Functional interaction of lithocholic acid conjugates with M3 muscarinic receptors on a human colon cancer cell line
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Abstract

Lithocholic acid (LA) conjugates interact with M3 receptors, the muscarinic receptor subtype that modulates colon cancer cell proliferation. This observation prompted us to examine the action of bile acids on two human colon cancer cell lines: H508, which expresses M3 receptors, and SNU-C4, which does not. Cellular proliferation was determined using a colorimetric assay. Interaction with muscarinic receptors was determined by measuring inhibition of muscarinic radioligand binding and changes in cellular inositol phosphate (IP) formation. Lithocholyltaurine (LCT) caused a dose-dependent increase in H508 cell proliferation that was not observed in SNU-C4 cells. After a 6-day incubation with 300 \textmu M LCT, H508 cell proliferation increased by 200\% compared to control. Moreover, in H508 cells, LCT caused a dose-dependent inhibition of radioligand binding and an increase in IP formation. LCT did not alter the rate of apoptosis in H508 or SNU-C4 cells. These data indicate that, at concentrations achievable in the gut, LA derivatives interact with M3 muscarinic receptors on H508 human colon cancer cells, thereby causing an increase in IP formation and cell proliferation. This suggests a mechanism whereby alterations in intestinal bile acids may affect the growth of colon cancer cells.

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1. Introduction

In the United States, colorectal cancer is the second most common cause of cancer death [1]. Experimental data support the concept that most of these cancers arise as a consequence of progression from normal colonic mucosa to adenomatous polyp to cancer, associated with the accumulation of somatic genetic alterations [2]. These alterations include mutations of both oncogenes and suppressor genes [2]. Environmental factors, such as dietary components and fecal bile acid concentrations, may play an important promoting role in this process [3–6].

Epidemiological studies in humans have associated the development of colorectal cancer with elevations in fecal bile acid concentration, particularly lithocholic acid (LA) [3–5] (although the term bile acid refers to the protonated form and bile salt the ionized form of these molecules, in this paper, as is common in the literature, these terms will be used interchangeably, and bile salt nomenclature will conform to recommendations by Hofmann et al. [7]). It has been reported that the ratio of lithocholate to deoxycholate (LA/DCA ratio) is 2-fold greater in persons with colon cancer compared to controls [8]. There has been a suggestion that by altering the delivery of conjugated bile acids to the colon, particularly secondary bile acids to the cecum and ascending colon (increased LA/DCA ratio), cholecystectomy increases the incidence of colon cancer [9–11]. Animal data, particularly from rats treated with carcinogens, indicate that direct instillation, or other means of increasing fecal bile acids, augments the development of colon cancer [12–14], and that the timing of interventions is important to the outcome [15]. In some studies, mucosal toxicity was noted following intrarectal...
instillation of bile acids. In rats fed LA, the risk of aberrant colonic epithelial growth is increased compared to those fed deoxycholic acid (DCA) [16]. The major beneficial effects of dietary elements like fiber and calcium are thought to result from binding of bile acids in the gut lumen, thereby inhibiting their damaging effects on epithelial cells and potentiating actions on experimental colon cancer [17–19]. Absent from this body of largely circumstantial evidence is a defined mechanism whereby bile acids promote the development or progression of colon cancer.

Recently, experimental findings in our laboratories suggested a possible mechanism that explains how bile acids might stimulate colon cancer cell proliferation. In the course of examining the actions of bile acids on pepsinogen secretion from gastric chief cells, Raufman et al. [20] reported that taurine conjugates of LA, but not other bile acids, bind to muscarinic receptors, increase cellular inositol phosphates (IP), and stimulate secretion by a cholinergic mechanism. Molecular cloning studies revealed the existence of five muscarinic receptor genes, designated M1–5 based on their order of cloning [21,22]. Because gastric chief cells express M3 muscarinic cholinergic receptors [23,24], we concluded that these actions were mediated by interaction of lithocholyltaurine (LCT) with that receptor subtype. These findings have been confirmed in preliminary studies using Chinese hamster ovary cells transfected with the gene for the M3 muscarinic receptor [25]. Frucht et al. [26,27] reported that several colon cancer cell lines express M3 receptors, and that activation of these receptors with cholinergic agonists stimulates an increase in IPs and cell proliferation. Together, these observations suggest the possibility that the proliferative effects of fecal bile acids, particularly LA conjugates, on colonic neoplasia are mediated by cholinergic actions.

