Deoxycholic Acid Induces Intracellular Signaling through Membrane Perturbations*

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Secondary bile acids have long been postulated to be tumor promoters in the colon; however, their mechanism of action remains unclear. In this study, we examined the actions of bile acids at the cell membrane and found that they can perturb membrane structure by alteration of membrane microdomains. Depletion of membrane cholesterol by treating with methyl-β-cyclodextrin, and staining for cholesterol with filipin showed that DCA caused a marked rearrangement of this lipid in the membrane. Likewise, DCA was found to affect membrane distribution of caveolin-1, a marker protein that is enriched in caveolae membrane microdomains. Additionally, fluorescence anisotropy revealed that DCA causes a decrease in membrane fluidity consistent with the increase in membrane cholesterol content observed after 4 h of DCA treatment of HCT116 cells. Significantly, by using radiolabeled bile acids, we found that bile acids are able to interact with and localize to microdomains differently depending on their physicochemical properties. DCA was also found to induce tyrosine phosphorylation and activate the receptor tyrosine kinase epidermal growth factor receptor in a ligand-independent manner. In contrast, ursodeoxycholic acid did not exhibit any of these effects even though it interacted significantly with the microdomains. Collectively, these data suggest that bile acid-induced signaling is initiated through alterations of the plasma membrane structure and the redistribution of cholesterol.

Bile acids are hydrophobic derivatives of cholesterol that play an important role in digestion and absorption of fats. They are synthesized in the liver, stored in the gallbladder, and excreted into the intestine as conjugated bile acids covalently linked to glycine and taurine. Bile acids serve many important physiological functions, including cholesterol homeostasis, lipid absorption, and generation of bile flow, that help in the excretion and recirculation of drugs, vitamins, and endogenous and exogenous toxins (1). Typically, after secretion into the intestines, bile acids are efficiently reabsorbed through enterohepatic circulation, although a small percentage (~4%) is known to escape into the colon (2, 3). There, the primary bile acids, cholic acid (CA),2 and chenodeoxycholic acid (CDCA), may be deconjugated and dehydroxylated by intestinal bacteria to form secondary bile acids, such as deoxycholic acid (DCA), lithocholic acid, and ursodeoxycholic acid (UDCA). More importantly, secondary bile acids such as DCA have been postulated to be tumor promoters in the colon (4). In fact, in vitro studies have revealed the cytotoxic effects of secondary bile acids on the colon further implicating them in tumorigenicity (5). Animal studies have shown that DCA can induce loss of colonic epithelium and a subsequent proliferative response (6). Several studies have shown a dose-dependent effect of bile acids and the incidence of tumors, all of which support a causal link of bile acids to cancer occurrence (7, 8). Furthermore, epidemiological studies have confirmed that consumption of a high fat/low fiber diet leads to elevated fecal concentrations of secondary bile acids and is associated with an increased incidence of colorectal cancer (9, 10). Finally, clinical studies have demonstrated that patients with colorectal cancer have higher levels of bile acids in the colonic lumen further supporting a role for bile acids as tumor-promoting agents (11–13).

In the liver, numerous studies have revealed that bile acids are signaling molecules that activate nuclear receptors and regulate many physiological pathways and processes to maintain bile acid and cholesterol homeostasis. Analyses of orphan receptors expression patterns in enterohepatic tissues have identified bile acids as ligands for the farnesoid X receptor (14). Particularly, the primary bile acids CDCA and to a lesser extent the secondary bile acids lithocholic acid and DCA have all been shown to be potent activators of farnesoid X receptor (15, 16). Additionally, studies by Stratvitz et al. (17) have shown that bile acids can transactivate protein kinase C in hepatocytes and repress the cholesterol 7α-hydroxylase enzyme CYP7A1. In addition, animal studies have also demonstrated a role for bile acids in the activation of inflammatory cytokines and the MAPK signaling pathways involving nuclear receptors such as HNF-4 (18). More recently, we have shown that hydrophobic bile acids such as DCA are able to activate signaling cascades in colon cancer cells (19). We showed that bile acids can stimulate intracellular signaling cascades, including those mediated by protein kinase C and mitogen-activated protein kinases (MAPK), two important signal transducers that activate intracellular signals that control cell growth and are involved in tumorigenesis (19, 20). Moreover, DCA appears to suppress the mitogenic activation of the p53 tumor suppressor (21). Hence, DCA can activate mitogenic signaling and inhibit tumor suppressor activity.

Studies done in our laboratory have shown a differential effect of bile acids based on their hydrophobicity. Highly hydrophobic bile acids such as DCA and CDCA are able to induce apoptosis rapidly, whereas UDCA, a less hydrophobic bile acid, is not cytotoxic and is able to inhibit fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; Mes, 4-morpholineethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
proliferation in colon cancer cell lines (22, 23). In fact, because of its hydrophilic nature UDCA has been found to inhibit DCA-induced apoptosis in rat hepatocytes through modulation of the mitochondrial transmembrane potential and through production of reactive oxygen species (24). The above findings not only strengthen a role for bile acids in signaling but also reveal a distinct mechanism of action of bile acids based on their physicochemical properties.

