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Research Paper

Altered expression of MRP2, MRP3 and UGT2B1 in the liver affects the disposition of morphine and its glucuronide conjugate in a rat model of cholestasis

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Abstract

Objectives The aim was to investigate the disposition of morphine and morphine-3-glucuronide (M3G) in a rat model of cholestasis induced by bile duct ligation (BDL).
Methods Morphine (15 mg/kg) was administered intravenously, and morphine and M3G concentrations in the plasma and urine measured by HPLC. Changes in the mRNA expression of multidrug resistance-associated protein (MRP)2, MRP3 and UDP-glucuronosyltransferase (UGT)2B1 in the liver were estimated using RT-PCR.
Key findings Although the plasma morphine concentrations declined exponentially, the elimination was delayed 3 and 5 days after BDL. Plasma M3G concentrations on day 1 after BDL were similar to those in the untreated control group, but were increased 3 and 5 days after BDL. Expression of MRP3 and UGT2B1 mRNA increased after BDL. The urinary excretion of M3G was increased significantly after BDL.
Conclusions Enhanced glucuronidation of morphine and transportation of M3G into the blood increased the plasma M3G concentration in the BDL groups. However, M3G disposition 1 day after BDL was similar to that in the untreated control group because urinary excretion of M3G increased.
Keywords bile duct ligation; disposition; morphine; morphine-3-glucuronide; transporter

Introduction

Morphine is a potent analgesic that acts by binding to μ-opioid receptors, used to relieve pain due to carcinomas and myocardial infarction. The binding of morphine to these receptors causes side-effects such as nausea, vomiting, constipation, drowsiness and respiratory depression. Reduced clearance of morphine in patients with severe liver disease increases the risk of these side-effects. Nevertheless, morphine is used for patients with serious liver dysfunction and to control pain due to cancers such as hepatoma. Therefore, characterising the disposition of morphine in liver disease may provide insights into ways to avoid these side-effects.

Drug metabolism occurs in two phases: phase 1 is catalysed by cytochrome P450 enzymes and phase 2 by enzymes such as UDP-glucuronyltransferase (UGT), sulfotransferase and glutathione-S-transferase. Approximately 70% of morphine entering the system is eliminated primarily in the bile after glucuronidation in the liver. In humans, approximately 55% of morphine is metabolised to morphine-3-glucuronide (M3G) and 15% to morphine-6-glucuronide in reactions mainly catalysed by UGT2B7. On the other hand, in rats, morphine is metabolised to M3G in a reaction mainly catalysed by UGT2B1. The pharmacological effects of M3G and M6G differ profoundly: M3G has no analgesic properties, whereas M6G is more potent than morphine itself.[1,2] These glucuronide conjugates are transported across the sinusoidal and canalicular membranes of hepatocytes by the ATP-binding cassette (ABC) membrane transporters, which belong to the multidrug resistance-associated protein (MRP) family. MRP2 transports morphine conjugates across the canalicular membrane into the bile, while MRP3 transports them across the sinusoidal membrane into the blood.[3,4]

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The function of hepatic transporters may be altered in diseases because of changes in the levels of several substances in the body. In this study, we investigated the disposition of morphine in cholestasis – the arrest of bile flow and the most common condition associated with liver disease. In cholestasis, bilirubin, biliary acid and cholesterol accumulate in hepatocytes, and their concentrations in the blood are elevated because biliary excretion is inhibited. There have been several reports of the altered expression of transporters and metabolic enzymes in rat models of cholestasis induced by bile duct ligation (BDL). Bohan and colleagues[5] demonstrated that the expression of MRP2 decreases and that of MRP3 increases, while that of the bile salt export pump (BSEP) remains unchanged. Furthermore, Dumont and colleagues[6] reported that expression of the organic anion-transporting polypeptide (OATP) and Na-taurocholate cotransporting polypeptide (NTCP) is down-regulated. Further investigations on the altered hepatic expression of transporters and metabolic enzymes in animal models of cholestasis are required to elucidate the mechanisms of morphine disposition in hepatic disorders. We used a rat model of cholestasis induced by BDL to study the disposition of morphine and M3G over a 5-day period after performing BDL.

