Ursodeoxycholic Acid Inhibits Ras Mutations, Wild-type Ras Activation, and Cyclooxygenase-2 Expression in Colon Cancer


INTRODUCTION

K-ras mutations occur frequently in colon cancer and contribute to autonomous growth. In the azoxymethane (AOM) model of colon cancer, in addition to K-ras mutations, we have shown that wild-type (WT) Ras can be activated by upstream pathways, including, e.g., signaling by ErbB receptors. Tumors with mutant or activated WT Ras had increased cyclooxygenase-2 (Cox-2) expression. We have also shown that ursodeoxycholic acid (UDCA) prevented AOM-induced colon cancer and suppressed Cox-2 induction. In this study, we examined the role of Ras in Cox-2 inhibition by UDCA. Rats were fed AIN-76A chow alone, or supplemented with 0.4% UDCA, and received 20 mg/kg AOM i.p. weekly × 2 weeks. At 40 weeks, rats were sacrificed, and tumors were harvested. K-ras mutations were assessed by primer-mediated RFLP, allele-specific oligonucleotide hybridization, and direct DNA sequencing. Ras was immunoprecipitated and defined as activated if [Ras – GTP/(Ras – GDP)] > 3 SD above normal values. Cox-2 mRNA was determined by reverse transcription-PCR, and protein expression was assessed by Western blotting and immunostaining. In the AOM alone group, Ras was activated by mutations in 8 of 30 (27%) tumors, and WT Ras was activated in 7 of 30 (23%) tumors. UDCA significantly suppressed the incidence of tumors with mutant Ras (1 of 31, 3.2%; P < 0.05) and totally abolished the development of tumors with activated WT Ras (0 of 10; P < 0.05). In the AOM alone group, Cox-2 was up-regulated >50-fold in tumors with normal Ras activity and further enhanced in tumors with mutant or signaling-activated Ras. UDCA significantly inhibited Cox-2 protein and mRNA levels in tumors with normal Ras activity. In summary, we have shown for the first time that UDCA suppressed the development of tumors with Ras mutations and blocked activation of WT Ras. Furthermore, UDCA inhibited Cox-2 induction by Ras-dependent and -independent mechanisms.

INTRODUCTORY

Colorectal cancer is the second leading cause of cancer-related deaths in both males and females in America, causing ~50,000 deaths each year (1). Colonic carcinogenesis is characterized by an accumulation of activating mutations in proto-oncogenes, such as K-ras, and inactivating mutations in tumor suppressor genes, including the adeno- matous polyposis coli gene (2–4). Epigenetic events involved in colonic carcinogenesis include activation of regulated K-Ras, the small GTP-binding protein encoded by WT3 K-ras, and induction of Cox-2, the rate-limiting enzyme for eicosanoid biosynthesis (5–8). Ras participates in diverse signaling pathways that control proliferation and apoptosis (9). This proto-oncogene is also an important regulator of Cox-2 expression (10). Cox-2 is intimately involved in colonic carcinogenesis, promoting angiogenesis, tumor invasion, and metastasis (11–13).

The AOM model of colon cancer recapitulates many of the clinical, histological, and molecular derangements of sporadic human colon cancer. As in human colon cancer, K-Ras (Ras) can be activated by mutations in AOM tumors (4, 14). We have demonstrated previously that WT Ras can also be activated by ErbB2 growth factor receptor signaling pathways in AOM-induced tumors (8). More recently, in preliminary studies, we found human colon cancers can express phosphorylated (activated) ErbB2 receptors and activated WT Ras, extending earlier studies of increased ErbB2 receptors in human colon cancer (15). In AOM tumors with mutant Ras or signaling-activated WT Ras, we showed that the Ras effector, MAPK, is also activated (8). Although Cox-2 was generally increased in AOM tumors, Cox-2 expression was further enhanced in tumors with mutant Ras or signaling-activated WT Ras (8).

Environmental factors are thought to contribute to the genetic and epigenetic alterations that drive tumor development. Environmental risk factors implicated in colon cancer include diets high in animal fat and low in vegetable fiber and micronutrients (16). These diets are associated with increases in secondary bile acids, such as deoxycholic acid and lithocholic acid, that act as tumor promoters in experimental and perhaps human colon cancer. In the AOM model, tumor-promoting high fat diets have also been shown to increase Ras activation and Cox-2 expression (17–19). Thus, alterations in Ras and Cox-2 induced signaling-activated WT Ras (8).

