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Biochemical Pharmacology 70 (2005) 1035–1047
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Bile acid-induced proliferation of a human colon cancer cell line is mediated by transactivation of epidermal growth factor receptors

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Received 10 June 2005; accepted 8 July 2005

Abstract

Although epidemiological studies indicate an association between elevations in fecal bile acids and the development of colorectal cancer, the cellular mechanism for the proliferative actions of bile acids is not clear. Studies from other laboratories indicate a paradoxical pro-apoptotic action of bile acids on cell culture lines. Our previous studies indicate that cholinergic agonist-induced proliferation of colon cancer cells that express M_3 muscarinic receptors (M_3R) is mediated by transactivation of the epidermal growth factor receptor (EGFR) and that bile acids stimulate proliferation of colon cancer cells that express M_3R. In the present study, we investigated the effects of bile acids on cell signaling and proliferation of a human colon cancer cell line (H508 cells) that abundantly expresses M_3R and EGFR. Treatment with taurine and glycine conjugates of lithocholic and deoxycholic acids stimulated reversible activation of the p44/42 MAP kinase signaling cascade and proliferation of H508 cells. Bile acids did not stimulate proliferation of SNU-C4 colon cancer cells that express EGFR but not muscarinic receptors. Atropine, a muscarinic receptor inverse agonist, blocked bile acid-induced H508 cell proliferation. At concentrations that stimulate cell proliferation, conjugated bile acids did not activate caspase-3, a key mediator of apoptosis. Conjugated bile acids stimulated phosphorylation of EGFR Tyr992, thereby implicating EGFR transactivation in the cellular mechanism underlying their proliferative actions. This was confirmed by observing that inhibitors of EGFR activation and antibodies to the ligand-binding domain of EGFR blocked both the signaling and proliferative actions of bile acids. Collectively, these results suggest that in this colon cancer cell line, bile acid-induced colon cancer cell proliferation is M_3R-dependent and is mediated by transactivation of EGFR.

Keywords: Bile acids; Colon cancer; Epidermal growth factor receptor; Muscarinic receptors; Acetylcholine; Signal transduction; Proliferation

1. Introduction

Experimental data support the concept that the majority of colon cancers arise as a consequence of progression from normal colonic mucosa to adenomatous polyp to cancer, associated with the accumulation of somatic genetic alterations that affect the regulation of cell proliferation, apoptosis and DNA repair [1]. These genetic alterations include mutations and/or methylation of oncogenes, and of tumor suppressor and mismatch repair genes [1,2]. There is compelling evidence that environmental factors, such as dietary components and fecal bile acids, play an important promoting role in this process [3–7]. For example, colonic grafts from mice with an APC gene mutation will not develop adenomas if they are removed from the fecal stream [8].

Epidemiological studies have documented an association between elevations in fecal bile acid[1] concentration,

Abbreviations: MAPK, mitogen activated protein kinase; M_3R, M_3 subtype muscarinic receptor; ACh, acetylcholine; p90RSK, p90 ribosomal S kinase; EGFR, epidermal growth factor receptor; DCT, deoxycholyltaurine; DCG, deoxycholylglycine; LCT, lithocholyltaurine; GPCR, G-protein coupled receptor; RTK, receptor tyrosine kinase

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[1] The term *bile acid* refers to the protonated form and *bile salt* the ionized form. In this report as is common in the literature, these terms are used interchangeably. Bile acid nomenclature conforms to recommendations by Hofmann et al. [9].

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doi:10.1016/j.bcp.2005.07.023

particularly lithocholic and deoxycholic acids, and the development of colorectal cancer [3–5]. In animal experiments using rodents treated with carcinogens, direct instillation or other means of increasing fecal bile acid concentrations augments the development of colon cancer [10–12]. Reducing fecal deoxycholic acid concentration by feeding rats ursodeoxycholic acid results in a decrease in colon tumor formation [13,14]. Likewise, in humans with inflammatory bowel disease, modulating fecal bile acids with oral ursodeoxycholic acid treatment reduces the incidence of colorectal dysplasia and cancer [15].

In summary, a large body of data indicates an important role for fecal bile acids in the genesis and progression of colon cancer. Absent from this largely circumstantial evidence is a defined mechanism whereby bile acids interact with colonic epithelial cells to promote the development or progression of colon cancer. Important studies by Martinez and colleagues initiated examination of the effects of deoxycholic acid on signal transduction and gene expression in colon cancer [16,17]. However, early work from this group indicated that deoxycholic acid did not stimulate proliferation of colon cancer cells and, in fact, that this agent stimulates apoptosis [16]. More recent studies indicated that deoxycholic acid stimulates proteosomal degradation of the tumor suppressor gene product p53, thereby resulting in decreased p53 protein levels and transcriptional activity [18]. These findings suggest a mechanism for the tumor-promoting, anti-apoptotic effects of the bile acid [18]. Pertinent to the current studies, activation of mitogen activated protein kinase (MAPK) signaling was implicated in mediating the apoptotic and anti-apoptotic actions of deoxycholic acid [18,19].

In colon cancer, activation of cell surface receptors has not been implicated in bile acid-induced proliferative signaling, nor have bile acid-activated pro-proliferative signaling cascades been elucidated. In this context, it is noteworthy that in other cells types (e.g. cholangiocytes) epidermal growth factor receptors (EGFR) have been implicated as important participants in bile acid-induced proliferative signaling [20] and that EGFR activation is important for colonic tumorigenesis [21,22]. Recently, it was suggested that deoxycholic acid-induced apoptosis is mediated in part by EGFR activation and that this effect can be reversed by adding ursodeoxycholic acid [23]. However, the latter findings appear somewhat paradoxical in that EGFR activation was linked to apoptosis rather than cell proliferation [23]. In general, and in the colon, many studies have shown that EFGR activation stimulates proliferation, not apoptosis [21].

For the past 5 years, our laboratory has explored the pathophysiological implications of the interaction of bile acids with muscarinic cholinergic receptors [24] (see Ref. [25] for review). Because of their high relative concentration in the lumen of the gastrointestinal tract and the studies reviewed above that associated changes in fecal bile acids with colon cancer risk, we focused on the proliferative effects of these agents on colon cancer cells. Frucht and colleagues demonstrated that all normal human colonic epithelial cells express muscarinic M_3 subtype receptors (M_3R) and that most human colon cancers over-express M_3R [26–28]. In collaboration with the Frucht laboratory, we demonstrated that secondary bile acids interact with M_3R and activate post-M_3R cellular signaling pathways in H508 human colon cancer cells, thereby stimulating cellular proliferation [29]. H508 cells are derived from a moderately differentiated cecal adenocarcinoma and were studied because they have high levels of M_3R expression [27].In H508 cells, a 5-day incubation with the taurine conjugate of a secondary bile acid, lithocholyltaurine (LCT), stimulated a 200% increase in cell proliferation compared to control [29]. These changes were accompanied by a modest increase in inositol phosphate formation. In a control cell line, that does not express M_3R, the bile acid did not alter cell proliferation or inositol phosphate formation [29]. Hence, the H508 cell line provides an excellent model to examine the effects of bile acids on interaction with cell membrane receptors, stimulation of signaling cascades, and functional actions on cell proliferation.

In subsequent studies we showed that, as observed for other G-protein coupled receptors (GPCR) [30,31] including M_3R in non-colonic tissues [32–34], the cellular mechanism underlying cholinergic agonist-induced H508 cell proliferation involves transactivation of EGFR. Inhibitors of EGFR activation abolished acetylcholine (ACh)- and EGF-induced increases in phosphorylation of EGFR, p44/42 MAPK (also referred to as ERK 1/2), and p90 ribosomal S kinase (p90RSK), a nuclear response protein downstream of p44/42 MAPK [35]. EGFR inhibitors also blocked ACh-induced stimulation of H508 cell proliferation [35]. Hence, our experimental observations indicate that ACh-induced proliferation of H508 cells is mediated by transactivation of EGFR [35]. Independent observations by another group using T84 colon cancer cells indicate that this mechanism is not limited to only one colon cancer cell line [36].

