Deoxycholic acid causes DNA damage while inducing apoptotic resistance through NF-κB activation in benign Barrett’s epithelial cells

Xiaofang Huo,1 Stefanie Juergens,5 Xi Zhang,1 Davood Rezaei,2 Chunhua Yu,1 Eric D. Strauch,6 Jian-Ying Wang,6,7,8 Edaire Cheng,3 Frank Meyer,5 David H. Wang,1,4 Qiuyang Zhang,1 Stuart J. Spechler,1 and Rhonda F. Souza1,4

Departments of 1Medicine, 2Research and Development, and 3Pediatrics, Veterans Affairs North Texas Health Care System, Children’s Medical Center, and University of Texas Southwestern Medical Center, 4Harold C. Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center, Dallas, Texas; 5Department of Surgery, Otto-von-Guericke University School of Medicine, Magdeburg, Germany; 6Cell Biology Group, Department of Surgery, and 7Department of Pathology, University of Maryland School of Medicine, and 8Baltimore Veterans Affairs Medical Center, Baltimore, Maryland

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Huo X, Juergens S, Zhang X, Rezaei D, Yu C, Strauch ED, Wang J, Cheng E, Meyer F, Wang DH, Zhang Q, Spechler SJ, Souza RF. Deoxycholic acid causes DNA damage while inducing apoptotic resistance through NF-κB activation in benign Barrett’s epithelial cells. Am J Physiol Gastrointest Liver Physiol 301: G278–G286, 2011. First published June 2, 2011; doi:10.1152/ajpgi.00092.2011.—Gastroesophageal reflux is associated with adenocarcinoma in Barrett’s esophagus, but the incidence of this tumor is rising, despite widespread use of acid-suppressing medications. This suggests that refluxed material other than acid might contribute to carcinogenesis. We looked for potentially carcinogenic effects of two bile acids, deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA), on Barrett’s epithelial cells in vitro and in vivo. We exposed Barrett’s (BAR-T) cells to DCA or UDCA and studied the generation of reactive oxygen/nitrogen species (ROS/RNS); expression of phosphorylated H2AX (a marker of DNA damage), phosphorylated IkBα, and phosphorylated p65 (activated NF-κB pathway proteins); and apoptosis. During endoscopy in patients, we took biopsy specimens of Barrett’s mucosa before and after esophageal perfusion with DCA or UDCA and assessed DNA damage and NF-κB activation. Exposure to DCA, but not UDCA, resulted in ROS/RNS production, DNA damage, and NF-κB activation but did not increase the rate of apoptosis in BAR-T cells. Pretreatment with N-acetyl-L-cysteine (a ROS scavenger) prevented DNA damage after DCA exposure, and DCA did induce apoptosis in cells treated with NF-κB inhibitors (BAY 11-7085 or AdIkBα superrepressor). DNA damage and NF-κB activation were detected in biopsy specimens of Barrett’s mucosa taken after esophageal perfusion with DCA, but not UDCA. These data show that, in Barrett’s epithelial cells, DCA induces ROS/RNS production, which causes genotoxic injury, and simultaneously induces activation of the NF-κB pathway, which enables cells with DNA damage to resist apoptosis. We have demonstrated molecular mechanisms whereby bile reflux might contribute to carcinogenesis in Barrett’s esophagus.

