Bile acid hydrophobicity is correlated with induction of apoptosis and/or growth arrest in HCT116 cells

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Faecal bile acids have long been associated with colon cancer; highly hydrophobic bile acids, which induce apoptosis, have been implicated in the promotion of colon tumours. The moderately hydrophobic chemopreventive agent ursodeoxycholic acid (UDCA) does not induce apoptosis; rather, it causes colon-derived tumour cells to arrest their growth. To investigate the relationship between bile acid hydrophobicity and biological activity we examined 26 bile acids for their capacity to induce apoptosis or alter cell growth. We found that the rapidity with which, and the degree to which, bile acids could induce apoptosis or growth arrest was correlated with their relative hydrophobicities. Of the bile acids tested, only deoxycholic acid (DCA) and chenodeoxycholic acid, the most hydrophobic bile acids tested, could induce apoptosis in less than 12 h in the human colon cancer cell line HCT116. The moderately hydrophobic bile acids hyoDCA, lagoDCA, norDCA, homoUDCA and

isoUDCA induced growth arrest at 12 h but longer incubations resulted in apoptosis. Conjugation of glycine or taurine to the bile acids decreased relative hydrophobicity and eliminated biological activity in our assays. In addition, we tested a subset of these bile acids for their ability to translocate across cell membranes. When ¹⁴C-labelled and ³H-labelled DCA, UDCA and lagoDCA were added to cell cultures, we found only minimal uptake by colon cells, whereas hepatocytes had considerably higher absorption. These experiments suggest that hydrophobicity is an important determinant of the biological activity exhibited by bile acids but that under our conditions these activities are not correlated with cellular uptake.

Key words: cellular uptake, colon cancer, deoxycholic acid, signal transduction pathways, ursodeoxycholic acid.

INTRODUCTION

A typical part of the Western diet is a high fat intake that leads to increased levels of faecal bile acids [1,2]. After synthesis by the liver and excretion into the digestive tract, bile acids are metabolized by enteric bacteria to produce secondary bile acids [3,4]. These secondary bile acids, primarily deoxycholic acid (DCA) in humans, are cytotoxic to colon cells and have been implicated as tumour promoters [5,6]. Although the mechanism by which bile acids function to promote colon tumorigenesis is not known, it has been suggested that relative hydrophobicities are an important determinant of the biological properties of these compounds [7]. Bile acids are natural detergents that aid in fat solubilization and absorption. It is hypothesized that bile acids with increased hydrophobicity have a greater capacity to perturb the structure of, or partly digest, cell membranes [7–9]. In addition, it has been postulated that highly hydrophobic bile acids are able to pass through the membrane and interact with DNA and intracellular molecules, thus directly causing DNA damage and activating signalling cascades [10].

Highly hydrophobic bile acids such as DCA and chenodeoxycholic acid (CDCA) are able to induce apoptosis rapidly; this activity might require protein kinase C and the activator protein AP-1 [11]. Interestingly, the chemopreventive agent ursodeoxycholic acid (UDCA), a less hydrophobic stereoisomer of CDCA, is not cytotoxic and is able to inhibit proliferation in colon cancer cell lines [12–14]. Here we examine the biological effects of 16 different unconjugated bile acids and 10 conjugated bile acids on

the human colon cancer cell line HCT116, to determine whether bile acid hydrophobicity is indeed an important predictive indicator of bile acid's ability to induce apoptosis or growth arrest.

MATERIALS AND METHODS

Cell culture

The human colon cancer cell lines HCT116 and HT29 were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). The human liver cancer cell line HepG2 was a gift from Dr David Lei (University of Arizona, Tucson, AZ, U.S.A.). All cell lines were grown at 37 °C and in a humidified air/CO₂ (19:1) atmosphere in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL), 100 i.u. of penicillin, 100 mg of streptomycin, 2 mM L-glutamine, 4 mM sodium pyruvate and 100 μM non-essential amino acids.

Bile acid hydrophobicity

HPLC of bile acids was performed on a Hewlett-Packard Series 1100 instrument equipped with a data system to store and analyze data. The chromatography was conducted on a Zorbax eclipse XBD-C₁₈ reversed-phase column (4.6 mm internal diam. × 25 cm; 5 μm particle size) and a Hewlett-Packard 1047A

Abbreviations used: CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; DMEM, Dulbecco's modified Eagle's medium; UDCA, ursodeoxycholic acid.

