Effects of bile acids on the muscle functions of guinea pig gallbladder

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Received 20 December 2001; accepted in final form 23 March 2002

Xiao, Zuo-Liang, Aloysius K. Rho, Piero Biancani, and Jose Behar. Effects of bile acids on the muscle functions of guinea pig gallbladder. Am J Physiol Gastrointest Liver Physiol 283: G87–G94, 2002. First published March 28, 2002; 10.1152/ajpgi.00536.2001.—Hydrophobic bile acids impair gallbladder emptying in vivo and inhibit gallbladder muscle contraction in response to CCK-8 in vitro. This study was aimed at determining the mechanisms of muscle cell dysfunction caused by bile acids in guinea pig gallbladders. Muscle cells were obtained by enzymatic digestion. Taurochenodeoxycholic acid (TCDC), a hydrophobic bile acid, caused a contraction of up to 15% and blocked CCK-induced contraction. Indomethacin abolished the TCDC-induced contraction. Hydrophilic bile acid tauroursodeoxycholic acid (TUDC) had no effect on muscle contraction but prevented the TCDC-induced contraction and its inhibition on CCK-induced contraction. Pretreatment with NADPH oxidase inhibitor Prt2I, xanthine oxidase inhibitor allopurinol, and free-radical scavenger catalase also prevented TCDC-induced contraction and its inhibition of the CCK-induced contraction. TCDC caused H2O2 production, lipid peroxidation, and increased PGE2 synthesis and activities of catalase and SOD. These changes were significantly inhibited by pretreatment of Prt2I or allopurinol. Inhibitors of cytosolic phospholipase A2 (cPLA2), protein kinase C (PKC), and mitogen-activating protein kinase (MAPK) also blocked the TCDC-induced contraction. It is concluded that hydrophobic bile acids cause muscle cell dysfunction by stimulating the formation of H2O2 via activation of NADPH and xanthine oxidase. H2O2 causes lipid peroxidation and activates cPLA2 to increase PGE2 production, which, in turn, stimulates the synthesis of free-radical scavengers through the PKC-MAPK pathway.

H2O2 production; oxidase inhibitors; smooth muscle

Bile acids are important for the maintenance of bile flow, lipid solubilization in bile, and intestinal fat absorption (3). Most of the bile acids in human bile are hydrophobic bile acids (3, 22). These bile acids, such as chenodeoxycholate, have been shown to affect the gastrointestinal tract causing bile esophagitis, gastritis, and cholelithiasis. They can also induce cholestasis in animal models as well as in humans (13). At the cellular level, hydrophobic bile acids seem to disrupt a variety of functions. They affect isolated cell membranes by inserting themselves into the lipid bilayer to activate phospholipase A2 (PLA2) and by generating free radicals (3, 11, 26). They can solubilize membranes by promoting the loss of cholesterol and phospholipids (14, 18), impair mitochondrial function by depleting ATP, and increase the permeability transition of mitochondrial membranes (18). In contrast, none of these deleterious effects is seen with hydrophilic bile acids, such as ursodeoxycholate, that can actually improve cellular functions in experimental animals in vivo (18). In some cell types, ursodeoxycholate prevented the damage to plasma membranes caused by hydrophobic bile acids, suggesting a direct interaction of ursodeoxycholate with those bile acids or with plasma membranes (11). In muscle strips from guinea pig gallbladders, hydrophobic bile acids such as taurochenodeoxycholic acid (TCDC) dose dependently inhibited CCK-induced muscle contraction. In contrast, the hydrophilic bile acid tauroursodeoxycholic acid (TUDC) pretreatment prevented the inhibition of TCDC on CCK-induced contraction (34). However, the mechanisms responsible for the different actions of hydrophobic and hydrophilic bile acids are unknown (13).