The observations that bile acids stimulate the proliferation of H508 human colon cancer cells [37] and that in this cell line muscarinic agonist-induced cell proliferation is mediated by transactivation of EGFR [35] prompted us to study the actions of bile acids on cellular signaling in H508 colon cancer cells. The primary objective of the present study was to ascertain whether in H508 colon cancer cells bile acids alter the activation of post-receptor signaling proteins, including p44/42 MAPK and p90RSK. We also investigated in this cell line whether bile acids induce transactivation of EGFR. As described herein, our findings support the novel hypothesis that bile acid-induced colon cancer cell proliferation is M_3R-dependent and mediated by transactivation of EGFR.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium, MEM non-essential amino acids, penicillin, streptomycin and G418 were from GibcoBRL; PD98059 and AG1478 from Alexis; PD168393 from Calbiochem; bile acids were from Sigma. Mouse monoclonal antiphospho-MAPK, rabbit polyclonal antiphospho-EGFR and rabbit polyclonal antiphospho-p90RSK were from Cell Signaling (Beverly, MA). EGFR antibody was from UBI (Lake Placid, NY) and rabbit polyclonal anti-MAPK antibody from Santa Cruz Biotechnology. All other chemicals were obtained from Sigma or Fisher.

2.2. Immunoblotting and immunoprecipitation

Phosphorylation of p44/42 MAPK, EGFR and p90RSK was determined by methods described previously [38]. Briefly, cells were subcultured in six-well plates (10^6 cells/well). After a 24 h incubation at 37 °C, the cells were serum-starved for an additional 24 h, washed with PBS, and allowed to recover in PBS for 30 min at 37 °C before adding test agents. After a 10 min incubation with test agents, the reaction was terminated by adding lysis buffer [150 mM NaCl, 10 mM Tris/HCl, 1% (w/v) deoxycholic acid, 1% (v) Nonidet-40, 0.1% (w/v) SDS, 4 mM EDTA, 1 mM Na_3VO_4, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 250 μg/ml nitrophenylphosphate and 1 mM phenylmethylsulfonyl fluoride, pH 8]. When inhibitors (e.g. PD98059, AG1478) were used, they were added 30 min before test agents. When antibodies (e.g. anti-EGFR) were used, they were added 2 h before test agents. Cell lysates were subjected to SDS-PAGE (10% gel, Invitrogen). Resolved proteins were transferred electrophoretically to nitrocellulose membranes (Micron Separations) and probed with antiphospho-p44/42 MAPK, antiphospho-EGFR or antiphospho-p90RSK. Bound antibody was detected by chemiluminescence (Supersignal kit, Pierce). To verify that equal amounts of protein were added to each lane, the blots used with antiphospho-p44/42 MAPK were stripped and re-probed with anti-p42.

For immunoprecipitation, H508 cells were subcultured in 10 cm plates (10^7 cells/plate). After 24 h incubation at 37 °C, cells were serum-starved for an additional 24 h, washed with PBS, and allowed to recover in PBS for 30 min at 37 °C before adding test agents. After incubation with test agents for 10 min at 37 °C and then for an additional 10 min on ice, cells were lysed by incubation for 10 min on ice in a solution containing 50 mM Hepes/Na, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM Na_3VO_4, 10 mM $Na_3P_2O_7$, 50 mM NaF, 1% Triton X-100, 10% glycerol, 2 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF (pH 7.4). EGFR in cell lysates was precipitated by incubation with mouse monoclonal anti-EGFR antibody (4 μg/ml, Upstate Labs, NY) overnight at 4 °C. Protein G-sepharose (Amersham Biosciences) was added followed by incubation for 2 h. Pellets were washed twice with lysis buffer and resuspended in SDS sample buffer. After boiling, samples were subjected to electrophoresis and immunoblotting with anti-EGFR or phospho-specific antibodies for EGFR phospho-tyrosines (all from Cell Signaling except phospho-EGFR Tyr1173 from Biosource).

2.3. Cell culture

H508 and SNU-C4 human colon cancer cell lines 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% CO_2 and 95% air. CHO cells expressing rat M_3 (rM_3R) subtype muscarinic receptors were obtained from ATCC. These cells do not express EGFR [39]. Cells were grown in DMEM supplemented with 10% FBS, 1× MEM non-essential amino acids, penicillin (50 units/ml), streptomycin (50 mg/ml) and G418 (0.1 mg/ml).

2.4. Cytotoxicity assays

Before proceeding with these studies, we evaluated potential cytotoxic actions of test agents on H508, SNU-C4 and CHO-rM_3R cells by trypan blue exclusion (Sigma assay kits). At concentrations used in the following experiments none of these agents altered trypan blue exclusion.

2.5. Cell proliferation assay

Cell proliferation was determined using the sulforhodamine B (SRB) colorimetric assay [40]. 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% CO_2 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 unbuffered 10 mM Tris base. Absorbance was measured at 560 nm using a computer-interfaced, 96-well microtiter plate reader.

2.6. Caspase-3 assay

To evaluate potential apoptotic actions of bile acids in H508 cells, we examined the activation of caspase-3. Cells ($(2–5) \times 10^5$ cells/ml) were seeded in T75 flasks. Growth medium was removed after 24 h and cells were incubated

with the indicated agent, containing no FBS, for 5 days at 37 °C in an atmosphere of 5% CO_2 and 95% air. After incubation, the 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, cells were incubated with 300 μM deoxycholic acid for 2 h. As a negative control, cells were incubated with a caspase-3 inhibitor provided by the manufacturer (Sigma).

2.7. Statistical analysis

All figures (e.g. immunoblots) show data representative of at least three separate experiments. All graphs show the mean and standard error of the mean of three separate experiments. Statistical calculations were performed using the Student's two-tailed unpaired *t*-test assuming normal distribution with equal variance. Statistical significance is given by the number of *asterisks* ($^{*}p < 0.05$; $^{**}p < 0.005$).

3. Results

3.1. ACh and conjugated secondary bile acids activate p44/42 MAPK signaling and colon cancer cell proliferation

In H508 human colon cancer cells that express M_3R, lithocholic acid conjugates stimulate post-receptor signaling and cellular proliferation [27,29]. These actions do not occur in SNU-C4 colon cancer cells that have negligible expression of muscarinic receptors [27,29]. In H508 cells, ACh activates the p44/42 MAPK signaling cascade [35]. Hence, to determine whether bile acids activate the same signaling pathways as conventional cholinergic agonists, we examined the actions of lithocholyltaurine (LCT) on phosphorylation of p44/42 MAPK and the downstream nuclear response protein, p90RSK, which has been linked to regulation of the cell cycle [41]. As shown in Fig. 1A, in H508 cells, as observed previously with ACh, LCT stimulates an increase in both p44/42 MAPK and p90RSK phosphorylation.

To explore further the mechanism underlying bile acid-induced colon cancer cell proliferation, we extended our studies to examine the actions of deoxycholic acid conjugates. Like the glycine and taurine conjugates of lithocholic acid, deoxycholylglycine (DCG) and deoxycholyltaurine (DCT) are secondary bile acids formed as a consequence of intestinal bacterial 7α-dehydroxylation of primary bile acids. However, compared to lithocholic acid conjugates, the concentration of deoxycholic acid conjugates in the intestinal lumen is an order of magnitude greater [42–44]. Hence, physiological or pathophysiological actions of deoxycholic acid conjugates are likely to be more important.