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refractive-index detector was employed for detection of the various bile acids. The bile acid (10 µg) was dissolved in 10 µl of methanol and then injected into the HPLC column. A solvent system consisting of methanol/water/acetic acid (10:30:0.1, by vol.) was used and the flow rate was maintained at 2 ml/min (operating pressure 20–30 MPa).

Bile acids

DCA, cholic acid, CDCA and hyoDCA were obtained from Sigma Chemical (St Louis, MO, U.S.A.) and UDCA was obtained from Calbiochem (La Jolla, CA, U.S.A.). All were maintained as 100 mM stock solutions in water. All other bile acids, including the radiolabelled bile acids, were synthesized by A. K. Batta (V.A. Medical Center, East Orange, NJ, U.S.A.). The bile acids, except for radiolabelled bile acids, were also stored as 100 mM stock solutions in water. The radiolabelled bile acids were stored as 200 µM stock solutions in PBS. On the addition of bile acids to medium, no change in pH was observed.

Apoptosis assays

For apoptosis assays 10⁵ HCT116 cells were added to 60 mm tissue culture plates and left to attach for 24 h. This concentration of cells produced 30–40 % confluence at the time that bile acids were added. The cells were treated with 500 µM bile acids for the durations indicated. The medium was removed and saved. Cells were then rinsed in PBS and then trypsin-treated and resuspended in the saved medium. Approximately 5 × 10⁵ cells/ml were treated with Acridine Orange and ethidium bromide, each at 100 µg/ml.

Viable cell nuclei stain green with Acridine Orange. Apoptotic cell nuclei, which are condensed and often fragmented, acquire a red colour from the ethidium bromide, which enters the nucleus owing to increased nuclear membrane permeability. Cells were viewed microscopically at the 60× dry objective under UV radiation. Cells were counted as either viable or apoptotic by morphological criteria.

Incorporation of [³H]thymidine

HCT116 cells were plated at a density of 3.5 × 10⁵ cells per well of a 24-well plate, in 1 ml of medium. After 20 h the cells were treated with 500 µM bile acid for 12 h. Medium was then removed and replaced with DMEM; 10 µCi/ml [³H]thymidine was added to each well. After 1 h the DMEM was removed and the cells were harvested and added directly to Whatman 2.4 cm GF/C glass microfibre filters in a Millipore manifold apparatus. The filters were rinsed with 5 ml of distilled water, then rinsed twice with 2 ml of 70 % (v/v) ethanol and once with 2 ml of acetone and allowed to dry in air. Radioactivity was quantified by counting in a Beckman LS 5000 TD scintillation counter.

Radiolabelled bile acids

Approximately 6 × 10⁴ cells per well were left to attach to a 24-well plate for 24 h. The medium was then removed and 10 µM radiolabelled bile acid (10⁵ d.p.m.) was added per well with 200 µl of DMEM. ¹⁴C-labelled DCA, lagoonDCA or ³H-labelled UDCA added to HCT116, HT29 or HepG2 cells for 0, 3, 6 or 24 h. The medium was then removed and the cells were rinsed twice with 1 ml of PBS; 500 µl of a 10 % (w/v) SDS/10 mM EDTA solution was added to solubilize the cells. Radioactivity in the entire cell lysate from each sample was measured with a Beckman scintillation counter.

RESULTS

Hydrophobic bile acids induce apoptosis

Previous work in our laboratory and by others has shown that relatively hydrophobic bile acids are able to induce apoptosis in colon cancer cell lines [14]. To determine whether this ability to cause an apoptotic response is inherent in bile acids on the basis of their chemical structure or hydrophobicity, we tested 16 unconjugated and ten conjugated bile acids to determine what structural factors favoured the ability to cause apoptosis. Different bile acids that were structurally similar to DCA, CDCA and UDCA, but differed in the presence of or the orientation of hydroxy groups at C-3, C-6, C-7 and C-12 and/or by shortening or lengthening the side chain, were tested for biological activity. A graphical representation of the basic bile acid structure and where modifications occurred is shown in Figure 1.

