

Microtubule-independent choleresis and anti-cholestatic action of tauroursodeoxycholate in colchicine-treated rat liver

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In order to cast light on the anti-cholestatic and cytoprotective properties of ursodeoxycholic acid (UDCA), intrahepatic transport and secretion of bile salts and biliary phospholipids were investigated by using isolated perfused livers from colchicine-pretreated rats. Administration of taurocholic acid (TCA) after colchicine pretreatment induced marked cholestasis. Tauroursodeoxycholic acid (TUDCA) treatment, in contrast, was associated with maintenance of bile flow, with excretion rates of bile acids and phospholipids similar to those in control animals. Furthermore, TCA-induced cholestasis in colchicine-treated rat livers was clearly decreased by co-administration of TUDCA. Although simultaneous addition of UDCA also showed slight improvement, with or without taurine pre-treatment, biliary bile-salt analysis also showed that cholestasis was markedly remitted as the excretion of taurine-conjugated UDCA was increased. The results suggest that the cytoprotective and anti-cholestatic effects of TUDCA may be linked to action at the intrahepatocyte level, represented by mild detergent effects on organelle lipids and preservation of intracellular transport even under microtubule-dysfunctional conditions. In addition, it was indicated that cytoprotective effects of UDCA may also be exerted after its conjugation with taurine inside hepatocytes.

INTRODUCTION

Ursodeoxycholic acid (UDCA) has been clinically utilized for treatment of primary biliary cirrhosis, drug-induced intrahepatic cholestasis, and chronic hepatitis [1–3]. Investigation of UDCA or its taurine conjugate (TUDCA), by using animal experimental models of intrahepatic cholestasis, has shown these compounds to improve cholestasis and to exert cytoprotective actions on hepatocytes [4–6]. It is particularly noteworthy that, although taurocholic acid (TCA) and taurochenodeoxycholic acid (TCDC), which are predominant in man and animals, induce cholestasis by themselves when they are administered at an excessive dose or to cases with hepatic disorders, UDCA can improve cholestasis. However, the exact mechanism and site of action of the toxicity of TCA or TCDC remain unknown. Moreover, it is also unclear why and how another bile acid, TUDCA, should decrease this toxicity. In the present investigation, isolated perfused rat livers after treatment with colchicine, an inhibitor of microtubule polymerization, were utilized to assess how cytotoxic and cytoprotective actions differ in association with intracellular interaction of bile acids and lipids.

MATERIALS AND METHODS

Materials

SD-strain male rats (8 weeks old and 240–280 g body wt.) were used. At 3 h before the start of perfusion of isolated liver preparations, 0.2 mg of colchicine/100 g body wt. was administered by intravenous injection to each rat. Control rats received no colchicine. Solid food and drinking water were given freely, and the animals were fasted for 12–18 h before experiments. TCA, UDCA and TUDCA were gifts from Tokyo Tanabe Pharmaceutical Co., Tokyo, Japan. Colchicine was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and taurine was from Wako Pure Chemicals Co., Tokyo, Japan. BSA was the powder fraction A from Armour Pharmaceutical Co., New York, NY, U.S.A.

Isolated liver preparation

Laparotomy was performed under ether anaesthesia, a PE-10 tube was inserted into the common bile duct, and the oesophagus and the pancreatico-duodenal vein were ligated. Heparin sodium (1000 units) was injected into the inferior vena cava; then a plastic tube (1.5 mm internal diam.) was inserted into the portal vein, and infusion of 500 ml of perfusate was started immediately. The liver itself was then isolated and transferred to the perfusion apparatus. The initial perfusion of up to 300 ml was done with a non-circulating system. Thereafter a circulating system was applied.

The perfusion system used was based on Miller's apparatus with a total of 200 ml of recirculating Krebs–Ringer bicarbonate buffer perfusate supplemented with 240 mg of glucose/dl and 0.83 g of BSA/dl and saturated with O₂/CO₂ (19:1). The inside of the perfusion apparatus was maintained at 37–38 °C. The pressure of perfusate flowing into the liver was kept at 16 cmH₂O, and the outflow from the portal vein was maintained at 60–70 ml/min.

