J. Biochem. 129, 683–689 (2001)

Effects of Deoxycholic Acid and Its Epimers on Lipid Peroxidation in Isolated Rat Hepatocytes

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Received November 24, 2000; accepted February 7, 2001

We studied the effects of deoxycholic acid and its three epimers with β-hydroxyl groups (3α,12β-, 3β,12α-, and 3β,12β-dihydroxy-5β-cholan-24-oic acids), which were hydrophilic and less cytotoxic, on lipid peroxidation to elucidate the relationship between structural features of bile acids and their effect on lipid peroxidation. Taurodeoxycholate markedly increased the production of thiobarbituric acid-reactive substances, end products of lipid peroxidation, in isolated rat hepatocytes, whereas epimers of taurodeoxycholate did not. Deoxycholic acid inhibited mitochondrial NADH dehydrogenase and NADH:ferricytochrome *c* oxidoreductase activities, leading to free radical generation, whereas epimers of deoxycholic acid had no effect on mitochondrial enzymes. These findings suggested that hydrophobic bile acids cause lipid peroxidation by impairment of mitochondrial function, leading to the generation of free radicals; and epimerization of α-hydroxyl groups in the steroid nucleus to β-hydroxyl groups results in a decrease of the toxic effects of deoxycholic acid on lipid peroxidation.

Key words: bile acid, hepatocyte, hepatotoxicity, hydrophilicity, lipid peroxidation.

Cholestasis is characterized by impairment of bile flow leading to consecutive hepatic accumulation of various compounds normally excreted into bile. Continuation of this disease for long periods results in both acute and chronic liver failure, finally leading to biliary fibrosis and cirrhosis (*1*, *2*). Effective methods for treatment of cholestasis have not been established because of complications of this disorder. Thus, understanding the mechanisms by which cholestasis leads to liver injury is important for development of new treatment methods for this disorder. Although several pathogenic pathways may occur during cholestasis, one of the final common events leading to liver injury is intracellular accumulation of hydrophobic bile acids (*3–5*), especially lipophilic monohydroxy and dihydroxy bile acids such as lithocholic acid, chenodeoxycholic acid and deoxycholic acid (DCA), which are biological detergents and damage biological membranes. Several *in vivo* and *in vitro* studies have shown that hydrophobic bile acids damage hepatocellular plasma membranes (*6*, *7*). However, these studies were performed with bile acid concentrations in the millimolar range, higher than the concentrations to which hepatocytes are expected to be exposed *in vivo* (*8*). Under physiological conditions, bile acid toxicity cannot be explained by its detergent effects alone.

Several lines of evidence suggest that hepatocyte lipid peroxidation occurs during cholestatic liver disease (*9–11*). Increases in plasma lipid peroxide levels have been reported in cholestatic children (*12*). Other studies have shown that hydrophobic bile acids cause isolated rat hepatocytes and mitochondria to generate a large amounts of hydroperoxides (*13*, *14*). These results indicated that lipid peroxidation is an important mechanism in the pathophysiology of cholestasis. Lipid peroxidation is a biological process involving free radicals, which are normally generated in neutrophils and mitochondria (*15*, *16*). Under physiological conditions, 2% of oxygen consumed in mitochondria is partially reduced to superoxide by electrons generated in mitochondria (*16*), and superoxide production by mitochondria increases under a variety of pathological conditions in which mitochondrial function is impaired (*17*, *18*). Further, recent studies revealed that hydrophobic bile acids caused mitochondrial dysfunction, and impairment of both electron transport and ATP synthesis (*19*, *20*). We hypothesized that hydrophobic bile acids may impair mitochondrial function, leading to the generation of oxygen free radicals, and as a result cause lipid peroxidation.

Molecular structure is an important determinant of physiological and toxic effects of bile acids. Generally, bile acids with β-hydroxyl groups, such as ursodeoxycholic acid (UDCA) and β-muricholic acid, are hydrophilic and of low cytotoxicity, and exhibit no effects on cholesterol 7α-hydroxylase (*21*, *22*), as opposed to hydrophobic bile acids such as DCA and chenodeoxycholic acid. In many cases, the physiological effects of bile acids have been shown to correlate closely with their molecular structure, especially the orientation of the hydroxyl groups. For example, UDCA is the 7β-hydroxylated epimer of chenodeoxycholic acid. Although these bile acids are identical in chemical structure except for the orientation of the hydroxyl group at C-7, UDCA is