Barrett’s esophagus; bile salts; gastroesophageal reflux

GASTROESOPHAGEAL REFUX DISEASE (GERD) and its sequela, Barrett’s esophagus, are well-established risk factors for esophageal adenocarcinoma (31). The gastroesophageal reflux of acid is thought to contribute to the development of this lethal tumor (36), but the incidence of esophageal adenocarcinoma continues to rise at an alarming rate, despite the widespread use of potent acid-suppressive medications, such as proton pump inhibitors (PPIs), for the treatment of GERD (25). This suggests that refluxed hydrochloric acid might not be the sole factor contributing to esophageal carcinogenesis in GERD. Indeed, there are data to suggest a carcinogenetic role for refluxed bile acids. Barrett’s-like intestinal metaplasia of the esophagus and esophageal adenocarcinoma develop in rat models of reflux esophagitis in which a surgeon creates an esophagojejunostomy to induce the reflux of acid and bile into the esophagus (6, 28). In one study, rats subjected to esophagojejunostomy combined with total gastrectomy developed esophageal adenocarcinomas at the same rate as those subjected to esophagojejunostomy without gastrectomy, showing that the reflux of bilious intestinal juice alone (without acid) is sufficient to cause esophageal cancer in rats (10). Patients with Barrett’s esophagus have significantly more esophageal exposure to bile and significantly higher esophageal luminal concentrations of bile salts than patients who have GERD without Barrett’s esophagus (23, 35). For patients with cancers in Barrett’s esophagus, a carcinogenetic role for bile reflux has been proposed (20).

Bile acids have been shown to cause DNA damage in cultures of Barrett’s epithelial cells and Barrett’s-associated esophageal adenocarcinoma cells and in biopsy specimens of Barrett’s epithelium maintained ex vivo (8, 9, 11, 17, 19). Severe DNA damage often triggers apoptosis (programmed cell death), which is an important mechanism to prevent the replication of cells with mutations that might result in cancer development. Indeed, to become malignant, cells must develop the ability to resist apoptosis (12). Ex vivo cultures of Barrett’s mucosa are more resistant to bile acid-induced apoptosis than normal esophageal squamous mucosa (9). This observation suggests that Barrett’s epithelial cells might be able to resist apoptosis in the setting of bile acid-induced DNA damage, an ability that might enable dangerous mutations to persist and facilitate neoplastic progression.

In earlier studies, we showed that nonneoplastic, telomerase-immortalized Barrett’s epithelial (BAR-T) cell lines exposed to UV-B radiation developed DNA injuries, but the cells resisted apoptosis by activating the NF-κB pathway (13). The present study was designed to explore DNA damage and the contribution of the NF-κB pathway to apoptotic resistance in BAR-T cell lines treated with deoxycholic acid (DCA), a toxic, hydrophobic bile acid, or ursodeoxycholic acid (UDCA), a hydrophilic bile acid. We also sought to confirm the validity of our in vitro findings by looking for DNA damage and for activation
of NF-κB pathway proteins in biopsy specimens of Barrett’s metaplasia taken from patients whose Barrett’s esophagus was perfused with DCA or UDCA during endoscopic examinations.

METHODS

Cell culture. We used two telomerase-immortalized Barrett’s epithelial cell lines (BAR-T and BAR-T10) that were created from endoscopic biopsy specimens of nondysplastic specialized intestinal metaplasia taken from patients with long-segment (>3 cm) Barrett’s esophagus (15, 36–38). As a control for cell phenotype, we used normal rat intestinal epithelial (IEC-6) cells (American Type Culture Collection, Manassas, VA). IEC-6 cells were cultured in DMEM with 10% FBS, and BAR-T cell lines were cocultured with a fibroblast feeder layer, as previously described (15, 26). All cell lines were maintained in monolayer culture at 37°C in humidified air with 5% CO2 in growth medium, as previously described. For individual experiments, the BAR-T cell lines were equally seeded into collagen IV-coated wells (BD Biosciences, San Jose, CA). Cellular morphology was documented using the Metamorph imaging system (Universal Imaging, Downingtown, PA).

Bile acid exposure. For individual experiments, the cells were cultured in collagen IV-coated wells 24 h before treatment with experimental media (all at pH 7.2) containing DCA (50 or 250 μM; Sigma, St. Louis, MO) or UDCA (50 or 250 μM; Sigma) or control medium without bile acids. Media were added for 5, 10, or 30 min to equally seeded wells of subconfluent cells and then removed, and the samples were collected for analyses (see below). Exposure durations, bile acid concentrations, and pH were chosen to simulate typical samples were collected for analyses (see below). Exposure durations, bile acid concentrations, and pH were chosen to simulate typical.
64.5 ± 2.8 (SE) yr old, who were taking PPIs] who had specialized intestinal metaplasia without dysplasia involving ≥2 cm of the distal esophagus and were scheduled for elective endoscopic examinations were invited to participate. During endoscopy, using a jumbo biopsy forceps (Olympus FB-50K-1) and a technique similar to that previously described by our laboratory (7, 23, 30), we obtained six biopsy specimens of Barrett’s metaplasia before and after perfusion of the distal esophagus with 10 ml of unconjugated DCA (5 patients) or conjugated UDCA (5 patients) at 250 μM over 5 min. For the in vivo perfusions, DCA and UDCA were dissolved in alcohol, resulting in solutions with pH 5.0 and 4.7, respectively. These pH levels are within the range of weakly acidic reflux episodes (pH 4–7), which commonly occur in patients who take PPIs (4).