An essential constituent of plasma membranes is cholesterol, which rigidifies the membrane and is an important structural component of membrane microdomains (25). Due to the fact that bile acids are cholesterol derivatives with detergent properties, it has been speculated that bile acids may alter the stability of the membrane lipid bilayer (26). In fact, it is hypothesized that bile acids with increased hydrophobicity have a greater capacity to perturb the structure of, or partly digest, cell membranes (27–29). Moreover, cholesterol has been shown to play an important role in the functioning of receptor-mediated signaling (30), and cholesterol has also been shown to play a critical role in organizing membrane microdomains, such as rafts and caveolae through binding of caveolin proteins (31, 32). Because bile acids are cholesterol derivatives and cholesterol is enriched in microdomains and able to modulate receptor activity in rafts and caveolae, we decided to investigate the effects of bile acids on the membrane. We found that hydrophobic bile acids have a marked effect on membrane structure and activation of intracellular signaling. The role that these activities may have in bile acid-mediated tumorigenesis is discussed.

**EXPERIMENTAL PROCEDURES**

**Bile Acids**

DCA, CA, and CDCA were obtained from Sigma, and UDCA was obtained from Calbiochem. The stock solutions for these bile acids were maintained as 100 mM stock solutions diluted in deionized water.

**Antibodies and the Reagents**

The β-actin and ERK antibodies were used at a 1:5000 dilution and were obtained from Sigma. Caveolin-1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). EGF receptor antibody and phospho-EGFR (Tyr-1068) were obtained from Cell Signaling. Anti-EGF neutralizing antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse monoclonal phosphorysine antibody was purchased from Santa Cruz Biotechnology. Mouse and rabbit secondary antibodies were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). The fluorescent probes 1,6-diphenyl-1,3,5-hexatriene, trimethylammonium-diphenylhexatriene (TMA-DPH), and Alexa-Fluor 594 secondary antibody were obtained from Molecular Probes (Eugene, OR). Vectashield mounting medium was purchased from Vector Laboratories (Burlingame, CA). The Prolong antifade kit was purchased from Molecular Probes (Eugene, OR). Cholesterol reagent was purchased from Raichem, division of Hemagen Diagnostics (San Diego). The ceramide (C-16 and C-18) and phosphatidylcholine standards were from Avanti Polar Lipids Inc. The cholesterol standard from Raichem cholesterol detection kit was subjected to cholesterol oxidation (Calbiochem) treatment to generate cholestaneone standard. BioSafe II scintillation fluid was purchased from Research Products International (Mount Prospect, IL). The LK5D Silica Gel TLC plates were purchased from Whatman (Mobile, AL).

Methyl-β-cyclodextrin, spheromyelin, genistein, menadione, filipin complex Streptomyces filipinensis, protease inhibitor mixture, the in vitro toxicity assay kit, ethidium bromide, and acridine orange were all purchased from Sigma. All other chemicals were obtained from Sigma.

**Cells**

The human colon adenocarcinoma cell lines HCT116 and the epidermoid carcinoma cell line A431 were purchased from the American Tissue Type Culture Collection (Manassas, VA). All cell lines were maintained in bulk culture in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 mM sodium pyruvate, 100 µM nonessential amino acids, 100 units of penicillin, and 100 mg of streptomycin in a 5% CO$_2$ atmosphere at 37 °C and passaged using 0.25% EDTA/trypsin.

**Detection of Phosphorysine-activated Proteins**—HCT116 cells were grown in 10-cm culture dishes in FBS supplemented DMEM to 90% confluence. Twelve hours prior to the study, the medium was replaced by serum-free DMEM. Then cells were treated with bile acids at 500 µM. Control cells (kept in serum-free media) and bile acid-treated cells were harvested and lysed in RIPA lysis buffer (0.05 M Tris, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) containing protease and phosphatase inhibitors (10 mM NaF, 10 mM sodium pyrophosphate, 10 mM Na$	ext{VO}_4$). Forty microliters of SDS loading buffer (4% SDS, 0.1 M Tris, pH 8.0, 2 mM EDTA, β-mercaptoethanol, 0.1% bromphenol blue) were then added to 100-µg samples, boiled for 5 min, loaded onto a 7.5% or other SDS-polyacrylamide gel, and subjected to electrophoresis at 60 V for 16 h. Proteins were electrophotorethetically transferred to nitrocellulose membranes for 3 h. The membranes were then blocked with 5% milk in TBS-T (Tris-buffered saline with Tween) and then incubated overnight with the primary antibody at 4 °C. Proteins were visualized by ECL reagents (Amershams Biosciences) and exposed to Kodak film.

**Apoptosis Assay**—Apoptosis was ascertained microscopically using ethidium bromide and acridine orange as described previously (33). Briefly, cells were seeded in 30-mm plates at 1 × 10$^5$ cells for 24 h until they reached confluence. They were then treated with various bile acids, genistein, or methyl-β-cyclodextrin for the times indicated. Both the floating and adherent cells were collected and centrifuged. They were then washed in PBS, pH 7.0, and resuspended in 1 ml of DMEM. To detect apoptosis, 10 µl of cells were mixed with ethidium bromide and acridine orange solution (100 µg/ml each in DMEM) and visualized for morphological changes. Acridine orange stains the nuclei green in viable cells, and the propidium iodide is excluded. However, in apoptotic or necrotic cells, the nuclei are stained red by the propidium iodide, and the morphology of the nucleus easily determines whether the cell is apoptotic or necrotic. A minimum of 200 cells was counted and the percentage of apoptotic cells determined. All experiments were performed in triplicate.