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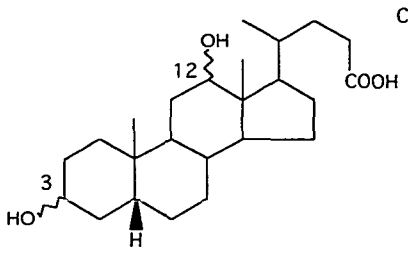
Abbreviations: DCA, deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid); UDCA, ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid); 3α,12β, 3α,12β-dihydroxy-5β-cholan-24-oic acid; 3β,12α, 3β,12α-dihydroxy-5β-cholan-24-oic acid; 3β,12β, 3β,12β-dihydroxy-5β-cholan-24-oic acid; TBA, thiobarbituric acid; TBARS, thiobarbituric acid–reactive substances; CMC, critical micellar concentration; LDH, lactate dehydrogenase.



C-3	C-12	
α	α	3α,12α-Dihydroxy-5β-chonan-24-oic acid:Deoxycholic acid (DCA)
α	β	3α,12β-Dihydroxy-5β-chonan-24-oic acid (3α,12β)
β	α	3β,12α-Dihydroxy-5β-chonan-24-oic acid (3β,12α)
β	β	3β,12β-Dihydroxy-5β-chonan-24-oic acid (3β,12β)

Fig. 1. **Chemical structures of deoxycholic acid (DCA) and its epimers.**

hydrophilic and nontoxic bile, whereas chenodeoxycholic acid is hydrophobic and cytotoxic, indicating that the orientation of the hydroxyl group in the steroid nucleus is the key factor determining their physiological effects.

We hypothesized that epimerization of α-hydroxyl groups of hydrophobic bile acids to β-hydroxyl groups would diminish the toxic effects on lipid peroxidation. To elucidate the relationship between the structural features of bile acid and the effects on lipid peroxidation, we synthesized three epimers of DCA by converting α-hydroxyl groups of DCA to β-hydroxyl groups (Fig. 1), and examined the effects of these epimers on production of lipid peroxides in isolated rat hepatocytes, mitochondrial function, and deoxyribose degradation caused by radical reaction.

MATERIALS AND METHODS

Materials—All chemicals used for preparation of the buffer solutions were of analytical grade and were obtained from the suppliers shown. All chemicals used for the isolation and culture of rat hepatocytes were obtained from sources described previously (*23*, *24*). β-NADH, β-NAD, cytochrome *c*, deoxyribose, thiobarbituric acid (TBA), and RPMI-1640 medium were purchased from Sigma Chemical (St. Louis, MO), and all other chemicals were of reagent grade.

Bile Acids—DCA was obtained from a commercial source. UDCA was supplied by Tokyo Tanabe (Tokyo). Epimers of DCA, 3α,12β-dihydroxy-5β-cholan-24-oic acid (3α,12β), 3β,12α-dihydroxy-5β-cholan-24-oic acid (3β,12α), and 3β,12β-dihydroxy-5β-cholan-24-oic acid (3β,12β) were synthesized from DCA by the methods described previously (*25*, *26*). Taurine conjugates of DCA, UDCA, 3α,12β, 3β,12α, 3β,12β (DC-tau, UDC-tau, 3α,12β-tau, 3β,12α-tau, 3β,12β-tau) were synthesized by the methods described previously (*27*). The purity of each bile acid was >98% as determined by GLC or HPLC. All bile acids were used as sodium salts.

Measurement of Rm Value and CMC—*Rm* value, which indicates lipophilicity (hydrophobicity) of bile acid, was measured by the method described previously (*28*). Measurement of critical micellar concentration (CMC) was performed spectrophotometrically by the dye solubilization method essentially as described by Roda (*29*). Orange OT, a water-insoluble dye, was used in this study. Relative solubility of Orange OT was calculated by taking the maximum absorbance as 100%.

Hepatocyte Isolation and Measurement of Lipid Peroxides—All rats received humane care, and the study protocol was carried out in accordance with the guidelines of our institutional committee. Hepatocytes from male Sprague-Dawley rats weighing 200–250 g were isolated by perfusion with 0.05% collagenase as described previously (*30*). Isolated hepatocytes were >90% viable as determined by trypan blue exclusion.