Statistical analyses. The data were collected from at least two independent experiments. Quantitative data are expressed as means ± SE. Statistical analysis was performed using ANOVA and the Student-Newman-Keuls multiple-comparison test with the Instat for Windows statistical software package (GraphPad Software, San Diego, CA). P < 0.05 was considered significant for all analyses.

RESULTS

DCA causes DNA damage but does not induce apoptosis in Barrett’s epithelial cells. BAR-T and BAR-T10 cells were grown to 70% confluence and then exposed to 50 or 250 μM DCA for 5–30 min. Cells were collected immediately after exposure, and DNA damage was assessed by expression of p-H2AX (5). Apoptosis was determined by optic morphology and cell death ELISA at 24 h after treatment with DCA. In both BAR-T cell lines, 50 and 250 μM DCA caused substantial increases in p-H2AX expression, even after only 5 min of exposure (Fig. 1). However, BAR-T and BAR-T10 cells treated with 250 μM DCA for up to 30 min exhibited no significant changes in their rates of apoptosis (Fig. 2). These findings demonstrate that DCA causes DNA damage in Barrett’s epithelial cells, but the cells resist apoptosis, despite their genotoxic injuries.

DCA does not cause DNA damage or induce apoptosis in normal intestinal cells. In contrast to the BAR-T cell lines, IEC-6 cells (normal rat small intestinal epithelial cells) did not exhibit phosphorylation of H2AX after exposure to 250 μM DCA for ≤30 min (Fig. 3A). IEC-6 cells also exhibited no significant changes in their rates of apoptosis after exposure to DCA (Fig. 3, B and C).

UDCA does not cause DNA damage or induce apoptosis in Barrett’s epithelial cells. We treated BAR-T and BAR-T10 cells with 50 or 250 μM UDCA for 5–30 min. In contrast to treatment with DCA, UDCA did not cause DNA damage at any dose or duration of exposure (Fig. 4). In addition, the cells exhibited no significant changes in their rates of apoptosis after exposure to UDCA (Fig. 5).
NF-κB pathway is activated by DCA, but not by UDCA, in Barrett’s epithelial cells. In Barrett’s-associated adenocarcinoma cells, DCA has been shown to activate NF-κB and to increase expression of NF-κB target genes, including IL-8 and IκB (1, 18). We sought to determine whether bile acids activate NF-κB pathway proteins in nonneoplastic Barrett’s epithelial cells. BAR-T or BAR-T10 cells were treated with 250 μM DCA or UDCA for 5 min, and Western blotting for p-IκBα and p-p65 was performed. DCA increased the expression of p-IκBα and p-p65 in both Barrett’s epithelial cell lines (Fig. 6A). In contrast, UDCA had no effect on the expression of these phosphoproteins. Activation and phosphorylation of p65 lead to its nuclear translocation. As shown in Fig. 6B, DCA increased nuclear expression of total p65 and p-p65 in both Barrett’s epithelial cell lines. DCA also increased expression of Bcl-2, a survival protein that is a downstream target of NF-κB, by 24 h (Fig. 6C). Taken together, these data suggest that the NF-κB pathway is activated by DCA in Barrett’s epithelial cells.