Acute cholecystitis tends to occur in patients with supersaturated bile with cholesterol, which is associated with a defective gallbladder muscle contraction and bile stasis (2, 6). These abnormalities appear to be reversible after treatment with ursodeoxycholic acid (27). Treatment with ursodeoxycholic acid also reduced the risk of acute cholecystitis in patients with symptomatic gallstones over an 18-yr period (28). These prophylactic effects of ursodeoxycholic acid were independent of gallstone dissolution, suggesting that they may due to an amelioration of the deleterious effects of bile constituents, to an improvement of gallbladder stasis, or both. It is thus conceivable that ursodeoxycholic acid prevents these complications, in part, by neutralizing the effects of hydrophobic bile acids.

These studies, therefore, were aimed at determining the effects and mechanisms of hydrophobic and hydrophilic bile acids on gallbladder muscle functions in guinea pigs.
MATERIALS AND METHODS

Animals. Adult male guinea pigs were purchased from Elm Hill Breeding Laboratory (Chelmsford, MA). The Animal Welfare Committee of Rhode Island Hospital approved their use. Animals were housed in thermoregulated rooms and had free access to food and water. After an overnight fast, the animals were anesthetized with an intramuscular injection of ketamine hydrochloride (30 mg/kg) followed by pentobarbital sodium (30 mg/kg ip). The gallbladder was removed and rinsed with ice-cold, oxygenated Krebs solution (in mM: 116.6 NaCl, 3.4 KCl, 21.9 NaHCO3, 1.2 NaH2PO4, 2.5 CaCl2, 1.2 MgCl2, and 5.4 glucose). The mucosa and serosa were carefully peeled off under a dissecting microscope. The muscle layer was further cleaned by gently removing the remaining connective tissue.

Isolation of muscle cells. Single muscle cells were obtained by enzymatic digestion (31, 32, 36–39). Gallbladder muscle layers were cut into 2-mm-wide strips and digested in HEPES buffer containing 0.5 mg/ml type F collagenase and 2 mg/ml papain (activity of ~13.9 U/mg protein) for ~20 min at 35°C in a shaking water bath. The buffer was gently gassed with 100% O2 during digestion. At the end of the digestive process, the tissue was filtered through Nitex mesh 200 (Tetko, Elmsford, NY) and rinsed with 20 ml HEPES buffer. The tissue remaining on the filter was collected and incubated in HEPES buffer at 35°C for 15 min to allow the free dispersion of cells.

Studies on muscle cell contraction. Muscle cell contraction was determined in cells treated with increasing concentrations of TCDC or TUDC for 15 min before treatment with CCK-8 (30 s) or inhibitors of protein kinase C (PKC), mito-

tizations of TCDC or TUDC for 15 min before treatment with

Measurement of PGE2 content. The PGE2 content was measured by using a radioimmunoassay kit from NEN Life Science Products (Boston, MA) (9). TCDC or oxidase inhibitors plus TCDC-treated muscle cells were homogenized in HEPES buffer containing EDTA/indomethacin to inhibit the metabolism of arachidonic acid to prostaglandins. The suspension was centrifuged at 10,000 g for 15 min. PGE2 was extracted from the supernatant by the methods of Kelly et al. (16). Extracted PGE2 was converted into its methyl oximate derivative using the methyl oximation reagent. The PGE2 content was determined by following the kit’s protocol and was expressed as picograms per milligram of protein.

Determination of H2O2 content. Determination was based on the oxidation of ferrous ions to ferric ions by H2O2 (15). Under acidic conditions, ferric ions bind with the indicator dye xylenol orange to form a stable colored complex, which can be measured at 560 nm. H2O2 was measured in homog-

epared plasma membranes.

Preparation of plasma membranes. Plasma membranes of gallbladder muscle cells were prepared and purified by sucrose gradient centrifugation as described previously (25, 30). TCDC or oxidase inhibitors plus TCDC-treated and untreated muscle cells were homogenized separately by using a tissue tearer (Biospec Products, Racine, WI) in 10 vol by weight of a sucrose-HEPES buffer. The homogenates were centrifuged at 600 g for 5 min, and the supernatant was collected in a clean centrifuge tube (Beckman Instruments) and centrifuged at 150,000 g for 45 min. The pellet was resuspended in sucrose-HEPES, layered over a linear 9–60% sucrose gradient, and centrifuged at 90,000 g for 3 h. The plasma membranes were collected at ~24% sucrose. They were then diluted and pelleted by centrifugation at 150,000 g for 30 min. The pellet of membranes was stored at ~70°C.