The resulting cell preparation was washed three times and resuspended at a concentration of 10^6 cells/ml in RPMI-1640 medium without phenol red. Incubation of freshly isolated rat hepatocytes in RPMI-1640 medium in the presence or absence of exogenous bile acids was performed by a modification of the method of Sokol *et al.* (*9*). One of several bile acids (or a similar volume of medium as a control) was added to the incubation mixture to a final concentration of 200 or 500 μM and incubated with hepatocytes for various times (30–240 min). Aliquots of hepatocyte suspension were removed from each incubation mixture at the indicated times and analyzed for the generation of lipid peroxidation products by a modification of the TBA-reactive substances (TBARS) method, as previously described (*9*, *31*, *32*). Briefly, hepatocyte suspension (0.5 ml) was added to a test tube containing 1.0 ml of trichloroacetic acid (10% w/v) and 50 μl of antioxidant, dibutylhydroxytoluene (2% w/v). After heating at 90°C for 10 min, the precipitate was removed by centrifugation. The supernatant was then added to 1.0 ml of TBA (1% w/v) and heated at 90°C for 15 min. After cooling, absorbance was determined at 532 nm. In another set of experiments, incubation was performed with DC-tau in the presence of 1% dimethylsulfoxide (DMSO), a hydroxy radical scavenger.

Isolation of Hepatic Mitochondria and Measurement of Enzyme Activities Involved in the Electron Transport Chain—Rat liver mitochondria were isolated by a modification of the method described previously (*33*, *34*). Rats were fasted overnight then killed by decapitation and their livers were quickly removed and washed in ice-cold sucrose buffer (250 mM sucrose, 10 mM HEPES-NaOH buffer, pH 7.4, 1 mM EDTA, 0.1% EtOH). The liver was minced and washed with sucrose buffer. Minced liver suspensions were prepared in a Potter-Elvejhem homogenizer with a loose-fitting pestle. Nuclei and cell debris were removed by centrifugation at 700 ×*g* for 10 min, and the mitochondrial fraction was isolated by centrifugation of the supernatant at 7,000 ×*g* for 10 min. The resulting mitochondrial pellet was washed twice with sucrose buffer and finally diluted to various concentrations.

Mitochondrial enzyme activities were measured spectrophotometrically at 30°C. NADH dehydrogenase activity was measured as described previously (*20*, *35*). Reaction mixtures contained (final concentration) potassium phosphate (20 mM, pH 7.4), EDTA (0.1 mM), ferricytochrome *c* (0.15 mM), bile acid (500 μM), and mitochondrial protein (0.1 mg/ml). After an equilibration period of 3 min, the reaction was started by adding NADH (0.28 mM) as a substrate, and changes in absorbance at 550 nm were moni-

tored.

NADH:ferricytochrome *c* oxidoreductase and ferrocytochrome *c*:oxygen oxidoreductase were determined according to the established spectrophotometric methods (*36*, *37*). Each assay was performed in the presence of 500 μM taurine-conjugated and unconjugated bile acids, after an equilibration period of 3 min.

Deoxyribose Degradation Assay—Deoxyribose degradation by hydroxy radicals generated by the Fenton reaction was performed essentially according to the method of Gutteridge (*32*). The hydroxy radical generating solution containing (final concentration) ascorbate (0.2 mM), EDTA (0.4 mM), and ferrous sulfate (0.3 mM) was added to deoxyribose solution (2.5 mM), and incubation was performed in the presence of bile acids or hydroxy radical scavenger DMSO at 1.0 mM (in the absence of bile acids and DMSO as a control). The amount of deoxyribose degradation in a 60-min incubation at 37°C was determined by the TBARS method as described above.

Lactate Dehydrogenase (LDH) Activity—Hepatocytes from male Sprague-Dawley rats weighing 200–250 g were isolated by perfusion with 0.05% collagenase and plated on 60-mm plastic culture dishes coated with rat tail collagen at a density of 3.4×10^6 cells/dish. Maintenance of hepatocytes was performed essentially as described by Bissell and Guzelian (*30*). All cultures were maintained in 3 ml of William's E medium supplemented with fetal calf serum (5%), insulin (0.25 unit/ml), penicillin (100 units/ml), thyroxine (1.0 mM), and dexamethasone (0.1 mM). Hepatocytes were incubated at 37°C in an atmosphere of 5% CO_2. After 4 h, medium was replaced with fresh medium, and after 20 h, fresh medium containing taurine-conjugated bile acids (1 mM) was added. After 120 min of incubation, medium was aspirated and LDH released into the medium was measured spectrophotometrically by monitoring the increase of NADH at 340 nm. Briefly, 150 μl of aspirated medium was added to 1.2 ml of Tris/lactate buffer (125 mM Tris-NAOH buffer, pH 9.5, 65 mM L-lactate, 194 mM KCl), and the reaction was started by addition of 150 μl of 20 mM β-NAD. The increase of absorbance at 340 nm was monitored for 3 min at 37°C.