**In Vitro Toxicology Viability Assay, MTT-based**—Cell viability was measured by using an in vitro toxicity assay kit from Sigma. HCT116 cells were seeded at 3 × 10$^4$ cells and allowed to grow for 24 h in 6-well plates until they reached confluence. Cells were then incubated in serum-free media containing 500 µM methyl-β-cyclodextrin for 12 h. They were then washed three times in PBS and re-incubated in phenol red-free media along with reconstituted MTT in an amount equal to 10% of the culture medium volume. Cells were then returned to a 37 °C incubator for 3 h. After the incubation period, the resulting formazan crystals were dissolved in an MTT solubilization solution in an amount equal to the original culture medium volume. To ensure complete dissolution of formazan crystals, the solution was gently pipetted up and down, and the absorbance was then read using a Max 200 microplate spectrophotometer at a wavelength of 570 nm. All experiments were performed in triplicate.

**Filipin Staining and/or Co-localization with Caveolin—**HCT116 cells seeded on coverslips were rinsed briefly three times in PBS and
then fixed with 4% paraformaldehyde in H$_2$O for 30 min at room temperature. Cells were then washed three times, quenched with 1.5 mg/ml glycerine in PBS for 10 min, permeabilized with 0.5% Triton X-100, and subsequently stained with 0.05 mg/ml filipin in PBS for 30 min. The cells were again washed three times with PBS and mounted with coverslips in 90% glycerol/PBS. The slides were then viewed on a fluorescent microscope using a 40X objective and a 4,6-diamido-2-phenylindole filter (340–380 nm excitation). For the localization of caveolin-1, HCT116 cells were seeded and fixed as described above. The cells were then rehydrated with PBS for 5 min before the addition of caveolin-1. Cells were then placed in a 32 °C incubator for 1 h and subsequently washed three times with PBS. An Alexa Fluor 594-conjugated secondary antibody was then added to the cells for 30 min. Subsequently, cells were washed three times with PBS, mounted with a slow anti-fading agent kit, and observed using a Nikon PCM2000 confocal microscope and a 60X, 1.4NA Plan Apo objective using a Green-He/Ne laser (633 nm).

Membrane Fluidity Measurements—HCT116 cells seeded on coverslips were incubated overnight in serum-free medium or in serum-free medium containing 500 μM methyl-β-cyclodextrin. Cells were then incubated at 37 °C with 500 μM fluorescent DCA in serum-free medium for various times. Next, coverslips were washed three times in PBS and then fixed with 4% paraformaldehyde in H$_2$O for 30 min at room temperature. Cells were again washed three times in PBS and mounted with Vectashield mounting medium. For the co-localization of fluorescent bile acids and caveolin-1, cells were treated with salt DCA and UDCA for 1 h at 37 °C, washed three times in PBS, and fixed with 4% paraformaldehyde. Cells were then permeabilized with 0.5% Triton X-100 and incubated with cavolin-1 in a 32 °C incubator for 1 h and later washed three times with PBS. An Alexa Fluor 594-conjugated secondary antibody was then added to the cells for 30 min along with 500 μM fluorescent DCA. Subsequently, cells were washed three times with PBS, mounted with a slow anti-fading agent kit, and observed using a 40X objective on a Nikon confocal microscope with an argon filter (590–617 nm excitation). Particle percentage analysis was performed using the Image J program from the National Institutes of Health.

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- The synthesis of fluorescently tagged deoxycholic acid derivative 2 followed the procedure described by Mills et al. (35, 36) for the synthesis of cholyllysylfluorescein 1. Deoxycholic acid was coupled with N$^\text{δ}$-benzoxycarbonyl lysine methyl ester using N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline to give a product in 98% yield. Saponification of the methyl ester gave a new product in 62% yield, and removal of the benzoxycarbonyl group via hydrolysis gave deoxycholyllysyl formate in 98% yield. Conversion to the sodium carboxylate salt was followed by reaction with fluorescein isothiocyanate (FITC) in aqueous sodium bicarbonate buffered to pH 9.5. Following purification by reverse phase column chromatography (37–53 μM C-18 silica eluted with MeOH/H$_2$O, 1:1), the desired compound 2 (Scheme 1) was obtained in 35% yield. The corresponding fluorescently tagged ursodeoxycholic acid derivative 3 (37) was prepared in a similar fashion (complete synthesis scheme available upon request).

- Purification of Caveolin-enriched Membrane Fractions—Total cell membranes were isolated by the method of Song et al. (38) with slight modifications. Briefly, HCT116 cells were homogenized in 1 ml of homogenization buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 200 mM sucrose, 1 mM phenylmethylsulfonyl fluoride). The nuclei and cell debris were removed from the homogenate by centrifugation at 900 × g for 10 min at 4 °C. The resulting lysate was then sonicated, adjusted to 45% sucrose by the addition of sodium carbonate and 2 ml of 90% sucrose prepared in MBS (25 mM Mes, pH 6.5; 0.15 M NaCl), and placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient was formed above (4 ml of 5% sucrose, 4 ml of 35% sucrose, both in MBS containing 250 mM sodium carbonate) and centrifuged at 39,000 rpm for 16–20 h in an SW40 Ti rotor. Twelve 1-ml fractions were collected and analyzed by SDS-PAGE and/or used in cholesterol analysis. In addition, whole cell lysate of A431 cells was also prepared by a nondetergent purification method in order to obtain a more purified raft fraction (38).