In the present study, we tested the hypothesis that conjugated derivatives of LA interact with M3 muscarinic receptors on colon cancer cells, thereby stimulating proliferation. The specific aims of this study were to test the ability of LA conjugates to bind to M3 receptors on H508 colon cancer cells, to activate post-receptor signaling mechanisms, and to stimulate cell proliferation. These actions were compared to those of known cholinergic agents and to the actions of the LCT metabolite, 3-O-sulfate LCT (S-LCT). The SNU-C4 colon cancer cell line, which does not express muscarinic receptors [27], was used as a control. Our results support the hypothesis that chronic exposure of colon cancer cells to fecal bile acids may promote growth of neoplastic cells by a cholinergic mechanism.

2. Materials and methods

2.1. Cell lines and bile acids

Colon cancer cell lines (H508 and SNU-C4) were available in the investigator’s laboratories. Cancer cells were grown in RPMI 1640 (ATCC) supplemented with 10% fetal bovine serum (FBS) (Biowhittaker). Adherent cultures were passaged weekly at subconfluence after trypsinization. Cultures were maintained in incubators at 37 °C in an atmosphere of 5% CO₂ and 95% air. LCT was from Sigma. Lithocholylglycine (LCG) was from Steraloids. Stock solutions of LCT and LCG (100 mM) were prepared using DMSO (100%). The highest DMSO concentration in solutions incubated with cells was 0.6%. Carbachol was from CalBiochem. All other chemicals were obtained from Sigma or Fisher.

2.2. Cytotoxicity assays

Potential cytotoxic actions of bile acids and other test agents on H508 and SNU-C4 cells were examined by trypan blue exclusion.

2.3. Cell proliferation assay

Cell proliferation was determined using the sulforhodamine B (SRB) colorimetric assay [28]. Cells were seeded in 96-well plates (Corning Glass Works, Corning, NY) at approximately 10% confluence and allowed to attach for 24 h. The growth medium was removed and fresh medium without FBS and containing the indicated concentration of test agent was added. Cells were incubated for the described period of time at 37 °C in an atmosphere of 5% CO₂ and 95% air. After incubation, cells were treated for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Protein-bound dye was extracted with 10 mM unbuffered Tris base. Absorbance was measured at 560 nm using a computer-interfaced, 96-well microtiter plate reader.

2.4. Caspase-3 assay

Caspase-3 activation is a key step in the regulation of apoptosis [29,30]. H508 and SNU-C4 cellular caspase-3 activity was measured using a kit from Sigma. Cells (2–4 × 10⁵ cells/ml) were seeded in T75 flasks. Growth medium was removed after 24 h and cells were incubated with the indicated agent, containing no added FBS, for 3 or 6 days at 37 °C in an atmosphere of 5% CO₂ and 95% air. After incubation, cells were washed with PBS and lysed. Caspase-3 activity was examined with 96-well plates in duplicate for 90 min and absorbance was measured at 405 nm using a computer-interfaced 96-well microtiter plate reader. As a positive control, H508 and SNU-C4 cells were incubated with DCA (250 µM) for 1.5 h and 30 min, respectively.

2.5. Radioligand binding

Binding of radioligand to muscarinic receptors on test cell lines was examined using N-[¹H-methyl]scopolamine (¹H-NMS, 82 Ci/mmol) (New England Nuclear, Boston, MA). The ¹H-NMS binding assay was performed as

described previously [20]. Cells (28 × 10^6 cells/ml) were incubated for 45 min at 22 °C with 0.6 nM [3H]-NMS alone or with unlabeled ligands in an incubation solution containing 50 mM Tris (pH 7.4), 5 mM MgCl₂, 130 mM NaCl, 7.7 mM KCl, 1 mM EGTA, 4 μg/ml leupeptin, 0.1% bacitracin and 0.1% BSA. Nonsaturable binding was determined in the presence of 10 μM unlabeled NMS and was < 20% of total binding in all experiments. The reaction was terminated by centrifuging 500 μl of cell suspension (10,000 × g) for 7 min at room temperature. Supernatant (100 μl) was sampled for determination of free ligand concentration, and the remaining liquid was carefully decanted. The cell pellet was washed, drained, and dissolved in 100 μl Soluene 350. Ecoscint A was added, and the radioactivity in the tubes was measured in a liquid scintillation counter (1214 Rackbeta, LKB/Wallac, Gaithersburg, MD). Values shown represent binding with radioligand alone (total binding) minus nonsaturable binding. The concentration of agent that caused 50% inhibition of binding (IC₅₀) was determined by using a nonlinear, least-squares curve fitting program [31].