Statistical Analysis—Data are presented as means ± SD. Statistical significance of the differences between experimental groups was determined by one-way ANOVA utilizing Scheffe's F test.

RESULTS

Physicochemical Properties of Individual Bile Acids—Rm value was the highest for DC-tau, and decreased in the order 3β,12α-tau > 3α,12β-tau > 3β,12β-tau > UDC-tau, indicating that epimerization of DC-tau decreased its hydrophobicity (Table I). Similar results were obtained with unconjugated bile acids (*Rm* values were as follows: DCA, 4.579; 3β,12α, 4.339; 3α,12β, 4.263; 3β,12β, 4.263; UDCA, 4.087; data are not shown in table). This result indicated that unconjugated bile acids are more hydrophobic than those with conjugated taurine. Relative solubility of Orange OT was also decreased by epimerization (Table I and Fig. 2), but the order (DC-tau > 3β,12β-tau > 3α,12β-tau > 3β,12α-tau > UDC-tau) did not completely agree with the order of *Rm* value. CMC of DC-tau was calculated at 2.49 mM, but CMC of epimers of DC-tau and UDC-tau could not be calculated in the range of 0–5 mM (Fig. 2), and are expected to be much higher than this range.

TABLE I. **Physicochemical properties of UDC-tau, DC-tau, and its epimers.**

Bile acid	*Rm* value	CMC (mM)	Relative solubility[a] of Orange OT (%)
DC-tau	2.043	2.49	100
3α,12β-tau	1.322	>5.0	19.31
3β,12α-tau	1.807	>5.0	13.97
3β,12β-tau	1.247	>5.0	25.16
UDC-tau	1.066	>5.0	9.08

[a]Data were calculated by taking the maximum absorbance as 100%.

Effects of Individual Bile Acids on Leakage of LDH—LDH leakage was measured to examine the cytotoxicity of individual bile acids. DC-tau (1 mM) significantly increased release of LDH into the medium compared with its epimers and UDC-tau (Fig. 3). 3β,12α-Tau also significantly increased release of LDH, but to a lesser extent than DC-tau (Fig. 3).

Effects of DC-tau and Its Epimers on Lipid Peroxidation in Isolated Rat Hepatocytes—To examine the effects of DC-

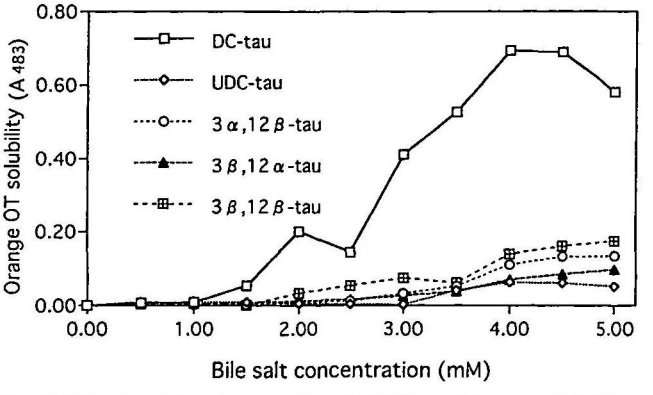


Fig. 2. **Effects of taurine-conjugated bile acids on solubilization of Orange OT.** The amount of dye solubilized in relation to bile salt concentration was determined spectrophotometrically. Orange OT was added to bile salt solutions followed by incubation for 3 days at 37°C. Absorbance of the supernatant was measured at 483 nm.

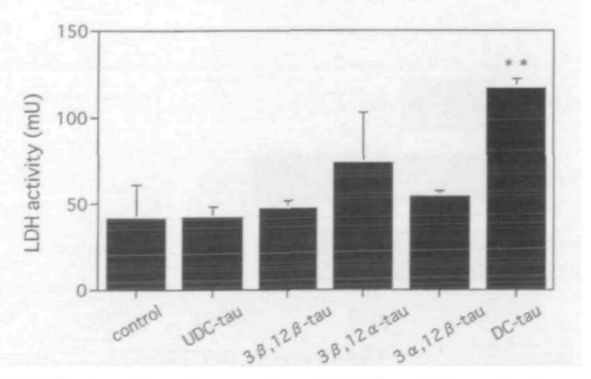


Fig. 3. **Effects of taurine-conjugated bile acids on leakage of lactate dehydrogenase in primary cultured rat hepatocytes.** Primary cultured rat hepatocytes were incubated with bile acids at 1 mM. After 120 min of incubation, LDH released into medium was measured spectrophotometrically. Values represent means ± SD ($n = 4$). $^{**}p < 0.005$ *vs.* control.

tau on hepatocyte lipid peroxidation, isolated rat hepatocytes were incubated with DC-tau, and production of thiobarbituric acid–reactive substances (TBARS) was measured. DC-tau stimulated the production of TBARS in a time-dependent manner (Fig. 4). At a concentration of 500 μM, TBARS production was markedly increased (Fig. 4).

