controls while in advanced primary biliary cirrhosis (stage IV), protein expression levels of MR2 were decreased, indicating similar regulation events as in rats.
However, data regarding phase I and phase II enzymes are completely lacking and hence our results cannot be directly extrapolated to the human situation.

In summary, we have shown that biliary cirrhosis can result in elevated tissue binding of activated PhIP metabolites in the cirrhotic liver, the target organ of PhIP carcinogenesis in rats. This is primarily the result of deaccelerated and reduced metabolism as well as reduced elimination in the liver, but also demonstrates the remote effects of liver disease on other organs such as the kidney and colon. There are substantial differences in PhIP metabolism between rats and humans which has led to the conclusion that PhIP mediated human carcinogenesis is underestimated in rats. In humans, higher levels of adducts forming in colon tissue have been observed when directly compared with rats. The relation between activation (N-hydroxylation) and detoxification (4′-hydroxylation) is much more unfavourable in humans than in rats (97:1 v:3:113). Additionally, the presence of polymorphisms in human genes affecting enzyme activity58 may result in specific subsets of patients with an increased cancer susceptibility in extrahepatic tissue in general, and the colon in particular. In combination, these facts may lead to magnification of the effects seen in the present study in rodents. The extent of PhIP induced carcinogenesis in humans is still debatable but PhIP is one xenobiotic model compound, representing numerous other genotoxic substances whose metabolism might also be reduced in liver cirrhosis. Systematic epidemiological studies are therefore warranted to study the clinical importance of liver cirrhosis in extrahepatic carcinogenesis.

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Authors’ affiliations

C G Dietrich, A Geier, H E Wasmuth, S Matern, C Gartung, Department of Gastroenterology and Hepatology, Aachen University, Aachen, Germany

D R de Vaart, R P O J O Elferink, Amsterdam Liver Centre, Academic Medical Centre, Amsterdam, the Netherlands

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