- Cholesterol/Cholestenone Extraction and Detection—To measure the increase in cell surface cholesterol, HCT116 cells were grown to 85–90% confluence and treated with DCA for the indicated times. Cells were washed with PBS, harvested by scraping, and fixed in 1% glutaraldehyde for 10 min. Cells were again washed and subjected to 4 units of
chol {\text{-}4}} \text{cholesterol oxidase in PBS at 25 °C for 30 min. Cells were then lysed in 0.5% Triton X-100, and total lipids were extracted according to the Bligh and Dyer method (39). Briefly, 100 μl of cell lysate was mixed with 3:7:5 μl of chloroform/methanol (1:2) followed by 30 s of vortexing. Subsequently, 125 μl of chloroform and 125 μl of 1 M NaCl were added while mixing. The contents were then centrifuged to separate the organic phase from the aqueous phase. The organic fraction was then collected and evaporated in a speed vacuum centrifuge. The dried extracts were dissolved in chloroform/methanol (1:1) and were subsequently applied to an LK5D Silica Gel 150A TLC plate (Whatman). The samples along with cholesterol and cholesterol standards were separated on TLC plates using chloroform/methanol (100:2) as running solvent. The detection and densitometry were carried out as described by Herget et al. (40). Briefly, the dried plates were exposed to a solution containing 10% cupric acid and 8% phosphoric acid, and the plates were charred at 150 °C for 10 min until the cholesterol and cholestene bands were visible. The plates were then scanned, and densitometry was carried out. The density values were normalized per microgram of protein for plotting graphs.

**Detection of Ceramide and Sphingomyelin**—To detect cellular changes in sphingomyelin and ceramide, HCT116 cells were treated with DCA for the indicated times and harvested as described above. The cells were lysed in 0.5 Triton X-100, and total lipids were extracted according to a modified Bligh and Dyer method (41). Again, 100 μl of cell lysate was added to 120 μl of chloroform and 120 μl of methanol (mixed with 2% acetic acid) and vortexed three times for 10 s. The subsequent steps were essentially similar to the method described above except that the phosphatidylincholine, ceramide (C-16 and C-18), and sphingomyelin markers were run on an LK5D Silica Gel 150A TLC plate with the samples, while the phospholipids were resolved in a solvent system containing chloroform/acetone/methanol/acidic acid/water (10:4:3:2:1, v/v). The dried plates were then exposed to a solution containing 10% cupric acid and 8% phosphoric acid, and the plates were charred at 150 °C for 10 min until the sphingomyelin, ceramide, and phosphatidylincholine bands were visible. The plates were then scanned, and densitometry was carried out. The density values were normalized per microgram of protein for plotting graphs.

**Detection of ['H]DCA and ['H]UDCA in Raft Microdomains**—HCT116 cells were seeded at 1.0 × 10⁶ cells in a 60-mm plate until 80% confluency was reached. Cells were then incubated in serum-free media for 12 h along with the EGF neutralizing antibody at 10 μg/ml. After 12 h, cells were subsequently treated with either EGF (10 μg/ml) or bile acids (50 μM) at various time points. Cells were then harvested and lysed with RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride, 100 mM sodium orthovanadate, and protease mixture inhibitors. Cells were agitated in lysis buffer (50 mM Tris-Cl, pH 7.4, 0.25 M NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and mixture protease inhibitors (Sigma)) at 4 °C for 1 h, and the supernatant was then collected. Protein concentrations were determined, and 100 μg of protein was loaded onto a 7.5% polyacrylamide gel. Western blot protocol was followed (as described previously) and pEGFR (Tyr-1068), EGFR, and actin were subsequently detected using these specific antibodies.