After treatment with DMSO, a scavenger of hydroxy radicals, the stimulatory effect of DC-tau on TBARS production was slightly but significantly decreased (Fig. 5). This indicated that the effect of DC-tau on hepatocyte lipid peroxidation partially requires free radicals. Epimers of DC-tau and UDC-tau also stimulated TBARS production in isolated rat hepatocytes (Fig. 6). These stimulatory effects were significant, but much smaller than that of DC-tau.

Effects of Individual Bile Acids on Mitochondrial Enzymes—NADH dehydrogenase activity was decreased in the presence of DCA by 49% as compared to control, whereas other unconjugated bile acids showed no inhibitory effects on NADH dehydrogenase activity (Table II). Of the taurine-conjugated bile acids, in contrast to the unconjugated bile acids, hydrophobic DC-tau and 3β,12α-tau showed no inhibitory effect on NADH dehydrogenase. However, hydrophilic bile acids UDC-tau, 3α,12β-tau, and 3β,12β-tau decreased NADH dehydrogenase activity by 32, 26, and 32%, respectively.

NADH:ferricytochrome *c* oxidoreductase activity was decreased in the presence of DCA by 47%. Other unconjugated and taurine-conjugated bile acids showed no inhibitory effects (Table II).

Ferrocytochrome *c*:oxygen oxidoreductase activity was not affected by any of the bile acids examined (data not shown).

Effects of Individual Bile Acids on Fenton Reaction—The Fenton reaction generates hydroxy radicals *in vitro*. To investigate the effects of bile acids on radical reaction, we performed the Fenton reaction in the presence of 1 mM bile acid, and determined whether this reaction induced deoxyribose degradation by measurement of TBARS production. In the control group, deoxyribose degradation occurred, and in the Fe(−) group, containing no ferrous ions, deoxyribose degradation was not observed (Fig. 7). Addition of bile acids

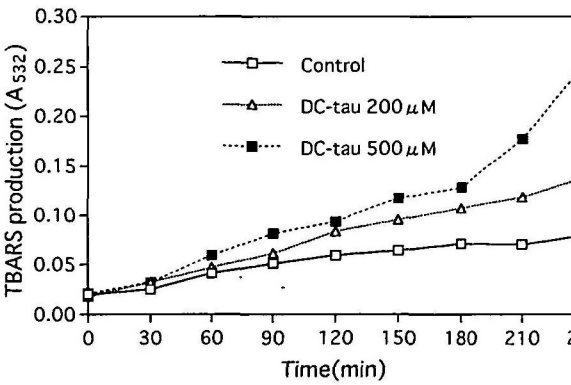


Fig. 4. **Time course of TBARS production in the presence of DC-tau in isolated rat hepatocytes.** Isolated rat hepatocytes were incubated with DC-tau (200 or 500 μM), and aliquots of hepatocytes were removed from incubations at the indicated times. TBARS production in hepatocytes was determined by measurement of absorbance at 532 nm.

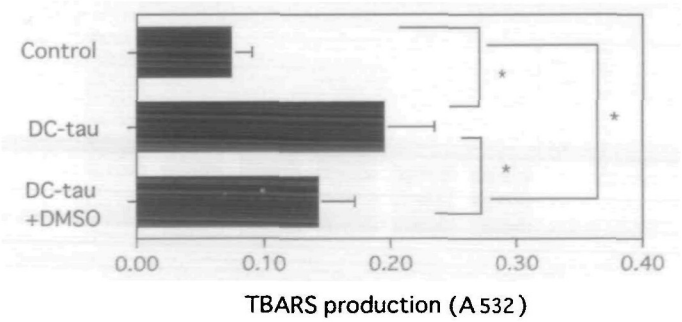


Fig. 5. **Inhibitory effect of DMSO on DC-tau induced TBARS production in isolated rat hepatocytes.** Isolated rat hepatocytes were incubated with 500 μM DC-tau in the presence of DMSO (1%). After 240 min of incubation, TBARS production was determined by measurement of absorbance at 532 nm. Values represent means ± SD (n = 4). $^{*}p < 0.001$.

