Comparative studies on bile flow and biliary lipid excretion after bile-acid loading in normal and partially hepatectomized rats

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This study was performed to investigate sequential changes in bile secretion and biliary lipids after taurocholic acid (TCA) loading of regenerating rat liver. TCA was administered intravenously at stepwise-increasing doses to groups of non-operated control and partially hepatectomized rats, 24, 72 and 168 h after surgery. Bile flow, bile-acid output (BAO) and phospholipid output (PLO) (expressed per gram of liver) in partially hepatectomized rats increased more than in the controls. Using an isolated perfusion rat-liver system, TCA infusion was also carried out on groups of non-operated control and hepatectomized rats

72 h after operation. Again bile flow, BAO and PLO (expressed per gram of liver) were significantly higher in the partial hepatectomy case, mirroring the results obtained *in vivo*. When horseradish peroxidase (HRP) was pulse-loaded in isolated perfusion preparations, the second peak of biliary HRP secretion in hepatectomized rats was significantly higher than in controls. We conclude that increased bile-acid flow in partially hepatectomized rats is dependent upon acceleration of vesicular transport accompanying or following proliferation in regenerating livers.

INTRODUCTION

The liver differs from most other organs in that it can withstand extensive necrosis or resection, as it has a large functional reserve capacity and regenerative ability. It is well known that in both experimental animals and man the liver mass is fully restored rapidly after partial hepatectomy (PH) [1,2]. In the rat, for example, following two-thirds hepatectomy, normal weight and volume are restored within 7–10 days [2]. Although liver functions also recover quickly with liver restoration, it appears that they do not necessarily correlate with regenerating liver size. From the point of cholestasis, it is of interest to understand changes in bile secretion, which is one of the most important functions of the liver, during hepatic regeneration after PH. The major driving force for bile flow is secretion of bile acids into bile canaliculi, with transport at the canalicular membrane being thought of as a rate-limiting step in transhepatocellular bile-acid transport.

Exogenous or endogenous bile-acid loading has been performed clinically as a liver-function test for chronic liver diseases. When bile acids are infused into the jugular vein in rats in a stepwise-increase fashion, bile flow and bile-acid output (BAO) into bile are gradually increased. However, above a certain dose, decreases in these two parameters occur [3–5] due to the fact that each bile acid has both primary choleric and secondary cholestatic effects. The mechanism of bile-acid passage across the liver cell from sinusoid to bile canaliculus is still a matter of controversy, but recent evidence suggests that, in addition to cytosolic binding-proteins, a vesicle pathway is involved [6–10].

In this study, in order to gain information on bile-acid transport ability in the regenerating rat liver after PH, we investigated bile flow, biliary excretion of bile acids and phospholipids following stepwise loading of taurocholic acid (TCA) *in vivo* and in an isolated liver perfusion system. The role of the vesicle transport system in the intrahepatocytic transport of bile

acid was also assessed using horseradish peroxidase (HRP) [11], a marker for microtubule-dependent vesicle transport.

MATERIALS AND METHODS

Animals and materials

Male Sprague–Dawley rats weighing 230–280 g (SLC, Shizuoka-jikken Doubutsu, Hamamatsu, Japan) were allowed free access to water and Purina rodent chow (Oriental Co., Tokyo, Japan) prior to experimental use. Animals were maintained in temperature-controlled rooms under a constant light cycle. Partial hepatectomies (70%) were performed under ether anesthesia according to the method of Higgins and Anderson [12]. The animals were allowed to recover with free access to water and food after the surgical procedure. A preliminary experiment was conducted to confirm that there is no difference between non-operated and sham-operated rats in both basal level and TCA loading-values for bile flow, BAO and phospholipid output (PLO). Therefore, non-operated rats were used as controls in all experiments.

Sodium taurocholate and purified HRP (Type II) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan) or Daiichi Chemicals (Tokyo, Japan). The study protocol was approved by the animal care committee of Nagoya City University.

Studies *in vivo*

Under pentobarbital anesthesia (50 mg/kg body weight, intraperitoneally), the right jugular vein was cannulated with PE-50 tubing (Clay Adams, Parsippany, NJ, U.S.A.) for infusions. After tracheal tubing with PE-240 tubing, a median laparotomy was performed and the common bile duct was cannulated with

Abbreviations used: TCA, taurocholic acid; BAO, bile-acid output; PLO, phospholipid output; HRP, horseradish peroxidase; PH, partial hepatectomy; IPRL, isolated perfused rat-liver.