Individual bile salts were added 45 min after the start of the perfusion. TCA or TUDCA (each 36 μmol) was initially assessed in terms of choleric action on normal and colchicine-treated livers. In addition, TCA was added in combination with UDCA or TUDCA (in each case 36 μmol), and their effects were similarly studied. To examine the significance of taurine conjugation, 360 μmol of taurine was first added 30 min after the start of perfusion, then UDCA and TCA (both 36 μmol) were added simultaneously 15 min later. Bile samples were collected at intervals of 5 min or 10 min after the start of bile-salt addition. The weight of secreted bile was measured, and the bile flow was expressed as μl/h per g of liver, taking the specific density of bile to be 1.0 g/ml.

Assay of bile salts

Total bile acid levels were assayed by an enzymic method using an Enzabile Kit (Dai-ichi Chemicals Co., Tokyo, Japan).

Abbreviations used: UDCA, ursodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; Tβ-MCA, tauro-β-muricholic acid; TCDC, taurochenodeoxycholic acid; TCA, taurocholic acid; GOT, glutamate-oxaloacetate transaminase.

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To examine changes with time in bile-salt composition, bile samples at 0–5, 10–15 and 30–35 min after bile-salt addition were used for assay by a combined approach using h.p.l.c. and immobilized 3α -hydroxysteroid dehydrogenase in column form [7]. Each bile sample was diluted 100-fold with methanol, then centrifuged for 10 min at 2000 *g*, and a 10 μ l portion of the supernatant was taken for assay. A total of 18 compounds, namely UDCA, cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid and β -muricholic acid, and their taurine- and glycine-conjugated forms, all received as gifts from Tokyo Tanabe Pharmaceutical Co., were used as standard bile salts.

Assay of phospholipids

Phospholipids in bile samples were assayed by an enzymic method using the Phospholipids C-test (Wako Pure Chemicals Co.).

Enzyme activities in bile and perfusate samples

To determine the release of liver enzymes into the perfusate, samples were collected 5, 30 and 60 min after bile-acid addition, and the glutamate-oxaloacetate transaminase (GOT) activity was measured by the GOT-UV Test (Wako). To determine the excretion of biliary enzymes into bile, bile samples were collected over 30 min from the addition of bile salt. Activities of γ -glutamyl transpeptidase were assayed with a Hitachi autoanalyser (Japan) and expressed as units/ μ mol of bile acid output.

Statistics

All data were statistically analysed by Student's *t* test, values of $P < 0.05$ or $P < 0.01$ being regarded as significant.

RESULTS

Choleretic actions of individual bile salts in normal and colchicine-treated livers

(1) **Groups receiving TCA alone.** In the control group, the bile flow, bile acid output and phospholipid output were rapidly increased by TCA addition, to reach in 10 min peak values of $183.0 \pm 18.7 \mu\text{l/h}$ per g of liver, $4.61 \pm 0.82 \mu\text{mol/h}$ per g of liver and $(32.3 \pm 11.7) \times 10^{-2} \text{mg/h}$ per g of liver respectively, and then decreased gradually to their basal levels (see Fig. 1). In the colchicine-treated group, on the other hand, the bile flow was increased only slightly up to a peak value of $69.8 \pm 13.3 \mu\text{l/h}$ per g of liver immediately after TCA addition, and then markedly declined after 10 min to a value significantly ($P < 0.01$) lower than that of the control group, indicating a cholestatic state. The bile acid output and the phospholipid outputs were also decreased to significantly ($P < 0.01$) lower levels than those in the control group, their peak values being $0.73 \pm 0.38 \mu\text{mol/h}$ per g of liver and $(8.25 \pm 1.74) \times 10^{-2} \text{mg/h}$ per g of liver respectively.

(2) **Groups receiving TUDCA alone.** The bile flow, bile acid output and phospholipid output were rapidly increased immediately after TUDCA addition in the control group to peak values of $157.3 \pm 60.9 \mu\text{l/h}$ per g of liver, $3.46 \pm 1.44 \mu\text{mol/h}$ per g of liver and $(34.6 \pm 12.7) \times 10^{-2} \text{mg/h}$ per g of liver respectively after 10 min, thereafter returning gradually to their basal levels within 40 min (see Fig. 2). In the colchicine-treated group, the bile flow, bile acid output and phospholipid output were similarly increased by TUDCA addition to peak values of $137.0 \pm 38.3 \mu\text{l/h}$ per g of liver, $3.26 \pm 1.45 \mu\text{mol/h}$ per g of liver and $(24.9 \pm 11.1) \times 10^{-2} \text{mg/h}$ per g of liver respectively, with subsequent decrease to their basal levels by the end of the experiment.