Materials and Methods

Materials

Morphine was obtained from Takeda Chemical Inc. Ltd (Osaka, Japan). M3G was a gift from Shionogi Co., Ltd (Osaka, Japan). Naloxone was obtained from Daiichi Sankyo Co., Ltd (Tokyo, Japan). Sepasol RNA I Super was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Glycogen solution and the SuperScript III first-strand synthesis system for RT-PCR were from Invitrogen Life Technologies Inc. (Tokyo, Japan) and the KOD Dash kit from Toyobo Inc. (Osaka, Japan). Sense and antisense primers for MRP2 and UGT2B1 were obtained from Sigma Inc. (Kanagawa, Japan) and those for MRP3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Bex Inc. (Tokyo, Japan). All the other reagents were of molecular biology grade and the highest quality available.

Animals

Twenty-five 6-week-old male Donryu rats were purchased from Japan SLC Inc. (Shizuoka, Japan). They were housed for 1 week to exclude any abnormal animals. Rats in the BDL group were anaesthetised with pentobarbital and the bile duct ligated at two sites, and the region between these sites transected to create a gap. Control rats were subjected to a sham operation; untreated controls did not receive any treatment. The control rats were killed under anaesthesia 5 days after the sham operation. The rats in the BDL group were killed under anaesthesia at 1, 3 and 5 days after the surgery (BDL1, BDL3 and BDL5 groups, respectively). Liver tissue and blood samples were collected and stored at −80°C until analysis. The blood samples were centrifuged at 12 000 rpm for 10 min to obtain plasma, which was sent to Falco Biosystems Ltd (Kyoto, Japan) for measurement of total and conjugated bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

All rats were handled in accordance with the guidelines for the care of laboratory animals established by Kobe Gakuin University. The protocol for this animal study was approved by the Animal Experimentation Ethics Committee of Kobe Gakuin University.

Analysis of mRNA expression by RT-PCR

Total RNA was extracted from the rat liver tissue (100 mg) using Sepasol RNA I Super, chloroform and isopropanol. cDNA was synthesised by performing RT-PCR with the SuperScript III first-strand synthesis system. Synthetic cDNA (0.5 μl) and sense and antisense primers were added to a 24.5 μl reaction mixture containing KOD Dash DNA polymerase. Denaturation, annealing and extension were performed for 23–35 cycles using the iCycler system.

Degenerate PCR primers were designed and chemically synthesised on the basis of the amino acid sequences of rat UGT2B1 (255 bp; sense primer, 5′-TGT TGG TAT TCC CTT GTT TGC-3′; antisense primer, 5′-GTG CTT GGC TCC TTT GTG ACG-3′); MRP2 (421 bp; sense primer, 5′-ATC CTC AGC TGC TGA AGT TG-3′; antisense primer, 5′-CTG ATC TTG GAT GCC AGA AC-3′); MRP3 (423 bp; sense primer, 5′-TCA AAG AGG AGA TCG CAG AG-3′; antisense primer, 5′-AGC ATG AGG ATG GGG GCC AG-3′); and GAPDH (576 bp; sense primer, 5′-CCA TCA CCA TCT TCC AGG AG-3′; antisense primer, 5′-CCT GCT TCA CCA CCT TCT TG-3′).

The PCR products were resolved by electrophoresis on 2% agarose gels for 50 min. The agarose gels were then stained with ethidium bromide. The bands were visualised using a UV transilluminator (Atto Corp., Tokyo, Japan), and specific bands were quantified by densitometric analysis.

Disposition of morphine and its glucuronide conjugate

Three or four rats from each group were given 15 mg/kg morphine, administered by continuous intravenous infusion over 5 min via the lower limb arteries; 0.5 ml blood samples were collected from the arteries of the contralateral limb under isoflurane anaesthesia and 0.5 ml saline injected to maintain the blood flow. Samples were obtained at baseline (t = 0) and at 5, 10, 15, 25, 45, 65, 95, 125, 185, 245 and 365 min after morphine administration. Samples were centrifuged at 12 000 rpm for 10 min to obtain the plasma. Urine excreted during the experiments was collected and bladder urine collected directly after the experiments. Plasma and urine samples were stored at −80°C until analysis.