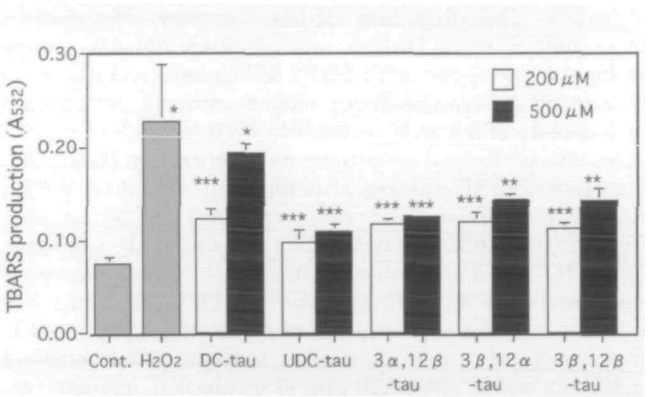


Fig. 6. **Effects of taurine-conjugated bile acids on lipid peroxidation in isolated rat hepatocytes.** Isolated rat hepatocytes were incubated with 200 or 500 μM taurine-conjugated bile acids or 0.1% hydrogen peroxide (H_2O_2). TBARS production in isolated rat hepatocytes was determined by measurement of absorbance at 532 nm. Values represent means ± SD (n = 4). $^{*}p < 0.0001$ *vs.* control, $^{**}p < 0.001$ *vs.* control, $^{***}p < 0.05$ *vs.* control.

TABLE II. **Effects of taurine-conjugated and unconjugated bile acids on NADH dehydrogenase and NADH:ferricytochrome *c* oxidoreductase activities in rat liver mitochondria.**

Bile acid	NADH dehydrogenase activity (% of control)	NADH:ferricytochrome *c* oxidoreductase activity (% of control)
Unconjugated bile acid		
DCA	51.3 ± 4.4[a]	52.9 ± 20.7[a]
UDCA	99.3 ± 9.5	95.1 ± 5.4
3α,12β	96.1 ± 4.9	105.7 ± 1.8
3β,12α	99.3 ± 3.0	92.6 ± 2.3
3β,12β	95.4 ± 24.4	91.9 ± 5.9
Taurine-conjugated bile acid		
DC-tau	101.6 ± 9.4	92.7 ± 8.1
UDC-tau	67.9 ± 9.9[a]	97.1 ± 5.7
3α,12β-tau	73.9 ± 6.2[b]	90.8 ± 5.4
3β,12α-tau	101.6 ± 5.3	93.7 ± 5.5
3β,12β-tau	67.9 ± 10.0[a]	86.4 ± 4.2

NADH dehydrogenase and NADH:ferricytochrome *c* oxidoreductase activities were determined spectrophotometrically by measurement of absorbance at 550 nm. Isolated rat liver mitochondria were incubated with 500 μM unconjugated and taurine-conjugated bile acids. Values represent means ± SD (n=4). $^{a}p < 0.0001$ *vs.* control; $^{b}p < 0.0005$ *vs.* control.

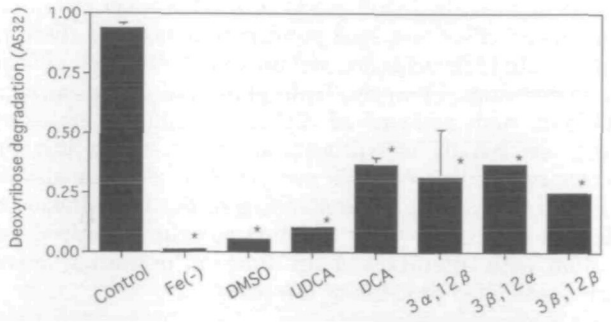


Fig. 7. **Inhibitory effects of bile acids and DMSO on deoxyribose degradation caused by Fenton reaction.** The hydroxy radical-generating system (Fe^{2+}EDTA-ascorbate) was added to deoxyribose solution. Incubation was performed in the presence of 1 mM bile acids and 1 mM DMSO, or in the absence of bile acid alone (control). Fe(−) contained no ferrous ions. Deoxyribose degradation was determined by measurement of absorbance at 532 nm. Values represent means ± SD ($n = 4$). $^*p < 0.0001$ *vs.* control.

to Fenton reaction mixtures markedly reduced the level of deoxyribose degradation (Fig. 7). This indicated that bile acid molecules themselves prevent radical reaction *in vitro*.