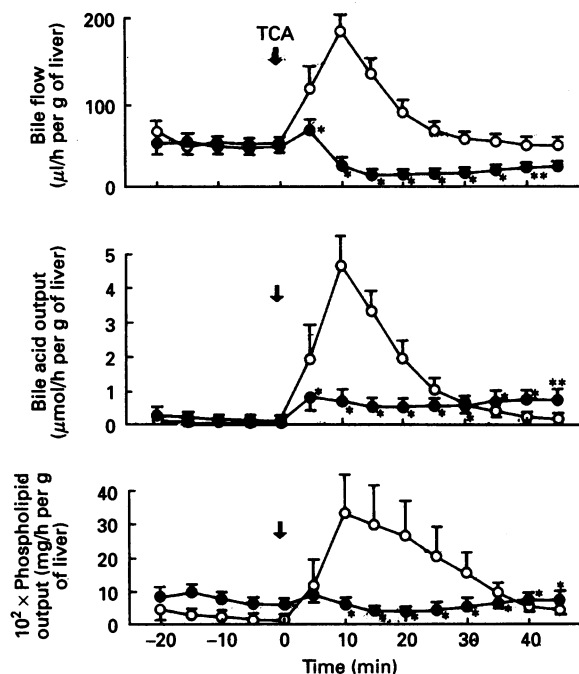


Fig. 1. Sequential changes in bile flow rate (upper panel), bile acid excretion rate (middle panel) and phospholipid excretion rate (lower panel) after addition of 36 μmol of TCA to control (○) or colchicine-treated (●) rats

Results are expressed as means \pm s.d. from 5 experiments, with indication of significant differences at ** $P < 0.05$ or * $P < 0.01$ versus control rat values.

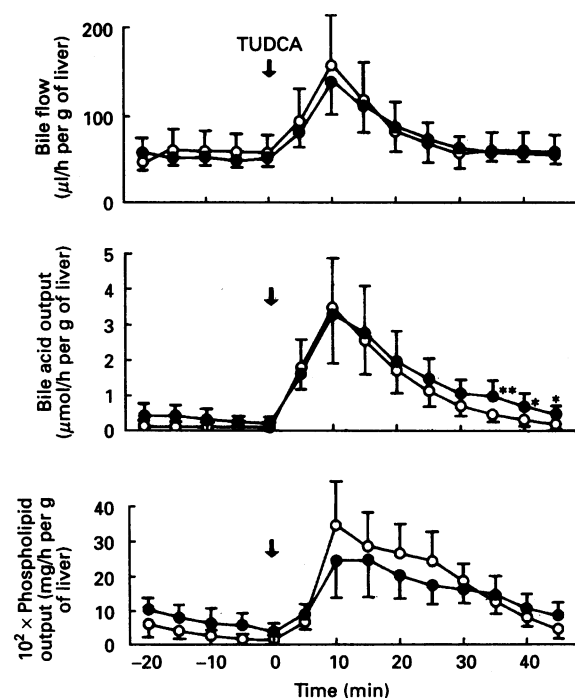


Fig. 2. Sequential changes in bile flow rate (upper panel), bile acid excretion rate (middle panel) and phospholipid secretion rate (lower panel) after addition of 36 μmol of TUDCA to control (○) or colchicine-treated (●) rats

Results are expressed as means \pm s.d. from 5 experiments, with indication of significant differences at ** $P < 0.05$ or * $P < 0.01$ versus control rat values.

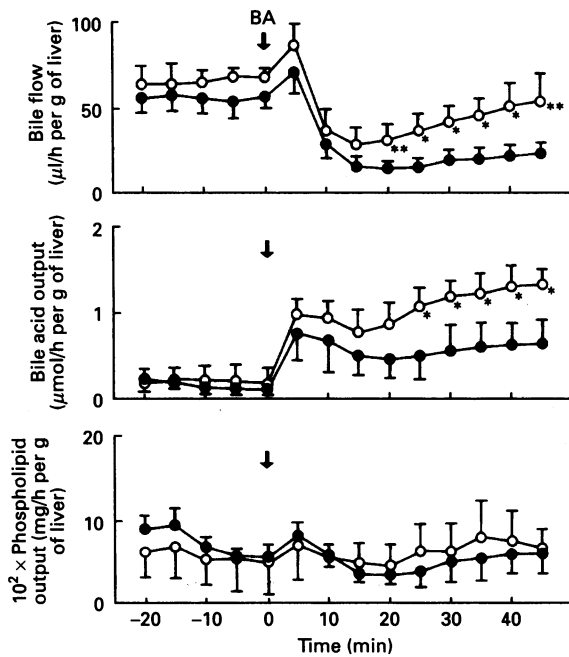


Fig. 3. Sequential changes in bile flow rate (upper panel), bile acid excretion rate (middle panel) and phospholipid secretion rate (lower panel) after addition of bile acids (BA: TCA + UDCA, ○; TCA alone, ●) each at 36 μmol to control or colchicine-treated rats