**RESULTS**

**Bile Acids Cause Capping and Formation of Large Aggregates at the Plasma Membrane of HCT116 Cells**—Although numerous studies have shown that bile acids can activate signaling molecules at the membrane, the mechanism by which this process occurs remains elusive. To determine the nature of the interaction of bile acids with the plasma membrane, fluorescent-tagged bile acids were generated. FITC-bile acids were tested for signal activation by examining ERK phosphorylation, and we found that FITC-DCA was able to activate ERK as do the parent bile acids (Fig. 1). Next, we added FITC-bile acids to HCT116 live cells for various times, and membrane localization was visualized through confocal microscopy. As seen in Fig. 2A, FITC-DCA did not distribute uniformly in the membrane but instead accumulated in a punctuated pattern in the membrane. In addition, the size of the patches was seen to increase with time. This was quantitated by analysis of the aggregates using ImageJ software. This revealed a time-dependent increase in the size of the aggregates suggesting a transition of microag-
FIGURE 2. Membrane capping and aggregate formation in response to DCA. HCT116 cells were treated with FITC-DCA or FITC-UDCA as described under “Experimental Procedures.” Cells were fixed with 4% paraformaldehyde and washed with PBS, and confocal images were then captured in order to visualize the plasma membrane (A, top). HCT116 cells were treated with 500 μM FITC-DCA at the indicated times. Bottom, bar graph depicting the percentage of aggregate formation that was calculated using Image J software (B). HCT116 cells treated for 12 h with 500 μM MβCD, washed in serum-free media, and then treated with 500 μM FITC-DCA for the same time points as described above (C). HCT116 cells treated with 500 μM FITC-UDCA for 5, 15, and 30 min and 1, 2, and 4 h and the percentage of aggregate formation were quantitated as mentioned above (D). HCT116 cells were treated for 12 h with 500 μM MβCD, washed in serum-free media, and then treated with 500 μM FITC-UDCA for the same time points as described above. The arrows depict regions where patches were observed. Micrographs are representative of three separate experiments.
UDCA, a chemopreventive bile acid, is known to inactivate specific signaling pathways induced by DCA (45). Consequently, we sought to examine the interaction of this bile acid with the membrane. Surprisingly, UDCA exhibited little capping at the membrane but rather was endocytosed, a pattern different from that observed at the membrane by DCA (Fig. 2C). Additionally, analysis of the size of the aggregates revealed that UDCA was found primarily in microaggregates, and the cells did not show the transition pattern toward macroaggregates as was observed previously in FITC-DCA-treated cells. In a parallel experiment, we treated HCT116 cells with MβCD for 12 h as described above and treated the cells with FITC-UDCA, and we again noticed that microaggregates overwhelmingly outnumbered macroaggregates (Fig. 2D). Overall, these data suggest that the mechanism of action of bile acids could be through clustering of membrane components, which could lead to specific activation of signaling pathways. This may be dependent on their chemical structure.

Bile Acids Induce Cholesterol Clustering in HCT116 Cells—Because bile acids are cholesterol derivatives, they may have the ability to intercalate into the lipid bilayer of the membranes and compete with, mimic, or displace cholesterol at the plasma membrane (46). To ensure that the effects we observed using FITC-labeled bile acids on cholesterol and the membrane reflected the behavior of these components when treated with unmodified bile acids, we stained with filipin, which binds cholesterol with high affinity and which fluoresces (at 340–380 nm), allowing us to visualize membrane cholesterol in bile acid-treated cells (47). HCT116 cells were initially stained with filipin and then observed over time (Fig. 3A). DCA causes cholesterol to aggregate into macroscopic domains that first appear after a 1-h treatment (Fig. 3B). The mottled pattern of cholesterol aggregates persisted and became highly evident after 4 h of treatment with DCA.

To determine whether the cholesterol aggregation was bile acid-specific, we repeated the same set of experiments with UDCA (Fig. 3C) and CA (Fig. 3D) in HCT116 cells. With UDCA no cholesterol aggregates were detected in the cell membranes. Likewise, CA, which appears to be biologically inert, did not affect cholesterol in the membrane. Because UDCA is hydrophilic and DCA is relatively hydrophobic, we conclude that bile acids may have different effects at the plasma membrane that is dependent on their biophysicochemical properties, which is consistent with our results published previously (48). Specifically, DCA may cause redistribution of cholesterol at the plasma membrane, although UDCA maintains the normal distribution pattern of cholesterol at the membrane.

Cholesterol Redistribution at the Membrane Is Specifically Induced by DCA—We postulate that DCA causes its cytotoxic effects by disrupting the membrane structure. However, it is conceivable that the changes in membrane cholesterol distribution that occurs when cells are incubated with DCA could be because of the apoptotic process and not because of the bile acid. To test this possibility, we induced apoptosis using menadione (a synthetic form of vitamin K) that is known to cause apoptosis through oxidative processes (49). HCT116 cells were treated with 60 μM menadione, and apoptosis was quantified as described previously (4). In a parallel experiment, menadione-treated HCT116 cells were also labeled with filipin in order to examine the cholesterol status in the cells. Interestingly, filipin staining revealed that the membrane was intact even after 4 h of menadione treatment when the quantity of apoptotic cells had reached 40% (Fig. 4, inset). Hence, no apparent cholesterol...
Activity of DCA in HCT116 Cells

FIGURE 4. Characterization of the effects of DCA at the membrane. HCT116 cells were serum-starved for 12 h and then treated with 60 μM of menadione for various times as indicated. Apoptosis was then determined using ethidium bromide and acridine orange in a morphological assay as described under “Experimental Procedures.” Inset depicts HCT116 cells treated with menadione for 4 h and then stained with 0.05 mg/ml filipin. Arrows indicate regions of filipin staining with no detection of cholesterol aggregation. The results are shown as the mean ± S.D. from three independent experiments. * indicates significantly different values (p < 0.01) as compared with the corresponding values obtained for control cells.

