Ca\(^{2+}\) - and PKC-dependent stimulation of PGE\(_2\) synthesis by deoxycholic acid in human colonic fibroblasts

YINGTING ZHU,1 PING HUA,2 SHAZIA RAFIQ,1 ERIC J. WAFFNER,1 MICHAEL E. DUFFEY,1,2 AND PETER LANCE1,2

1Department of Medicine, Veterans Affairs Medical Center and 2Department of Physiology and Biophysics, University at Buffalo, Buffalo, New York 14215

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Zhu, Yingting, Ping Hua, Shazia Rafiq, Eric J. Waffner, Michael E. Duffey, and Peter Lance. Ca\(^{2+}\) - and PKC-dependent stimulation of PGE\(_2\) synthesis by deoxycholic acid in human colonic fibroblasts. Am J Physiol Gastrointest Liver Physiol 283: G503–G510, 2002. First published May 1, 2002; 10.1152/ajpgi.00525.2001.—We investigated prostanoid biogenesis by human colonic fibroblasts (CCD-18Co cells and nine primary fibroblast cultures) exposed to a primary (cholic, CA) or a secondary (deoxycholic, DCA) bile acid. Basal PGE\(_2\) levels in CCD-18Co cultures and fibroblast strains initiated from normal and adenocarcinomatous colon, respectively, were 1.7 ± 0.3, 4.0 ± 2.0, and 15.0 ± 4.8 ng/mg protein. Peak levels 24 h after exposure to DCA (300 \(\mu\)M) rose, respectively, seven-, six- and sevenfold, but CA elicited no such responses. Increases in PGE\(_2\) synthesis were preceded by sequential increases in PGH synthase-2 mRNA and protein expression and were fully prevented by a nonselective (indomethacin) or a selective (cecloxib) nonsteroidal anti-inflammatory drug. DCA, but not CA, caused abrupt, transient increases in fibroblast intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) ~1 min after exposure. Increased [Ca\(^{2+}\)]\(_i\) was required for DCA-mediated induction of PGE\(_2\) synthesis, and protein kinase C was a further essential component of this signaling pathway. Colonic fibroblasts may be a major target for prostanoid biogenesis induced by fecal bile acids and, potentially, other noxious agents of these agents. colorectal neoplasms; feces; mesenchymal cells; prostaglandin H synthases

PGE\(_2\) AND OTHER prostanooids are generated through activity of the bifunctional prostaglandin H synthase (PGHS)-1 and -2 enzymes (18), colloquially often referred to as cyclooxygenase (Cox)-1 and -2. PGHS-1 is constitutively expressed in a wide range of tissues, and there is little modulation in the levels of expression. PGHS-2, in contrast, is highly inducible by cytokines and other participants in inflammatory and neoplastic processes (22).

PGHS-2 mRNA and protein expression are increased in colorectal adenocarcinomas compared with levels in adjacent normal tissue (23), and a causal role for PGHS-2 in murine colorectal carcinogenesis was demonstrated experimentally in vivo (32); the biological effects of PGHS-2 upregulation were mediated predominantly through increased PGE\(_2\) production. Interestingly, the site of Cox-2 upregulation in the earliest adenomas in this model was subepithelial, that is, in mesenchymal cells. Subsequent studies of the Min mouse (40) and carcinogen-induced tumors in interleukin (IL)-10-deficient mice (41) have provided further evidence of PGHS-2 expression by stromal cells in experimental animal models. Less is known of the source of PGHS-2 expression in nonneoplastic human colorectal tissue.

Powell and colleagues (3, 34, 35) described a population of specialized subepithelial “myofibroblasts” with pleiotropic capabilities, including the ability to modulate intestinal secretory responses to inflammatory mediators by releasing PGE\(_2\). We reported that IL-1\(\beta\) or tumor necrosis factor-\(\alpha\) could induce synthesis of PGE\(_2\) by as much as 25-fold in a human neonatal colonic fibroblast strain (CCD-18Co) and four human adult primary colorectal fibroblast strains that we initiated from colonoscopic pinch biopsies of normal mucosa (24).

The primary bile acids, cholic (CA) and chenodeoxycholic acid, are synthesized in hepatocytes from cholesterol and conjugated with glycine or taurine before their secretion in the bile (20, 21). After bacterial deconjugation in the distal small intestine, most bile acids are absorbed and returned to the liver in the enterohepatic circulation. Unabsorbed, now unconju-
gated bile acids are dehydroxylated by colonic bacteria to become secondary bile acids, such as deoxycholic acid (DCA), which is the predominant fecal bile acid. Secondary bile acids cause diarrhea by stimulating colonic secretion of electrolytes and water, effects that we showed to be mediated by release of intracellular Ca\(^{2+}\) (9).