We have demonstrated previously that another bile acid, UDCA, is chemopreventive in the AOM model (20). UDCA is the 7β-epimer of tumor-promoting chenodeoxycholic acid. This dihydroxy bile acid is derived from chenodeoxycholic acid by bacterially mediated epimerization within the colon and accounts for ~4% of the bile acid pool. UDCA is absorbed in the intestine and undergoes enterohepatic circulation. UDCA is widely used to treat cholestatic liver disorders. Compared with chenodeoxycholic acid and deoxycholic acid, UDCA is relatively hydrophilic, a chemical property suggested to contribute to its cytoprotection against these more hydrophobic membrane-damaging bile acids (21). Numerous potentially overlapping activities of UDCA have been postulated to contribute to its cytoprotective effects, including immune modulation, anti-inflammatory properties, membrane stabilization, and increased cholestasis (21). Increases in intracellular calcium, protein kinase C-α, and MAPK activities have been implicated in one or more of these events (22–24). In hepatocytes, UDCA inhibited formation of reactive oxygen intermediates and blocked mitochondrial membrane permeability transitions, while preventing apoptosis induced by more hydrophobic bile acids (25, 26). More recently, we have shown that UDCA inhibited the overexpression of cyclin D1 and Cox-2 in AOM tumors (27). Because Ras is an important positive regulator of cyclin D1 and Cox-2 in colon cancer (8), we examined the role of Ras in Cox-2 suppression by...
UDCA. Our findings, and their implications for colon cancer chemoprevention, form the basis for the current report.

MATERIALS AND METHODS

Materials. UDCA was generously provided by Dr. Horst-Dietmar Tauschel of Falk Pharma GmbH (Freiburg, Germany). AOM was obtained from Sigma (St. Louis, MO). Rats were purchased from Harlan Sprague Dawley. Rat chow diets of AIN-76A and AIN-76A supplemented with 0.4% (w/w) UDCA were prepared fresh each month by ICN (Aurora, OH). PCR primers for Cox-1 and Cox-2 were provided by Life Technologies, Inc. (Gaithersburg MD). Polynucleotide anti-Cox-2 antibodies were obtained from Cayman Chemical (Ann Arbor, MI). Polynucleotide anti-Cox-1 and monoclonal Y13–259 anti-Ras antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Electrophoretic grade acrylamide, bis acrylamide, Tris, SDS, and prestained molecular weight markers were from Bio-Rad Laboratories (Richmond, CA). Kodak (Rochester, NY) supplied the X-OMAT blue film. Polyvinylidine difluoride membranes (Immobilon-P) were purchased from Millipore, Inc. (Bedford, MA). Nytan maximum strength nylon membranes were obtained from Schleicher & Schuell (Keene, NH) The restriction enzymes, BstNI and BglI, were obtained from New England Biolabs, Inc. (Beverly, MA). QiAquick PCR purification columns were obtained from Qiagen, Inc. (Santa Clarita, CA). New England Nuclear (Boston, MA) provided [γ-32P]ATP. RNA/DNA/protein isolation reagent (TRI Reagent) and other reagents for glycine-glycine or mutant for codon 12 (GAA, GAG) or codon 59 as described previously (29). For exon-1, the sequences of these polymers were of the highest quality available and obtained from Sigma, unless otherwise noted.

Experimental Animal Protocol. Tumor induction with AOM and dietary supplementation with 0.4% UDCA in AIN-76A chow were carried out exactly as described in a previous study (27). Control colonocytes from saline-treated (AOM-vehicle) rats that were unsupplemented or supplemented with UDCA chow were isolated as described previously and stored frozen at −80°C. Separately, a small portion of each tumor was fixed in 10% buffered formalin to classify tumor histology as adenomas or adenocarcinomas as described previously (8).

K-ras Mutations Detected by ASOH. AOM causes δ2-methylguanine adduct formation that leads to G→A transitions in codons 12, 13, or 59. These mutations in turn activate mutant Ras. To identify these mutations, tumor samples were mechanically disrupted and DNA extracted using TRI Reagent. K-ras exon 1, containing codons 12 and 13, and exon 2, containing codon 59, were amplified by PCR. Amplified DNA was neutralized and cross-linked to Nytan Maximum-Strength nylon membranes. ASOH was carried out using primers and 32P-labeled oligonucleotides that flanked K-ras codons 12 and 13 or codon 59 as described previously (29). For exon-1, the sequences of these oligonucleotides were WT for exon 12 (GGT) and exon 13 (GCC) coding for glycine-glycine or mutant for exon 12 (GAT) or exon 13 (GAC), each of which coded for an aspartic acid substitution. For exon 2, the sequences of these oligonucleotides were WT for exon 59 (GCA) coding for alanine or mutant for exon 59 (ACA), coding for threonine.