2.6. Inositol phosphates

Inositol phosphates were measured by previously described methods [32]. Cells were seeded onto 6-well plates at a density of 10^6 cells/well. After 18 h, growth medium was removed from subconfluent cell monolayers, and the cells were incubated with RPMI 1640 containing myo-[2-3H(N)]-inositol (1 μCi/ml, New England Nuclear), 2% FBS at 37 °C for 24 h. Before the addition of agents to be tested, cells were treated with 20 mM LiCl in PBS for 30 min. Phosphoinositide hydrolysis was initiated with the addition of PI buffer (135 mM NaCl; 20 mM HEPES; 2 mM CaCl₂; 1.2 mM MgSO₄; 1.0 mM EGTA; 20 mM LiCl; 11.1 mM glucose and 0.05% BSA) containing various concentrations of test agents. The incubation was allowed to proceed at 37 °C for 30 min. The incubation was stopped by adding 2 ml MeOH/HCl to each well. Total IPs (inositol 1-phosphate, inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate) were purified by chromatography with AG1-X8 resin (BioRad) and eluted with 1 mM ammonium formate and 100 mM formic acid [33,34]. Hydrofluor was added and radioactivity determined in a liquid scintillation counter.

2.7. Statistical analysis

All data are reported as mean ± SE of at least three independent experiments. Statistical analysis was performed using unpaired Student’s t-tests to determine significance between two means. Statistical significance was set at P values less than 0.05.

3. Results

3.1. Effects of bile acids on colon cancer cell proliferation

To determine whether, as observed with other cholinergic agonists [27], LCT would stimulate proliferation of H508 cells, the cell lines were incubated with increasing concentrations of the bile acid for 6 days. Over the course of a 6-day incubation, LCT caused a dose-dependent increase in H508 cell proliferation (Fig. 1). Fig. 1a shows the time-
course for the effect on proliferation with increasing concentrations of the bile acid. With 100 and 300 μM LCT, a significant increase in cell proliferation was first detected at 3 and 2 days, respectively. With the highest concentration tested, 300 μM, the proliferative effects of LCT appeared to plateau after the 5th day of incubation, achieving an approximately 3-fold increase in proliferation. In the presence of 1 μM atropine (a concentration that did not significantly alter basal proliferation), after the 5th day of incubation, LCT (300 μM)-induced cell growth was inhibited to basal levels (data not shown). Fig. 1b shows the dose–response curve for LCT after 6 days of incubation. In the H508 cell line, significant stimulation was observed with LCT concentrations greater than 30 μM. In contrast, the SNU-C4 cells showed no significant change in cellular proliferation.

3.2. Lack of cell toxicity with tested concentrations of bile acids

To exclude cell damage as a reason for the differences in cell proliferation, we evaluated potential toxic effects of bile acid derivatives and DMSO, used as the solvent. We examined the actions of the bile acids and cholinergic agents on the exclusion of trypan blue from H508 and SNU-C4 cells. At concentrations used in the following experiments, none of these agents altered this measure of cell damage. Using conditions similar to those for cell proliferation and IP formation, no trypan blue staining was observed in 200 counted cells. Hence, there was no evidence of cell damage with these agents.

3.3. Effect of bile acids on apoptosis in H508 and SNU-C4 cells

To exclude apoptosis as a potential reason for the differences in cell proliferation, we evaluated the effects of bile acid derivatives and the other agents tested on caspase-3 activity in H508 and SNU-C4 cells. Caspase-3 plays a critical role in apoptosis [29] and caspase-3 activity has been used as an index of apoptosis by several investigators [30]. As shown in Fig. 2, after 3 and 6 days of incubation, in both colon cancer cell lines, DCA, used as a positive control [35], caused a 15- to 43-fold increase in apoptosis. This increase was abolished by addition of a caspase-3 inhibitor. In contrast, DMSO, carbachol and LCT had no effect on caspase-3 activity in H508 or SNU-C4 cells. S-LCT caused a small but significant increase in caspase-3 activation in H508, but not SNU-C4 cells. These results indicate that apoptosis is not the cause of the changes in cell proliferation observed with the LA derivatives.