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Table 1 Serum biochemical data during liver regeneration after partial hepatectomy in rats

Statistically significant difference from control value: * $P < 0.05$; ** $P < 0.01$. GPT, glutamate pyruvate transaminase; ALP, alkaline phosphatase.

Groups	Total bile acid ($\mu\text{mol/l}$)	Total bilirubin (mg/l)	GPT (Karmen units)	ALP (King Armstrong units)
Control	30.2 $\pm$ 5.9	0.5 $\pm$ 0.6	26.8 $\pm$ 4.8	27.8 $\pm$ 7.3
24 h after PH	147.8 $\pm$ 65.8*	3.0 $\pm$ 1.8*	1186.5 $\pm$ 603.6**	48.4 $\pm$ 18.8
72 h after PH	177.6 $\pm$ 101.5*	2.0 $\pm$ 1.4	118.3 $\pm$ 42.8**	48.0 $\pm$ 9.4*
168 h after PH	30.4 $\pm$ 14.1	0.3 $\pm$ 0.5	39.3 $\pm$ 4.5**	38.8 $\pm$ 12.8
336 h after PH	47.9 $\pm$ 19.4	0.8 $\pm$ 0.5	40.0 $\pm$ 2.2**	32.3 $\pm$ 5.0

PE-10 tubing. In order to make a buffered 50 mM TCA solution, TCA was dissolved in a mixture of the same volumes of 0.15 M NaCl and 0.1 M Na_2CO_3 [13]. This was adjusted to pH 8.3 with 5 M HCl and then sterilized by passage through a millipore filter (0.45 μm). Body temperature was maintained at 37.0–37.5 °C during the experiments, controlled using a heating table and lamp. Bile samples of non-operated controls and partially hepatectomized rats 24, 72 and 168 h after operation were collected every 10 min to measure bile flow, BAO and PLO. After collection of two initial bile samples, TCA loading was started through the jugular vein at a rate of 0.5 $\mu\text{mol/min}$ per 100 g body weight and increased stepwise by 0.5 $\mu\text{mol/min}$ per 100 g body weight every 20 min to a final rate of 2.0 $\mu\text{mol/min}$ per 100 g body weight.

Isolated perfused rat-liver (IPRL) studies

IPRL preparations were made from groups of non-operated control and hepatectomized rats 72 h after PH. While each rat was under pentobarbital anesthesia (50 mg/kg body weight, intraperitoneally), the common bile duct was cannulated with PE-10 tubing. The pancreaticoduodenal branch of the portal vein was then ligated and the portal vein was cannulated with a 14-gauge Teflon intravenous catheter (Critikon Inc., Tampa, FL, U.S.A.). The liver was perfused immediately at a constant flow rate of 20 ml/min with oxygenated (95% O_2 /5% CO_2) Krebs–Ringer bicarbonate, pH 7.4, transferred to a heated perfusion chamber after cannulation of the superior vena cava and then perfused in a single-pass system at a constant flow rate of 40 ml/min. TCA was administrated with a stepwise increase at rates of 1.0, 2.0 and 3.0 $\mu\text{mol/min}$ for 20 min to non-operated control and 0.67, 1.33 and 2.00 $\mu\text{mol/min}$ to partially hepatectomized rats, 72 h after surgery (almost the same infusion dose per gram of liver for two groups). Bile samples were collected every 5 min to measure bile flow, BAO and PLO. In addition, to assess the involvement of the vesicular transport system in bile secretion by the regenerated liver, HRP (25 mg) was pulse-loaded in a single-pass system perfused with Krebs–Ringer bicarbonate buffer to groups of non-operated control, and hepatectomized rats 72 and 168 h after PH. Ten bile samples were collected every 2 min after HRP injection and then a further ten bile samples were collected at 5 min intervals.