K-ras Mutations Detected by PM-RFLP. To confirm the ASOH results, PM-RFLP was used to detect K-ras mutations in codons 12 and 13. Briefly, mismatched 5′ primers created restriction sites for BstNI or BglI in WT K-ras that were abolished by G→A mutations in codons 12 or 13, respectively. Restriction sites for BstNI or BglI incorporated into the 3′ primer were cleaved in both WT and mutant K-ras and served as internal controls for digestion. The PCR conditions and restriction digests were described previously (8, 30).

Codon 12 K-ras Mutations Detected by Enriched PM-RFLP. To amplify low abundance codon 12 K-ras mutant forms a procedure of enriched PM-RFLP was carried out as described previously (8). Tumors with WT K-ras yielded a BstNI-digested fragment of 53 bp, whereas K-ras codon 12 GGT→GAT mutations abolished the BstNI site, resulting in an undigested 82-bp product.

DNA Sequencing. K-ras mutations and sequences for Cox-1 and Cox-2 RT-PCR products were confirmed by direct sequencing of amplified DNA using automated fluorescent DNA sequencing. PCR products were purified using a QiAquick PCR purification column. Purified products were sequenced using the ABI Prism dRhodamine terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA) on an ABI Prism 377 sequencer.

Measurement of Ras-bound GTP and GDP. To assess the activation of Ras, we measured the amount of GTP and GDP bound to Ras as described previously (8, 31). Frozen tumor specimens, or colonocytes from saline-treated (AOM-vehicle) control rats, were rapidly minced and extracted in a Dounce homogenizer in ice-cold modified RIPA buffer (50 mM HEPES (pH 7.4), 10 mM MgCl2, 150 mM NaCl, 1% NP40, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin). After a 10,000 × g centrifugation, the soluble fraction was divided into two aliquots that were incubated with 3 μg of the anti-Ras antibody Y13–259 or 3 μg of nonimmune rat IgG. To each aliquot, goat anti-rat IgG and protein G agarose were added, followed by NaCl, SDS, and deoxycholate to final concentrations of 500 mM, 0.05%, and 0.5%, respectively. After gentle shaking for 1 h at 4°C, the immunoprecipitates were washed four times in RIPA buffer containing 500 mM NaCl, 0.05% SDS, and 0.5% deoxycholate and then washed twice in 20 mM Tris-phosphate buffer (pH 7.4). To elute the Ras-bound guanine nucleotides, the immunoprecipitates were resuspended in 30 μl of 5 mM Tris-phosphate (pH 7.4), 2 mM DTT, and 2 mM EDTA; heated to 100°C for 3 min; and then cooled on ice and centrifuged at 10,000 × g for 2 min. Using coupled reactions, eluted GTP was quantitatively converted to ATP by nucleoside diphosphate kinase and ADP (31). To measure GDP, this nucleotide was quantitatively converted to GTP using pyruvate kinase and phosphoenolpyruvate. The ATP was measured by light emission using a luciferin/luciferase system with standard curves to quantitate the Ras-GTP and Ras-GDP normalized to DNA content as described previously (31). Ras was defined as activated if the ratio (Ras → GTP/[Ras → GTP + Ras → GDP]) exceeded the mean ± 3 SD of that of normal colonocytes as described (8).

Immunostaining. For Cox-1 and Cox-2 immunostaining, antigens were retrieved by microwave heating in 0.01 m citrate buffer (pH 7.0). Sections were incubated overnight at 4°C with 1:50 dilution of primary antibodies, followed by incubation with 1:200 dilution of biotinylated antirabbit antibodies at room temperature. Antibody-antigen complexes were detected with the ABC kit (Vector Laboratories), using 3,3′-diaminobenzidine as substrate as described (8). For negative controls, sections were processed in the absence of primary antibodies. A semiquantitative scale was used to assess Cox-2 immunoreactivity of epithelial and stromal cells: 0, nonreactive; 1+, <10% reactive; 2+, 10–50% reactive; and 3+, >50% reactive.