The hydrophobicity of each of the 16 unconjugated bile acids was measured by HPLC and an ordered list was constructed on the basis of retention times (Table 1). Increased retention times indicate increased hydrophobicity. The bile acids were ordered on the basis of HPLC retention times and biological activity as determined in subsequent experiments. Previous work in the area of bile acid chromatography indicates that the order of bile acids

Figure 1 Bile acid primary structure

Diagram of a generalized bile acid structure. Modifications occurring in each of the bile acids tested are indicated by the labelled R groups. Glycine or taurine conjugations were made at the side chain labelled R^{*}.

Table 1 Unconjugated bile acids arranged in order of increasing HPLC retention times/increasing hydrophobicity with consideration to biological activity

Bile acid	HPLC retention time (min)
DCA	31.68
CDCA	24.48
NorDCA	19.44
HomoUDCA	14.54
LagoDCA	12.88
HyoDCA	12.12
UDCA	10.52
IsoUDCA	9.7
Cholic acid	15.26
IsoCDCA	14.76
NorCDCA	14.76
Murocholic acid	8.32
β-Muricholic acid	7.65
ω-Muricholic acid	7.18
NorUDCA	6.27
Urocholic acid	5.01

Figure 2 Apoptosis in HCT116 cells by bile acids

Unconjugated bile acids (500 μ M) were added to HCT116 cells for 12 h; apoptosis was measured by a visual evaluation of cell morphology. Values are means $\pm$ range for at least two samples. Cells treated with cholic acid showed no apoptosis.

with intermediate hydrophobicity varies, depending on the particular method or solvent used [15].

Next, each of the bile acids was tested for the capacity to induce apoptosis by incubating HCT116 cells with an unconjugated bile acid (500 μ M) for 12 h. HCT116 cells have an approx. 1% basal rate of apoptosis when measured by this assay. The results in Figure 2 show that only the most hydrophobic of the bile acids tested, CDCA and DCA, induced apoptosis, at levels of approx. 60%.

Bile acids conjugated with taurine and glycine occur naturally in humans and are less hydrophobic than their unconjugated counterparts [15,16]. Taurine and glycine conjugations were added to the side chains of DCA, CDCA, UDCA, hyoDCA and cholic acid and were tested for their ability to induce apoptosis in HCT116 cells. Incubation with conjugated bile acids (500 μ M) for 48 h did not induce apoptosis (results not shown). The covalent linkage of either amino acid to the bile acid side chain therefore nullified the capacity of DCA and CDCA to induce apoptosis. These results are consistent with the notion that hydrophobicity is important in determining the biological activity of these compounds. We observed a close relationship between the biological activity of a bile acid and its relative hydrophobicity. However, we could find no apparent link between a particular structural modification and biological activity.

Intermediate biological activities of moderately hydrophobic bile acids

During the course of our experiments we noticed that although some moderately hydrophobic bile acids did not induce apoptosis at 12 h, they did cause apoptosis after extended incubation with HCT116 cells. To examine the timing of apoptosis in more detail, several bile acids, including DCA, lagoonDCA, norDCA, isoUDCA, hyoDCA, homoUDCA and UDCA, were added to HCT116 cells at 500 μ M for 0, 3, 6, 12, 24 and 48 h (Figure 3). As expected, none of the bile acids except DCA induced apoptosis at 12 h. However, when the incubations were continued, apoptotic cells were detected at 24 and 48 h in all samples except those treated with UDCA. One of the bile acids, lagoonDCA, induced as much apoptosis as DCA. The sole exception, UDCA, did not

Figure 3 Effects of long-term exposure to moderately hydrophobic bile acids

Highly to moderately hydrophobic unconjugated bile acids were added at 500 μ M to HCT116 for 0, 3, 6, 12, 24 and 48 h; apoptosis was determined by cellular morphology. Values are means $\pm$ S.D. for at least three samples. Symbols: ▲, lagoonDCA; ○, DCA; ■, norDCA; △, isoUDCA; ●, hyoDCA; □, homoUDCA; ◆, UDCA.

Figure 4 Effects on cell growth by unconjugated bile acids

Cell growth after treatment with bile acid, measured by the incorporation of [^{3} H]thymidine. HCT116 cells were treated with 500 μ M unconjugated bile acids for 12 h. Values are shown as percentages of thymidine incorporation in an untreated control. Any bile acid that decreased cell growth by at least 60% was classified as causing growth arrest. Results for DCA and CDCA are not included because these bile acids induced high degrees of apoptosis at this time point. Values are means $\pm$ range for at least two samples.

induce apoptosis in cells even when incubation times were extended to 72 h (results not shown).