Analytical methods

The volume of bile was determined by weight, assuming a density of 1.0 g/ml, and bile-flow was expressed as $\mu\text{l/min}$ per gram of liver weight. Total phospholipids were assayed using kits supplied by Wako Pure Chemical Industries (Osaka, Japan). Serum and biliary total bile acids were measured by enzymic assay using

Enzabale 2 (Daiichi Pure Chemicals, Tokyo, Japan). The concentrations of HRP in bile were determined spectrophotometrically by measuring the rate of oxidation of 4-aminoantipyrine at 510 nm as described previously [14].

Statistical analysis

Data from the physiological experiments are presented as arithmetic means $\pm$ S.E. and were compared at individual time-points. In the HRP experiments, the area under the HRP secretion curve was calculated as total HRP output. Because biliary excretion of HRP appeared in two peaks that were separated by approximately 10 min after pulse loading, the area under the first peak was defined as 0–10 min, and the area under the second peak was defined as 10–50 min. The significance of differences between means was determined using Student's *t* test.

RESULTS

Serum biochemical changes after PH

Total serum bilirubin and alkaline phosphatase (ALP) activity showed a slight increase after PH but total serum bile acids and glutamate pyruvate transaminase activity at the 24 and 72 h time-points were appreciably increased as compared with non-operated control values, returning to near the normal after 168 h (Table 1).

Bile flow, BAO, PLO and the PLO:BAO molar ratio after step-wise increase in TCA loading *in vivo*

Bile flow and bile-acid excretion into bile in hepatectomized rats were significantly higher than respective values in non-operated controls before and after TCA infusion, 24, 72 and 168 h after PH, decreasing with time (Figures 1a and 1b). PLO into bile also increased in hepatectomized rats, and the peak value 72 h after PH was approximately 245% of that of control rats (Figure 1c). Bile-flow and PLO in control livers began to decrease at the 30 min time-point when 0.5 $\mu\text{mol/min}$ per 100 g body weight of TCA was infused, but in livers investigated 24 and 72 h after PH, showing higher values before TCA infusion, the increase was demonstrated to continue till the 50 min time-point (Figures 1a and 1c). A plateau of BAO into bile was attained in all groups when infusion rates of TCA exceeded 1.0 $\mu\text{mol/min}$ per 100 g body weight. Maximum secretion of TCA 24–168 h after PH was significantly higher than that in non-operated controls (Figure 1b). The mean PLO:BAO molar ratio 72 h after PH also showed

Figure 1 Effects of TCA administration on bile flow (a), BAO (b) and PLO (c) during liver regeneration 24 (▲), 72 (■) and 168 h (●) after PH *in vivo*

TCA was infused at rates of 0–2.0 $\mu\text{mol}/\text{min}$ per 100 g body weight (b.wt.). Bile flow and PLO 24 and 72 h after PH were significantly higher than in controls (○). Maximum secretory rates of TCA 24, 72 and 168 h after PH were also increased. Values for each time-point represent the means $\pm$ S.E. of data for five different rats.

a significant increase compared with control values. However, the levels at 24 and 168 h after PH were not different from those of controls (Table 2). Bile flow, PLO and the PLO:BAO molar

Figure 2 Effects of TCA administration on bile flow, BAO and PLO in livers from non-operated control rats (a) and partially hepatectomized rats 72 h after surgery (b) in the isolated liver perfusion system

TCA was infused with a stepwise increase 1.0, 2.0 and 3.0 $\mu\text{mol}/\text{min}$ for 20 min to non-operated control and 0.67, 1.33 and 2.00 $\mu\text{mol}/\text{min}$ to partially hepatectomized rats (almost the same infusion dose per gram of liver for two groups). Bile flow and PLO of the hepatectomized rats before and after TCA administration were significantly higher than those of the controls and BAO of the hepatectomized rats increased more after TCA infusion at rates of $> 1.33 \mu\text{mol}/\text{min}$. Values for each time-point represent the means $\pm$ S.E. of data for five different rats.

ratio during TCA loading at 336 h after PH were essentially the same as in controls (results not shown).

Bile flow, BAO, PLO and the PLO:BAO molar ratio after step-wise increase in TCA loading in IPRL

Before and after TCA administration, bile flow and PLO 72 h after PH, as expressed per gram of liver, were significantly higher than the control levels. After TCA loading, bile flow 72 h after PH increased to 130–340% of the control value and PLO increased approximately 3.4–4.7-fold (Figures 2a and 2b). Thirty

Table 2 Effects of TCA administration on the mean molar ratio of PLO:BAO during liver regeneration 24, 72 and 168 h after PH *in vivo*

Each value represents the mean $\pm$ S.E. of data for five different rats. Statistically significant difference from control value: ** $P < 0.01$.