Assessment of lipid peroxidation. Purified plasma membranes were resuspended with 1.15% KCl (23) and mixed with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid solution (pH 3.5), and 1.5 ml of 0.8% aqueous solution of thioarbitu-

cid acid. This mixture was added to a volume of 4 ml with distilled water. The sample was heated at 95°C for 60 min. After the sample was cooled, 1 ml of distilled water and 5 ml of the mixture of n-butanol and pyridine [15:1 (vol/vol)] were added and shaken vigorously. The organic layer was taken after it was spun at 2,000 g for 10 min, and its absorbance (red pigment) was measured at 532 nm. 1,1,3,3-Tetramethoxy propane was used as an external standard.

The level of lipid peroxides was expressed as nanomoles of malondialdehyde (MDA; secondary product of lipid peroxidation) per 100 mg protein.

Measurement of SOD activity. The total SOD activity was measured by using a spectrophotometric assay kit (R&D systems, Minneapolis, MN) (20). Muscle cells pretreated with TCDC or oxidase inhibitors plus TCDC were homogenized in HEPES buffer. The supernatant was obtained after the suspension was centrifuged at 8,500 g for 10 min at 4°C. Ice-cold extraction reagent [400 μl; ethanol/chloroform, 62:5:37.5 (vol/vol)] was added to 250 μl of the supernatant, vortexed for at least 30 s, and centrifuged at 3,000 g for 10 min at 4°C. Then, the aqueous upper layer was collected for assay of the SOD
activity. The determination of SOD activity was achieved by following the kit’s protocol. Data were expressed as units per milligram of protein.

**Protein determination.** The protein content of the plasma membranes was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Melville, NY). Values for each sample were means of triplicate measurements.

**Drugs and chemicals.** H$_2$O$_2$ (30% solution) was obtained from Fisher Scientific (Pittsburgh, PA). H$_2$O$_2$-560 quantitative assay kit was from OXIS International. SOD spectrophotometric assay kit was from R&D systems. PGE$_2$ radioimmunoassay kit was obtained from NEN Life Science Products. Oxidase inhibitors Pt2I and allopurinol, type F collagenase, papain, as well as other reagents were from Sigma.

**Data analyses.** One- and two-factorial repeated-measures ANOVA and Student’s t-test were used for statistical analyses. $P < 0.05$ was considered to be statistically significant.

**RESULTS**

**Bile acids and muscle contraction.** Single muscle cells were obtained from guinea pig gallbladders by enzymatic digestion. The average resting length of intact cells was 59.8 ± 1.5 μm, and of control cells treated with buffer, average resting length was 59.6 ± 0.7 μm. No significant differences in resting cell length were observed among them (1-factor ANOVA). A 15-min treatment with TCDC caused a dose-dependent muscle contraction, and maximal contraction of 14.9 ± 0.6% was achieved with a 5 μM dose. Increasing concentrations of TUDC had no effect on muscle cell contraction (Fig. 1; *$P < 0.001$ vs. TCDC-treated group by ANOVA).

Preincubation of muscle cells with 5 μM of indomethacin for 15 min before TCDC inhibited the maximal contraction of 14.9 ± 0.6% to 2.1 ± 1.1% induced by this bile acid. These results suggest that TCDC-induced muscle contraction is mediated by prostaglandins (Fig. 2; *$P < 0.001$ by ANOVA). TCDC also blocked the CCK-induced contraction. Increasing concentrations of TCDC gradually reduced the contraction caused by CCK-8 (10$^{-8}$ M). The maximal effect of TCDC at a dose of 10$^{-5}$ M reduced the CCK-induced contraction from 19.9 ± 1.6% for CCK-8 to 15.8 ± 0.8%, which was not different from that induced by TCDC alone (*$P < 0.01$ vs. CCK-8 alone by Student’s t-test). The TCDC blockade of CCK-induced contraction can be better appreciated when muscle cells were pretreated with indomethacin (5 μM) for 15 min, which eliminated the TCDC contribution. The magnitude of contraction induced by CCK-8 was reduced from 20.2 ± 1.1 to 2.3 ± 1.3% after pretreatment with indomethacin plus TCDC ($P < 0.01$ vs. CCK-8 alone by Student’s t-test).