DCA, but not UDCA, generates ROS/RNS in Barrett’s epithelial cells. In Barrett’s-associated adenocarcinoma cells, treatment with antioxidants has been shown to prevent DCA-induced DNA damage and expression of NF-κB-dependent genes, which suggests a role for ROS/RNS in triggering these events (17, 19). Using fluorescence microscopy, we determined the effect of 250 μM DCA or UDCA on ROS/RNS production by our nonneoplastic Barrett’s epithelial cells. In agreement with the data on Barrett’s cancer cells, we found that exposure to DCA for 5, 10, or 30 min induced ROS/RNS production in BAR-T and BAR-T10 cells (Fig. 7A; data not shown for BAR-T). In contrast, exposure to 250 μM UDCA for up to 30 min did not induce the production of ROS/RNS in either Barrett’s epithelial cell line (Fig. 7A; data not shown for BAR-T).

NAC prevents DNA damage and activation of the NF-κB pathway in Barrett’s epithelial cells exposed to DCA. To explore whether the DCA-induced production or ROS/RNS was responsible for DNA damage and activation of the NF-κB pathway we observed in BAR-T and BAR-T10 cells, we treated the cells with 250 μM DCA for 5 min in the presence of 10 mM NAC. NAC prevented the DCA-induced increase in p-H2AX and p-p65 expression, suggesting that ROS/RNS produced in response to DCA exposure are responsible for the genotoxic injury and activation of the NF-κB pathway in Barrett’s epithelial cells (Fig. 7B).

In patients with Barrett’s esophagus, esophageal perfusion with DCA, but not UDCA, causes DNA damage and activation of the NF-κB pathway. Having found that exposure to DCA, but not UDCA, increases phosphorylation of H2AX and activation of NF-κB pathway proteins in our Barrett’s epithelial cell lines, we sought to confirm that these same effects occur in patients with Barrett’s esophagus in vivo by taking endoscopic

**Fig. 3.** DCA does not increase p-H2AX expression or apoptosis in IEC-6 small intestinal cells. A: representative Western blot demonstrating no change in p-H2AX expression following exposure to DCA in IEC-6 cells. β-Tubulin served as a loading control. B and C: effects of DCA exposure on apoptosis in IEC-6 cells as determined by optic morphology and cell death ELISA, respectively. Values are means ± SE of 2 individual experiments.

**Fig. 4.** Ursodeoxycholic acid (UDCA) does not increase p-H2AX expression in Barrett’s cell lines. Representative Western blots demonstrate p-H2AX expression following exposure to 50 or 250 μM UDCA for 5, 10, or 30 min in BAR-T and BAR-T10 cells. β-Tubulin served as a loading control.
biopsy specimens of Barrett’s epithelium before and after a 5-min perfusion of the esophagus with 250 μM DCA or UDCA. In agreement with our in vitro data, we found increased p-H2AX, p-IκBα, and p-p65 expression in biopsy specimens of Barrett’s metaplasia from all five patients whose esophagus was perfused with DCA (Fig. 8A), but in none of the five patients whose esophagus was perfused with UDCA (Fig. 8B).

**Inhibition of the NF-κB pathway sensitizes Barrett’s cells to DCA-induced apoptosis.** Having found that DCA activates the NF-κB pathway in Barrett’s epithelial cells in vitro and in vivo, we next sought to determine whether that activation contributes to their resistance to apoptosis. BAR-T and BAR-T10 cells were treated with BAY 11-7085 at 5 μM (a concentration that we previously found to inhibit NF-κB activity and induce apoptosis in BAR-T cells) and then exposed to 250 μM DCA for 5 min (13). We confirmed inhibition of the NF-κB pathway by performing Western blotting for p-IκBα in the presence and absence of DCA (Fig. 9A). DCA induced a significant increase in apoptosis in both cell lines treated with BAY 11-7085 (Fig. 9B). Moreover, cells treated with BAY 11-7085 and exposed to DCA became small and shrunken, a morphology that suggests an apoptotic phenotype (Fig. 9C). To confirm these findings,
we infected the cells with the AdIκB-SR construct (containing a mutant form of IκB) and determined the rate of apoptosis following exposure to DCA. Infection with AdIκB-SR alone caused no change in basal rates of apoptosis, but the infection significantly increased apoptosis after exposure to DCA (Fig. 10). These data suggest that the apoptosis induced by DCA in the infected cells was indeed due to inhibition of the NF-κB pathway and not a nonspecific effect of the AdIκB-SR.