DISCUSSION

Recently, many investigators have reported that bile acid toxicity is associated with lipid peroxidation (*9–14*). The present study also showed that the hydrophobic bile acid DC-tau stimulated TBARS production time- and dose-dependently in isolated rat hepatocytes (Fig. 4). This result was in good agreement with many previous reports suggesting that hydrophobic bile acids may cause lipid peroxidation (*13*). In this study, we determined production of TBARS, mainly malondialdehyde (MDA), as an indicator of lipid peroxidation. MDA is an end product of decomposition of lipid peroxides (*38*), and the production of MDA is a relatively late reaction in the process of lipid peroxidation (*38*). However, MDA level was reported to be correlated well with lipid peroxidation and decreases in cell viability (*9*, *39*). Thus, we used the TBARS method to monitor lipid peroxidation.

TBARS production is an indirect indicator of lipid peroxidation involving free radical generation (*40–42*). To obtain more direct evidence of free radical genaration in our study, we examined the effects of DMSO, an antioxidant that scavenges hydroxy radicals, on TBARS production induced by DC-tau. In DMSO-treated hepatocytes, TBARS production induced by DC-tau was significantly decreased (Fig. 5). This indicated that lipid peroxidation caused by DC-tau requires free radicals such as reactive oxygen species.

In our study, isolated hepatocytes were used in preference to primary cultured hepatocytes for two reasons. First, the capacity for uptake of bile acid is well preserved in isolated hepatocytes, whereas this function is significantly decreased in cultured hepatocytes. Previous studies suggested that, in primary cultured rat hepatocytes, the mRNA level of the bile salt uptake transporter Na^+-taurocholate−cotransporting polypeptide (ntcp) decreased spontaneously, followed by a decrease in bile salt uptake (*43*). Second, the response of primary cultured rat hepatocytes to concentration of exogenous bile acids was lower than that of isolated hepatocytes (*9*).

To investigate the correlation between the effects on lipid peroxidation and bile acid structural features, we synthesized three epimers of DCA with β-hydroxyl groups and examined their effects on TBARS production in isolated rat hepatocytes. Taurine conjugates of these epimers also caused TBARS production. However, the level of production was much lower than that induced by DC-tau (Fig. 6). TBARS production in the presence of epimers of DC-tau and UDC-tau may be caused by spontaneous oxidative stress in isolated hepatocytes. Figure 4 shows that hepatocyte auto-oxidation occurred in the control group despite the absence of bile acids. This auto-oxidation may have been due to a gradual decrease in cellular antioxidation capacity in isolated hepatocytes (*44*). Thus, isolated hepatocytes may tend to be oxidized in the presence of less toxic bile acids such as epimers of DC-tau and UDC-tau, and these β-hydroxylated bile acids have less toxic effects on lipid peroxidation than DC-tau.

To understand the mechanisms of bile acid–induced hepatocyte lipid peroxidation, we studied the effects of bile acids on mitochondrial oxidases. Previous studies indicated that mitochondrial dysfunction, depletion of ATP and generation of superoxide radicals were caused by hydrophobic bile acids during cholestatic liver disease (*19*, *45*). We examined the effects of epimers of DCA on mitochondrial enzymes. In isolated rat liver mitochondria, NADH dehydrogenase activity decreased in the presence of DCA, whereas other unconjugated bile acids such as epimers of DCA and UDCA showed no inhibitory effects on NADH dehydrogenase acitivity (Table II). This inhibitory effect of DCA on NADH dehydrogenase activity may have been due to the potent detergent effect of DCA. Previous studies indicated that hydrophobic bile acids with potent detergent effects such as DCA may cause dysfunction of membrane-bound proteins by incorporation into the membrane bilayer or by alteration of membrane lipid composition (*20*, *46*). In our study, DCA was the most hydrophobic bile acid, with the highest *Rm* value and the lowest CMC, indicating that it has a strong detergent effect on biological membranes. DCA may alter the lipid composition of the mitochondrial membrane and affect the activity of membrane-bound proteins such as NADH dehydrogenase. DCA also decreased NADH:ferricytochrome *c* oxidoreductase activity, probably by a similar mechanism to that in the case of NADH dehydrogenase (Table II). Hydrophilic bile acids such as UDCA and epimers of DCA had no effect on these oxidase activities in the unconjugated form. However, in the taurine-conjugated form, unexpected results were obtained. Hydrophilic bile acids such as UDC-tau, 3α,12β-tau, 3β,12β-tau decreased NADH dehydrogenase activity slightly, although DC-tau did not (Table II). These results were contradictory to the hypothesis that the inhibitory effects of hydrophobic bile acids on membrane bound proteins are due to a potent detergent effect (hydrophobicity). In our study, LDH leakage from primary cultured hepatocytes was not affected in the presence of UDC-tau or three epimers (Fig. 3), indicating that the effects of these β-hydroxylated bile acids on biological membranes are moderate. However, these β-hydroxylated bile acids decreased NADH dehydrogenase activity despite their hydrophilicity and modest detergent effects. Although taurine-conjugated bile acids are less hydrophobic than unconjugated bile acids, UDCA, 3α,12β,