3.4. Effects of bile acids on binding of ³H-NMS to H508 cells

To determine the ability of LA conjugates to inhibit binding of a known muscarinic receptor ligand to M3 receptors, we used a radioligand binding assay. Binding of ³H-NMS to H508 cells was examined alone, and in the presence of increasing concentrations of cholinergic agonists and conjugated derivatives of LA. Results of radioligand binding are not shown for SNU-C4 cells because, as published previously [26], ³H-NMS does not bind to SNU-C4 cells. As shown in Fig. 3, acetylcholine (ACh), carbamylcholine (carbachol), LCT and LCG significantly inhibited binding of the cholinergic radioligand. In contrast, in the presence of S-LCT, the major human metabolite of LCT [36], binding of the radioligand was the same as the control. Inhibition of ³H-NMS binding was detectable with 50 μM LCT or 120 μM LCG. Inhibition of binding observed with the maximal concentrations of LCT and LCG used, 250 and 600 μM, respectively, was approximately 25–30% of that observed with maximal concentrations of ACh or carbachol. Using a nonlinear, least-squares curve fitting program, LIGAND [31], the concentrations of ACh, carbachol, LCT and LCG that caused half-maximal inhibition of ³H-NMS binding were 0.2, 0.3, 1.0 and 4.4 mM, respectively. These results indicate that, in terms of inhibition of ³H-NMS binding, the bile acids are approximately 5 to 20 times less potent than ACh.
As shown in Fig. 4, although compared at different times, the pattern of interaction for 100 μM carbachol, LCT, and S-LCT was the same when comparing inhibition of 3H-NMS binding to stimulation of cellular proliferation. That is, 100 μM carbachol and LCT caused similar inhibition of 3H-NMS binding and stimulation of cellular proliferation, whereas the same concentration of S-LCT caused a significantly lesser effect on either parameter. This observation supports the hypothesis that LCT-induced stimulation of H508 cell proliferation is mediated by interaction with M3 muscarinic receptors. Neither LCT nor S-LCT (0.1–1000 μM) altered proliferation in the SNU-C4 cell line (data not shown).

3.5. Effects of bile acids on cellular formation of IPs

To confirm that interaction of bile acids with muscarinic receptors on H508 cells activates post-receptor signaling pathways, we examined the actions of increasing concentrations of LCT and bile acids on carbachol- and ACh-induced increases in IPs.

Fig. 4. Comparison of the actions of LCT on inhibition of 3H-NMS binding to H508 cells and cell proliferation. (a) Actions of 100 μM carbachol, LCT and S-LCT on 3H-NMS binding to H508 cells after 45 min incubation. (b) Actions of 100 μM carbachol, LCT and S-LCT on H508 cell proliferation after 6 days incubation. In each experiment, results given are means from at least three separate experiments. (*, **) Indicates that response with S-LCT is significantly less (P < 0.05 and 0.001, respectively) than that observed with carbachol or LCT. Vertical bars, SE.
and LCG on ACh- and carbachol-induced increases in cellular IPs (Fig. 6). We hypothesized that if these agents were interacting with the same receptors, the less efficacious agents (LCT and LCG) would inhibit the actions of sub-maximal concentrations of the more efficacious agents (ACh and carbachol). As shown in Fig. 6a, increasing concentrations of LCT caused a progressive decrease in ACh- and carbachol-induced IP production. A similar effect was observed with LCG (Fig. 6b). In contrast, S-LCT did not alter carbachol-induced IP production (data not shown). These results support the hypothesis that the glycine and taurine conjugates of LA interact with the same receptors on H508 cells as ACh and carbachol.

4. Discussion

In animal models, increased concentrations of secondary bile acids are associated with increased proliferation of colorectal epithelium and the promotion of colon tumor growth [14]. Beneficial properties of agents like calcium and estrogens, that have been evaluated for chemopreven-

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**Fig. 5.** Actions of ACh, carbachol, LCT, and LCG on H508 cellular IP formation in H508 and SNU-C4 cells. Cells (10⁶ cells/well) were preincubated with myo-[2-³H(N)]-inositol for 24 h at 37 °C, and the medium was replaced with a PBS solution containing 20 mM LiCl for 30 min. Cells were then incubated in PI buffer, alone or with test agents for 30 min at 37 °C. Data are expressed as dpm in the IP fraction after separation by ion-exchange chromatography. (a) Effect of increasing concentrations of ACh and carbachol on H508 and SNU-C4 cellular formation of IPs. (b) Effect of increasing concentrations of LCT and LCG on H508 and SNU-C4 cellular formation of IPs. Results given are means from 3 to 11 separate experiments. (*, **) Indicates values that are significantly greater (P < 0.05 and 0.001, respectively) than control. Vertical bars, SE.