TUDC by itself causes no muscle contraction and had no effect on CCK-8-induced contraction at a dose of <50 μM (Fig. 3). However, increasing concentrations of TUDC caused a dose-dependent inhibition of the max-

![Fig. 1. Dose-response relationships of taurochenodeoxycholic acid (TCDC) and tauroursodeoxycholic acid (TUDC) on gallbladder muscle cells. TCDC caused a dose-dependent muscle contraction of up to 15% ($P < 0.001$ vs. TUDC group by ANOVA). TUDC had no effect on muscle cell contraction. Values are means ± SE of 3 experiments.](http://ajpgi.physiology.org/)

![Fig. 2. Effect of indomethacin on TCDC-induced gallbladder muscle contraction and on its blockade of CCK-induced contraction. Muscle contraction induced by increasing concentrations of TCDC was blocked by pretreatment with indomethacin (Indo; 5 μM; *$P < 0.001$ by ANOVA). TCDC blocked the expected contraction induced by CCK-8 (*$P < 0.01$ by Student’s t-test). Because TCDC by itself causes contraction, its blockade of CCK-induced contraction can be better seen when cells were pretreated with Indo (5 μM; # $P < 0.01$ by Student’s t-test). Values are means ± SE of 3 experiments.](http://ajpgi.physiology.org/)

![Fig. 3. Effect of TUDC on CCK-induced muscle contraction. TUDC by itself caused no contraction and had no effect on CCK-induced contraction. Values are means ± SE of 3 experiments.](http://ajpgi.physiology.org/)
Mechanism of TCDC-induced contraction. H₂O₂ content was measured to determine whether the contraction induced by TCDC and its inhibition on CCK-induced muscle contraction were mediated by generation of free radicals (Fig. 5). Muscle cells were treated with the NADPH oxidase inhibitor (Pit2I) or with the xanthine oxidase inhibitor allopurinol for 15 min before treatment with TCDC for 15 min. Pit2I and allopurinol by themselves had no effect on H₂O₂ production (Fig. 5A). The magnitude of the H₂O₂ generation increased significantly from 2.2 ± 0.5 to 11.4 ± 2.2 μM/mg protein after TCDC treatment (Fig. 5B; **P < 0.001 vs. control group in Fig. 5A by Student’s t-test). Pit2I or allopurinol pretreatment blocked TCDC-induced increase in H₂O₂ content (***P < 0.01 vs. TCDC alone by Student’s t-test). These data suggest that increased generation of free radicals through NADPH oxidase and xanthine oxidase pathways play a role in the TCDC-induced muscle contraction.

The effects of the NADPH oxidase inhibitor Pit2I and of the xanthine oxidase inhibitor allopurinol on muscle contraction were studied to further support these findings. Pretreatment with Pit2I blocked the TCDC-induced contraction (Fig. 6A; *P < 0.001 vs. TCDC alone by Student’s t-test). Pit2I also prevented the inhibitory action of TCDC on CCK-8-induced contraction from 14.2 ± 0.9 to 19.8 ± 1.1%, which was not different from that induced by CCK-8 alone (Fig. 6B; **P < 0.05 vs. TCDC + CCK group by Student’s t-test). Similar results were obtained in xanthine oxidase inhibitor allopurinol-pretreated muscle cells. Pretreatment with allopurinol blocked TCDC-induced contraction (Fig. 7A; *P < 0.001 vs. TCDC alone by Student’s t-test). It also reduced the inhibitory effect of TCDC on CCK-8-induced contraction, the magnitude of contraction induced by TCDC plus CCK-8 increased from 14.2 ± 0.9 to 17.3 ± 2.1%, which was not different from that induced by CCK-8 alone (Fig. 7B; **P < 0.05 vs. TCDC + CCK group by Student’s t-test).
The level of lipid peroxidation (expressed as MDA) was determined as a measure of damage to the integrity of plasma membranes after exposure to TCDC (Fig. 8). TCDC markedly increased the levels of lipid peroxidation in plasma membranes (*P < 0.001 vs. control by Student’s t-test). The increase in lipid peroxidation caused by TCDC was blocked by pretreatment with Pt2I or with allopurinol from 425 ± 75 to 235 ± 41 and 275 ± 42 nmol MDA/100 mg protein, respectively. (**P < 0.01 vs. TCDC group by Student’s t-test). These data suggest that TCDC caused damage to the plasma membrane by generating free radicals.