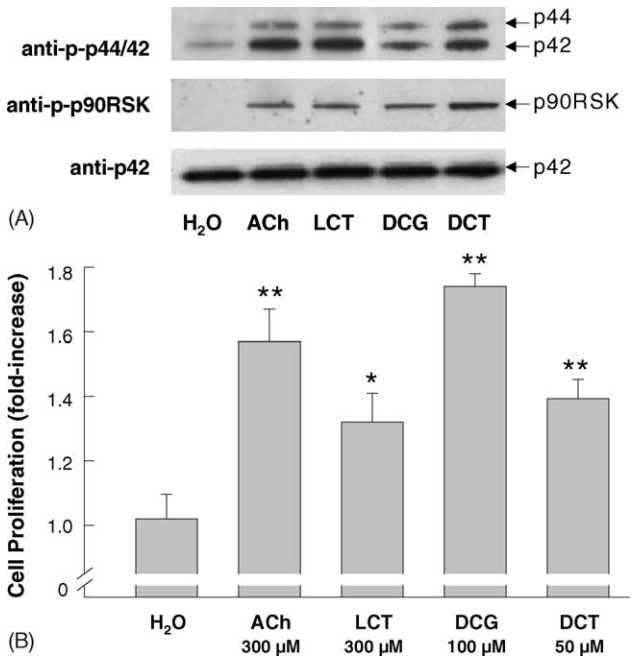

Fig. 1. Acetylcholine (ACh) and conjugated secondary bile acids [lithocholyltaurine (LCT), deoxycholyltaurine (DCT), deoxycholylglycine (DCG)] stimulate colon cancer cell proliferation and p44/42 MAPK phosphorylation. (A) H508 cells were treated with water, ACh (300 μM), and bile acids (300 μM) for 10 min at 37 °C. p44/42 MAPK and p90RSK activities were determined by immunoblotting with antibodies specific for phosphorylated MAPK and p90RSK. The quantity of protein added was verified by immunoblotting with antibodies specific for non-phosphorylated p42 MAPK. Results are representative of three separate experiments. (B) H508 cells were treated with water, ACh (300 μM), LCT (300 μM), DCG (100 μM) and DCT (50 μM) for 5 days at 37 °C. Cellular proliferation was determined by the sulforhodamine blue (SRB) colorimetric assay [40]. Results are mean ± S.E.M. of three experiments. Asterisks (*,**) indicate value significantly different than that observed in the presence of water ($p < 0.05$ and 0.005, respectively, unpaired Student's *t*-test).

Previous work indicated that, as observed with lithocholic acid derivatives, glycine and taurine conjugates of deoxycholic acid bind to M_3R [45]. However, in CHO cells that express muscarinic receptors, the interaction of deoxycholic acid conjugates with M_3R does not stimulate post-receptor signaling [46].

To determine whether, as observed with lithocholic acid derivatives [29], deoxycholic acid conjugates stimulate proliferation of colon cancer cells, we examined their actions on H508 cells. In preliminary experiments (not shown), we determined the dose-dependence and maximal concentrations for the proliferative effects of deoxycholic acid conjugates. Experiments examining H508 cell proliferation were conducted for 5 days for two important reasons: (1) Previous experiments [29] demonstrated that bile acid-induced proliferation was maximal after 5 days. Preliminary experiments with DCG and DCT indicated similar time-courses for stimulated cell proliferation (not shown). (2) We also showed previously that in serum-free medium, unstimulated H508 colon cancer cells grow slowly with a doubling time of approximately 5–6 days [29].

As observed with ACh and LCT, a 5-day incubation of H508 cells with maximal concentrations of deoxycholic acid conjugates resulted in robust stimulation of cell proliferation (approximately 70% and 50% increase with100 μM DCG and 50 μM DCT, respectively, compared to basal proliferation) (Fig. 1B). The magnitude of the increase in cell proliferation observed with the deoxycholic acid conjugates was similar to that observed with ACh. Moreover, as observed with ACh and LCT, incubation of H508 cells with deoxycholic acid conjugates activated post-receptor signal transduction, as evidenced by phosphorylation of p44/42 MAPK and p90RSK (Fig. 1A).

To examine further activation of the p44/42 MAPK signaling cascade, we used an inhibitor of MEK (MAPK kinase), the kinase immediately upstream of p44/42 MAPK. As shown in Fig. 2A, addition of PD98059 inhibited both LCT- and DCG-induced phosphorylation of p44/42 MAPK and p90RSK. Likewise, addition of PD98059 inhibited both LCT- and DCG-induced H508 cell proliferation (Fig. 2B). These findings indicate that in H508 colon cancer cells, conjugated secondary bile acids activate post-receptor signaling via the p44/42 MAPK signaling cascade, thereby stimulating an increase in cell proliferation. With regard to reports that pro-apoptotic bile acids activate c-Jun related signaling [47], the bile acids tested in the present study did not alter c-Jun phosphorylation (not shown).

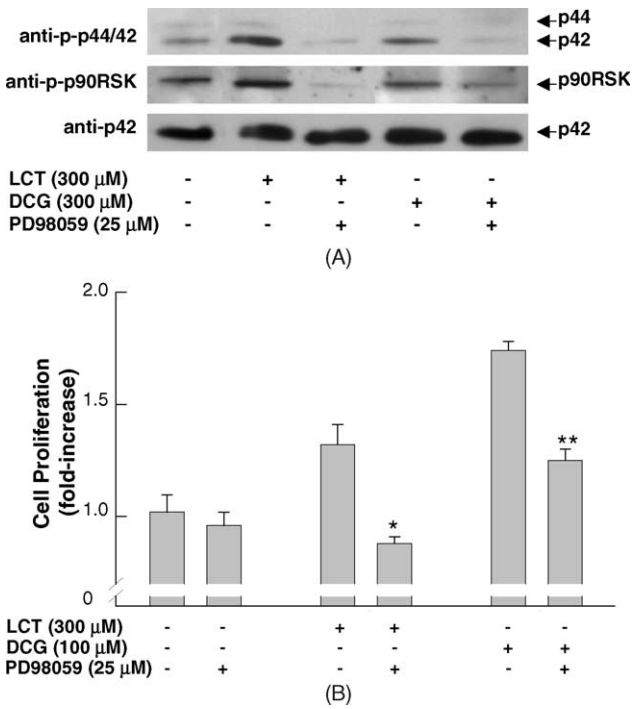

Fig. 2. Actions of a MEK inhibitor on bile acid-induced p44/42 MAPK phosphorylation and H508 cell proliferation. (A) H508 cells were treated with water, LCT (300 μM) and DCG (300 μM) with or without the MEK inhibitor, PD98059, for 10 min at 37 °C. p44/42 MAPK and p90RSK activities were determined by immunoblotting with antibodies specific for phosphorylated MAPK and p90RSK. The quantity of protein added was verified by immunoblotting with antibodies specific for non-phosphorylated p42 MAPK. Results are representative of three separate experiments. (B) H508 cells were treated with water, LCT (300 μM), and DCG (100 μM), with or without PD98059, for 5 days at 37 °C. Cellular proliferation was determined by the sulforhodamine blue (SRB) colorimetric assay [40]. Results are mean ± S.E.M. of three experiments. Asterisks (*,**) indicate value significantly less than those observed in the absence of the MEK inhibitor ($p < 0.05$ and 0.005, respectively, unpaired Student's *t*-test).