Results are expressed as means ± S.D. from 5 experiments, with indication of significant differences at ***P* < 0.05 or **P* < 0.01 versus TCA-alone values.

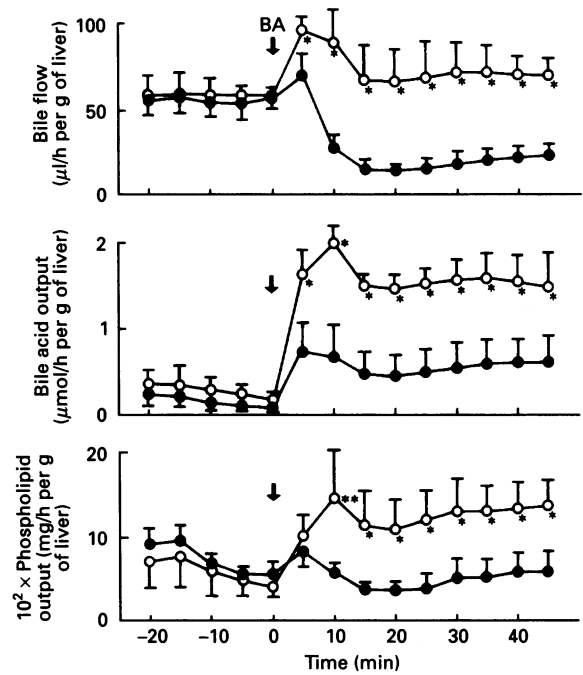


Fig. 5. Sequential changes in bile flow rate (upper panel), bile acid excretion rate (middle panel) and phospholipid secretion rate (lower panel) after addition of bile acids (BA: TCA alone, ●; TCA + TUDCA; ○) each at 36 μmol to colchicine-treated rats

Results are expressed as means ± S.D. from 5 experiments, with indication of significant differences at ***P* < 0.05 or **P* < 0.01 levels versus control rat values.

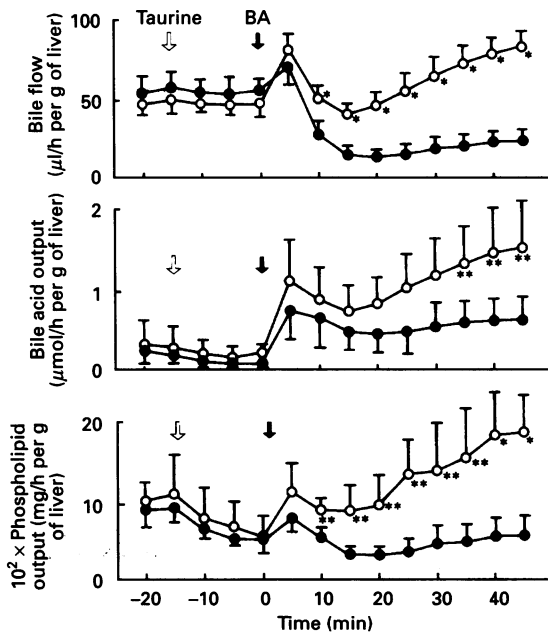


Fig. 4. Sequential changes in bile flow rate (upper panel), bile acid excretion rate (middle panel) and phospholipid secretion rate (lower panel) after addition of bile acids (BA: TCA alone, ●; TCA with UDCA after 15 min pre-perfusion after administration of 360 μmol of taurine, ○) each at 36 μmol to colchicine-treated rats after taurine pretreatment

White and black arrows respectively indicate addition of taurine and bile acids. Results are expressed as means ± S.D. from 5 experiments, with indication of significant differences at ***P* < 0.05 or **P* < 0.01 versus control rat values.