Solid-phase extraction was performed using Inertsep C18-C FF cartridges (GL Science Inc., Tokyo, Japan). The cartridges were conditioned with 3 ml methanol, 3 ml water, 2 ml acetonitrile in 10 mmol/l phosphate buffer (10 : 90 v/v; pH 2.1), and 3 ml 0.5 mol/l ammonium sulfate buffer (pH 9.3). Following conditioning, 200 μl of sample was added to 20 μl naloxone (internal standard) and loaded onto the cartridge. The plasma samples were loaded undiluted; urine samples were first diluted with saline. The cartridges were then washed twice with 2 ml 5 mmol/l ammonium

sulfate buffer (pH 9.3). The samples were eluted with 2 ml acetonitrile in 10 mmol/l phosphate buffer (10 : 90 v/v; pH 2.1). The eluent was evaporated at 50°C and the residue dissolved in 300 μl mobile phase; 50 μl was injected onto the HPLC system. Morphine was detected using an electrochemical detector (Coulochem II, Esa Inc., MA, USA), and M3G using a UV-visible spectrophotometer (214 nm; SPD-10A, Shimadzu Inc., Kyoto, Japan.). The reverse-phase column (Inertsil ODS-3, GL Sciences Inc., Tokyo, Japan) was maintained at 50°C. The mobile phase was a solution of 0.1 mol/l phosphate buffer (pH 2.1), acetonitrile and methanol (72 : 24 : 2 by volume), delivered at a flow rate of 1 ml/min, for a total run time of 70 min.

The elimination half-life of morphine ($T_{1/2\beta}$) and total clearance (CL_{tot}) were calculated from the plasma concentration–time profiles, using the formulae $T_{1/2\beta} = 0.693/\beta$ (min) and CL_{tot} = dose/AUC (ml/min), where β is the kinetic constant for the disappearance phase in a two-compartment model and AUC is the area under the plasma concentration–time curve (μg min/ml).

Statistical analyses

The data are presented as means ± SD. Non-parametric comparisons were performed using the Kruskal–Wallis test. Significant differences were evaluated using Dunnett's test if the Kruskal–Wallis test was significant. Values of $P < 0.05$ were considered statistically significant.

Results

Analysis of mRNA expression by RT-PCR

Concentrations of total and conjugated bilirubin, AST and ALT in the plasma were measured to determine whether BDL had successfully induced cholestasis (Table 1). The total bilirubin concentration was higher in the BDL groups than in the control group. A similar trend was noted for the concentration of conjugated bilirubin. The plasma concentrations of AST and ALT were higher in the BDL1 group than in the control group but lower in the BDL3 group than in the BDL1 group.

We investigated the mRNA expression of UGT2B1, which metabolises morphine into M3G; MRP2, which transports M3G from hepatocytes into bile; and MRP3, which transports M3G from hepatocytes into plasma. The corresponding electrophoretic profiles and band strengths are shown in Figure 1. The mRNA expression of UGT2B1 was increased approximately 1.7-fold in the BDL1 group compared with the control group (Figure 1b). The mRNA expression of MRP2 did not differ between the BDL groups whereas the mRNA expression of MRP3 increased with time after BDL. The mRNA expression of MRP3 was increased approximately 3.7-fold in the BDL5 group compared with the control group (Figures 1c and 1d).

Disposition of morphine and its glucuronide conjugates

The plasma concentration–time profiles of morphine and M3G are shown in Figure 2. The concentration of unchanged morphine declined exponentially. The distribution phase was rapid, and the subsequent elimination phase was slower (Figure 2a). The plasma concentration–time profile of morphine was similar in the BDL1 and untreated control groups. The elimination phase was slower in the BDL3 and BDL5 groups than in the BDL1 and untreated control groups. The final concentrations were approximately 10-fold higher in the BDL3 and BDL5 groups than in the untreated control and BDL1 groups. Calculated values of $T_{1/2\beta}$, AUC and CL_{tot} are shown in Table 2. $T_{1/2\beta}$ values were similar in the BDL1 and untreated control groups, but were increased in the BDL3 and BDL5 groups. AUC and CL_{tot} values in the BDL1 and untreated control groups were similar. AUC values were higher in the BDL3 and BDL5 groups than in the untreated control group, while the CL_{tot} values were lower.