and 3β,12β decreased NADH dehydrogenase activity in taurine-conjugated form, but not in unconjugated form. The reasons for these conflicts in NADH dehydrogenase measurement are not clear. However, a previous study revealed a selective increase of hydrophilic phospholipid secretion into bile *via* the hepatocyte canalicular membrane during intravenous infusion of UDC-tau (*47*), indicating that UDC-tau shows high affinity to hydrophilic phospholipid. In contrast to other biological membranes, mitochondrial membrane lipids are composed predominantly of phospholipid (to approximately 95% of the total membrane lipid content); and the majority of fatty acyl chains are unsaturated hydrophilic species (*48*, *49*), and lipid composition of this membrane is critical for normal function of the mitochondria (*50*, *51*). Hydrophilic bile acids such as UDC-tau and epimers of DC-tau may have high degree of affinity with mitochondrial membranes enriched in hydrophilic phospholipid and affect membrane fluidity by selective alteration of membrane lipid composition. As a result, these hydrophilic bile acids may have caused functional alteration of membrane-bound protein in this study.

DC-tau markedly increased TBARS production in isolated rat hepatocytes (Fig. 3), but had no effect on NADH dehydrogenase or NADH:ferricytochrome *c* oxidoreductase activity (Table II), and UDC-tau only slightly increased TBARS production in isolated rat hepatocytes despite inhibition of NADH dehydrogenase activity. The relative contribution of mitochondrial dysfunction to lipid peroxidation is unknown. DC-tau may affect other mitochondrial oxidases, or cytosolic enzymes such as xanthine oxidase. Xanthine oxidase also generates reactive oxygen species (*52*, *53*). DC-tau may alter the conformation of various enzymes such as xanthine oxidase directly or indirectly, and as a result stimulate the generation of reactive oxygen species leading to lipid peroxidation.

Further experiments were performed to examine whether bile acids acted as direct or indirect oxidants in the process of lipid peroxidation. The Fenton reaction is one of the most common reactions that generates free radicals, mainly hydroxy radicals, and the free radicals generated attack various biological molecules including deoxyribose (*32*). A previous study showed that cholic acid inhibited deoxyribose degradation caused by the Fenton reaction (*31*). We examined the effects of bile acids on Fenton reaction-induced deoxyribose degradation and found that all bile acids examined exhibited inhibitory effects (Fig. 7). This indicated that the bile acid molecule itself acts as an antioxidant, and that bile acids impair the free radical reaction *in vitro* and do not oxidize biological membrane lipids in cholestatic liver disease. Within cells, however, bile acids are indirect oxidants that induce lipid peroxidation by stimulation of various sources of free radical generation. The biological oxidants involved in hepatocyte lipid peroxidation are not bile acids but sources of cellular free radical generation such as mitochondrial oxidases and xanthine oxidase.

This study indicated that hydrophobic bile acids stimulate lipid peroxidation in an indirect manner. Hydrophobic bile acids induce the generation of reactive oxygen species by stimulation of the various sources of free radical generation in hepatocytes. Membrane damage caused by hydrophobic bile acids may lead to hepatocelluar lipid peroxidation during cholestasis. Structural features and hydrophobicity of bile acids are related to lipid peroxidation. Epimerization of the α-hydroxyl group to a β-hydroxyl group decreased the effects on lipid peroxidation, because these β-hydroxylated bile acids showed no severe effects on biological membranes. However, hydrophilic bile acids such as UDC-tau and epimers of DC-tau exhibited inhibitory effects on NADH dehydrogenase in our study, but the mechanism of this effect is not yet clear. Further studies will lead to a better understanding of the mechanisms of bile acid-induced lipid peroxidation and the structural correlation with alteration of the function of various membrane-associated proteins by bile acid.

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