Effects of UDCA and TUDCA on TCA-induced choleresis in colchicine-treated liver

(1) **Simultaneous addition of UDCA and TCA.** When UDCA and TCA were simultaneously added to colchicine-treated livers, bile flow first slightly increased, and then decreased rapidly, with a gradual return towards basal levels thereafter (see Fig. 3). The peak value of the bile acid excretion rate was $86.7 \pm 12.3 \mu\text{mol/h per g of liver}$, which was higher than the basal level. Both bile flow and bile acid excretion rate were significantly greater in the group receiving UDCA and TCA than in the group receiving TCA alone, whereas the phospholipid excretion rate was not changed.

(2) **Simultaneous addition of UDCA and TCA after pre-perfusion of taurine-rich buffer.** For 20 min of perfusion after taurine administration, little change was noted for bile flow, bile acid output or phospholipid output (see Fig. 4). When UDCA and TCA were simultaneously added, however, the bile flow, bile acid excretion rate and phospholipid excretion rate immediately increased, then began to decrease, but began increasing again after 20 min to reach peak values of $80.0 \pm 9.7 \mu\text{l/h per g of liver}$, $1.47 \pm 0.6 \mu\text{mol/h per g of liver}$ and $(18.3 \pm 4.7) \times 10^{-2} \text{ mg/h per g of liver}$ respectively, after 45 min. These values were significantly higher, not only than those in the TCA-alone control group (*P* < 0.01) but also than their basal values (*P* < 0.05). Thus simultaneous addition of UDCA and TCA after taurine pre-treatment prevented the development of choleresis and was associated with choleresis.

(3) **Simultaneous addition of TUDCA and TCA.** The bile flow, bile acid excretion rate and phospholipid excretion rate were increased by simultaneous addition of TUDCA and TCA to peak values of $96.2 \pm 6.4 \mu\text{l/h per g of liver}$, $19.2 \pm 0.21 \mu\text{mol/h per g of liver}$ and $(14.5 \pm 5.8) \times 10^{-2} \text{ mg/h per g of liver}$ respectively (see Fig. 5). Although they then decreased to some extent, all values exceeded basal levels throughout the exper-

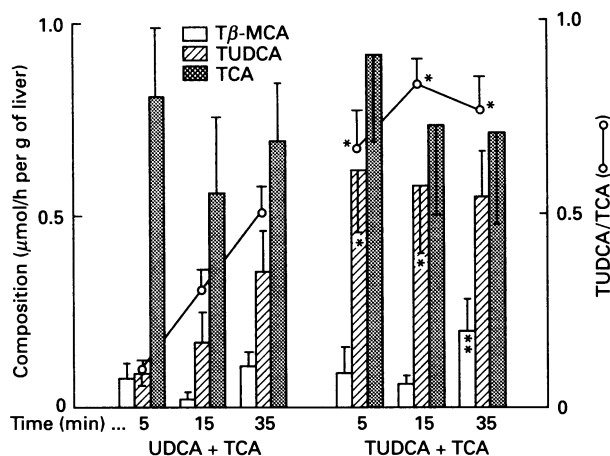


Fig. 6. Bile acid composition in 5 min samples collected 5, 15 or 35 min after administration of various bile acids to colchicine-treated rats (left, TCA + UDCA; right, TCA + TUDCA)

Asterisks indicate significant differences at $**P < 0.05$ or $*P < 0.01$ versus TCA + UDCA respectively.

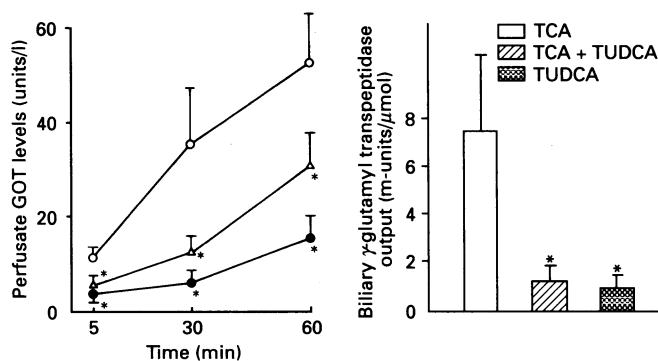


Fig. 7. (a) Changes in perfusate GOT in samples collected 5, 30 and 60 min after administration of various bile acids at $36 \mu\text{mol}$ to colchicine-treated rats (\circ ; TCA alone; \bullet , TCA + TUDCA; \triangle , TUDCA alone); (b) biliary enzyme output over 30 min after addition of bile acids each at $36 \mu\text{mol}$ to colchicine-treated rats

(a) Results are expressed as means \pm S.D. from 5 experiments, with indication of significant differences at $*P < 0.01$ versus TCA-alone values. (b) Results are expressed in m-units/ μmol of bile acid output, as means \pm S.D. from 5 experiments, with indication of significant differences at $*P < 0.01$ versus TCA-alone values.

imental period. Hence the development of cholestasis could be significantly prevented ($P < 0.01$).