3.2. Role of M_3R in mediating the signaling and proliferative actions of bile acids

Our previous studies indicated that ACh-induced H508 colon cancer cell proliferation is mediated by transactivation of EGFR and activation of post-EGFR signaling [29,35]. To determine whether expression of both M_3R and EGFR is required to mediate bile acid-induced actions on colon cancer cells, we compared the actions of a deoxycholic acid conjugate on p44/42 MAPK phosphorylation in cell lines with varying expression of M_3R and EGFR. In these experiments, to increase the certainty of detecting bile acid-induced effects on p44/42 MAPK phosphorylation, a relatively high concentration of the bile acid (300 μM) was used. In CHO cells transfected with rM_3R (CHO-rM_3R), DCG did not alter p44/42 MAPK phosphorylation (Fig. 3A). CHO-rM_3R cells do not express EGFR [35]. Control experiments in CHO-rM_3R cells demonstrated that ACh, but not EGF, stimulates p44/42 MAPK phosphorylation (Fig. 3A). As shown in Fig. 3A, in SNU-C4 human colon cancer cells that express EGFR but not M_3R [26,27], deoxycholic acid conjugates did not alter p44/42 MAPK phosphorylation. However, as expected in SNU-C4 cells, addition of EGF stimulated robust p44/42 MAPK phosphorylation. Comparison of p44/42 MAPK phosphorylation observed with ACh, EGF, and bile acids in H508 cells (Fig. 3A) shows that the level of bile acid-induced phosphorylation is less than that observed with ACh and EGF. Nonetheless, compared to control (water), the bile acid-induced clear and reproducible phosphorylation of the kinase. Consistently, we observe less basal and stimulated phosphorylation of p44 compared to p42 [35]. We do not know the reason for the differential phosphorylation of these MAPK components. This may result from a disparity in the relative abundance of the proteins or from an actual difference in the respective levels of phosphorylation.

In support of the hypothesis that deoxycholic acid conjugates interact with M_3R, neither ACh nor the secondary bile acids altered cell proliferation of SNU-C4 cells that express EGFR but not muscarinic receptors (Fig. 3B). Moreover, addition of atropine inhibited both ACh- and DCT-induced H508 cell proliferation, thereby providing additional evidence that the proliferative effects of bile acids are mediated by a muscarinic receptor-dependent mechanism (Fig. 3C) [29]. Overall, these findings provide evidence that co-expression of both M_3R and EGFR is required for bile acids to induce stimulation of p44/42 MAPK signaling and cell proliferation. Moreover, the

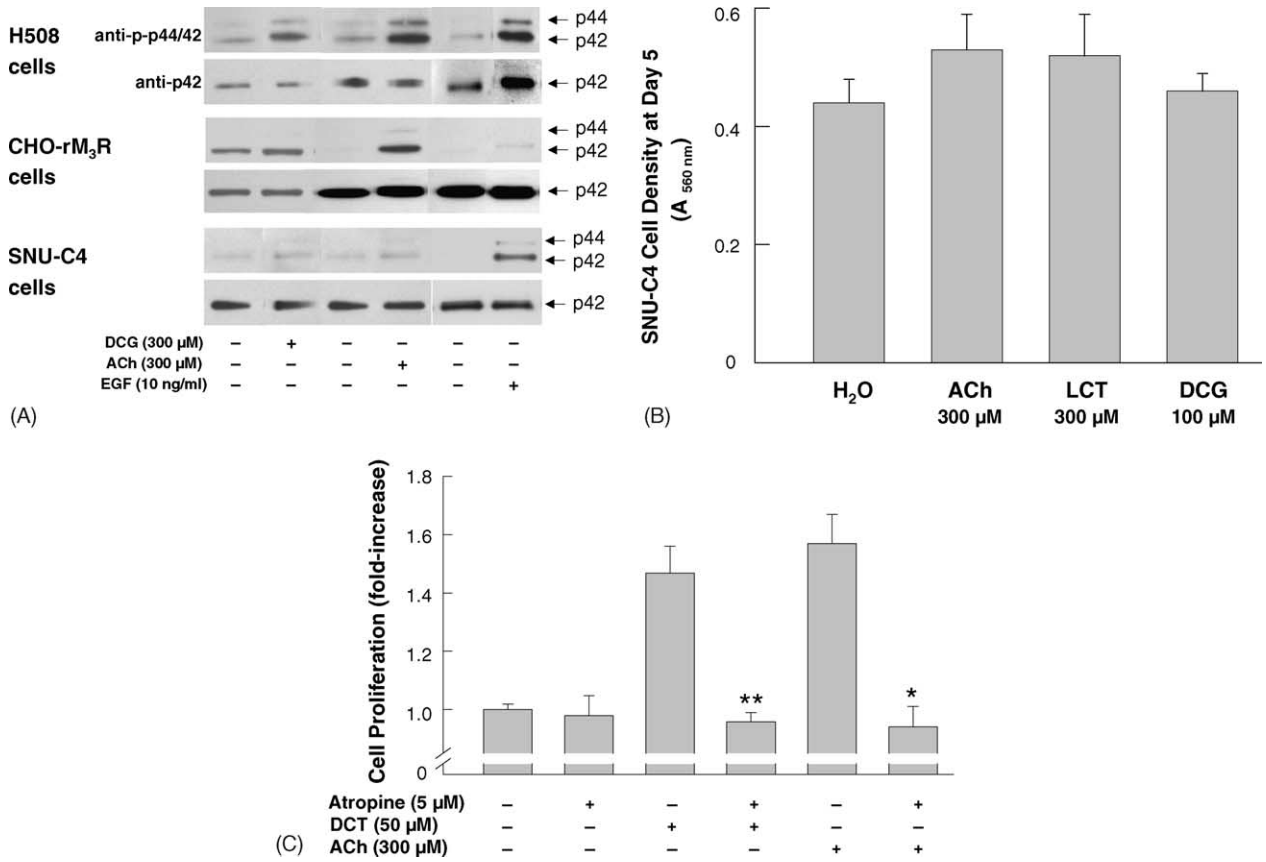

Fig. 3. Bile acid-induced colon cancer cell proliferation requires activation of muscarinic receptors. (A) H508, SNU-C4, and CHO-M_3R cells were treated with water, DCG (300 μM), ACh (300 μM), and EGF (10 ng/ml) for 10 min at 37 °C. Results are representative of 3 separate experiments. (B) SNU-C4 cells were treated with water, ACh (300 μM), LCT (300 μM), and DCG (100 μM) for 5 days at 37 °C. Results are mean ± S.E.M. of three experiments. (C) H508 cells were incubated with water, DCT (50 μM), and ACh (300 μM), alone or in the presence of atropine (5 μM). Results are mean ± S.E.M. of three experiments. Asterisks (*, **) indicate values significantly less than those observed with agonist alone ($p < 0.05$ and 0.005, respectively).

failure of bile acids to stimulate signaling or proliferation of SNU-C4 cells indicates that these agents are not EGFR ligands per se.

3.3. Actions of bile acids on caspase-3 activation

Other investigators have noted that deoxycholic acid stimulates cellular apoptosis [16]. Deoxycholic acid (300 μM) caused a striking increase in caspase-3 activation, a marker of apoptosis (Fig. 4). Adding a caspase-3 inhibitor blocked the effects of deoxycholic acid (Fig. 4). In contrast, DCT at concentrations up to 1 mM did not alter caspase-3 activity (Fig. 4). The highest concentration of EGF used (10 ng/ml) caused a very small, yet statistically significant increase in caspase-3 activity. These findings indicate that in H508 cells, at the concentrations used in our experiments, in contrast to the parent molecule conjugates of deoxycholic acid do not stimulate apoptosis.

3.4. Reversibility of bile acid-induced p44/42 MAPK activation

Compared to ACh, bile acids possess bulky lipophilic ring structures that may cause prolonged interaction with plasma membrane-anchored receptors. To determine whether bile acid-induced phosphorylation of p44/42 MAPK is reversible, we examined the effects of washing H508 cells after a 10-min incubation with DCT (Fig. 5).