Group	Control	24 h after PH	72 h after PH	168 h after PH
Mean PLO:BAO molar ratio ($\times 10^{-2}$)	6.24 $\pm$ 0.71	6.57 $\pm$ 1.07	9.71 $\pm$ 0.76**	6.86 $\pm$ 0.15

Figure 3 Effects of liver regeneration after partial hepatectomy on the biliary excretion of HRP after a pulse-loading of HRP (25 mg)

The first peak of HRP excretion 72 h after PH was markedly increased and the second peak of HRP excretion 72 and 168 h after PH was also significantly higher than the control level. Values for each time-point represent the means $\pm$ S.E. of data for 3–5 different rats. Statistically significant difference from control value: * $P < 0.05$.

minutes after the start of TCA infusion, increasing the infusion rate of TCA inhibited the TCA-induced increase in bile flow, BAO and PLO in control livers (Figure 2a). However, this was not the case with hepatectomized rat livers, both BAO and PLO continuing to rise until nearly the end of the experiments (Figure 2b). When TCA was infused with a stepwise increase, mean values for the PLO:BAO molar ratio 72 h after PH were also significantly elevated over the control level. Thus, in both *in vivo* and IPRL cases, bile flow, BAO, PLO and PLO:BAO molar ratio during TCA infusion 72 h after PH were increased similarly.

Biliary excretion of HRP after pulse-loading with HRP in IPRL

When HRP (25 mg) was infused for 1 min as a pulse to IPRL from non-operated control and hepatectomized rats 72 and 168 h after PH, a pattern of a first peak within 6–8 min and a second peak within 20–25 min was observed in each case (Figure 3). For livers taken 72 h after PH, marked increases in both peaks were apparent. The first peak value for 168 h after PH was not significantly different from that in controls, being if anything slightly lower, but the second peak value demonstrated a significant increase. The area under the HRP excretion curve, calculated as total HRP output, for the first peak (defined as 0–10 min) was 5.87 ± 1.38 ng/g of liver at 72 h after PH, more than 3.5-fold that in controls (1.66 ± 0.30). In contrast, at 168 h after surgery, the area under the first peak was significantly decreased although that under the second peak was still elevated. Values of the area under the second peaks were 8.18 ± 0.45 , 17.48 ± 3.18 , 12.83 ± 3.23 ng/g of liver, for control and hepatectomized rat livers 72 and 168 h after PH, respectively.

DISCUSSION

The present experiments demonstrate that, in contrast to the cholestasis observed at an early stage when TCA is infused in a stepwise fashion to control rats *in vivo*, hepatectomized rats, especially 24 and 72 h after PH, are resistant to TCA-associated cholestatic effects, with marked relative increases in BAO and PLO. The increase of the PLO:BAO molar ratio apparent for 72 h after PH suggests that the bile canaliculi membrane might be involved. This is in line with the consideration that acceleration

of PLO without cholestasis implies a sufficient resupply of phospholipid vesicles to the bile canaliculi membrane [15].

The maximum secretion rate [5] of TCA was clearly increased in the present hepatectomized rats. Although the mechanisms by which regenerated or regenerating livers can transfer larger amounts of bile acids have not been defined, cellular changes following or accompanying cell division are presumably involved. It is generally recognized that hepatocytes in zone I, the area near the portal vein, deal with bile-acid dependent flow and hepatocytes in zone III, around the central vein deal with bile-acid independent flow in the intact liver [16]. In the regenerating liver, mitosis in zone I is thought to occur earlier than in the other areas [17,18]. Thus, almost all of the parenchymal cells in zone I should have already finished mitosis 30 to 48 h after PH and the percentage of hepatocytes dealing with bile-acid transport therefore increases. This physiological phenomenon might result in an increase in the maximum secretory rate of TCA. A second possibility is that differences in roles between zones I and III, may tend to disappear with proliferation so that almost all hepatocytes become capable of dealing with bile-acid dependent flow, namely bile-acid transport, and this would naturally be reflected in increased TCA transport.

