Bile salt exposure increases proliferation through p38 and ERK MAPK pathways in a non-neoplastic Barrett’s cell line

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Gastroesophageal reflux disease (GERD) is a strong risk factor for EAC, presumably because GERD causes Barrett’s esophagus (29). Compared with asymptomatic control subjects in the general population, patients with GERD symptoms like heartburn and regurgitation are eight times more likely to develop EAC (9). The major harmful components of refluxed gastric material are acid and bile salts. The role of acid reflux in the pathogenesis of Barrett’s esophagus is widely accepted, but the role of bile reflux remains controversial (28). Bile reflux is more common and intraesophageal bile acid concentrations are higher in patients with Barrett’s esophagus than in those with uncomplicated GERD (16, 34). Studies have suggested that bile and acid have synergistic effects in causing esophageal damage and that bile may play an important role in the malignant transformation of Barrett’s epithelium (7, 16, 33).

Refluxed gastric juice contains both conjugated and unconjugated bile salts. In patients with Barrett’s esophagus, glycine- and taurine-conjugated bile salts are more prevalent than unconjugated ones (16). These patients often are treated with proton pump inhibitors (PPI), and, consequently, they commonly reflux gastric material with a pH between 4 and 7 (34). Although conjugated and unconjugated bile salts are poorly soluble in acid, they are soluble at the relatively neutral gastric pH levels caused by PPI therapy. These soluble bile salts have the potential to exert damaging effects on the esophageal epithelium.

The molecular mechanisms by which bile salts might promote carcinogenesis in Barrett’s esophagus are poorly understood. One potential mechanism involves MAPK pathways, which are known to have proliferative and antiapoptotic effects in a number of tissues (3, 18, 25). In one study (28), acid exposure was found to induce proliferation in a Barrett’s-associated adenocarcinoma cell line through activation of the p38 and ERK MAPK pathways. However, this study, like most cell culture investigations on Barrett’s esophagus, used a cancer cell line that had sustained extensive genetic alterations during its malignant transformation. The utility of such transformed cells for determining the early carcinogenetic events in Barrett’s esophagus is limited. We explored the effects of bile salts on a primary, nonmalignant Barrett’s cell line. These cells were immortalized, but not transformed, through the forced expression of telomerase. We hypothesized that bile exposure would activate p38 and ERK MAPK pathways to promote growth in these metaplastic cells.

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METHODS

Cell culture. All cells were maintained using standard culture techniques in a humidified 37°C, 5% CO2 incubator. The human, telomerase-immortalized, Barrett’s-derived, non-neoplastic cell line (BAR) (15) was maintained in coculture with mitomycin C-treated Swiss 3T3 feeder cells, which were removed with 0.02% EDTA before maintenance subculturing. BAR cells were maintained in keratinocyte basal media (KBM-2) from Cambrex (Walkersville, MD) supplemented with 5% FBS (Atlanta Biologicals; Norcross, GA), 0.1 mM cholaer toxin (Calbiochem; San Diego, CA), 100 U/ml penicillin-streptomycin (GIBCO-BRL; Gaithersburg, MD), 70 µg/ml bovine pituitary extract (Hammond Cell Technologies), 400 ng/ml hydrocortisone, 20 ng/ml EGF, 10 mg/ml adenine, 5 µg/ml insulin, and 5 µg/ml transferrin (all from Sigma; St. Louis, MO). Before the experimental exposures, BAR cells were cultured in a growth reduced media, which we define as KBM-2 supplemented with hydrocortisone, adenine, penicillin-streptomycin, and transferrin in the same concentrations as described above with additional 0.07 mM Ca2+ and 0.05 µg/ml insulin. Twenty-four hours before the experiments on MAPK activation, cell number, DNA synthesis, and TdT-mediated dUTP nick-end labeling (TUNEL) assays, BAR cells were cultured in this growth-reduced media. For annexin V assays, BAR cells were placed in growth reduced media lacking insulin for 4 h before the experimental exposures. For MAPK Western blot analysis, feeder layer cells were removed with 0.02% EDTA washes 6 h before bile exposure.