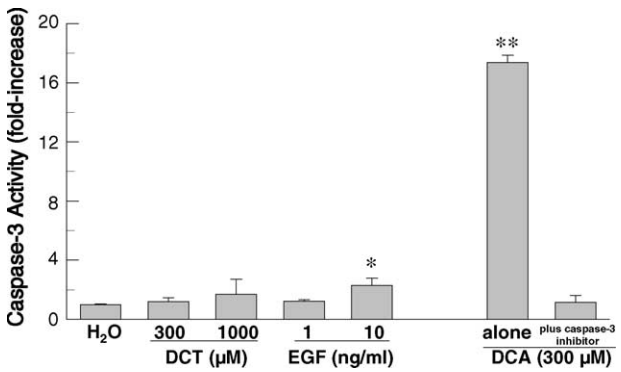

Fig. 4. Effect of deoxycholic acid conjugates and EGF on caspase-3 activation. H508 cells were incubated with water, DCT (300 and 1000 μM) and EGF (1 and 10 ng/ml) for 5 days at 37 °C and caspase-3 activation was determined. Deoxycholic acid (DCA, 300 μM), alone or in the presence of a caspase-3 inhibitor, was used as a positive and negative control, respectively. Results are mean ± S.E.M. of three experiments. Asterisks (*, **) indicate values significantly greater than those observed with water alone ($p < 0.05$ and 0.005, respectively).

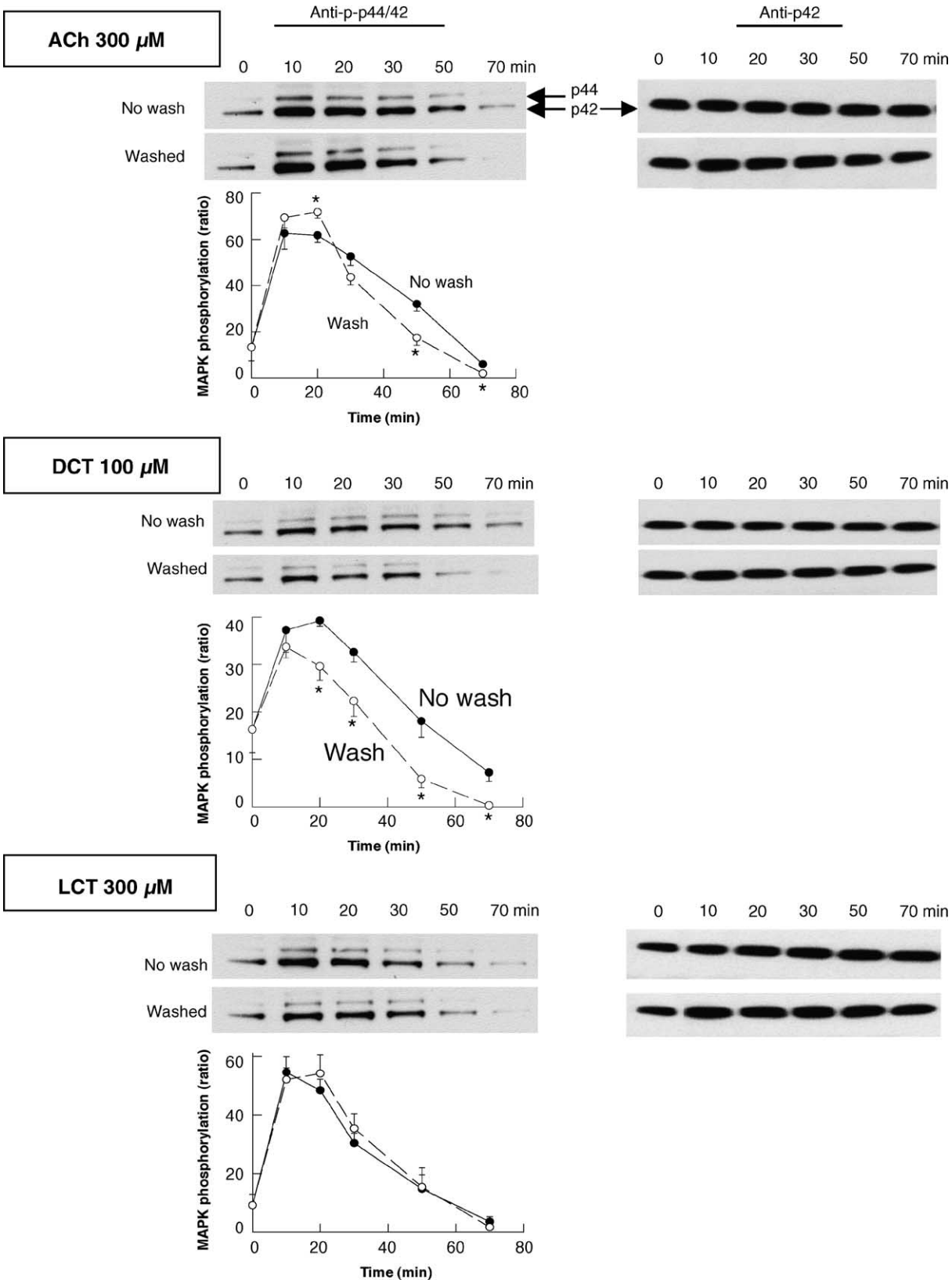

Fig. 5. Reversibility of ACh-, DCT-, and LCT-induced p44/42 MAPK phosphorylation. H508 cells were incubated at room temperature with ACh (300 μM), DCT (100 μM), and LCT (300 μM) for 70 min (no wash, closed circles) or for 10 min followed by washing twice with phosphate buffered saline (pH 7.4) (wash, open circles). Cell samples were assayed for p44/42 MAPK phosphorylation at the times indicated. To control for loading, membranes were stripped and re-probed with antibody to non-phosphorylated p42 MAPK. Data are representative of three separate experiments. Graphs represent densitometry values of immunoblots (mean ± S.E.M.) from at least three separate experiments. The densitometry values shown are a summation of p42 and p44 intensities. *Values significantly different than the corresponding value without washing ($p < 0.05$, unpaired Students t-test).

For comparison we examined the effects of the same conditions on cells incubated with ACh and LCT. The actions of ACh were reversible as demonstrated by a reduction in p44/42 MAPK phosphorylation after washing (Fig. 5, top panel). Control experiments, performed by stripping and re-probing the membranes with anti-p42 MAPK, showed that equal amounts of sample were loaded (Fig. 5, top-right panel). As observed with ACh, in cells incubated with DCT and then washed twice with phosphate-buffered saline (pH 7.4), compared to control (no wash), p44/42 MAPK phosphorylation was reduced at 20, 30, 50 and 70 min (Fig. 5, middle panel). These results indicate that, as observed with ACh, DCT-induced signaling is reversible. In contrast, when cells were incubated with LCT and subsequently washed, phosphorylation was not reduced at any time point compared to control (no wash) (Fig. 5, bottom panel). One possible explanation for these findings is that, in contrast to water-soluble ACh and the less lipophilic monohydroxy deoxycholic acid conjugate, lipophilic LCT remains anchored to the lipid bilayer of the plasma membrane. The finding that, like ACh, DCT causes reversible activation of post-receptor signaling is compatible with a similar mechanism of action for these agents.

3.5. Deoxycholic acid conjugates cause transactivation of EGFR in H508 colon cancer cells

To determine whether, as observed previously with ACh [35], bile acid-induced p44/42 MAPK signaling is dependent on transactivation of EGFR, we examined DCT-induced phosphorylation of tyrosine moieties in EGFR, and the effects of adding an EGFR inhibitor on DCT-induced signaling and cell proliferation. In preliminary experiments, to evaluate ACh-induced transactivation of EGFR, we observed cholinergic agonist-induced phosphorylation of only one tyrosine residue in EGFR, Tyr992 [35]. Phosphorylation of other EGFR tyrosine residues (Tyr845, Tyr1045, Tyr1068) was not detected [35].