FIGURE 5. Cholesterol depletion suppresses DCA-induced apoptosis. A, HCT116 cells were serum-starved and treated with increasing concentration of MβCD for 12 h and then treated with 500 μM DCA for 4 h. Apoptosis was then determined using ethidium bromide and acridine orange in a morphological assay. B, HCT116 cells were treated as described above but washed twice in serum-free media and then treated with 500 μM DCA for 4 h, and apoptosis was quantified using the method described above. C, cell viability assay showing percentage of viable HCT116 cells in cells treated with MβCD for 12 h and untreated cells. The results are shown as the mean ± S.D. obtained from three independent experiments. * indicates significantly different values (p < 0.01) as compared with the corresponding values obtained for control (Ctrl) cells.
Because bile acids are cholesterol derivatives, we presumed that MβCD could also be sequestering DCA and negating its effect. To address this issue, we again pretreated HCT116 cells for 12 h with MβCD, washed the cells twice with serum-free media, and then treated the cells with DCA. As predicted, pre-washing with serum-free media did have a significant effect on the amount of apoptosis, and only a 15% reduction could now be observed (Fig. 5B). Nevertheless, cells treated with 500 μM MβCD for 12 h and then DCA were found to have a statistically significant reduction in DCA-induced apoptosis. We assessed the viability of MβCD-treated cells and found that over 90% of the cells were viable and metabolically active as compared with the nontreated cells (Fig. 5C). Hence, the observed decrease in apoptosis confirms our FITC-bile acid confocal microscopy data and suggests that cholesterol may be important in mediating the biological effects of DCA.

**DCA Induces Changes in the Membrane Fluidity of HCT116 Cells**—Cholesterol with its polyhydrocarbon ring structure plays a distinct role in modulating membrane fluidity. It is an important component of the plasma membrane, and changes in plasma membrane cholesterol content can alter the physical state of the plasma membrane (51). In addition, the interaction of cholesterol with other membrane constituents has been shown to affect both membrane stability and rigidity (52). Because we have shown that DCA causes a redistribution of cholesterol at the plasma membrane, we decided to investigate the effects of bile acids on the fluidity of the membranes in HCT116 cells. Fluorescence polarization is known to be one of the most sensitive, reproducible, and convenient means of probing the fluidity and organization of membranes and therefore was used to analyze the effects of bile acids on membrane fluidity (53). 1,6-Diphenylhexatriene is a hydrophobic probe known to incorporate inside the hydrophobic core of all membrane bilayers. Its cationic derivative, TMA-DPH only inserts into the outer leaflet of the plasma membrane (54). Table 1 summarizes the results of the calculated anisotropy. Fluorescence anisotropy was used as a measure of membrane fluidity and is defined as the reciprocal of the membrane fluidity. DCA, which causes cholesterol to aggregate, resulted in a decrease in membrane fluidity. UDCA and CA both of which showed little or no cytotoxicity nor cholesterol aggregation had no effect on membrane fluidity. This result is consistent with our previous data showing a distinct effect on cholesterol by DCA as opposed to UDCA and CA, which do not alter the membrane composition.

**DCA Causes an Increase of Cholesterol Content in the Plasma Membrane**—Our filipin results prompted us to analyze further the changes that were occurring at the membrane. Because membrane cholesterol is redistributed with filipin treatment and MβCD causes a decrease in apoptosis, we decided to quantify the cholesterol in the membranes of bile acid-treated HCT116 cells using an enzymatic and colorimetric assay that utilizes cholesterol oxidase (42, 55). HCT116 cells were treated with DCA for 4 h or UDCA for 16 h, and a purified caveolin-enriched membrane was obtained. Following fractionation, cholesterol analysis revealed that an increase in cholesterol mostly occurred in those fractions where caveolin-1 was also detected (sucrose density fractions 4–6) (Fig. 6A). The DCA-treated cell gradients showed a 2.0-fold increase in cholesterol in the light fractions (fraction 4–6) as compared with the untreated or those treated with UDCA. Additionally, this increase in cholesterol was confirmed by oxidizing cell surface cholesterol using cholesterol oxidase, which showed a time-dependent increase in cholesterol in response to DCA treatments (Fig. 6C). These results not only support our filipin studies but also demonstrate that DCA is able to alter the state of cholesterol at the plasma membrane and consequently may affect membrane-associated proteins.

Because we have shown that DCA causes a redistribution of cholesterol and caveolin is known to bind cholesterol, we reasoned that redistribution of caveolin-1 might also be induced. To test whether membrane cholesterol redistribution by DCA has a significant effect on microdomains, we examined the localization of caveolin-1, a caveoleae marker, in response to the treatment of bile acids by using confocal microscopy. HCT116 cells were treated with DCA for 1 h, and localization of caveolin-1 was assessed by using immunofluorescence. As shown in Fig. 7, treatment with different bile acids caused a change in the localization of caveolin-1. Treatment with DCA for 1 h (Fig. 7, panel 3) caused internalization of the protein, although control and UDCA treatment (Fig. 7, panels 2 and 4) showed no internalization but rather an increase in the quantity of caveolin-1 at the membrane. In addition, Western blot analysis of whole cell extracts revealed that the total amount of caveolin-1 protein remained the same after DCA treatment (data not shown). Taken together, these results suggest that bile acids can indeed perturb the membrane, specifically microdomains, which may explain why bile acids are able to activate various intracellular signaling.