The plasma concentration–time profiles of M3G in the BDL1 and untreated control groups were similar, the M3G concentration increasing initially and decreasing thereafter. Concentrations of M3G were significantly higher in the BDL3 and BDL5 groups than in the untreated control group, and this trend persisted (Figure 2b). The maximum drug concentration (C_{max}), as calculated from the plasma concentration–time profiles of M3G, time taken to achieve C_{max} (T_{max}) and AUC are shown in Table 3. The C_{max} and AUC values were similar in the BDL1 and untreated control groups, whereas C_{max} had increased 3 and 5 days after BDL (BDL3 and BDL5 groups). T_{max} was higher in the BDL1 group than in the untreated control group but the difference was not significant. T_{max} was significantly higher in the BDL3 and BDL5 groups than in the untreated control group.

The volume of urine was lower in the BDL1, BDL3 and BDL5 groups (1.6 ± 0.5, 1.6 ± 0.7 and 2.0 ± 1.1 ml, respectively) than in the untreated control group (3.4 ± 2.4 ml). Figure 3 shows the urinary excretion ratios of morphine and M3G (defined as the molar ratio of urinary excretion per dose applied). The morphine and total excretion ratio did not differ significantly between the untreated control and BDL groups, while that for M3G was significantly higher in the BDL1 and BDL3 groups than in the untreated control group.

Table 1 Concentrations of total and conjugated bilirubin, AST and ALT in plasma

	Control	BDL1	BDL3	BDL5
AST (IU/l)	90.0 ± 24.3	732.3 ± 72.7*	406.3 ± 125.5*	591.0 ± 31.3*
ALT (IU/l)	39.7 ± 6.0	308.7 ± 55.3*	106.7 ± 18.0	151.0 ± 12.3*
Total bilirubin (mg/dl)	0.1 ± 0.0	2.0 ± 0.3	3.6 ± 0.2*	3.4 ± 0.1*
Conjugated bilirubin (mg/dl)	ND	1.9 ± 0.2	3.0 ± 0.4*	3.1 ± 0.2*

Values are means ± SD ($n = 3$). *$P < 0.05$ vs untreated control rats. BDL1, 3, 5 are the groups 1, 3, 5 days after bile duct ligation (BDL). ALT, alanine aminotransferase; AST, aspartate aminotransferase; ND, not detected.