Moderately hydrophobic bile acids can inhibit proliferation

Because the moderately hydrophobic UDCA inhibits cell growth in HCT116 cells, we wished to determine whether other bile acids had similar abilities. We treated HCT116 cells with various bile acids. The results showed that higher relative hydrophobicities were correlated with decreases in cell growth as indicated by decrease in [^{3} H]thymidine incorporation. The results of treating HCT116 cells with different bile acids are shown in Figure 4.

Figure 5 Effects of conjugated bile acids on cell growth

Conjugated bile acids (500 μ M) were added to HCT116 cells for 48 h; cell growth was measured by the incorporation of [^{3} H]thymidine. Values are shown as percentages of thymidine incorporation in an untreated control. Values are means $\pm$ range for two samples, except for taurine-conjugated DCA and taurine-conjugated UDCA, which each contained results from one sample. Abbreviations: G-, glycine-conjugated; T-, taurine-conjugated.

Results are expressed as percentages of the untreated control. DCA and CDCA seemed to cause a marked decrease in the [^{3} H]thymidine incorporation that we attribute to cell loss due to the induction of high levels of apoptosis. UDCA, lagoonDCA, hyoDCA, homoUDCA, norDCA, isoDCA and isoUDCA caused a 65–80 % decrease in thymidine incorporation without inducing apoptosis at this time point (see Figure 2). These results indicate that these more moderately hydrophobic bile acids are able to induce growth arrest after 12 h of exposure.

We next examined whether the conjugation of amino acids to the side chain had any effect on the capacity of bile acids to induce growth arrest. Conjugated bile acids (500 μ M) were added for 48 h to HCT116 cells. Figure 5 shows that the conjugated bile acids tested had little or no capacity to decrease cell proliferation, in contrast with their unconjugated counterparts (see Figure 4). Although glycine-conjugated DCA did decrease cell growth by approx. 50 %, showing that it had some biological activity, the addition of glycine to DCA markedly altered its affect on cells. These experiments show that the addition of amino acids, which decreases the hydrophobicity of a bile acid, also abrogates or changes their activity.

Bile acids associate only moderately with colon cells

Several theories have suggested that bile acids can change the biological functions of colon and other cell types by passing through the cellular membrane and disrupting intracellular functions. Because this property would be expected to be related to hydrophobicity, we tested whether several bile acids had the ability to enter colon and liver carcinoma cell lines. HepG2, HCT116 and HT29 cells were treated with [^{14} C]-labelled UDCA or [^{3} H]-labelled DCA or lagoonDCA for 0, 3, 6 and 24 h. The cells were harvested and the quantity of radioactivity associated with the cells was measured (Figure 6). All cell types were also treated with [^{35} S]methionine for the same time course: all cells showed uptake in a time-dependent manner (results not shown). HepG2 cells are known to contain transmembrane bile acid transport proteins and nuclear receptors for bile acids. Unsurprisingly, when these cells were treated with radiolabelled bile acids they

Figure 6 Uptake of radiolabelled bile acids into HepG2, HCT116 and HT29 cells

Radiolabelled DCA (A), lagoonDCA (B) and UDCA (C) were added to HCT116 (■), HT29 (▲) or HepG2 (●) cells for 0, 3, 6 and 24 h in serum-free DMEM. Sample values represent total radioactivity (c.p.m.) associated with the cells in each sample.

showed a time-dependent uptake of the bile acids. However, the colon cancer cell lines HCT116 and HT29 showed minimal uptake of DCA and UDCA and no measurable uptake of lagoonDCA. In addition, the quantity of bile acids associated with colon cells did not increase over time. Instead, the amount of bile acid associated with colon cells remained constant between the 6

and 24 h time points. Therefore, although bile acids such as DCA and UDCA have marked effects on cell physiology, hydrophobicity is not correlated with increased cellular uptake of these drugs and uptake was not correlated with their biological activities.