Telomerase-immortalized normal squamous esophageal (NES) cells (14) were maintained in coculture with mitomycin C-treated Swiss 3T3 feeder cells in a 3:1 mixture of DMEM-Ham’s F-12 medium (GIBCO-BRL) supplemented with 5% FBS, 0.4 µg/ml hydrocortisone, 5 µg/ml transferrin, 2 × 10−11 M 3,3,5-triiodo-l-thyronine (Sigma), 5 µg/ml insulin, 20 ng/ml recombinant EGF, 180 µM adenosine, 0.1 mM cholaer toxin, and 100 U/ml penicillin-streptomycin. NES cells were cultured in growth reduced media defined as DMEM-F-12 with hydrocortisone, transferrin, triiodothyronine, adenosine, cholaer toxin, and penicillin-streptomycin at the concentrations described above with additional 0.05 µg/ml insulin. Feeder cells were removed with brief 0.02% EDTA washes before maintenance subculturing. BAR and NES cells were grown in coculture with feeder cells for both maintenance and experimental purposes. To create feeder layer cells (1–3 × 10⁶ cells/100-mm dish), Swiss 3T3 cells were treated with mitomycin C (10 µg/ml for 2 h) as previously described(21).

The human Barrett’s-derived esophageal adenocarcinoma SEG-1 cell line (27) was maintained in DMEM (low glucose, 1-glutamine, 110 mg/l sodium pyruvate, and pyridoxine hydrochloride, from GIBCO-BRL) supplemented with 10% FBS and 1% antibiotic-antimycotic solution containing 10,000 U/ml penicillin sodium, 10,000 µg/ml streptomycin sulfate, and 25 µg/ml amphotericin B in 0.85% saline (GIBCO-BRL). Before the experimental exposures, SEG-1 cells were cultured in growth reduced media consisting of DMEM with 1% antibiotic.

Bile salts. All bile salts were obtained from Sigma and dissolved in sterile growth reduced media. All bile salts were used at a pH of 7.4. The unconjugated bile salt chenodeoxycholic acid (CDA) and the conjugated bile salt taurocholic acid (TCA) were used at concentrations for initial examination of the effects of bile salt exposure on cell number in BAR cells. BAR cells were exposed to bile for 5 min to simulate an episode of reflux. For subsequent cell count, proliferation, and apoptosis studies, a 5-min GCDA (200 µM) exposure was uniformly used as our model of bile reflux.

Determination of cell number. BAR, SEG-1, or NES cells (15,000–30,000 cells) were plated onto 24-well plates in the optimal growth conditions described above. Twenty-four hours before the experimental exposures, cells were placed in growth reduced media. Cells were exposed to bile salts for 5 min followed by an incubation for 24 h at 37°C and 5% CO2. Cells were harvested with either 0.05% trypsin (for BAR and NES cells) or 0.25% trypsin (for SEG-1 cells). Changes in cell number were examined using a Coulter Z-1 particle counter.

MAPK Western blot analysis. BAR cells (500,000 cells) were equally seeded onto 100-mm plates in optimal media conditions. Twenty-four hours before the experimental exposures, cells were placed in growth reduced media with 0.5 µg/ml insulin. Six hours before the experimental exposures, feeder cells were removed with multiple 0.02% EDTA washes, and BAR cells were returned to growth reduced media. Cells were exposed to 200 µM GCDA for 5–20 min, followed by cold lysis buffer (Cell Signaling Technology; Beverly, MA). Samples were sonicated, centrifuged, and stored at −70°C until assayed. Proteins were separated by SDS-PAGE using a 10% gel and transferred to a polyvinylidene difluoride membrane by electroblotting. Membranes were blocked in 5% milk with Tris-buffered saline-Tween (TBST) and incubated overnight at 4°C with primary antibody, either a 1:1000 dilution of rabbit anti-human total p38 or total p44/42 (both from Cell Signaling Technology). Membranes were washed and incubated with a secondary antibody of either a 1:2000 dilution of rabbit anti-human phosphorylated p38 (BioSource; Camarillo, CA) or a 1:1000 dilution of rabbit anti-human phosphorylated p44/42 (Cell Signaling Technology). Secondary antibody was then added, and chemiluminescence was determined as per the manufacturer’s instructions (Amersham Biosciences; Bucking- hamshire, UK). Western blots were quantitated with densitometry utilizing Quantity One software from Bio-Rad Laboratories.