Western Blotting. Proteins from control colonocytes and tumors were extracted in SDS-containing Laemml buffer. Proteins were subjected to quantitative Western blotting analysis as described previously (8). Briefly, proteins (50 μg) were separated by SDS-PAGE using a 10% resolving polyacrylamide gel and electrobotted. Blots were incubated overnight at room temperature with specific primary antibodies anti-Cox-1 (1:3000), anti-Cox-2 (1:10,000), or anti-beta-actin (1:5000), followed by 2-h incubation with appropriate peroxidase-dase-coupled secondary antibodies and subsequent detection by enhanced chemiluminescence.

Reverse Transcription and PCR. Control colonocytes and tumors were mechanically disrupted, and RNA was extracted using TRI Reagent. After DNase treatment, first-strand cDNA synthesis and PCR amplification were carried out using a Perkin-Elmer kit following the protocol recommended by the manufacturer. Briefly, 1 μg of total RNA was reverse transcribed to cDNA using random hexamers in the final volume of 40 μl, and an aliquot of the cDNA was amplified for 30 cycles using specific primers for Cox-1 and Cox-2. RT-PCR reactions were normalized to 18S RNA abundance as an internal standard (Ambion). The oligonucleotide primer sequences were as follows: (a) Cox-1: sense 5′-ACACTTATCATCGCCATCC-3′ and antisense 5′-4GAAGGACACCTTTACATT-3′, which were predicted to amplify a product of 584 bp; and (b) Cox-1: sense 5′-AGCCCCCTATCCACCTATT-3′ and antisense 5′-CCAGGAGCCTGGTCTACTGG-3′, which were predicted to amplify a product of 561 bp. The cycling parameters were the following: (a) 1.5 min at 94°C for denaturation; (b) 1.5 min at 56°C for primer annealing; and (c) 1.5 min at 72°C for extension. A single 10-min elongation period at 72°C completed the PCR amplification. Products of the predicted size were identified by electrophoresis on a 2% agarose gel containing ethidium bromide and sequenced to authenticate the product. The quantitative assays used 10-fold dilutions of cDNA that we determined in preliminary studies ensured the PCR products were within the linear amplification range. RNA samples subjected to the PCR procedure without previous reverse transcription served as negative reverse transcriptase controls to assess genomic DNA contamination. Appro-
private blanks, lacking cDNA templates, were included to serve as controls for cDNA contamination.

Statistical Methods. Data were expressed as means ± SD. Incidences of Ras mutations were compared by the Fisher exact test. Differences in the incidence of tumors with activated versus nonactivated Ras were determined by $\chi^2$ analysis. Levels of Ras activation and Cox-2 expression were compared by ANOVA. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Effects of UDCA on K-ras Mutations and Ras Activation in AOM Tumors. We and others (20, 27, 32) have shown previously that UDCA is chemopreventive in the AOM model of colon cancer. In this model, AOM methylates guanine bases, leading to G to A transitions. In agreement with others, we have demonstrated that AOM tumors, like human colon cancers, frequently possess K-ras mutations (14, 33). To extend these studies, we have examined the effect of UDCA supplementation on AOM-induced K-ras mutations. In the AOM alone and AOM + UDCA groups, there were ~20% adenocarcinomas and 80% adenomas. We classified these tumors by mutational status using three complementary methodologies: (a) ASOH; (b) PM-RFLP; and (c) direct DNA sequencing. Using a very sensitive enriched PM-RFLP method, we also examined these tumors for codon 12 G → A transitions, the most common K-ras mutation in this model. There was complete agreement among these methods in identifying tumors with WT and mutant K-ras. A representative gel of K-ras exon 1 by PM-RFLP is shown in Fig. 1, and the results of the K-ras mutational analysis are summarized in Table 1. There were K-ras mutations in 9 of 49 AOM tumors (18.4%) in the AOM alone group, compared with only 1 K-ras mutation in 31 tumors (3.2%; $P < 0.05$) from carcinogen-treated animals supplemented with UDCA. These findings indicate that UDCA inhibits the development of tumors with K-ras mutations.