**DCA Modulates the Levels of Sphingomyelin and Ceramide in HCT116 Cells**—It is well established that lipid rafts and caveoleae are enriched in both sphingomyelin and ceramide. Moreover, studies by Slotte and Bierman (58) have shown that degradation of sphingomyelin in cultured fibroblasts led to a dramatic redistribution of cholesterol. Because the majority of cholesterol resides at the plasma membrane and DCA is affecting the levels of plasma membrane cholesterol, we reasoned that DCA may also be affecting the levels of sphingomyelin. Likewise, the known apoptotic lipid secondary messenger ceramide was also examined because of its apoptotic inducing effect in lipid membrane domains. Indeed, treatments of HCT116 cells with DCA caused a 50% decrease in the levels of sphingomyelin.

### Table 1

| Treatments | DPH          | TMA-DPH       |
|------------|--------------|---------------|
| Control    | 0.144 ± 0.027 | 0.286 ± 0.181 |
| CA         | 0.131 ± 0.017 | 0.298 ± 0.016 |
| UDCA       | 0.114 ± 0.009 | 0.297 ± 0.015 |
| DCA        | 0.228 ± 0.092 | 0.322 ± 0.062 |

**Activity of DCA in HCT116 Cells**

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**TABLE 1**

Bile acids affect membrane fluidity of HCT116 cells differently

HCT116 cells were serum-starved for 12 h and then treated with various bile acids (CA, UDCA, and CA) for 1 h. DPH and TMA-DPH were both used at 1 μM/ml to probe the membranes of the cells, and fluorescence anisotropy values were obtained with an 8600 Perkin Elmer spectrofluorometer (PerkinElmer Life Sciences). The results are shown as the mean ± S.D. obtained from three independent experiments.
although the total levels of phosphatidylcholine remained constant (Fig. 8A). In addition, treatments with DCA also showed a significant drop in the levels of ceramide after 4 h (Fig. 8B). Collectively, the above results support our hypothesis that bile acids are perturbing membrane-associated lipids, specifically those signaling lipids associated with raft and caveolae domains.

*Bile Acids Interact with Microdomains Differently*—Because different bile acids exert distinctly different biological effects (59, 60), we postu-
lated that these differences might be due to interaction with different membrane components. To examine the interaction of bile acids with membrane microdomains, HCT116 cells were treated with [3H]DCA and [3H]UDCA, respectively, and membrane localization was assessed using density sucrose gradient. Fig. 9B showed that UDCA, a less hydrophobic bile acid, is distributed in the same gradient fractions as caveolin-1 suggesting that it can interact with the caveolae domain. DCA, on the other hand (Fig. 9A), showed minimal interaction with the caveolae domain. Overall, these results illustrate that different bile acids interact differently with the plasma membrane. This may be dependent on their chemical structure and is likely to be important for activation of intracellular signaling.

**DCA Activates Receptor Tyrosine Kinases in a Ligand-independent Manner**—Previously, we published that DCA is able to activate receptor tyrosine kinases such as the EGFR (45). The two plausible mechanisms by which this can occur is either through specific interaction of ligands with EGFR or through membrane perturbation that could eventually cause the receptors to autophosphorylate. Initially, we sought to determine the localization of EGFR in HCT116 cells; however, because of the low abundance of proteins in this cell line, we opted to use A431 cells, an epidermoid carcinoma cell line that overexpresses EGFR. A431 cells were treated with DCA or UDCA, and a density sucrose gradient was performed to assess the localization of EGFR in response to bile acids. Fig. 10 shows that untreated cells or cells treated with UDCA displayed normal levels of EGFR, which co-fractionates in the same region as caveolin-1. On the other hand, cells treated with DCA showed a decrease in the amount of EGFR as compared with the controls. This is similar to what we observed with caveolin.

We next examined the mechanism by which DCA causes EGFR activation, we treated HCT116 cells with an EGF neutralizing antibody and then examined the activation of EGFR after exposure to DCA. Fig. 11A showed that the neutralizing antibody abrogated the phosphorylation of EGFR induced by its natural ligand, EGF. In contrast, the neutralizing antibody (Fig. 11B) was unable to abrogate phosphorylation of EGFR in cells treated with DCA supporting the idea that DCA-induced signaling is ligand-independent.

**DISCUSSION**

Although many studies have implicated bile acids as activators of signal transduction pathways, their mechanism of action remains enigmatic. We present evidence suggesting that hydrophobic bile acids can have profound effects on the cell membrane and that this may lead to initiation of signaling induced by these bile acids.

