Dihydroxy Bile Acids Activate the Transcription of Cyclooxygenase-2*

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Bile acids, endogenous promoters of gastrointestinal cancer, activate protein kinase C (PKC) and the activator protein-1 (AP-1) transcription factor. Because other activators of PKC and AP-1 induce cyclooxygenase-2 (COX-2), we determined the effects of bile acids on the expression of COX-2 in human esophageal adenocarcinoma cells. Treatment with the dihydroxy bile acids chenodeoxycholate and deoxycholate resulted in an ~10-fold increase in the production of prostaglandin E2 (PGE2). Enhanced synthesis of PGE2 was associated with a marked increase in the levels of COX-2 mRNA and protein, with maximal effects at 8–12 and 12–24 h, respectively. In contrast, neither cholic acid nor conjugated bile acids affected the levels of COX-2 or the synthesis of PGE2. Nuclear run-off assays and transient transfections with a human COX-2 promoter construct showed that induction of COX-2 mRNA by chenodeoxycholate and deoxycholate was due to increased transcription. Bile acid-mediated induction of COX-2 was blocked by inhibitors of PKC activity, including calphostin C and staurosporine. Treatment with bile acid enhanced the phosphorylation of c-Jun and increased binding of AP-1 to DNA. These data are important because dihydroxy bile-acid-mediated induction of COX-2 may explain, at least in part, the tumor-promoting effects of bile acids.

Recent studies have established that there are two distinct isoforms of cyclooxygenase (COX), both of which catalyze the formation of prostaglandins from arachidonic acid. COX-1 is thought to be a housekeeping gene with essentially constant levels of expression (1). In contrast, the gene for COX-2 is an early-response gene that, like c-fos and c-jun, is induced rapidly following stimulation of quiescent cells. The expression of COX-2 is stimulated by growth factors, cytokines, oncoproteins, phorbol esters, and carcinogens and by overexpression of PKC (2–9). The different responses of the genes for COX-1 and COX-2 reflect, in part, differences in the regulatory elements in the 5′-flanking regions of the two genes (10).

A large body of data from a variety of experimental systems suggest that COX-2 is important for tumor formation. Oshima et al. (11) have reported, for example, that a null mutation of COX-2 is associated with a marked reduction in the number and size of intestinal polyps in Apc<sup>−/−</sup> (adenomatous polyposis coli) knockout mice, which are a model for human familial adenomatous polyposis. Treatment with a selective inhibitor of COX-2 also reduced the number of polyps in Apc<sup>−/−</sup> mice (11). COX-2 deficiency also appears to protect against the formation of tumors in other epithelia because COX-2 knockout mice develop fewer chemically induced skin papillomas than control mice (12). Although the exact causal link(s) between the activity of COX-2 and carcinogenesis remain uncertain, there are several different mechanisms that can account for this linkage (13). Prostaglandins, e.g., PGE2, affect cell proliferation and inhibit immune surveillance; thus, overproduction of prostaglandins could favor malignant growth (14). Overexpression of COX-2 inhibits apoptosis and increases the invasiveness of malignant cells (15, 16). Amounts of COX-2 are increased in transformed cells and tumors (7, 17–19), which results in enhanced synthesis of prostaglandins in malignant tissue (20, 21). Given these data, a reasonable strategy for inhibiting carcinogenesis is to suppress the expression of COX-2 (22, 23). It therefore becomes important to identify the factors that modulate the expression of COX-2.

In this regard, bile acids are known promoters of gastrointestinal cancer in vivo and enhance cell transformation in vitro (24–28). The precise mechanism(s) by which bile acids promote tumor formation is not known. But like tumor-promoting phorbol esters (29), bile acids activate PKC (30–32) and induce AP-1 activity (33). The latter effect may be especially important because inhibitors of AP-1-mediated transcription have anti-cancer properties (34). To further examine the mechanism by which bile acids promote tumor formation, we have examined their effects as inducers of COX-2. The rationale for this approach to defining the bile acid-cancer connection is that phorbol esters are prototypic inducers of COX-2 (2, 4, 5). Our data show that selected bile acids enhance the transcription of COX-2 and that inhibitors of PKC activity block this effect.