Biliary bile acid composition (colchicine-treated livers)

The percentage values for TUDCA in the group receiving TUDCA and TCA were 37% or more in the 5, 15 and 35 min fractions, these also being significantly ($P < 0.01$) greater than those in the UDCA plus TCA group. Absolute amounts of TUDCA excreted in the 5 and 15 min fractions in the group to which TUDCA and TCA were simultaneously added were about $0.5 \mu\text{mol/h}$ per g of liver in each case, significantly ($P < 0.01$) greater than those in the UDCA plus TCA group ($0.09 \pm 0.03 \text{ mmol/h}$ per g of liver and $0.12 \pm 0.08 \mu\text{mol/h}$ per g of liver respectively). Percentage composition values for UDCA and glyoursodeoxycholic acid were very small (results not shown). The amount of tauro- β -muricholic acid (T β -MCA) in the 35 min fraction of the group receiving TUDCA and TCA simultaneously was significantly ($P < 0.05$) higher than in the UDCA plus TCA group (see Fig. 6).

In the group to which UDCA and TCA were simultaneously applied after taurine pretreatment, the TUDCA component of total bile acids was significantly increased in the 15 min and 35 min fractions. The values were only slightly less than those in the TUDCA plus TCA group. A significant increase in T β -MCA in the 35 min fraction was also observed (results not shown in Fig. 6).

Changes in GOT in perfusates after addition of each bile acid (colchicine-treated livers)

After TCA administration, the mean GOT level in perfusates was markedly increased from 11.1 ± 1.9 units/l (5 min) to 35.0 ± 12.0 units/l (30 min) and to 52.2 ± 9.9 units/l (60 min) (see Fig. 7a). In contrast, after TUDCA loading only slight increases, namely from 3.7 ± 1.6 units/l (5 min) to 6.1 ± 2.8 units/l (30 min) and to 15.4 ± 4.6 units/l (60 min), were evident, all these values being significantly lower than those in the TCA group ($P < 0.01$). After simultaneous administration of TUDCA and TCA, the values were 6.1 ± 1.5 units/l (5 min), 12.8 ± 2.9 units/l (30 min) and 31.0 ± 6.0 units/l (60 min), which were also significantly ($P < 0.01$) smaller than those in the TCA alone group.

Biliary enzyme output relative to bile acid output (colchicine-treated livers)

Amounts of γ -glutamyl transpeptidase excreted into the bile over 30 min after administration of TUDCA or TUDCA plus TCA were 0.97 ± 0.61 m-units/ μmol and 1.19 ± 0.42 m-units/ μmol respectively, significantly ($P < 0.01$) lower than that in the TCA group (7.61 ± 3.06 m-units/ μmol) (Fig. 7b).

DISCUSSION

Colchicine is well known to exert inhibition effects on microtubule formation [8,9]. It is also reported that colchicine blocks secretion into the blood of albumin [10], fibrinogen [11] and clotting factors 5 and 7 [12], all synthesized in hepatocytes, and the transport of dimeric IgA to the biliary canaliculus [13–15]. In general, biliary lipid precursors are thought to originate from the endoplasmic reticulum of hepatocytes [16–18] and are transported via the microtubular system. Biliary lipid secretion is therefore also influenced by colchicine [19–22]. The driving force for lipid transport is provided by the so-called micelle-forming bile acids, such as TCA and TCDCA, and not taurodehydrocholate.

Barnwell and colleagues [20,21] first showed marked cholestasis after TCA administration to colchicine-treated rat liver, and speculatively proposed a cholestatic mechanism involving initial excretion of TCA into the bile canaliculus lumen, where it exerts a detergent action on bile-canaliculus membranes drawing phospholipids from the membranes into the bile. However, because of colchicine-induced dysfunction of microtubules, the bile canaliculus would receive an insufficient supply of phospholipid-containing vesicles to repair the canaliculus membrane, and thereby cholestasis would be the result. They thus hypothesized that this continuous transport of vesicles was needed to maintain the integral function of bile-canaliculus membranes [20,21].