It is known that hepatocyte swelling occurs in regenerating livers after PH [19]. Häussinger et al. reported that liver cell-volume was one important factor determining bile flow and biliary taurocholate excretion, and that cell swelling increased the V_{max} of transcellular taurocholate transport in perfused rat liver [20]. Canaliculi excretion of bile acids is the rate-limiting step for their overall transport from blood to bile, and recent studies [21,22] have indicated that ATP-dependent canaliculi transporters play an important role. Häussinger et al. [23] have hypothesized that the swelling-induced stimulation of taurocholate excretion into bile is due to a microtubule-dependent insertion of bile-acid transporter molecules into the canaliculi membrane.

The observed acceleration of BAO accompanied by an increase in PLO, furthermore, leads us to consider the following: larger amounts of TCA can be transported despite detergent effects of the bile acid on bile canaliculi membranes, since phospholipid vesicles from the endoplasmic reticulum are sufficiently resupplied. In other words, even if phospholipids are lost due to the TCA loading, the presence of a sufficient resupply would prevent cell damage. It is well known that production of cholestasis and liver dysfunction by hydrophobic bile acids reflects disruption of hepatocellular membranes and, possibly, the canaliculi membrane in particular [24].

Houweling et al. [25] reported that biosynthesis of phosphatidylcholine, a major structural phospholipid in biomembranes, is accelerated after PH because uptake of [3 H]choline to phosphatidylcholine is increased and intrahepatic CTP:phosphocholine cytidyltransferase, which is the rate-limiting enzyme of phosphatidylcholine biosynthesis, is induced at 48–96 h. Furthermore, Evans [26] mentioned vesicles of large diameter in the pericanaliculi plasma-membrane vicinity during compensatory regeneration in partially hepatectomized animals. These findings both suggest increased functional capacity in terms of bile flow.

Total biliary cholesterol output was not changed during the regeneration phase in the present study, so the total cholesterol output:PLO molar ratio (results not shown) decreased from 24 to 72 h after PH compared with control rats, especially at the 72 h time-point. Collard et al. [27] reported that total cholesterol:phospholipid molar ratios in plasma membranes of hepatocytes 24 h after PH was 0.38–0.47, whereas this ratio in non-operated rats was 0.60–0.71, and they demonstrated a possibly associated increase in membrane fluidity.

Since the increase of bile flow, BAO and PLO after TCA loading was observed not only *in vivo* but also in the IPRL study, the underlying mechanisms are not dependent on hormonal factors from organs other than the liver. To examine whether the resupply of phospholipid vesicles played a pivotal role the rate of vesicular transport was assessed. In regenerative livers, biliary excretion of HRP, a fluid-phase marker that has been used extensively in IPRL to label the transcytotic vesicle pathway of hepatocytes [28], was clearly accelerated in hepatectomized rats 72 and 168 h after PH. Both the early peak, generally thought to represent paracellular movement and/or a rapid transcellular microtubule-independent vesicle pathway, and the larger later peak that represents microtubule-dependent vesicle movement from the sinusoidal membrane to the canalicular domain [14,29,30], were affected. This result thus provides direct evidence for elevated activity of the microtubule-dependent vesicular transport system.

Bile acids are normally thought to be transported within the cytoplasm in association with bile acid-binding proteins. However, some reported findings and our previous data [31,32] have suggested that the vesicular pathway may be more important for intrahepatocytic transport of more hydrophobic bile acids and/or in cases of greater loading. While there remains a possibility that the acceleration of vesicular transport implies an increased transportation of vesicles containing bile-acid transporters rather than bile acid itself [23], the present results for HRP transport are interesting in terms of the higher capacity for TCA transport after PH. Moreover, the fact that the first peak of HRP excretion 72 h after PH was markedly elevated indicates an increase in the paracellular pathway, which might be in line with the reported reduction of adhesion molecules [33] or gap junction proteins in plasma membranes [34] during regeneration. Poucell et al. [35] has stated, on the basis of freeze-fracture examinations, that tight junctional strand counts, density and orientation parallel to canaliculi, were all reduced 24 h after PH.

In conclusion, the observed marked increases in bile flow and BAO after TCA loading of livers 24 to 168 h after PH, along with elevated PLO and second-peak biliary HRP excretion values suggested an acceleration of vesicle-associated transport.

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