Inhibition of p38 and ERK MAPK activation. When the effects of MAPK pathway inhibition on cell number, proliferation, or apoptosis were determined, cells were pretreated with 20 µM PD-98059, an ERK pathway inhibitor, or 5 µM SB-203580, a p38 inhibitor (both from Calbiochem; San Diego, CA), for 15 min before a 5-min bile salt exposure.

Determination of cell proliferation. Cell proliferation was measured using a 5-bromo-2-deoxuridine (BrdU) incorporation assay (Roche Molecular; Indianapolis, IN). BAR cells (8,000 cells/well) were evenly plated onto 96-well microtiter plates in full growth conditions. Cells were placed in growth-reduced media for 24 h before the experimental exposures. BAR cells were exposed to 200 µM GCDA with and without MAPK inhibitors, after which cells were returned to growth reduced media. Six hours after the experimental exposure, 100 µM BrdU substrate [in PBS (pH 7.4)] was added for a 6-h pulse. Media and BrdU-labeling solution were then removed, and the cells were fixed and DNA denatured. Cells were incubated with an antibody to BrdU for 60 min. The commercially provided substrate reacted with the anti-BrdU immune complexes to give a measurable product. Spectrophotometer readings were done at 450 nm.

Determination of apoptosis. Apoptosis was determined by labeling of DNA nicking by TUNEL immunohistochemistry (BD Biosciences) and by annexin V flow cytometry (BD Biosciences). For the TUNEL assay, 150,000 BAR cells were evenly plated onto 35-mm plates containing sterile coverslips. Twenty-four hours before the experimental exposures, cells were placed in growth reduced media with 0.05 µg/ml insulin. Because the spontaneous apoptosis rate of BAR cells is low, we used UV-B irradiation to induce apoptosis. We then examined the inability of 200 µM GCDA preexposure to prevent induced apoptosis. Twenty-four hours after GCDA and UV exposure, cells were fixed with 4% formaldehyde, made permeable with 0.1% Triton X-100 in 0.1% Na-citrate, and labeled with a commercial TUNEL reaction mixture for 1 h at 37°C. DNAase-treated (400 U/ml, Ambion; Austin,TX) cells were used as a positive control. TUNEL-positive cells were photographed using Metamorph (Universal Imaging; Downingtown, PA) imaging technology and manually counted.

For annexin V staining, 360,000 BAR cells were plated evenly onto 60-mm plates. Four hours before the experimental exposures, BAR
cells were placed in growth reduced media without any insulin. Cells were then exposed to 200 µM GCDA with and without a 15-min pretreatment with MAPK inhibitors, followed by UV-B exposure. Cells were returned to growth reduced media for 24 h before being harvested with 0.05% trypsin. Samples were resuspended in a commercial binding buffer (10 mM HEPES-NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl2) and filtered. Approximately 1 × 10^6 cells were labeled with 5 µl (50 µg/ml) propidium iodine (PI) and 5 µl annexin V. Samples were evaluated by flow cytometry using a FITC filter for annexin V.

Statistical analysis. GraphPad Prism version 3.00 for Windows (GraphPad Software; San Diego, CA) was used for all statistical analysis. ANOVA followed by Tukey’s multiple-comparison test was used to determine significance for all cell counting and proliferation experiments. Apoptosis data were analyzed by an unpaired Student’s t-test.

RESULTS

Conjugated bile salts caused greater increases in cell number than unconjugated bile salts. As shown in Fig. 1, BAR cells exposed for 5 min to the unconjugated bile salt CDA at a 50 µM concentration showed a 28% increase in cell number (P < 0.05). At higher CDA concentrations, however, total cell numbers decreased, such that at 500 µM, cell counts were reduced to 38% of control. In contrast, conjugated bile salt exposure with either TCDA or GCDA at all concentrations ranging from 50 to 500 µM resulted in a dose-dependent increase in cell number. At each concentration, GCDA produced a statistically significant (P < 0.05) cell number increase over control. GCDA exposure also induced a greater cell number increase than TCDA at the same concentrations. At the median concentration of bile found in Barrett’s patients of 200 µM (16), GCDA produced a statistically significant cell number increase of 30%. Although 200 µM TCDA also increased cell number by 12% over control, this increase was not statistically significant. Given these findings, we selected 200 µM GCDA as our model treatment for the remainder of our experiments.