We then assumed that TCDC increases PGE2 production, because its contraction is blocked by indomethacin, and that it generates free radicals that are known to increase prostaglandin synthesis (26, 33). PGE2 content was therefore measured in muscle homogenates after TCDC treatment by radioimmunoassay. TCDC significantly increased the PGE2 production (Fig. 9; *P < 0.01 vs. control group by Student’s t-test), which was reduced by Pt2I or allopurinol pretreatment (**P < 0.05 vs. TCDC group by Student’s t-test). These data suggest that the increased PGE2 production induced by TCDC is mediated by free radicals.

The activities of free-radical scavengers SOD and catalase were determined to further support these assumptions. TCDC treatment increased SOD activity (Fig. 10; *P < 0.01 vs. control group by Student’s t-test), which was inhibited by Pt2I or allopurinol (***P < 0.01 vs. TCDC group by Student’s t-test). Similarly, catalase activity was also increased by TCDC treatment (Fig. 11; *P < 0.01 vs. control group by Student’s t-test) and was inhibited by Pt2I or allopurinol pretreatment before TCDC (***P < 0.01 vs. TCDC group by Student’s t-test).
Moreover, pretreatment with free-radical scavengers blocked the TCDC-induced contraction and its inhibition on CCK-8-induced contraction. Catalase, but not SOD, blocked TCDC-induced contraction (\(*^* P < 0.01\) vs. TCDC group by Student’s t-test). Pretreatment with catalase prevented the TCDC inhibition on CCK-induced contraction (\(** P < 0.01\) vs. TCDC + CCK group by Student’s t-test).

Pathways involved in the actions of TCDC. The signal-transduction pathways, whereby TCDC caused muscle contraction, were determined using inhibitors of PKC, MAPK, and cPLA2 (Fig. 13). PKC inhibitor chelerythrine, MAPK inhibitor PD-98059, and cPLA2 inhibitor arachidonyl trifluoromethyl ketone blocked TCDC-induced contraction (\(*^* P < 0.01\) vs. TCDC group by Student’s t-test), but they did not eliminate the TCDC inhibition on CCK-induced contraction (\(** P < 0.01\) vs. TCDC + CCK group by Student’s t-test).

DISCUSSION

The present studies showed that the hydrophobic bile acid TCDC induces muscle contraction and inhibits CCK-induced contraction in isolated muscle cells. These findings are in agreement with a previous study (34) that showed that hydrophobic bile salts impair the contraction of gallbladder muscle strips in response to CCK and electrical field stimulation. They may also explain, in part, the increase in gallbladder volume after treatment with hydrophobic bile acids in patients with gallstones (34).

In contrast, TUDC had no effects on muscle cells, but it prevented the deleterious actions that are observed in enzymatically dissociated muscle cells induced by TCDC. This hydrophilic bile acid therefore appears to act at the cellular level by directly inhibiting the actions of hydrophobic bile acids on plasma membranes (12). These results may explain, in part, the prophylactic actions of TUDC in patients with symptomatic gallstones, in whom there was a significant reduction in the incidence of biliary colicky pain and acute cholecystitis (28).