Examination of membrane fractions from DCA-treated cells revealed an increase in the quantity of cholesterol in the plasma membrane, which was accompanied by an overall decrease in membrane fluidity. The membrane cholesterol in DCA-treated cells was seen to aggregate into large patches suggesting that DCA caused the rearrangement and perhaps solubilization of membrane cholesterol. This is consistent with the suggestion that the hydrophobic nature of bile acids enables them to solubilize the cell membrane and act as detergents (27–29, 61). Additionally, membrane capping was observed in cells treated with FITC-DCA and in the cholesterol of cells treated with DCA suggesting that bile acids-mediated receptor activation could occur as a result of bile acid interaction with the membrane. This stands in agreement with the notion that patches of aggregated receptors can migrate and coalesce to form a cap in a process involving cytoskeleton reorganization (62). Additionally, the observed microdomain interaction of radiolabeled bile acids suggests that depending on their physical property, bile acids are able to affect membrane activity differently. Interestingly, the membrane changes...
we observed in DCA-treated cells were not observed either when cells were treated with either CA or UDCA or when cells were treated with menadione, another apoptosis-inducing agent. Hence, the changes in membrane structure or composition that we observed were specific to DCA and appeared to be caused by DCA and are not a consequence of the apoptotic process. This could explain the differences in biological activities observed for different bile acids (22). Previous studies from our laboratory testing the activities of bile acids in vitro and in animal studies where bile acids were tested for tumor promoting activity showed that bile acids can exhibit oppos-
ing biological activities (4, 22, 63). Collectively, these observations suggest that bile acid interaction with the plasma membrane and with cholesterol may be an important step in the manifestation of the biological effects of these compounds. Consistent with this we found that depletion of membrane cholesterol using methyl-β-cyclodextrin led to reduced DCA-induced apoptosis. Hence, changing the composition of the plasma membrane can influence the ultimate biological outcome resulting from exposure to DCA.

Numerous studies have shown that bile acids activate intracellular signaling and that activation of these pathways is a key step in the induction of apoptosis and/or mitogenesis (64–66). Detailed analysis of activation of one of these, the MAPK pathway, shows that signaling is stimulated by DCA through activation of the EGF receptor (67). Because bile acids bear little resemblance to the natural ligand of the receptor, epidermal growth factor, the question arises is how can DCA activate receptors to induce intracellular signaling? One possibility is that the alterations in membrane structure caused by DCA may lead to stimulation of transmembrane receptor activity. It is well established that transmembrane receptors are anchored in specialized lipid microdomains in the plasma membrane known as caveolae or lipid rafts (68). These lipid microdomains are enriched in sphingomyelin, receptors, and cholesterol and represent platforms for conducting cellular functions such as vesicular trafficking and signal transduction (69). Additionally, studies have found the EGF receptor to be localized to the caveolae/rafts domains; consequently, this receptor could be activated by the effects of DCA on the cell membrane (70). We find that DCA causes an exit of caveolin, the major protein component of caveolae microdomains, from the plasma membrane and localization to the cytoplasm confirming that DCA does affect the quantity and positioning of caveolae in the membrane. Furthermore, fractionation of membranes from DCA-treated cells on sucrose gradients showed that there was a marked increase in the quantity of cholesterol in caveolin-containing fractions suggesting that not only does DCA change the membrane distribution of microdomains but also appears to alter their composition. This change in composition was further verified by thin layer chromatography which showed a decrease in both sphingomyelin and ceramide, both lipids localized to membrane microdomains. In fact, studies by Dobrowsky and co-workers (71) showed displacement of cholesterol in response to treatments with the apoptotic inducer ceramide. Thus it is conceivable that just like ceramide, DCA is also able to modulate the levels of cholesterol as well as that of membrane-associated proteins and lipids, and this may consequently lead to intracellular signaling.

Previously, we showed that DCA could activate the MAPK pathway; therefore we decided to analyze activation of EGF on the tyrosine residue involved in MAPK signaling (21). Significantly, phosphorylation on tyrosine 1068 of the EGF receptor was not suppressed by an EGF-inactivating antibody in cells that were treated with DCA. This indicates that activation of EGF by DCA occurs in an EGF-independent manner and is in agreement with previous work by Qiao et al. (72) that demonstrated that bile acids are able to activate the c-Jun NH2-terminal kinase pathway in a ligand-independent manner in hepatocytes. Collectively, our results strongly suggest that the EGF may be activated as a consequence of the changes in membrane composition and structure that are brought about by DCA. There is precedent for this type of mechanism in the observation that cholesterol depletion with methyl-β-cyclodextrin can lead to EGF dimerization, an indication of receptor activation (26). Additionally, based on our FITC-bile acids data, which shows that DCA causes membrane capping, it is conceivable that DCA-induced signaling occurs through membrane patching involving receptors localized in caveolae microdomains. It has been suggested that membrane components such as ceramides can re-organize into lipid patches capable of fusing into larger platforms (73). Hence, we propose that the signaling activities induced by DCA, and perhaps other bile acids, originate from the biochemical interaction between DCA and the lipids of the plasma membrane and cholesterol.

Although we chose EGF as a specific example receptor based on our previous studies, it seems unlikely that the activities exhibited by DCA are because of stimulation of this receptor alone. Indeed it is unlikely that the activity of any single receptor will likely explain bile acid activity. This is suggested by others and our experiments that examine tyrosine phosphorylation which shows that DCA has a pleiotropic effect with regard to.
Activity of DCA in HCT116 Cells

receptor activation.3 We found that several membrane proteins are affected and could become activated in response to DCA, and we are in the process of investigating the pathways affected by these transmembrane proteins. Because transmembrane receptors (i.e. EGFR and IGF1R) are localized to microdomains, we conclude that multiple signaling receptors are activated by DCA. Consistent with this are reports in the literature showing that DCA can activate EGFR (67), death receptors (72), and protein kinase C (64, 65, 74). Hence, it seems likely that the ultimate biological effect that bile acids exhibit is because of receptors activated as a consequence of perturbations in membrane structure and biophysical properties.

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