In our colchicine-treated rat livers, the bile flow, bile acid excretion rate and phospholipid excretion rate were found to be transiently increased in the 5 min fraction (immediately after addition of TCA), then declining markedly as cholestasis was induced. This TCA-induced cholestasis in colchicine-treated rat livers can also be explained well by the above hypothesis. In contrast, in the case of TUDCA administration, the bile secretion, including phospholipid excretion, in colchicine-treated rat livers was maintained at levels similar to those in control livers,

indicating an independence from colchicine-impaired microtubule function. Since, as shown in our control liver preparation, TUDCA causes choleresis, this bile acid belongs to the so-called micelle-forming bile acid group, similar to TCA or TCDCA but not taurodehydrocholate. According to the hypothesis of Barnwell [20], the application of TUDCA to colchicine-treated rat livers should disturb microtubule-dependent vesicular transport of phospholipids and should induce cholestasis. However, this was clearly not the case in the present experiment.

Although the reason why TUDCA sustains normal choleresis even under conditions of microtubule dysfunction is not clear, possible mechanisms can be discussed in view of the action of bile acids on intracellular formation and transport of biliary lipid precursors. The action of detergent of TUDCA on organelle lipids may be more mild than that of TCA, resulting either in vesicles whose nature is appropriate for intracellular transport under this condition or in a beneficial decrease in toxicity, preserving canalicular membrane function [23].

The most remarkable preventive effect observed in the present experiment concerned simultaneous administration of TUDCA with TCA. However, also in the case of UDCA with and without taurine pre-treatment, slight and delayed improvement of the cholestasis was evident, as we have preliminarily shown [24]. The analytical data for biliary bile acids demonstrated that, although biliary TUDCA levels were high from the starting fraction in the TUDCA-administration group, in the other two groups the initial level was very low, with a subsequent increase over time as TUDCA output increased. The unconjugated UDCA administered may have been conjugated during the first 20–30 min, then revealing the anti-cholestatic action. A parallel rise in T β -MCA excretion in each group was also observed, indicating that this bile salt might exert effects similar to those of TUDCA. In fact, we have reported previously that T β -MCA demonstrates characteristic choleric and hepatocyte protective actions in the same model [25]. Cytoprotective effects of TUDCA were also conspicuous from the data regarding excretion of γ -glutamyl transpeptidase into the bile or release of GOT into the perfusate.

One of the possible mechanisms underlying the cytoprotective effects of TUDCA may be linked to its characteristic influence on intracellular transport of biliary lipid precursors. TUDCA may compete with TCA in the step of interaction between organelle lipids for formation of biliary lipid precursors [26] and, as a result, a constant supply of biliary lipid vesicles would be sustained to preserve canalicular membrane function, as described above. The other possibility is a physicochemical interaction between the relatively hydrophilic TUDCA and the hydrophobic TCA, decreasing the latter's toxic influence against both the organelle lipid layer and the bile-canalicular membrane. In both cases excretion of cytotoxic TCA into the bile would be facilitated by TUDCA.

Cytoprotective action for bile acids, including TUDCA and T β -MCA, on cholestasis induced by excess dose-administration of more hydrophobic bile acids such as TCA or TCDCA has also been demonstrated in normal liver without colchicine treatment [5,27]. In this case the cholestasis has been thought to be due to harsh detergent action, mainly against canalicular membranes. Although the co-existence of both bile acids and their co-secretion were proposed as important factors for the observed cytoprotective actions in a recent report [27], the exact mechanism and site of action in the hepatocyte remain unclear. Recently, replacement of or decrease in relatively toxic bile acids by the less toxic UDCA in the bile acid pool has been considered to be responsible for the effect of UDCA therapy [28,29]. However, the most recent report by Heuman *et al.* [30] also showed a protective

action of TUDCA against taurodeoxycholate-induced hepatocyte necrosis, with the authors speculating that this effect may occur directly at the liver cell level.

In this context, the present study clearly showed an inhibitory action of TUDCA on TCA-induced cholestasis in the colchicine-treated model, indicating that the site of action of TUDCA is at the hepatocyte level rather than operating through a relative decrease in TCA from the bile acid pool. In addition, an important role for taurine-conjugation in determining cytoprotective potential was also suggested.

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