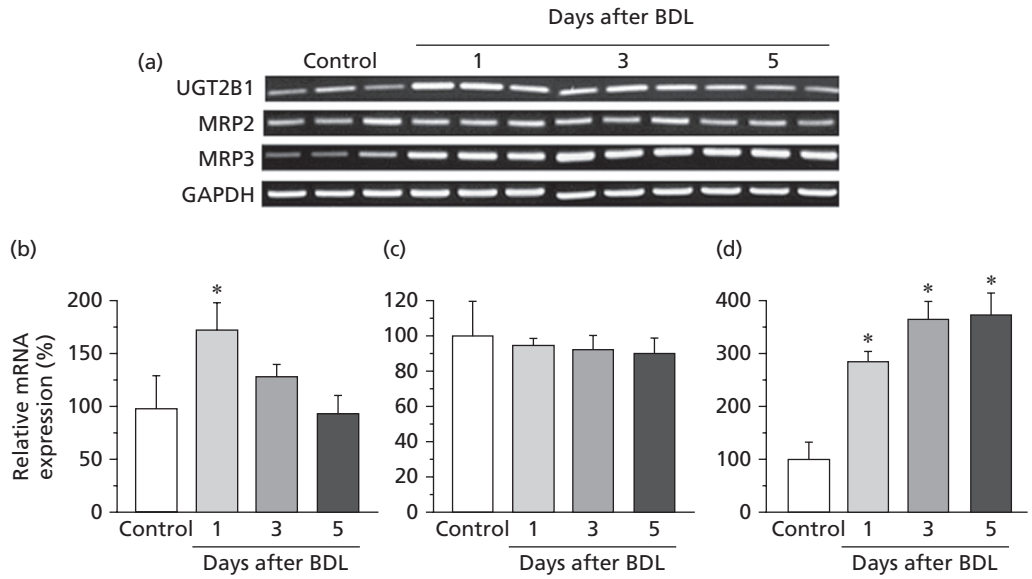

Figure 1 (a) Electrophoretic profiles of the mRNA expression of UGT2B1, MRP2 and MRP3 in the liver after bile duct ligation. Expression of (b) UGT2B1, (c) MRP2 and (d) MRP3 mRNA (expressed as a percentage of the control value). Data are means ± SD (n = 3 animals, experiments performed in triplicate). *P < 0.05 vs control rats. BDL, bile duct ligation.

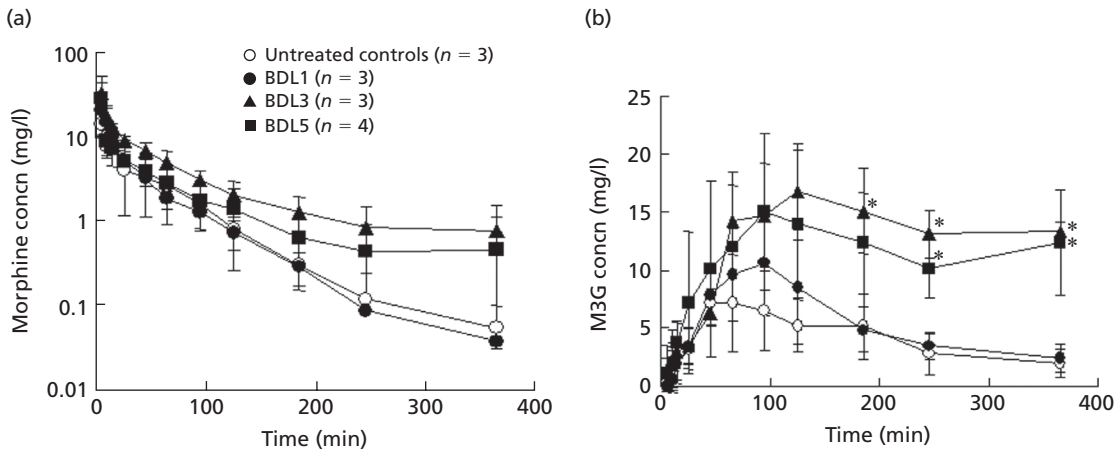

Figure 2 Plasma concentrations of (a) morphine and (b) morphine-3-glucuronide following administration of morphine 15 mg/kg by intravenous infusion over 5 min. BDL1, 3 and 5 are the groups 1, 3 and 5 days after bile duct ligation (BDL). Data are means ± SD. *P < 0.05 vs untreated control group. M3G, morphine-3-glucuronide.

Table 2 Pharmacokinetic parameters of morphine in plasma

	Untreated controls (n = 3)	BDL1 (n = 3)	BDL3 (n = 3)	BDL5 (n = 4)
$T_{1/2\beta}$ (min)	43.1 ± 1.4	45.6 ± 18.3	195.3 ± 262.9	94.7 ± 108.6
$AUC_{0\rightarrow\infty}$ (μg min/ml)	506.7 ± 150.5	528.0 ± 180.0	1522.0 ± 847.8	820.5 ± 400.8
CL_{tot} (ml/min per kg)	314 ± 9.4	30.9 ± 11.5	11.7 ± 5.0	22.2 ± 10.7

Elimination half-life ($T_{1/2\beta}$), AUC, and total clearance (CL_{tot}) were calculated from the plasma concentration–time profiles of morphine (Figure 2a). BDL1, 3 and 5 are the groups 1, 3 and 5 days after bile duct ligation (BDL). Data are means ± SD.