**DISCUSSION**

We have shown that DCA causes DNA damage and induces phosphorylation of proteins in the NF-κB signaling pathway in Barrett’s epithelial cells in vitro and in vivo. In two Barrett’s epithelial cell lines, we have found that DCA-induced DNA damage and NF-κB pathway activation are mediated by the generation of ROS/RNS. In addition, we have demonstrated that DCA-mediated activation of the NF-κB pathway allows Barrett’s epithelial cells to resist apoptosis in the setting of DNA injury, events that might contribute to neoplastic progression in Barrett’s esophagus.

In an earlier study, ex vivo cultures of Barrett’s mucosa were found to be more resistant to bile acid-induced apoptosis than esophageal squamous mucosal cultures (9). From that study, however, it was not clear whether resistance to bile acid-induced apoptosis was a unique feature of Barrett’s cells or whether the differences in apoptotic resistance were merely due to differences in cell phenotypes (i.e., intestinal-type columnar cells vs. squamous cells). Barrett’s epithelial cells share phenotypic features with small intestinal epithelial cells. Therefore, we exposed normal rat intestinal epithelial (IEC-6) cells to DCA and assessed DNA damage and apoptosis. Unlike our Barrett’s cell lines, we found that IEC-6 cells did not develop DNA damage with DCA exposure. In the absence of DNA damage, it was not surprising that DCA also had no effect on apoptosis in IEC-6 cells. Thus, despite some similar phenotypic features, the Barrett’s cells and small intestinal cells responded very differently to bile acid exposure. Epithelial cells of the small intestine regularly are exposed to high concentrations of bile acids, yet small bowel cancers are uncommon. The differences we observed between small intesti-
tinal and Barrett’s cells in their susceptibility to DCA-induced DNA damage might contribute to the rarity of cancer in the small intestine and the frequency of cancer in Barrett’s esophagus.

In earlier studies, we demonstrated that Barrett’s epithelial cells resist apoptosis due to UV-B-induced DNA injury by activating the NF-κB pathway (13). Although UV-B is a well-established, potent inducer of genomic injury, it is not a physiologically relevant agent for the esophagus. In the current study, we exposed our Barrett’s cells to DCA at physiological concentrations (50 and 250 μM) for 5–30 min to simulate episodes of gastroesophageal reflux (7, 23, 35). We selected DCA on the basis of clinical data demonstrating that concentrations of DCA are especially high in the gastric refluxate of patients with Barrett’s esophagus who take PPIs (23, 29, 32).

In patients who take PPIs, 80% of reflux episodes are either weakly acidic (pH 4–7) or weakly alkaline (pH >7) (4). Thus we elected to use bile acid solutions at pH >4.0 for the in vitro and in vivo studies. DCA, at neutral and acidic pH, has been shown to induce DNA damage in Barrett’s-associated adenocarcinoma cells (8, 17). In agreement with these data in cancer cells, we found that DCA induced genomic injury, as determined by the increase in H2AX phosphorylation in Barrett’s epithelial cells in vitro and in vivo.

We used phosphorylation of H2AX as a marker for DNA damage. H2AX becomes phosphorylated in response to various types of DNA damage, including single- and double-stranded breaks (DSBs) (5). DSBs are among the most serious forms of DNA damage, because cells with persistent DSBs have been found to develop chromosomal abnormalities that can contribute to genomic instability and cancer formation (5, 22). In fact, agents that cause DSBs, such as ionizing radiation and UV light, are considered carcinogens (2, 22). If the DCA-induced DNA damage we observed in Barrett’s cells included DSBs, then this bile acid would be considered a carcinogen in Barrett’s esophagus.