In previous studies, we have shown that some AOM tumors possess WT Ras activated by upstream signaling (8). We operationally defined WT Ras as activated if the ratio [Ras - GTP/ (Ras - GTP + Ras - GDP)] exceeded the mean + 3 SD above the mean of this ratio for normal colonocytes (8). There were no differences in Ras activation in colonocytes from saline-treated rats on the UDCA-supplemented diet, compared with those on the unsupplemented diet. As summarized in Table 2, tumors with mutant K-ras, as expected, had significantly higher Ras activation ratios, 12.6 ± 5.8 (n = 8), compared with control colonocytes (3.5 ± 1.8; n = 6; $P < 0.05$), or tumors with WT nonactivated Ras (4.2 ± 1.7; n = 25; $P < 0.05$). In the AOM alone group, there were eight tumors with WT activated Ras, with activation levels of 12.7 ± 4.6 (Table 2). In contrast, there were no tumors with activated WT Ras in the AOM + UDCA group (Table 2). The mean Ras activation ratios for tumors with mutant K-ras and signaling-activated WT Ras did not differ significantly, with values of 12.6 ± 5.8 versus 12.7 ± 4.6, respectively (Table 2). There were significantly more tumors with activated Ras in the AOM alone group (mutant + signaling, n = 15 of 30 tumors), compared with the AOM + UDCA group (Table 2; n = 1 of 11 tumors; $P < 0.01$). Thus, UDCA inhibited the development of tumors with activated (WT and mutant) Ras and also blocked development of tumors with K-ras mutations. FTP catalyzes the farnesylation of Ras, a requirement for Ras membrane association and activation (34). Because dietary fish oil supplementation prevented tumors and limited Ras activation in this model while concomitantly inhibiting FTP expression (35), we investigated the effect of UDCA on FTP. In agreement with others (35), we observed that AOM induced the expression of FTP in tumors, but UDCA supplementation did not alter this up-regulation as assessed by immunohistochemical staining (data

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Incidence of tumors with WT and mutant K-ras</th>
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<tr>
<td>Groups</td>
<td>Tumors</td>
</tr>
<tr>
<td>AOM</td>
<td>49</td>
</tr>
<tr>
<td>AOM + UDCA</td>
<td>31</td>
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* Tumors were examined in Tri Reagent, and K-ras exon-1 and exon-2 were amplified by PCR. Mutations for codons 12, 13, and 59 were evaluated by ASOH and confirmed by PM-RFLP (codons 12 and 13) and direct sequencing as described in “Materials and Methods.”

b $P < 0.05$ compared with AOM alone (Fisher’s exact test).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Incidence of activated and nonactivated Ras in AOM tumors</th>
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<tr>
<td>Group</td>
<td>Total no.</td>
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<td>---------</td>
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</tr>
<tr>
<td>Control Colon</td>
<td>3</td>
</tr>
<tr>
<td>Control Colon + UDCA</td>
<td>3</td>
</tr>
<tr>
<td>AOM</td>
<td>30</td>
</tr>
<tr>
<td>AOM + UDCA</td>
<td>11</td>
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* Tumors from AOM-treated rats and control colonocytes from saline-treated rats were extracted in modified RIPA buffer. Ras was immunoprecipitated with anti-Ras or nonimmune antibodies. After washing, GTP and GDP bound to Ras were eluted and quantified by coupled reactions as described in “Materials and Methods.”

* Ras was defined as activated if the ratio [Ras - GTP/(Ras - GDP + Ras - GTP)] exceeded the mean + 3 SD of normal colonocytes.

* Normal colonos from the saline-treated unsupplemented and UDCA-supplemented groups and tumors from the AOM and AOM + UDCA groups.

* $P < 0.05$ compared with Ras activation in normal colonocytes.

* $P < 0.05$ compared with the number of tumors with activated Ras in the AOM alone group.
Table 3. Cox-2 expression by immunostaining in AOM tumors

<table>
<thead>
<tr>
<th>Group</th>
<th>Epithelium</th>
<th>Stroma</th>
<th>Epithelium</th>
<th>Stroma</th>
<th>Epithelium</th>
<th>Stroma</th>
</tr>
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<tbody>
<tr>
<td>AOM</td>
<td>1⁺</td>
<td>0⁻</td>
<td>2⁻–3⁻</td>
<td>3⁻</td>
<td>2⁻–3⁻</td>
<td>1⁺</td>
</tr>
<tr>
<td>AOM + UDCA</td>
<td>0⁻</td>
<td>0⁻</td>
<td>1⁺</td>
<td>1⁺</td>
<td></td>
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</tr>
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</table>