Discussion

Cholestasis induces jaundice and liver disease because of the accumulation of bile components in hepatocytes. In cholestasis, bile salts, bile acid, bilirubin and fatty acids accumulate in the liver. These compounds are the major ligands of MRP2/3 and UGT, and thus the expression of these transporters and enzyme may change as a result of the accumulation of the compounds in cholestasis.[7] Because MRP2/3 and UGT play important roles in the disposition of

Table 3 Pharmacokinetic parameters of morphine-3-glucuronide in plasma

	Untreated controls (n = 3)	BDL1 (n = 3)	BDL3 (n = 3)	BDL5 (n = 4)
C_{max} (mg/l)	8.6 ± 3.1	11.3 ± 3.7	17.2 ± 4.0	17.0 ± 5.2
T_{max} (min)	46.7 ± 11.5	80.0 ± 17.3	140.0 ± 34.6	172.5 ± 125.8*
$AUC_{0\rightarrow365min}$ (μg min/ml)	1506.3 ± 759.9	1907.6 ± 589.0	4639.1 ± 565.0*	4121.3 ± 1328.8*

Maximum drug concentration (C_{max}) was calculated from the plasma concentration–time profiles of morphine-3-glucuronide (Figure 2b); time taken to achieve C_{max} (T_{max}) and AUC were calculated over 365 min. BDL1, 3 and 5 are the groups 1, 3 and 5 days after bile duct ligation (BDL). Values are means ± SD. *$P < 0.05$ vs untreated controls.

morphine and its glucuronide conjugates, we investigated alterations in their expression of in cholestasis.

The normal bilirubin concentration in human plasma is 0.2–1.2 mg/dl and mostly comprises unconjugated bilirubin. The plasma concentration of conjugated bilirubin markedly increases with jaundice and liver disease. Thus, measurement of the plasma bilirubin concentration is clinically important for the diagnosis of liver disease.[8] In clinical practice, deviations in the levels of hepatic enzymes such as AST and ALT are widely used as an index of liver function. In this study, liver disease was induced in rats in the BDL groups, indcated by increased concentrations of total and conjugated bilirubin, AST and ALT in the BDL groups (Table 1). These results are in good agreement with those of a previous study involving the use of a rat model of cholestasis induced by BDL.[9] The total bilirubin concentration increased up to day 3 after BDL and remained high thereafter. Therefore, we defined the BDL1 group as the initial phase of cholestasis and the BDL3 and BDL5 groups as the chronic phase. These can be defined in a similar manner on the basis of changes in the concentrations of AST and ALT. Although the plasma concentrations of AST and ALT in the BDL1 group were elevated as liver injury progressed through the initial phase of cholestasis, plasma concentrations of AST and ALT were lower in the BDL3 and BDL5 groups, reflecting the chronic phase of liver injury.

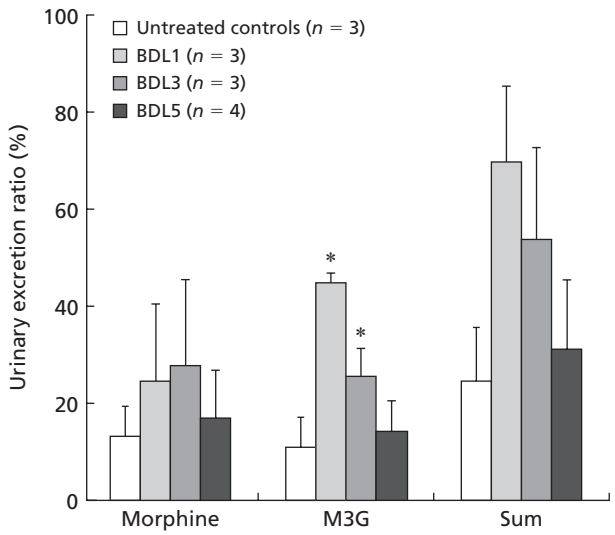

Figure 3 Urinary excretion ratios over 360 minutes for morphine and morphine-3-glucuronide. Results are expressed as a percentage in relation to the dose of morphine administered (15 mg/kg by intravenous infusion over 5 min). The sum values shown indicate the total excretion rate of morphine and morphine-3-glucuronide (M3G). BDL1, 3 and 5 are the groups 1, 3 and 5 days after bile duct ligation (BDL).