DISCUSSION

Experimental evidence has suggested a link between bile acid exposure and changes in cell growth. Hydrophobicity has long been considered a marker of the biological activity of bile acids. Here we have tested 26 bile acids for their ability to induce apoptosis and found that only DCA and CDCA, the two most hydrophobic, induced apoptosis within 12 h. However, we also found that moderately hydrophobic bile acids, such as isoUDCA, norDCA, ligoDCA, homoUDCA and hyoDCA, also induced apoptosis but required much longer to accomplish this effect. These results were consistent with the notion that the ability and rapidity with which bile acids caused apoptosis were dependent on hydrophobicity. Conjugations of either taurine or glycine with biologically active bile acids, which decrease hydrophobicity, also eliminated or altered biological activity. Interestingly, our experiments with the moderately hydrophobic bile acids indicate that the interaction between bile acids and cells could be complex because they had the ability to decrease [³H]thymidine incorporation. In particular, these bile acids did not cause significant levels of apoptosis until 24 h after exposure, indicating that the growth-arrest phenomenon preceded the induction of apoptosis. Hence these moderately hydrophobic bile acids might require more time to achieve the same effect as that of more hydrophobic bile acids such as DCA. Only UDCA, which is less hydrophobic than the other biologically active bile acids, was unable to induce apoptosis.

Our biological results suggest that there are four distinct subclasses of bile acid. The first class seems to have no discernible biological effect and includes cholic acid, β - and ω -muricholic acid, murocholic acid, norCDCA, norUDCA and ursocholic acid. In addition, none of the conjugated bile acids, with the exception of glycine-conjugated DCA, seem to have detectable biological activity on colon cells in our assays. The second subclass of bile acids includes hyoDCA, homoUDCA, UDCA, ligoDCA and norDCA. These bile acids are able to suppress cell growth after 12 h of treatment. However, extended exposure results in apoptosis. The third category is the bile acids that rapidly induce apoptosis without first causing growth suppression; they include DCA and CDCA. The final subtype of bile acids is those that cause growth arrest but do not subsequently cause apoptosis. This subtype is represented by the chemopreventive agent UDCA and by the glycine-conjugated DCA.

Work by Rao et al. and others has shown that, *in vitro*, bile acids are able to bind directly to DNA and protein kinase C, which might lead to the activation of gene expression [17,18]. Hydrophobicity has been hypothesized to impart biological activity to bile acids by allowing them to solubilize and then cross the cell membrane. Hepatocytes, such as HepG2 cells, contain transmembrane bile acid transport proteins and bile acid nuclear receptors [19,20]. These cells absorb considerable quantities of bile acids in a time-dependent manner. Hence, in liver-derived cells, elevated bile acid concentrations result in the activation of signalling pathways by binding directly to intracellular components of these pathways. However, we have found that only very small quantities of the bile acids become associated with colon-tumour-derived cells even after 24 h of treatment. Additionally, little increase in the amount of bile acids was detected between the 6 h and 24 h time points. We have shown that the

levels of bile acids absorbed by colon cells do not correlate with cellular response. This result indicates that bile acids do not activate biological responses by interacting directly with intracellular molecules in colon cells. Although we cannot exclude the possibility that small quantities of bile acids might enter colon cells and then be secreted back into the medium, there are no reports of known bile acid transporters in HCT116 cells. One possible explanation for the effect that bile acids have on cells is that they might solubilize the cell's membrane and that the perturbation of cell surface structures results in activation of membrane receptors. More hydrophobic bile acids might be more effective in causing this perturbation than less hydrophobic bile acids.

DCA-treated colon cells showed minimal uptake of the bile acid at 3 h in both HCT116 and HT29 cells. Both of these cell types displayed high levels of apoptosis at this time point (Figures 2 and 6). Our results also illustrate that increases in hydrophobicity among the bile acids tested do not increase bile acid uptake by colon cells. Although DCA is more hydrophobic than UDCA, DCA does not show a greater ability to enter the colon tumour cells. These results suggest that it is not the intracellular concentrations of bile acids that trigger the unique biological responses of DCA and UDCA. Recent work by Schlottman et al. [21] showed that DCA is able to activate caspase 8 in colon cell lines. It is known that caspase 8 is generally activated by signalling through death receptors. Other studies have shown in hepatocytes that bile salts signal apoptosis through the Fas receptor [22–24]. It is therefore tempting to speculate that DCA signals by activating death-receptor-mediated signal transduction pathways to induce apoptosis.

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