Bile salt exposure induced greater increases in cell number in BAR and SEG-1 cells than in NES cells. We examined the effects of bile salts on three cell lines: NES, BAR, and SEG-1 cells (Fig. 2). In the progression of NES epithelium to Barrett’s metaplasia and finally to adenocarcinoma, bile salt exposure may affect each type of cell differently. In NES cells, we found that conjugated bile exposure did not result in significant increases in cell number. In contrast, both BAR and SEG-1 cells demonstrated dose-dependent, statistically significant increases in cell number with conjugated bile salt exposure. In general, the cell number changes in SEG-1 cells paralleled those in metaplastic BAR cells. With 200 µM GCDA, NES cells did not exhibit a statistically significant increase in cell number, whereas both BAR and SEG-1 cells showed increases of 31% and 35%, respectively (both P < 0.05). At very high bile salt concentrations (1,000 µM), both BAR and SEG-1 cells showed decreases in cell number, which were not observed in NES cells. In contrast to our findings with BAR and SEG-1 cells, exposure to the unconjugated bile salt CDA produced cell number increases in NES cells at 200 and 500 µM CDA (data not shown).

GCDA exposure increased p38 and ERK phosphorylation. To determine MAPK activation, the levels of phosphorylated p38 and ERK were examined after a time course of GCDA exposure ranging from 5 to 20 min (Fig. 3A). BAR cells exposed for 5 or 10 min to 200 µM GCDA demonstrated a two- to threefold increase in both phosphorylated p38 and ERK levels. GCDA exposures for more than 10 min did not cause a further increase in phosphorylated MAPK levels. Time course experiments demonstrated that phosphorylated MAPK levels return toward baseline within 15 min of GCDA exposure.

We also examined MAPK levels with 50 µM CDA and 500 µM TCDA, both of which were associated with significant cell number increases in BAR cells (Fig. 3B). The response to the
conjugated bile salt TCDA was similar to that of GCDA; there
was an increase in both phosphorylated p38 and ERK. Inter-
estingly, the unconjugated bile salt CDA did not increase
MAPK levels despite increasing cell numbers.

**GCDA-induced cell number increases were MAPK dependent.** To examine whether the cell number increase was MAPK dependent, Coulter counter cell counts were done with BAR cells exposed to 200 μM GCDA for 5 min with and without a 15-min pretreatment with MAPK inhibitors (Fig. 4). SB-203580 (5 μM) was used to inhibit the p38 MAPK pathway, whereas the MEK inhibitor PD-98059 at 20 μM was used to inhibit the ERK MAPK pathway. SB-203580 (5 μM) produced a 79% inhibition of the GCDA-induced cell number increase (131% control to 107% control, P < 0.05). Similarly, with 20 μM PD-98059, the GCDA-induced cell number increase was completely blocked. Treatment of BAR cells with MAPK inhibitors alone did not produce any significant cell number decreases compared with untreated, control cells.

**GCDA exposure increased cell number by increasing proliferation through p38 and ERK MAPK pathway activation.** To determine whether the GCDA-induced cell number increase was due to increased proliferation or decreased apoptosis, we assessed proliferation by a BrdU incorporation assay (Fig. 5). This colorimetric assay measures the incorporation of the pyrimidine analog BrdU during DNA synthesis, thus giving an

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**Fig. 4.** Cell counts showing that the GCDA-induced cell number increase was mediated by MAPK. A 5-min exposure to 200 μM GCDA produced a 31% cell number increase over the media-only control (P < 0.05). A 15-min pretreatment with the p38 inhibitor SB-203580 (SB) or the MEK-1 inhibitor PD-98059 (PD) changed cell number to 107% and 96% of control, respectively (**P < 0.05 vs. GCDA**). Inhibitors alone did not significantly change cell numbers relative to the control. n = 11.