As shown in Fig. 6A, following incubation of H508 cells with either DCT or EGF, immunoblotting with antibodies for specific EGFR phospho-tyrosines revealed phosphorylation of EGFR Tyr992. As observed with ACh [35], immunoblotting with antibodies specific for EGFR phospho-Tyr845, Tyr1045, Tyr1068 and Tyr1173 did not reveal DCT-induced phosphorylation of these sites (not shown). Addition of PD168393, an inhibitor of EGFR activation [48–50], blocked bile acid- and EGF-induced Tyr992 phosphorylation (Fig. 6A). To increase the sensitivity of immunoblotting to detect phosphorylation of additional EGFR tyrosines, we repeated this experiment after immunoprecipitation with anti-EGFR antibody. Again, immunoblotting of resulting immunoprecipitates with antibodies specific for EGFR phosphotyrosines (Tyr845, Tyr992, Tyr1045, Tyr1068 and Tyr1173) revealed only DCT-induced phosphorylation of EGFR Tyr992 (Fig. 6B).

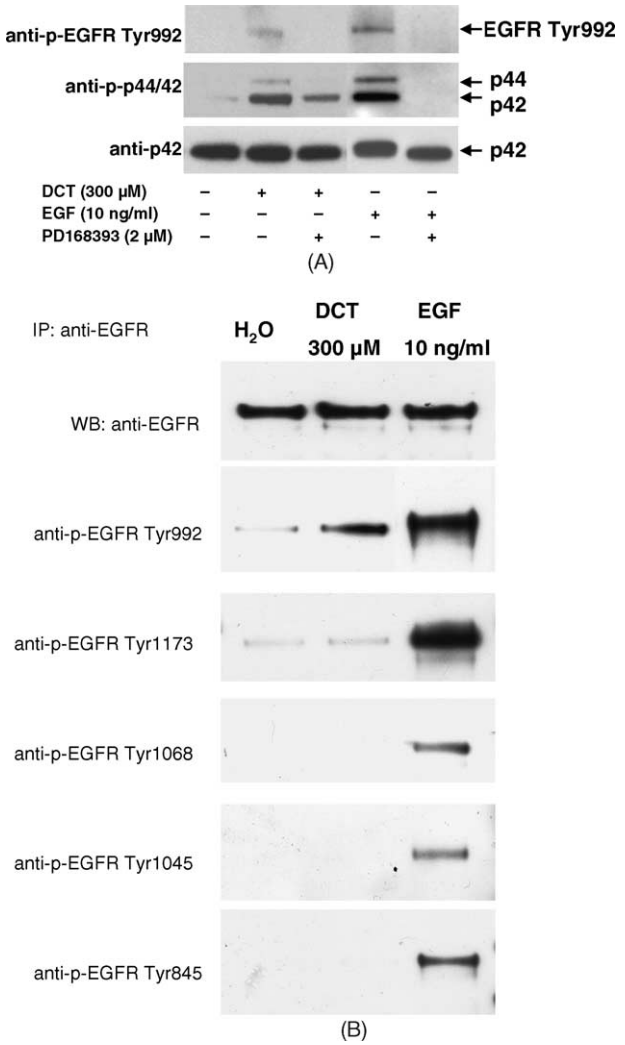

Fig. 6. DCT and EGF activate EGFR in colon cancer cells—effect of EGFR inhibitors on agonist-induced EGFR Tyr992 and p44/42 MAPK phosphorylation. (A) H508 cells were treated with water, 300 μM DCT or 10 ng/ml EGF for 10 min at 37 °C with or without PD168393 (2 μM). Transactivation of EGFR was determined by immunoblotting with antibodies specific for phosphorylated EGFR Tyr992 and examining the actions of an EGFR inhibitor (PD168393) on EGFR Tyr992 and p44/42 MAPK phosphorylation. Results are representative of three separate experiments. (B) H508 cells were treated with water, 300 μM DCT or 10 ng/ml EGF for 10 min at 37 °C. Immunoprecipitation of EGFR in protein extracts was performed as described in Section 2 using mouse anti-EGFR antibody. Phosphorylation of specific EGFR tyrosines was determined by immunoblotting with rabbit antibodies specific for total EGFR and for phosphorylated EGFR Tyr845, Tyr992, Tyr1045, Tyr1068, and Tyr1173. Results are representative of three separate experiments.

Whereas EGF, used as a positive control, stimulated phosphorylation of all the EGFR tyrosine residues, we did not observe bile acid-induced phosphorylation of EGFR Tyr845, Tyr1045, Tyr1068 or Tyr1173 (Fig. 6B). EGFR Tyr992, a high-affinity binding site for SH2 domains of phospholipase-Cγ [51,52], is an important effector of EGFR activation [53]. The finding of DCT-induced EGFR Tyr992 phosphorylation that is reduced in the presence of an EGFR inhibitor provides additional evidence in H508 colon cancer cells of bile acid-induced transactivation of EGFR.

3.6. EGFR inhibitors block DCT- and EGF-induced p44/42 MAPK phosphorylation and cell proliferation

To confirm that transactivation of EGFR plays a role in mediating the actions of DCT, we examined the effects on bile acid-induced p44/42 MAPK phosphorylation and cell proliferation of adding two EGFR inhibitors, PD168393 and AG1478 [48–50]. As shown in Fig. 7A, DCT- and EGF-induced p44/42 MAPK phosphorylation was blocked in the presence of either EGFR inhibitor. Likewise, inhibition of EGFR activation greatly reduced the magnitude of DCT-induced cell proliferation (Fig. 7B). Collectively, the observations depicted in Figs. 6 and 7 provide evidence that, in colon cancer cells, EGFR activation mediates the proliferative actions of bile acids.

3.7. EGFR antibody blocks DCT- and EGF-induced p44/42 MAPK phosphorylation and cell proliferation

Bile acid-induced activation of EGFR may be mediated directly by actions of the agents on the receptor or by the release of EGFR ligands. As noted above, the failure of bile acids to stimulate post-receptor signaling or proliferation in SNU-C4 cells that express EGFR provides evidence that bile acids are not EGFR ligands (Fig. 3A and B). The EGFR ligand family comprises six members; EGF, transforming growth factor-α (TGF-α), amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), betacellulin and epiregulin, that are synthesized as pro-ligands anchored to cell membranes [54,55].

To determine whether DCT-induced transactivation of EGFR is dependent on binding of an EGFR ligand, we examined the effects of blocking EGFR with an antibody directed to the EGFR ligand-binding domain. As shown in Fig. 8A, addition of EGFR antibody (0.1 μg/ml) reduced DCT- and EGF-induced phosphorylation of p44/42 MAPK. Moreover, as shown in Fig. 8B, addition of the EGFR antibody inhibited DCT-induced H508 cell proliferation. In these experiments, adding 0.01 μg/ml of anti-EGFR antibody did not significantly reduce the proliferative actions of EGF (not shown) and 0.03 μg/ml of the antibody caused only a modest reduction in EGF-induced

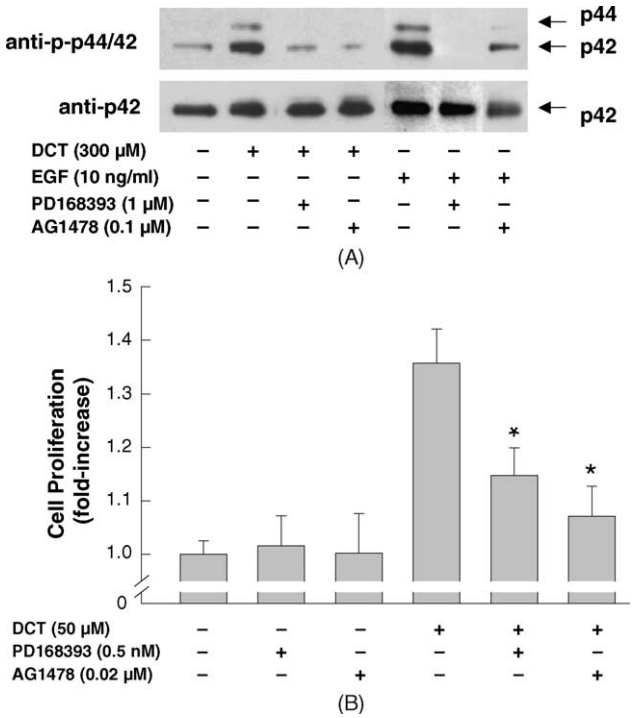

Fig. 7. Effect of EGFR inhibitors on bile acid-induced MAPK phosphorylation and H508 cell proliferation. (A) H508 cells were treated with water, DCT (300 μM), and EGF (10 ng/ml) for 10 min at 37 °C with or without EGFR inhibitors [PD168393 (1 μM) and AG1478 (0.1 μM)]. p44/42 MAPK activity was determined by immunoblotting with antibodies specific for phosphorylated MAPK. The quantity of protein added was verified by immunoblotting with antibodies specific for non-phosphorylated p42 MAPK. Results are representative of three separate experiments. (B) H508 cells were incubated with water and DCT (50 μM) for 5 days at 37 °C, with or without EGFR inhibitors. Cellular proliferation was determined by the sulforhodamine blue (SRB) colorimetric assay [40]. Results are mean ± S.E.M. of three experiments. *Values significantly less than that observed in the presence of DCT alone ($p < 0.05$, unpaired Student's t-test).