However, the therapeutic effects of TUDC in vivo may involve additional mechanisms. It may replace more apolar bile acids in bile and serum, inhibit the reabsorption of toxic bile acids in the intestine, and stimulate the exocytosis and choleretic leading to increased elimination of apolar bile acids and toxins from the liver and bile (13).
Our studies reveal some of the mechanisms whereby hydrophobic bile acids affect the functions of gallbladder muscle cells. Their initial effect appears to be confined to the plasma membrane and may also involve the mitochondria. In agreement with previous studies (3, 26), TCDC generates free radicals that explain its two major actions on muscle cells. They affect membrane phospholipids leading to lipid peroxidation as demonstrated by the increase in MDA and generation of active phospholipids by nonenzymatic actions such as platelet-activating factor-like lipids that can stimulate cPLA₂ and produce prostaglandins, particularly PGE₂ (10, 24). Increased prostaglandins explain the muscle contraction induced by TCDC, which is mediated by the PKC and MAPK pathway. This conclusion is supported by the finding that antagonists of cyclooxygenase, PKC, MAPK, and cPLA₂ block the TCDC-induced contraction. These findings are in agreement with a previous report (5) that hydrophobic bile acids can increase membrane phospholipid turnover and free-radical production that can lead to tissue injury by attacking membrane lipids causing lipid peroxidation, which, in turn, causes the accumulation of highly toxic metabolic byproducts (18).

Increased generation of free radicals may also explain the TCDC inhibition of the CCK-induced muscle contraction, because they damage unprotected transmembrane receptors, as demonstrated by the reduced 125I-labeled CCK-8 binding to gallbladder muscle cells pretreated with H₂O₂ (29). This conclusion is supported by the finding that catalase, a free-radical scavenger, blocks the TCDC-induced inhibition of the contraction caused by CCK-8. It has been reported that incubation of cultured endothelial cells with catalase prevented cell damage and lipid peroxidation caused by oxidative stress (1, 7). Because H₂O₂ crosses the plasma membrane freely, endogenously generated H₂O₂ induced by TCDC may be inactivated by catalase outside the cell (35).

The TCDC inhibition of the contraction induced by CCK-8, due to damage to the CCK-A receptors, is better appreciated when muscle cells are incubated with indomethacin before their exposure to TCDC and CCK-8. This is because TCDC causes simultaneous muscle contraction as well as complete inhibition of the CCK-8-induced contraction. Inhibition of prostanoid formation blocks the TCDC-induced contraction and unmasks its full effect on the CCK-8 actions.

Furthermore, the mechanism whereby TCDC generates free radicals is not known. Free radicals are generated in the mitochondria and, perhaps more degradable, in the plasma membrane by the actions of NADPH oxidase (8, 17, 19). It is conceivable that hydrophobic bile acids stimulate these enzymes, because their inhibitors blocked all the deleterious effects caused by TCDC, including generation of free radicals, lipid peroxidation, increased PGE₂ synthesis, and muscle contraction. These conclusions are also supported by the finding that catalase, a scavenger of free radicals, had similar protective actions against the TCDC effects.

The finding that gallbladder muscle cells are subjected to oxidative stress when exposed to TCDC suggests that hydrophobic bile acids may contribute to the pathogenesis of acute experimental cholecystitis in the guinea pig and possibly in humans. Muscle cells from acutely inflamed gallbladders exhibit an increase in reactive oxygen species (H₂O₂), lipid peroxidation, increased PGE₂ content, and activities of scavengers of free radicals, abnormalities that were demonstrated in the TCDC-treated muscle cells (31).

In summary, TCDC contracts muscle cells by generating free radicals that cause muscle contraction by stimulating the synthesis of PGE₂, which activates the PKC-MAPK pathway. Free radicals also inhibit the CCK-induced contraction by damaging plasma membrane receptors. These effects are prevented when muscle cells are pretreated with TUDC. PGE₂ appears to stimulate the activities of free-radical scavengers, suggesting that it may have a cytoprotective role. Moreover, the actions of TCDC reproduce all the muscle changes observed in muscle cells from animals with acute cholecystitis or when normal muscle cells are treated with free radicals (H₂O₂). Therefore, these findings suggest that they may contribute to the pathogenesis of acute cholecystitis by generating reactive oxygen species.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK-27389. These data were partially presented at the annual meeting of the American Gastroenterological Association in May, 1999, Orlando, FL.

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