The association between a high-fat diet and increased risk for colorectal cancer (37) is thought in part to be caused by the high levels of fecal bile acids, particularly DCA, that are generated by such diets (1, 43). Dihydroxy bile acids, such as DCA, have been shown to activate the transcription of PGHS-2 in an esophageal cancer cell line (47).

We hypothesized that bile acids might stimulate PGE\(_2\) synthesis in fibroblasts from nonneoplastic colon through upregulation of PGHS-2 expression. We report here that DCA, but not CA, can dramatically stimulate PGE\(_2\) synthesis in human colonic fibroblasts through induction of PGHS-2. This selective effect is mediated by a Ca\(^{2+}\)-dependent protein kinase C (PKC) transduction pathway.

MATERIALS AND METHODS

Fibroblast cultures. CCD-18Co, a colonic fibroblast strain derived from normal human fetal colon, was purchased from the American Type Culture Collection (Manassas, VA).

Primary fibroblast cultures were initiated from pinch biopsies obtained, with approval of the Institutional Review Board of the Buffalo Veterans Affairs Medical Center, at colonoscopies performed in the course of routine clinical care. One strain was initiated from biopsies of normal mucosa in each of four patients with a normal colon. One strain was also initiated from sampling of a colonic adenocarcinoma in each of five different patients.

Fresh biopsies were placed immediately in DMEM supplemented with penicillin (500 U/ml) and streptomycin (500 \(\mu\)g/ml). The tissue was minced with small sharp scissors and incubated in collagenase (0.75 mg/ml) at 37°C for 90 min. The resulting small pieces of tissue were washed four times in DMEM, placed in 24-well plates, and cultured at 37°C in a humidified 5% CO\(_2\) incubator in MCDB 153 medium (Sigma, St. Louis, MO) supplemented with 20% FBS, penicillin (200 U/ml), streptomycin (200 \(\mu\)g/ml), epidermal growth factor (10 ng/ml), and insulin-transferrin-selenium-X. Cultures were maintained under these conditions, with two times weekly changes of medium until cells with fibroblast-like morphology were clearly visible under an inverted microscope, indicating probable establishment of a fibroblast strain. Immunohistochemical stains were performed on representative cells from each putative strain to confirm its consistency with a fibroblast phenotype: positive vimentin stain and negative factor VIII, anticytokerin, \(\alpha\)-antitrypsin, and S-100 stains.

CCD-18Co or primary fibroblast strains were cultured in DMEM, supplemented with 10% FBS, nonessential amino acids (0.1 mM), and sodium pyruvate (1 mM). Tissue culture reagents were purchased from Gibco-BRL (Grand Island, NY). Studies were performed on fibroblast strains from the third to the sixth passage in culture.

\(\text{PGE}_2\) assay. Fibroblasts were seeded in 24-well plastic culture plates at a density of \(5 \times 10^5\) cells/cm\(^2\) and were cultured for 1 wk in DMEM supplemented with 10% FBS. It is well recognized that serum is a potent inducer of PGHS-2 and, thus, PGE\(_2\) (25). Therefore, 24 h before administering cytokine or other treatments to fibroblast cultures, medium was replaced with DMEM supplemented with 1% FBS, penicillin (200 U/ml), streptomycin (200 \(\mu\)g/ml), and nonessential amino acids (0.1 mM). To determine the effects of bile acids, selected cultures were incubated in medium supplemented with CA or DCA. Bile acids were purchased from Sigma.

For the final 30 min before harvesting of cultures, medium was replaced with PBS supplemented with 1% gelatin. Test compounds were present during this final incubation period. PGE\(_2\) levels in harvested PBS-gelatin were determined using radioimmunoassay kits (Amersham, Arlington Heights, IL) according to the manufacturer’s instructions. Protein content in the harvest was determined by the method of Bradford (5). All determinations of PGE\(_2\) levels were derived from experiments in four separate fibroblast cultures.

A nonselective PGHS inhibitor (indomethacin, 10 \(\mu\)M) or a highly selective PGHS-2 inhibitor (celecoxib, 5 \(\mu\)M) was combined with DCA in selected fibroblast cultures. Indomethacin was purchased from Sigma, and celecoxib was provided by Pfizer (St. Louis, MO).