Our finding that DCA induces DNA damage in Barrett’s epithelial cells has interesting clinical implications. In an effort to prevent cancer in Barrett’s esophagus, clinicians commonly prescribe PPIs. However, PPI therapy has been associated with increases in the concentration of toxic, unconjugated bile acids, such as DCA, in the gastric juice of Barrett’s patients (29, 32). Although PPIs clearly heal reflux esophagitis, an effect that would be expected to protect against cancer in Barrett’s esophagus, PPI effects on bile acids could conceivably contribute to cancer formation.

In Barrett’s-associated cancer cell lines, DCA has been shown to cause ROS/RNS production, DNA damage, and NF-κB activation, which can be prevented by treatment with antioxidants (16, 17, 19). However, it has not been clear that...
these DCA effects in cancer cells are applicable to benign Barrett’s cells. We have found that DCA increases ROS/RNS production in nonneoplastic Barrett’s epithelial cells. We also have found that treatment with a ROS/RNS scavenger (NAC) prevents the development of DNA damage and the phosphorylation of p65 when BAR-T cells are exposed to DCA. These findings demonstrate that, as in cancer cells, DCA causes DNA damage and NF-κB pathway activation that is mediated by ROS/RNS production in benign Barrett’s epithelial cells.

The mechanism underlying the apoptotic resistance of Barrett’s mucosa is not known, but circumstantial evidence has suggested a role for the NF-κB pathway. For example, expression of NF-κB has been found in 40–60% of biopsy specimens of benign Barrett’s epithelium and in 61–80% of esophageal adenocarcinomas, but in only 13% of biopsy specimens of reflux-injured squamous epithelium (1, 24). DCA has been shown to activate NF-κB and increase expression of the NF-κB target genes IL-8 and IkB in Barrett’s-associated adenocarcinoma cells (1, 18). We have reported that activation of the NF-κB pathway allows benign Barrett’s epithelial cells to resist apoptosis in the setting of DNA injury induced by UV-B irradiation (13). In the present study, we have shown that DCA increases the expression of activated NF-κB proteins (p-IκBα and p-p65) in Barrett’s epithelial cell lines in vitro and in biopsy specimens from patients with Barrett’s esophagus in vivo. We also have found that DCA increases nuclear expression levels of total p65 and p-p65 in Barrett’s cell lines, which also express higher levels of Bcl-2. This indicates that DCA activates the NF-κB pathway. In addition, we have found that inhibition of the NF-κB pathway with BAY 11-7085 or an IκB superrepressor construct abolishes resistance to apoptosis after DCA exposure in BAR-T cells. Taken together, these data demonstrate that the NF-κB pathway mediates the apoptotic resistance of Barrett’s epithelial cells.

UDCA is a hydrophilic bile acid that has been used to treat cholestatic liver diseases (21) and has been reported to reduce the development of colon cancer in patients with ulcerative colitis (34). In hepatoma cells, UDCA is less genotoxic than DCA (3), and, in Barrett’s epithelial cells exposed to a combination of acid and bile salts, the addition of UDCA decreases DNA damage and ROS generation (11). We have shown that, unlike DCA, UDCA does not increase the expression of p-H2AX, p-IκBα, or p-p65 in Barrett’s epithelial cells in vitro or in patients with Barrett’s esophagus in vivo. We also have found that UDCA does not induce ROS/RNS production in BAR-T cells, which may account for the absence of DNA damage and NF-κB activation. Although we did not explore a specific protective effect for UDCA in preventing bile acid damage, UDCA had no apparent ill effects in Barrett’s epithelial cells.

In conclusion, we have shown that DCA causes genotoxic injury in nonneoplastic, Barrett’s epithelial cell lines, which are able to resist the apoptosis that ordinarily follows severe DNA damage by activating the NF-κB pathway. Furthermore, we have verified our in vitro findings by demonstrating the development of DNA damage and phosphorylation of NF-κB pathway proteins in biopsy specimens of Barrett’s esophagus from patients who had DCA perfused into the esophagus for 5 min during endoscopic examinations. These findings suggest a molecular pathway whereby bile reflux, by inducing DNA damage in cells that can resist apoptosis, may contribute to carcinogenesis in Barrett’s esophagus.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

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