AOM tumors were fixed in 10% buffered formalin, and 5-μm sections were immunostained for Cox-2 protein as described in “Materials and Methods.” Cox-2 staining was graded using a semiquantitative scale. 0, negative; 1⁺, <10% reactive; 2⁻, 10–50% reactive; 3⁻, >50% reactive. n = 4–6 tumors analyzed in each group except for AOM + UDCA group, which contained only one tumor with mutant K-ras. Colonic mucosa from saline-treated unsupplemented (n = 3) and UDCA-supplemented rats (n = 3) were negative for Cox-2 staining.

not shown). In preliminary studies in colon cancer-derived Caco-2 cells, preincubation with UDCA blocked epidermal growth factor-induced Ras activation. Ras-GTP was assessed by a pull-down assay using a glutathione S-transferase fusion protein containing the Ras-binding domain of c-Raf (data not shown). These results suggest that UDCA might inhibit growth factor-induced activation of WT Ras in these tumors.

Cox-2 Expression in AOM Tumors. As shown in Fig. 2 and summarized in Table 3, we demonstrated by immunostaining that Cox-2 expression was increased in AOM tumors and further enhanced by Ras activation in agreement with our previous studies (8). Cox-2 was both nuclear and cytoplasmic in distribution and expressed in both stromal and epithelial cells. Although there was some heterogeneity in Cox-2 immunostaining, overall, the Cox-2 expression levels in tumors with mutant and activated WT Ras appeared comparable. Because there was only one tumor with mutant K-ras and no tumors with WT activated Ras in the AOM + UDCA group, we restricted our subsequent comparisons of Cox-2 expression in the AOM alone and AOM + UDCA groups to tumors with nonactivated WT Ras. As shown in Table 2, the Ras activation levels that were used for this study to compare Cox-2 expression levels were comparable in the AOM alone and AOM + UDCA tumors, with values of 4.1 ± 1.7 and 4.4 ± 1.8, respectively. Because the AOM alone and AOM + UDCA tumor subgroups possessed comparable Ras activity, any differences in Cox-2 expression between these groups could not be attributed to differences in Ras activation.

In tumors with normal Ras activity, UDCA supplementation inhibited Cox-2 expression (Fig. 2). Results of UDCA supplementation on Cox-2 expression in tumors as assessed by immunostaining are summarized in Table 3. We confirmed these effects of UDCA dietary supplementation on Cox-2 expression in carcinogen-induced tumors by quantitative Western blotting. As shown in Fig. 3A, Cox-2 protein expression was increased 50-fold in AOM-induced tumors, compared with normal colonocytes. UDCA supplementation significantly inhibited this Cox-2 up-regulation by >60% (P < 0.05). We next examined Cox-2 mRNA by semiquantitative RT-PCR to assess the role of alterations in Cox-2 mRNA in these changes in Cox-2 protein. As shown in Fig. 3B, Cox-2 message was significantly increased >20-fold in AOM tumors (n = 13), compared with normal colonic mucosa (n = 6, P < 0.05). Supplementation with UDCA, moreover, significantly inhibited Cox-2 mRNA up-regulation by 50% in AOM-induced tumors (n = 14, P < 0.05). In contrast to Cox-2, and in agreement with other studies (7), the expression of Cox-1 mRNA and protein was not altered in AOM tumors. Thus, taken together with our findings of UDCA inhibition of Ras activation (Table 2), UDCA...
inhibited Cox-2 expression by both Ras-dependent and -independent mechanisms.

**DISCUSSION**

We have shown previously that UDCA is a chemopreventive agent in the AOM model (20). In this study, we have extended our studies, demonstrating that UDCA inhibited the development of AOM tumors with activated WT or mutant Ras. Furthermore, UDCA inhibited Cox-2 induction by Ras-dependent and -independent pathways.

Potential mechanisms by which UDCA might block Ras mutations include inhibition of DNA methylguanine adduct formation or enhancement of adduct repair. In preliminary analysis of tumors from a recent study (36), K-ras mutations were present in 2 of 10 tumors in a group receiving UDCA only during AOM administration, whereas there were no mutations in 10 tumors from a group supplemented with UDCA beginning weeks after carcinogen treatment. These results suggest that UDCA does not block adduct formation and Ras mutations in the initiation phase but rather inhibits the expansion of clones with Ras mutations when supplemented during the tumor promotion/progression phase. Moreover, the observation that chemopreventive agents structurally unrelated to UDCA, such as piroxicam, limit the incidence K-ras mutations suggests a more generalized mechanism may suppress the development of tumors with mutant ras (30). Interestingly, several of these agents, including UDCA, are antiproliferative in this model (27, 30). UDCA might also inhibit Ras mutations by limiting the colonic concentration of deoxycholic acid (37), because this tumor-promoting secondary bile acid increases the incidence of AOM tumors with K-ras mutations (38).