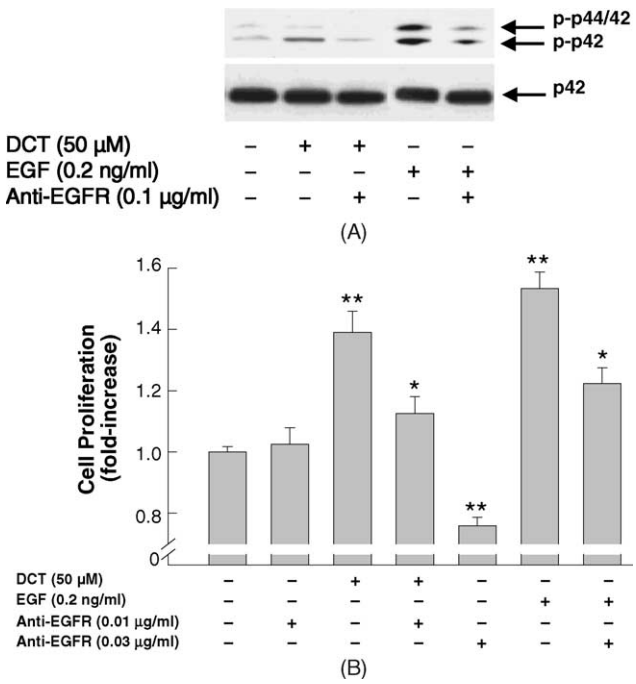

Fig. 8. Addition of EGFR antibody blocks the actions of EGF and DCT. (A) H508 cells were incubated with water, DCT (50 μM) and EGF (0.2 ng/ml) for 10 min at 37 °C, with or without EGFR antibody. p44/42 MAPK activity was determined by immunoblotting with antibodies specific for phosphorylated MAPK. The quantity of protein added was verified by immunoblotting with antibodies specific for total p42 MAPK. Results are representative of three separate experiments. (B) H508 cells were incubated with water, DCT (50 μM), and EGF (0.2 ng/ml) for 5 days at 37 °C, with or without the indicated concentrations of antibody to EGFR. Cellular proliferation was determined by the sulforhodamine blue (SRB) colorimetric assay [40]. Results are mean ± S.E.M. of three experiments. *Values significantly less than those obtained without the EGFR antibody ($p < 0.05$, unpaired Student's t-test). **Values significantly different than that observed in the presence of water ($p < 0.005$, unpaired Student's t-test).

cell proliferation (Fig. 8B). This is in contrast to the demonstrated potency of the antibody for inhibiting the actions of DCT (Fig. 8B). These findings suggest that an EGFR ligand other than EGF mediates the actions of the bile acid. Overall, these findings are consistent with the hypothesis that bile acids stimulate transactivation of EGFR by inducing the release from H508 cells of an EGFR ligand.

4. Discussion

The striking finding in the present communication is that in human colon cancer cells, conjugated secondary bile acids, which were shown previously to act as muscarinic receptor agonists [24,29,45], stimulate colon cancer cell proliferation. This is in marked contrast to the parent lithocholic and deoxycholic acids that induce apoptosis [16,23]. Moreover, we provide evidence that in colon cancer cells, lithocholic and deoxycholic acid conjugates activate post-receptor signaling, including activation of a nuclear response protein, p90RSK; that these signaling and proliferative effects require co-expression of M_3R and EGFR; and that they require activation of EGFR. These observations are illustrated in Fig. 9 with key phosphorylation events highlighted. As reported here, in H508 colon cancer cells, physiological concentrations of deoxycholic acid conjugates do not induce apoptosis. It is possible, if not likely, that bile acid-induced activation of EGFR signaling, as demonstrated in our experiments, provides

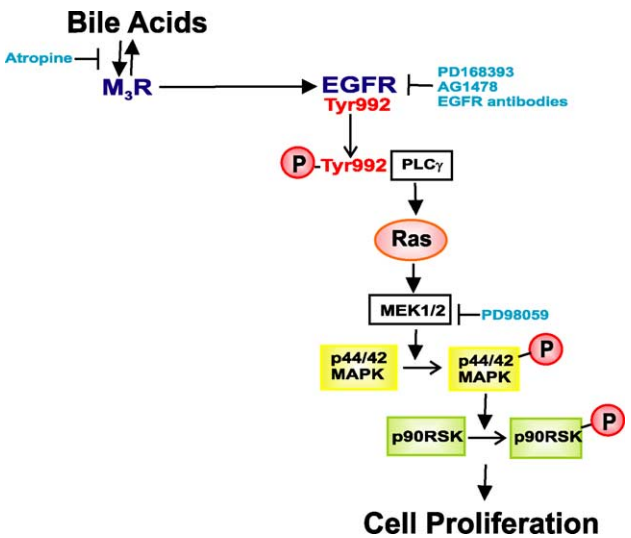

Fig. 9. Flow diagram showing bile acid signaling in H508 colon cancer cells. Conjugated secondary bile acids interact reversibly with M_3R, resulting in transactivation of EGFR (phosphorylation of EGFR Tyr992) and activation of PLCγ. These events activate a downstream signaling cascade that culminates in activation of p90RSK, a nuclear response protein that activates transcription factors that stimulate cell proliferation. Circled 'P' indicates phosphorylation events studied by immunoblotting with specific antibodies for phosphoproteins. Steps inhibited by atropine, EGFR activation inhibitors (PD168393, AG1478), EGFR antibodies, and a MEK inhibitor (PD98059) are shown.

the elusive mechanism underlying extensive epidemiological and experimental data that support the role of bile acids in promoting colon carcinogenesis [3–7].

Our findings support not only the concept that bile acids are signaling molecules that activate plasma membrane receptors, but they confirm that these effects depend strongly on the particular bile acid examined [24,45]. For example, our original observation of interaction of bile acids with muscarinic cholinergic receptors focused on lithocholic acid conjugates. Lithocholyltaurine stimulates muscarinic receptor-mediated pepsinogen secretion from guinea pig gastric chief cells [24] and proliferation of human colon cancer cells [29]. For both cell types, bile acid-induced responses depend upon the expression and activation of M_3R [24,27,29,56]. In the course of a systematic analysis of the interaction of the major human bile acids with CHO cells expressing each of the five muscarinic receptor subtypes, we observed that taurine and glycine conjugates of deoxycholic acid also bind to M_3R [45]. This observation is important because, in the gut, deoxycholic acid conjugates are present in much greater concentration compared to lithocholic acid conjugates. Equimolar concentrations of deoxycholic acid conjugates, which are less lipophilic than lithocholic acid conjugates, are also less likely to be cytotoxic [42–44]. Hence, receptor-mediated actions of deoxycholic acid conjugates are more likely to have physiological importance than those of less abundant, potentially more toxic, lithocholic acid conjugates.