The increased expression of UGT2B1 and MRP3 mRNA in the BDL groups was in good agreement with reports of a previous study.[5] That study also reported the marked upregulation of MRP2 mRNA expression in rats with induced cholestasis, whereas expression of MRP2 mRNA remained unchanged in our study (Figure 1). This difference might be explained by different periods from BDL to the measurement of the MRP2 mRNA expression: Bohan and colleagues measured MRP2 mRNA expression 14 days after BDL surgery, whereas we measured it 1, 3 and 5 days after surgery. Thus, downregulation of MRP2 mRNA is slow, which is why it did not differ between the control and BDL groups in our study, but did decrease significantly in the study of Bohan and colleagues.

It is well established that the roles of MRP2 and MRP3 are complementary in hepatocytes. In cholestasis, the MRP2-mediated excretion of bilirubin into the bile is compromised; the expression of MRP3 is therefore increased vicariously to facilitate the excretion of bilirubin into the blood.[10] In our study, the expression of MRP3 mRNA increased, whereas that of MRP2 remained unchanged. We assume that the expression of MRP3 changes quickly to excrete bilirubin that accumulates regardless of the expression of MRP2, because we had completely cut the bile duct in the present study. We also consider that the increase in UGT2B1 mRNA expression in the BDL1 groups occurred for the same reason.

Elimination of morphine into the bile was inhibited in the BDL1 group, but the increase in its metabolism to M3G was predictable because expression of UGT2B1 mRNA increased (Figure 1b). Consequently, the plasma concentration of morphine decreased, and the plasma concentration–time profiles of morphine in the BDL1 group were only slightly different from those in the untreated control group (Figure 2a). In contrast, in the BDL3 and BDL5 groups, the metabolism of morphine to M3G can be expected to have decreased (Figure 1b).

There is also the possibility of deterioration in renal function in rats in the BDL groups, because the urinary volume decreased in all these rats compared with the control rats. We presume that renal function decreases during the chronic phase of cholestasis, because glucuronide conjugate and bilirubin, which are excreted into the bile under normal conditions, were continually excreted into the blood in the rats with cholestasis. These phenomena induced by BDL may have delayed the elimination of morphine and prolonged the elimination half-life of morphine in the BDL3 and BDL5 groups (Table 2).

The final plasma M3G concentration was significantly higher in the BDL3 and BDL5 groups than in the untreated control group (Figure 2b). This was in good agreement with a previous study using mice that lacked expression of MRP2.[4] The explanation may be as follows. The excretion of bilirubin into the bile via MRP2 was impaired in the BDL groups; as a compensatory response, expression of UGT2B1 increased vicariously to reduce the toxicity of the bilirubin accumulated in the hepatocytes via glucuronate conjugation, and expression of MRP3 increased to facilitate bilirubin excretion into the blood. As a result, the production of M3G and its transport from hepatocytes into the blood were increased in the BDL groups. In addition, the decrease in urinary excretion of M3G induced by the decline in renal function during the chronic phase of cholestasis may have contributed to the high M3G concentrations (Figure 3). However, the plasma concentration–time profiles of M3G in the BDL1 group showed an initial increase followed by a decrease thereafter. An increase in urinary excretion in the BDL1 group could be related to the mechanism of the plasma M3G concentration–time profiles in the BDL1. On Day 1 after BDL (i.e. during the initial phase of cholestasis), the blood concentration of M3G increased rapidly because of increased expression of UGT2B1 and MRP3. However, the plasma concentration of M3G in the BDL1 group was in the same range as that in the untreated group because of the increased urinary excretion of M3G.

Conclusions

We investigated the disposition of morphine and its metabolites in a rat model of cholestasis, focusing on the altered hepatic expression of transporters and metabolic enzymes. During cholestasis, the expression of MRP3 and UGT2B1 in the liver was enhanced, and these alterations caused a change in the disposition of M3G. Furthermore, we postulated that together with the alteration in hepatic function shown in this study, altered renal function during cholestasis contributed to the changes in disposition of these metabolites.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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