EXPERIMENTAL PROCEDURES

Materials—DMEM, Opti-MEM, FCS, and Lipofectin were from Life Technologies, Inc. Enzyme immunoassay reagents for PGE2 assays were from Cayman Chemical Co., Inc. (Ann Arbor, MI). Bile acids, sodium arachidonate, staurosporine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; thiazolyl blue) and phorbol 12-myristate 13-acetate (PMA) were from Sigma. Calphostin C was from BIOMOL Research Labs Inc. (Plymouth Meeting, PA). [32P]CTP and [32P]UTP were from NEN Life Science Products. The AP-1 consensus oligonucleotide was obtained from Promega (Madison, WI). Random-priming kits were from Boehringer Mannheim. Reagents for the luciferase assay were from Analytical Luminescence Laboratory (San Diego, CA). Nitrocellulose membranes were from Schleicher & Schuell. The 18 S rRNA cDNA was from Ambion Inc. (Austin, TX). Rabbit polyclonal anti-human COX-2 antisera was from Oxford Biomedical Research,
Inc. (Oxford, MI). Goat polyclonal anti-human COX-1 and monoclonal anti-c-Jun (specific for the phosphorylated form of c-Jun) antiserum were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Western blot detection reagents (ECL) were from Amersham Corp. Fplasmid DNA was prepared using a kit from QIAGEN Inc. (Chatsworth, CA).

**Culture**—The SK-GT-4 cell line was established from a well differentiated adenocarcinoma arising in Barrett's epithelium of the distal esophagus (35). Cells were maintained in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin in a 5% CO2/water-saturated incubator at 37 °C. Treatments with vehicle (0.1% ethanol) or bile acid were carried out in 1% FCS. Cellular cytotoxicity was assessed by measurements of cell number, release of lactate dehydrogenase, trypan blue exclusion, and MTT assay. Levels of lactate dehydrogenase release were measured in the supernatants used for PGE2 analyses. MTT assay was performed according to the method of Denizot and Lang (36). Lactate dehydrogenase assays were performed according to the manufacturer's instructions. For trypan blue analysis, following treatment with bile acid for 12 h, cells were combined 1:1 with 0.4% trypan blue and examined for dye exclusion.

**PGE2 Production**—Cells (1 x 10^6/well) were plated in 24-well dishes and grown to 60% confluence in DMEM containing 10% FCS. The medium was then replaced with DMEM containing 1% FCS and vehicle (0.1% ethanol), CD (400 µM), or DC (400 µM) and grown to 60% confluence in DMEM containing 10% FCS. The medium was then replaced with fresh DMEM containing 1% FCS and vehicle (0.1% ethanol), and cell number, release of lactate dehydrogenase, and trypan blue exclusion were measured. After 30 min, the medium was collected for analysis of PGE2. The levels of PGE2 released by the cells were measured by enzyme immunoassay (7). Rates of production of PGE2 were normalized to protein concentrations.

**Western Blotting**—Lysates for the detection of COX-2 and COX-1 were prepared by treating cells with lysis buffer (150 mM NaCl, 100 mM sodium arachidonate (GCD), taurocholenodeoxycholate (TCD), or FAS (400 µM), 0.4% trypan blue, and examined for dye exclusion.

**Bile Acid**

24 h using equal cm²/ml of labeled nascent RNA transcripts for each treatment group. The membranes were washed twice with 2 x SSC for 1 h at 55 °C and then treated with 10 mg/ml RNase A in 2 x SSC for 30 min at 37 °C, dried, and autoradiographed.

**Plasmids**—The COX-2 promoter construct (−327/−59) was a gift from Dr. Tadashi Tanabe (National Cardiovascular Center Research Institute, Osaka, Japan) (10). The human COX-2 cDNA was generously provided by Dr. Stephen M. Prescott (University of Utah, Salt Lake City, UT). pSV-βgal was obtained from Promega.

**Transient Transfection Assays**—SK-GT-4 cells were seeded at a density of 5 x 10^4/well in 6-well dishes and grown to 50–60% confluence in DMEM containing 10% FCS. For each well, 1.8 µg of COX-2-luciferase construct and 0.2 µg of pSV-βgal were cotransfected into SK-GT-4 cells at a 1:4 ratio of DNA to Lipofectin following the manufacturer's instructions. After transfection, the medium was replaced with DMEM containing 10% FCS for 24 h. The cells were then treated with DMEM containing 1% FCS and vehicle (0.1% ethanol), CD (400 µM), or DC (400 µM). The activities of luciferase and β-galactosidase were measured in cellular extract 8 h after transfection. To adjust for differences in transfection efficiencies, the luciferase values were normalized using β-galactosidase.