**Fig. 5.** 5-Bromo-2'-deoxyuridine (BrdU) incorporation showing that GCDA exposure increased proliferation in a MAPK-dependent fashion. BAR cells were pulsed with BrdU between 6 and 12 h after bile salt exposure. A 5-min 200 μM GCDA exposure produced a 30% increase in BrdU incorporation, whereas pretreatment with 5 μM SB or 20 μM PD blocked this GCDA-induced increase (**P < 0.05 vs. GCDA**). SB or PD alone had a nonsignificant, baseline effect of 108% and 78% relative to the media-only control. n = 16.
estimate of cellular proliferation. BAR cells were exposed for 5 min to 200 μM GCDA with and without a 15-min pretreatment of 5 μM SB-203580 or 20 μM PD-98059. A 30% increase in BrdU incorporation was seen 12 h after cells were exposed to GCDA compared with a serum-free media control (P < 0.05). In the presence of SB-203580 or PD-98059, GCDA-induced BrdU incorporation was blocked (P < 0.05 vs. 200 μM GCDA). Inhibitor pretreatment alone did not significantly change BrdU incorporation compared with control (P > 0.05). These data suggest that GCDA-induced increases in cell number were due to increased proliferation of BAR cells.

**GCDA exposure had a minimal antiapoptotic effect.** We next examined the extent to which the GCDA-induced cell number increase was due to an antiapoptotic effect of GCDA. A TUNEL assay, which labels DNA nicks with a fluorescent tag, was done to identify apoptosis. At baseline, BAR cells exhibited minimal apoptosis (Fig. 6A) compared with DNAase-treated positive control cells (Fig. 6B). BAR cells treated with 200 μM GCDA for 5 min before a UV-B exposure of 600 J/m² (Fig. 6D) demonstrated 20% less apoptosis than the serum-free media-treated control (Fig. 6C; P < 0.05, n = 10). Although GCDA produced an antiapoptotic effect at lower UV doses, the results did not achieve statistical significance (Fig. 6E). Similarly, we also examined apoptosis using annexin V staining, in which early apoptotic cells are labeled with FITC-positive annexin V. BAR cells at baseline showed a 5.7% rate of annexin-positive, PI-negative, early apoptotic cells (Fig. 7A). On average, BAR cells exposed to 200 μM GCDA for 5 min before 400 J/m² UV-B exposure (Fig. 7C) demonstrated a 15% decrease in apoptosis relative to the serum-free media-treated control (Fig. 7B; P < 0.05). At 200 and 600 J/m² of UV-B irradiation, GCDA also decreased apoptosis relative to the control, but this did not reach statistical significance (Fig. 8).

**DISCUSSION**

We have shown that non-neoplastic Barrett’s cells exposed to the conjugated bile salt GCDA exhibit increased levels of both p38 and ERK MAPK phosphorylation and significant increases in cell number. Bile exposure increased BrdU incorporation levels, and this increase was blocked when cells were pretreated with inhibitors of p38 and ERK. These observations suggest that bile exposure enhances proliferation in Barrett’s cells through MAPK activation. Our observation that bile exposure protected against UV-induced apoptosis suggests that bile also has antiapoptotic effects. However, our finding that the baseline levels of apoptosis in this cell line are very low suggests that antiapoptotic effects did not contribute importantly to the overall bile-induced increase in cell number.