Initial experiments in CHO cells expressing M_3R suggested that deoxycholic acid conjugates were muscarinic receptor antagonists [45,46]. Maximal concentrations of these agents reduced maximal muscarinic receptor radioligand binding by 60%, but similar concentrations of the bile acids did not alter basal inositol phosphate formation or p44/42 MAPK phosphorylation [45,46]. As expected with a muscarinic receptor antagonist, increasing concentrations of deoxycholic acid conjugates progressively reduced ACh-induced inositol phosphate formation and MAPK phosphorylation [46]. The work presented here confirms our findings regarding the actions of deoxycholic acid conjugates on CHO cells that express M_3R. Clearly, the effects of deoxycholic acid conjugates vary depending on the cell type examined and, in particular, their actions depend on co-expression of M_3R and EGFR. Deoxycholic acid conjugates stimulate signaling and proliferation in H508 colon cancer cells that co-express M_3R and EGFR, but not in CHO cells that express only M_3R [39,57] or SNU-C4 cells that express only EGFR [27].

EGFR transactivation is thought to result from cross-talk between M_3R-activated signaling cascades that interposes an additional element of regulation and amplification between the signal (e.g. cholinergic agonists, including bile acids) and the cellular response (e.g. proliferation). In many cells, mitogenic signaling by ligands for G-protein coupled receptors (GPCR), like M_3R, requires transactivation of EGFR, a receptor tyrosine kinase [30,31]. Experimental

data indicate that this mechanism mediates proliferative actions of endothelin-1, lysophosphatidic acid, thrombin, ACh, and other GPCR ligands [30,31,35]. In terms of the potential for neoplastic transformation, cross-talk between GPCRs and receptor tyrosine kinases provides an additional level of control to prevent unregulated cell growth. With specific regard to the development and progression of colon cancer, activation of receptor tyrosine kinases is considered an important determinant of cancer cell proliferation [58,59] and a promising target for cancer chemotherapeutics [60].

It is of interest that immunoblotting of H508 cell extracts and of EGFR antibody immunoprecipitates revealed DCT-induced phosphorylation of only one EGFR tyrosine (Tyr992). In contrast, EGF, used as a positive control in these experiments, stimulated phosphorylation of EGFR Tyr845, 992, 1045, 1068 and 1173. By associating with specific downstream signaling molecules, each tyrosine phosphorylation site activates a particular post-EGFR signaling cascade [52]. For example, Cbl binds Tyr1045, grb2 binds Tyr1068 and 1086, shc binds Tyr1148, and Shp1, shc and phospholipase-Cγ bind Tyr1173 [52]. Our findings with bile acids add to recent intriguing evidence of ligand-specific phosphorylation of these sites [53]. EGFR Tyr992, the only EGFR tyrosine that is consistently and robustly phosphorylated by incubation with DCT, is a high-affinity binding site for SH2 domains of phospholipase-Cγ and is required for EGF-induced activation of this phospholipase C isozyme [51–53]. The present observation is consistent with previous evidence that M_3R-mediated transactivation of EGFR is calcium-dependent [35]. In any event, the finding of DCT-induced EGFR Tyr992 phosphorylation that is reduced in the presence of an EGFR inhibitor provides evidence in H508 colon cancer cells of bile acid-induced transactivation of EGFR. Moreover, this observation implicates activation of phospholipase C and calcium mobilization in bile acid-induced post-receptor signaling [61]. This will be explored in future studies.

Collectively, the data presented here provide compelling evidence that bile acid-induced proliferation of colon cancer cells is mediated by transactivation of EGFR. This includes the observations that bile acids stimulate EGFR tyrosine phosphorylation, activate post-receptor signaling and increase cell proliferation (Fig. 9). These actions are all blocked by addition of EGFR inhibitors and antibodies. Activation of downstream EGFR signaling cascades, presumably with consequent effects on gene transcription, results in cellular proliferation [62,63]. These bile acids are not EGFR ligands and do not directly activate EGFR. This is evidenced by the failure of these agents to stimulate MAPK signaling or proliferation in SNU-C4 colon cancer cells that express EGFR, but not M_3R. Hence, we conclude that co-expression of M_3R and EGFR is required for bile acid-induced colon cancer cell proliferation. Our work is now focused on identifying, in H508 colon cancer cells, the molecular mechanisms whereby bile acids cause transactivation of EGFR, and the role of M_3R in this process.

To draw conclusions regarding the clinical implications of our observations, it is necessary to demonstrate in vivo in the organ of interest (i.e. the colon) that lithocholic and/or deoxycholic acid derivatives achieve concentrations that are necessary for interaction with muscarinic receptors. However, exact bile acid concentrations in each human intestinal compartment are not known. Species differences in the bile acid composition of bile as well as differences in the intestinal flora that alter the ratio of secondary to primary bile acids and the ratio of conjugated to unconjugated bile acids, make it is difficult, if not impossible, to draw meaningful conclusions from animal data [44]. Nonetheless, in proximal animal and human colon, concentrations of lithocholic and deoxycholic acid derivatives are reported to be in the high micromolar to millimolar range [42,43], particularly if ileal damage prevents enterohepatic circulation or antibiotic treatment reduces bacterial populations in the colon [64]. Hence, it appears that the effective bile acid concentrations in our study are achieved in the human proximal colon. Since human colonic bacteria deconjugate secondary bile acids, we speculate that progressive deconjugation of bile acids as they pass from the right to left colon may explain why epidemiological findings most consistently associate changes in fecal bile acid concentration with the development of cecal and ascending colon cancers [3–5].

Our findings add to a growing body of work indicating that, in addition to their physiological actions in promoting lipid absorption and cholesterol excretion, bile acids are signaling molecules that activate plasma membrane and nuclear receptors [24,45,65–67]. To date the major consequences of bile acid interaction with nuclear receptors are limited to modulation of bile acid and cholesterol metabolism. For example, activation of the nuclear vitamin D receptor (VDR) has been implicated in colon carcinogenesis because lithocholic acid binding to VDR activates CYP3A, an enzyme that mediates the catabolism of this potentially carcinogenic bile acid [67]. However, at concentrations examined in the present communication, conjugated lithocholic and deoxycholic acids neither bind nor activate VDR [67]. Hence, there is no evidence that interaction of conjugated bile acids with nuclear receptors plays a role in mediating the proliferative actions we describe.

Potential consequences of bile acid signaling through plasma membrane receptors are much broader (also see review [25]). Our work suggests a mechanism whereby activation of plasma membrane muscarinic and EGF receptors by conjugated bile acids can stimulate colon cancer cell proliferation. Additional factors that support a role for bile acids as colon cancer promoters in vivo include: (a) the likelihood that fecal secondary bile acids are in contact with colonic epithelial cells for many years [the average age for developing colon cancer is older than

50 years [68]]; (b) lipophilic properties of monohydroxy lithocholic acid derivatives allow these agents to access muscarinic receptors in the lipid bilayer of the colon cancer cell membrane [for example, the novel bile acid:ACh hybrid molecule, lithocholylcholine, interacts with muscarinic receptors on a preparation of rat aortic strips [37]]; (c) neoplastic cells may lose cell membrane polarity, thereby leading to the expression of previously basolateral membrane receptors on the apical membrane; (d) colon cancer is associated with increased tight junction permeability [69], thereby increasing the likelihood that less lipophilic bile acids, like deoxycholic acid conjugates, will have access to basolateral membrane receptors (e.g. M_3R). The hypothesis that bile acid-induced transactivation of EGFR stimulates cell proliferation can be tested in rodent models of colon cancer [70,71]. In vivo confirmation of the findings presented in this paper and further elucidation of the underlying mechanisms may suggest novel molecular treatments for colon cancer.

Acknowledgements

This work was supported by a grant from the Office of Research and Development, Medical Research Service, Department of Veterans Affairs (JPR) and by NIH grant CA107345 (JPR).

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