**Electrophoretic Mobility Shift Assay**—SK-GT-4 cells were treated with DMEM containing 1% FCS and vehicle (0.1% ethanol) or CD or DC for 6 h, respectively. Cells were harvested and nuclear extracts were prepared as described previously (40). For binding studies, an AP-1 consensus oligonucleotide was used: 5′-GGCTTGTGATCGTGCCGC-GAA-3′ (sense) and 3′-GGCAACTACTCAGTCGGCCTT-GAG-5′ (antisense). The complementary oligonucleotides were annealed in 20 mM Tris (pH 7.6), 50 mM NaCl, 10 mM MgCl2, and 1 mM dithiothreitol. The annealed oligonucleotide was phosphorylated at the 5′-end with [γ-32P]ATP and T4 polynucleotide kinase. The binding reaction was performed by incubating 2 µg of nuclear protein in 20 µl HEPES (pH 7.9), 10% glycerol, 300 µg of bovine serum albumin, and 1 µg of poly(dI:dC) in a final volume of 10 µl for 10 min at 25 °C. The labeled Ap-1 consensus oligonucleotide was added to the reaction mixture and allowed to incubate for an additional 20 min at 25 °C. The samples were electrophoresed on a 4% nondenaturing polyacrylamide gel. The gel was then dried and subjected to autoradiography at −80 °C.

**Statistics**—Comparisons between groups were made by Student's t test. A difference between groups of p < 0.05 was considered significant.

**RESULTS**

**Effect of Bile Acids on the Synthesis of PGE2**—Fig. 1 shows the effects of several bile acids and bile acid conjugates on the production of PGE2. CD and DC caused a 3- and 10-fold in-
creases in the spontaneous production of PGE$_2$, respectively. Cholic acid and all conjugated bile acids, however, had no effect on rates of spontaneous synthesis of PGE$_2$. To examine the synthesis of PGE$_2$ in more detail, we also measured the effect of bile acids on the production of PGE$_2$ when an excess of arachidonic acid was added to the system. This experiment was done because PGE$_2$ production can be affected by changes in the activity of phospholipase A$_2$, which provides the substrate for COX-catalyzed reactions. Adding excess arachidonic acid minimizes any contribution of phospholipase A$_2$ activity to the rate of production of PGE$_2$. As for rates of spontaneous synthesis of PGE$_2$, treatment with CD or DC induced an ~10-fold increase in the synthesis of PGE$_2$ in the presence of excess arachidonate (Fig. 1). Neither cholic acid nor the conjugated bile acids affected the production of PGE$_2$ in the presence of excess arachidonic acid.

Cytotoxicity in these experiments was assessed by cell number, lactate dehydrogenase release, MTT assay, and trypan blue exclusion. No evidence of cellular toxicity was detected (data not shown).

**Dihydroxy Bile Acids Induce COX-2**—Western blotting was carried out to determine whether the above differences in PGE$_2$ production were related to differences in amounts of COX-2. We found that treatment with CD caused a dose-dependent induction of COX-2 protein with a peak effect at 400 $\mu$M CD (Fig. 2). Moreover, CD and DC were the only bile acids that induced COX-2 protein (Fig. 3), which is consistent with their effects on the production of PGE$_2$ (Fig. 1). We also compared the time course for induction of COX-2 by CD versus PMA. Maximal induction of COX-2 protein occurred after 12–24 h of treatment with CD (Fig. 4). In contrast, COX-2 was induced maximally by PMA after 8–12 h of treatment (Fig. 4). COX-1 was not detectable in this cell line by Western blotting under basal or stimulated conditions (data not shown).

To further elucidate the mechanism responsible for the changes in amounts of COX-2 protein, we measured the steady-state levels of COX-2 mRNA by Northern blotting. CD and DC stimulated the formation of COX-2 mRNA; maximal induction of COX-2 mRNA was observed after treatment for ~9 h (Fig. 5). Bile acid-mediated induction of COX-2 mRNA was still apparent after 24 h of treatment, which is consistent with the effects detected by Western blotting.

**Transcription of COX-2 Is Enhanced by Dihydroxy Bile Acids**—Differences in the levels of mRNA could reflect altered rates of transcription or stability of mRNA. Nuclear run-off assays were performed to distinguish between these possibilities. As shown in Fig. 6, we detected higher rates of synthesis of nascent COX-2 mRNA after treatment with CD or DC, consistent with the differences observed by Northern blotting. To further investigate the importance of bile acids in modulating the expression of COX-2, transient transfections were performed with a human COX-2 promoter-luciferase construct. Treatment with CD or DC led to approximately a doubling of COX-2 promoter activity (Fig. 7).