WT Ras is a major effector of ErbB receptors. Abnormalities in ErbB signaling have been implicated in colon cancer (8, 39–42). We reported previously the association of increased levels of ErbB2 receptors and activated WT Ras in AOM tumors (8). More recently, we observed phospho-active ErbB2 with activated WT Ras in human colon cancers. In addition, RasGAP was decreased in some AOM tumors with activated Ras (8). Compared with AOM tumors with nonactivated WT Ras, Cox-2 is 3-fold higher in tumors with activated Ras (8). Signaling by MAPK, a positive regulator of Cox-2 expression (44), was also enhanced in tumors with increased Ras activity (8). In the present study, we confirmed that Cox-2 expression was increased in AOM tumors possessing Ras activity comparable with normal colonocytes. Furthermore, we demonstrated that UDCA inhibited this Cox-2 up-regulation in tumors lacking increases in Ras and MAPK activities. Thus, UDCA inhibits Cox-2 expression in AOM carcinogenesis by Ras-dependent and -independent mechanisms that probably contribute importantly to the chemopreventive actions of UDCA. It is of interest that UDCA can increase intracellular antioxidants (45), which in turn have been shown to inhibit Cox-2 induction in colon cancer cells (46). Additional studies to assess the role of REDOX changes in the anticancer effects of UDCA will be of interest. In addition to Ras-dependent and -independent inhibition of Cox-2 expression, other mechanisms have been suggested for chemopreventive actions of UDCA (27, 45, 47–52).

Regardless of the mechanism(s) of chemoprevention, suppression of Cox-2 expression by UDCA in colonic carcinogenesis suggests an alternative, and possibly complementary, chemopreventive strategy to that of Cox-2 enzymatic inhibition. This is especially attractive because Cox-2-specific enzyme inhibitors block only the cyclooxygenase, but not the peroxidase, activity of Cox-2. Theoretically, persistent peroxidase activity, despite a Cox-2 inhibitor, could metabolize a
susceptible procarcinogen to active carcinogen. Some cyclooxygenase inhibitors, moreover, have been shown to increase Cox-2 expression in colon cancer cells (53). Such an increase of noninhibited Cox-2 peroxidase activity in the premalignant state is of concern in the setting of long-term use. Intermittent compliance with Cox-2 inhibitors, e.g., could lead to hazardous induction of Cox-2 protein. Because UDCA inhibits Cox-2, but not Cox-1 protein expression, this chemo-preventive bile acid would reduce both the peroxidase and cyclooxygenase activities of Cox-2, while avoiding the potential gastrointestinal toxicity associated with Cox-1 inhibition by NSAIDs. Low-dose NSAIDs, in combination with UDCA or NSAID-UDCA conjugates, have also been used successfully to prevent cancers in experimental models of chemical or genetic carcinogenesis with much lower risks of toxicity (54, 55).

In summary, we have shown that UDCA inhibited Ras mutations and WT Ras activation, as well as Cox-2 induction in AOM tumors. The UDCA inhibition of Cox-2 occurred at the mRNA and protein levels in these tumors and involved Ras/MAPK-dependent and -independent mechanisms. The excellent safety profile of UDCA and potential theoretical benefits over Cox-2 (enzyme) inhibitors, together with recent reports suggesting the efficacy of UDCA to prevent colonic neoplasia in humans (56–58), indicate that additional studies of this promising agent are warranted to further elucidate Ras-dependent and -independent mechanisms of its chemopreventive actions.

ACKNOWLEDGMENTS

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REFERENCES

The text seems to be a list of references or citations, possibly related to a scientific or medical field. Each entry typically includes information such as authors, journal titles, publication years, and page numbers. The text appears to be in English, and it seems to be discussing various scientific topics, possibly related to cancer, chemoprevention, and the effects of various substances on the body. The references are formatted in a standard academic citation style, which is common in scientific publications.
Ursodeoxycholic Acid Inhibits Ras Mutations, Wild-type Ras Activation, and Cyclooxygenase-2 Expression in Colon Cancer

Sharad Khare, Sonia Cerda, Ramesh K. Wali, et al.