**Fig. 6.** Effects of LCT and LCG on the increase in IPs observed with submaximal concentrations of carbachol (closed circles) and ACh (open circles). (a) Effects of increasing concentrations of LCT on ACh- and carbachol-induced IP formation. (b) Effects of increasing concentrations of LCG on ACh- and carbachol-induced IP formation. Results given are means from at least three separate experiments. (*, **) Indicates values that are significantly less (P < 0.05 and 0.001, respectively) than control. Vertical bars, SE.
tion of colon cancer in humans, result from the binding or reduced production of secondary bile acids [38]. Nevertheless, the mechanism whereby bile acids alter colonic epithelial-cell turnover has not been elucidated.

In the present study, we provide evidence that conjugates of LA stimulate colon cancer cell proliferation by muscarinic mechanisms. This includes the observations that LCT and LCG dose-dependently compete with a muscarinic radioligand for binding to M3 subtype receptors on cultured human colon cancer cells [27], and stimulate an increase in cellular formation of IPs. As predicted from the interaction at the same receptor, increasing concentrations of LCT or LCG inhibit IP formation by more efficacious cholinergic agonists (ACh or carbachol). More importantly, LCT at concentrations that inhibit 3H-NMS binding and stimulate IP formation, increases proliferation of H508 cells and this increase can be inhibited by the muscarinic receptor inverse agonist atropine. Similar concentrations of LCT did not alter proliferation of colon cancer cells (SNU-C4) that do not express M3 receptors [27]. Moreover, LCT did not alter apoptosis in either cell line.

To draw conclusions regarding the physiological or pathophysiological implications of our observations, it is necessary to demonstrate in vivo in the organ of interest that LA derivatives achieve concentrations necessary for interaction with muscarinic receptors. In fact, concentrations of LA derivatives in proximal animal and human colon have been reported in the high micromolar to millimolar range [39–41], particularly if ileal damage prevents enterohepatic circulation. Hence, concentrations of LA conjugates that interact with muscarinic receptors on H508 colon cancer cells may be achieved in the normal colon. Moreover, although the effects of LA derivatives on muscarinic radioligand binding and stimulation of IP formation in H508 cells are less than those observed with ACh, several factors argue for a potential pathological role for the bile acids in vivo. This includes: (a) the likelihood that fecal bile acids that are normally found in stool will be in contact with colonic epithelial cells for many years (the average age for developing colon cancer is greater than 50 years [1]); (b) LA derivatives do not contain an ester linkage and, consequently, will not be subject to hydrolysis by tissue cholesteryl esterases that rapidly inactivate ACh; and (c) lipophilic properties of monohydroxy LA derivatives allow these agents ready access to muscarinic receptors in the lipid bilayer of the colon cancer cell membrane.

Sulfation of LCT, a physiological detoxification mechanism [36], abolishes the molecule’s ability to interact with muscarinic receptors. LCT is hepatotoxic in species that lack the enzyme necessary for sulfation [42]. The present study suggests the possibility that alterations in the ability to sulfate LA conjugates may alter initiation or progression of colon cancer. It is of interest to note that a colon cancer cell line has been reported to sulfate LA [43]. Hence, it may be of interest to study possible genotypic or phenotypic variation in expression of hepatic and colonic bile acid sulfatases in subjects with and without advanced colon cancer. Moreover, situations associated with decreased LA conjugate sulfation, like advanced liver disease, may result in augmented cholinergic actions of LA derivatives.

Although it is possible that chronic muscarinic receptor stimulation may lead to colonic epithelial cell proliferation and neoplastic transformation [3,44,45] the present studies do not address this. We did not examine the effect of LA derivatives on normal colonic epithelium. Nonetheless, because we show that LCT stimulates proliferation of already neoplastic cells, our data are compatible with the hypothesis that LA derivatives enhance the growth of existent cancer cells. Hence, once neoplastic transformation occurs as a consequence of genetic mutation or other causes, fecal bile acids may augment cancer cell proliferation and consequently tumor growth. Whether the use of selective anti-cholinergic agents or other approaches to blocking potential interactions of bile acids with neoplastic colonic epithelium is a useful adjunct to colon cancer prevention or treatment remains to be determined.

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