**Mechanism of Bile Acid-mediated Induction of COX-2**—Bile acids have been reported to activate PKC. Therefore, to elucidate the signaling mechanism by which bile acids activate the transcription of COX-2, we measured the induction of COX-2 by CD in the presence of known inhibitors of PKC. Staurosporine and calphostin C were used as inhibitors. Both agents blocked the induction of COX-2 by bile acids (Fig. 8, A and B). Dexamethasone also blocked the induction of COX-2 by CD and PMA (Fig. 8C). Because activators of PKC modulate gene expression via AP-1, we determined the effects of CD on the phosphorylation state of c-Jun and AP-1 DNA-binding activity (Fig. 9). Treatment with CD increased the phosphorylation of c-Jun.
DISCUSSION

Bile acids are known tumor promoters that activate PKC, which may be the mechanism of bile acid-induced carcinogenesis (31–33). Dihydroxy bile acids are more effective in activating PKC than mono- and trihydroxy bile acids (32). The exact mechanism by which bile acids change the activity of PKC is uncertain. However, bile acids increase the activity of PKC, in part, by inducing the translocation of selected isoforms from cytosol to membrane fractions (31). It is also known, in the context of the tumor-promoting effects of bile acids, that dihydroxy bile acids stimulate the release of PGE2 by colonic epithelium (41); but, as for the underlying specific carcinogenic events modulated by PKC, the basis for the up-regulation of PGE2 synthesis by bile acids remains uncertain. Our prior work (7, 22, 23) and data from other laboratories (11–13, 15–19) provide evidence that COX-2, which is a PKC-dependent gene (9), is important for the genesis of cancer. Hence, there is evidence to link the activity of PKC to carcinogenesis, in part, via the induction of COX-2 and overproduction of prostaglandins. This work adds to this evidence by showing that bile acids up-regulate the expression of COX-2. Moreover, we found that only CD and DC induced COX-2, which is consistent with the greater effect of these two bile acids on activation of PKC versus c-Jun (Fig. 9A). Electrophoretic mobility shift assay revealed increased binding of AP-1 to DNA following treatment with CD (Fig. 9B).
cholic acid or conjugated bile acids (32). The present data therefore suggest a plausible mechanism to explain the promotion of esophageal (42–44) and colon (24–27) cancer by bile acids: induction of COX-2.

Bile acid-mediated induction of COX-2 can be important for tumorigenesis in the gastrointestinal tract because the products of COX-2 activity, e.g. prostaglandins, inhibit apoptosis, diminish immune surveillance, and increase the invasiveness of malignant cells (14–16). These conclusions are consistent with evidence that inhibitors of COX-2 are chemopreventive against cancers of the colon and upper gastrointestinal tract in experimental animals (45–48) and in humans (49, 50).

With regard to the mechanism by which bile acids induce COX-2, it is known that the human COX-2 promoter contains a cyclic AMP response element and sites for AP-2, NF-IL6 and NF-κB (10). Xie and Herschman (51) showed that the AP-1 transcription factor complex is important for activation of the murine COX-2 promoter via a cyclic AMP response element. This is consistent with other reports that AP-1 transcription factors can modulate transcription via a cyclic AMP response element (52). The current data show that bile acids increased the phosphorylation of c-Jun, an event known to be downstream from activation of the PKC signaling pathway (53). This is important because enhanced phosphorylation of c-Jun could potentiate its ability to activate transcription of COX-2. Suppression of bile acid-mediated induction of COX-2 by dexamethasone is consistent with prior reports that AP-1 activity can be regulated negatively by glucocorticoids (29).

AP-1 sites are only one of several cis-elements that mediate the effects of PKC. Other PKC-responsive cis-elements include the AP-2 recognition site (54) and the NF-κB-binding site (55). Irrespective of the exact mechanism, however, the results reported above suggest that inhibitors of PKC could be useful for down-regulating COX-2 and thereby preventing and/or treating cancer.

Although relatively high concentrations of bile acids were required to induce COX-2, similar concentrations of bile acids are found in bile (56) and the aqueous phase of the fecal stream (57). It will therefore be worthwhile to investigate whether bile acids also induce COX-2 in vivo. Further studies are also needed to confirm that induction of COX-2 by bile acids is mediated via AP-1 and to identify the responsible COX-2 promoter element(s). Given the already demonstrated effects of bile acids on the activity of PKC, it also becomes important to identify the specific isoforms of PKC that mediate the effects of bile acids on COX-2. A more detailed understanding of the precise mechanism(s) by which bile acids induce COX-2 should facilitate the development of chemopreventive strategies to diminish the risk of carcinogenesis within regions of the gastrointestinal tract exposed to bile acids.

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