Conjugated bile salts have been shown to induce proliferation in cholangiocyte and intestinal cells (1, 31, 36). Our experiments demonstrate that conjugated bile salts at neutral pH have a proliferative effect on non-neoplastic Barrett’s cell in vitro. Explants of Barrett’s esophagus exposed to a mixture of conjugated bile salts at neutral pH have also been found to exhibit proliferation, but these experiments used supraphysiological concentrations of bile acids (7). Unconjugated bile salts such as deoxycholate have been shown to induce proliferation in colon cancer models (12, 17). One study (32) found that Barrett’s adenocarcinoma cells exposed to short bursts of the unconjugated bile salt CDA also exhibited proliferation (32). However, the conclusions that can be drawn from this study regarding bile effects on benign Barrett’s esophagus are limited because the experiments were performed in an acidic environment (pH 4) in transformed cells. Furthermore, it is more disease relevant to use conjugated bile salts in Barrett’s esophagus models because esophageal aspiration studies have shown that conjugated bile salts predominate in these patients (16). In our cell culture model, glycine-conjugated bile salt exposure...
produced the highest cell number increase, at physiologically relevant bile salt concentrations of 200–500 μM. Interestingly, the proliferative effect of GCDA was seen in both BAR and SEG-1 cells but not in NES cells. Unconjugated bile salts induced cell number increases at low (50 μM) doses but not at higher doses in metaplastic and adenocarcinoma cells. In contrast, in NES cells, proliferation was seen only with high doses of unconjugated bile salts (200 and 500 μM). Given the prevalence of conjugated bile salts in refluxed material in patients with Barrett’s esophagus, the differential proliferative response between squamous and metaplastic esophageal epithelium to conjugated bile salts may be clinically significant. The replacement of normal squamous epithelium with metaplastic epithelium with increased sensitivity to conjugated bile reflux may contribute to hyperproliferation and malignant progression in Barrett’s esophagus.

We also found that pretreatment of Barrett’s cells with GCDA protected against UV-induced apoptosis. Although there are no previous studies that have examined the antiapoptotic effect of bile in Barrett’s esophagus, others have shown that TCDA exposure of cholangiocytes protects against apoptosis induced by vagotomy or carbon tetrachloride (10, 11). TCDA also provides an antiapoptotic signal in intestinal cells exposed to TNF-α (31). In contrast, GCDA is toxic to hepatocytes, inducing apoptosis through the Fas receptor pathway (5). Others have demonstrated that although GCDA promotes apoptosis in hepatocytes, both tauroursodeoxycholic acid and TCDA exposure inhibit apoptosis (24, 30). Our studies have...
shown that GCDA induces a small antiapoptotic effect when BAR cells are exposed to UV-B. However, given that baseline apoptosis in our Barrett’s esophagus model is very low, the antiapoptotic effect of GCDA may not be as important as its proproliferative effects.

GCDA exposure has also been shown in this study to activate both p38 and ERK MAPK signaling cascades. In adenocarcinoma models of Barrett’s esophagus, acid exposure and gastrin stimulation have also been reported to activate MAPK pathways (13, 28). CDA and, at higher concentrations, GCDA exposure in esophageal squamous cell lines have been shown to induce cyclooxygenase (COX)-2 through ERK activation (38). Pathways other than MAPK, such as phosphatidylinositol 3-kinase, COX-2, and PKC signaling mechanisms, may also be activated in response to bile exposure in Barrett’s esophagus (6, 8, 26, 39). Although the proximal signaling events linking bile-salt induced MAPK activation in Barrett’s esophagus is unknown, in other systems bile salts have been shown to activate MAPK signaling via the EGF receptor, Src kinases, and reactive oxygen species (4, 20, 22, 23, 37). Results from our studies have shown that in metastatic cells, GCDA and TCD exposure activates both p38 and ERK within 5 min of exposure and that inhibition of these pathways individually blocks GCDA-induced cell number increases. These findings suggest that both ERK and p38 pathways are important in GCDA-induced proliferation. Interestingly, low doses of the unconjugated bile salt CDA increased cell number but did not activate p38 or ERK signaling. This suggests that conjugated and unconjugated bile salts may induce proliferation by different mechanisms in Barrett’s esophagus.

In summary, we have found that conjugated bile salt exposure activates ERK and p38 MAPK pathways to produce a proproliferative effect in a non-neoplastic Barrett’s cell line. We also found that bile exposure may have a small antiapoptotic effect that contributes to the overall cell number increases. These findings suggest that bile exposure may contribute to the hyperproliferative state characteristic of Barrett’s epithelium. We speculate that prevention of bile reflux and its resultant proproliferative signaling might retard carcinogenesis in Barrett’s esophagus.

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