Cell viability. Cell viability was determined by trypan blue exclusion. Cells were seeded at the same time from a single parent culture. Cultures were incubated without (control) or with DCA (300 \(\mu\)M). Timing of the addition of DCA was staggered. Treated (12, 24, 36, 48, 72 h) and control cultures were harvested at the same time, after careful washing to remove cells that had detached during incubation. Harvested cells were incubated with trypan blue and counted using a hemacytometer. From each culture, four fields of duplicate preparations were counted for the percentage of cells that excluded the dye.

Isolation of fibroblast RNA and Northern analysis. Isolation of total cellular RNA, gel electrophoresis, Northern blotting, hybridization with cDNA probes, and quantitation of mRNA levels were performed as described (39). Fibroblasts were cultivated to confluence in 100-mm plastic culture plates, treated with test compounds, and harvested for isolation of total RNA. Near-full-length cDNA for human PGHS-2 (19) was used for hybridizations. The densities of the DNA-RNA hybrids were determined by scanning, and results were normalized by hybridization with \(\beta\)-actin cDNA (Oncor, Gaithersburg, MD).

Western analysis of PGHS-2 protein. PGHS-2 protein levels were determined as described (24) with modifications. Confluent cultured fibroblasts were shifted from medium supplemented with 10% FBS to medium containing 1% FBS for 24 h before and during treatment with test compounds. Lysates were electrophoresed on polyacrylamide gels in the presence of SDS (SDS-PAGE) and electroblotted to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was incubated for 4 h in PBS containing 10% nonfat milk and 0.1% Tween at pH 7.4. This was followed by a 2-h incubation at room temperature with goat anti-human PGHS-1 or rabbit anti-human PGHS-2 antibody (Cayman, Ann Arbor, MI) diluted 1:1,000 in PBS containing 10% nonfat milk and 0.1% Tween at pH 7.4. The membrane was washed three times in PBS containing 0.1% Tween at pH 7.4 and then was incubated for 1 h with horseradish peroxidase-conjugated rabbit anti-goat or goat anti-rabbit IgG (Sigma), respectively, for detection of PGHS-1 or -2 protein. The membrane was washed four times in PBS containing 0.1% Tween at pH 7.4. Bound antibodies were detected by chemiluminescence using reagents purchased from KPL (Gaithersburg, MD). The resulting bands were analyzed densitometrically.

Intracellular Ca\(^{2+}\) measurements. Cells on glass coverslips were loaded at room temperature with fura 2-AM (4 \(\mu\)M) for...
RESULTS

**DCA, but not CA, increases PGE2 synthesis.** As the predominant fecal bile acid, DCA is well suited for a role in colonic pathophysiology and has been implicated causally in experimental (38) and clinical studies (1) of colorectal neoplasia. There is no evidence that CA, minimally present in feces, contributes to colonic carcinogenesis. We (26) and others (27) have reported maximal effects of DCA in colonic epithelial cells in vitro at concentrations of 300–500 μM. Cell viability was unimpaired by DCA at concentrations in this range, and all effects were fully reversible (26). Depending on the method of measurement, bile acid concentrations of ~200–500 μM have been reported in human fecal water (36, 17). Therefore, DCA and CA were used at a concentration of 300 μM in the present study.

We measured PGE2 synthesis in untreated CCD-18Co cells and after exposure to DCA (Fig. 1) or CA, both at concentrations of 300 μM, for up to 72 h. The mean basal PGE2 level from four separate experiments was 1.7 ± 0.3 ng/mg protein. An increase in PGE2 level was first evident 6 h after exposure to DCA and rose sevenfold to a maximal level of 11.2 ± 2.5 ng/mg at 24 h. Exposure to CA elicited no increase in PGE2 synthesis (data not shown).

Results of treating primary fibroblast strains initiated from human colonoscopic biopsies are shown in Fig. 2. One strain was established from each of nine patients. Four of the strains were derived from normal colonic mucosa from patients with a normal total colonoscopy. Five of the strains were derived from biopsies of colonic adenocarcinomas. Mean basal levels in fibroblasts from normal and adenocarcinomatous tissue, respectively, were 4.0 ± 2.0 and 15.0 ± 4.8 ng/mg protein. Respective peak levels, occurring at 24 h in both normal and cancer-associated strains,
were 25.1 ± 10.5 and 105.0 ± 8.1 ng/mg protein. CA elicited no response in either strain.

Cell viability. Viability of CCD-18Co fibroblasts was determined by trypan blue exclusion after treatment with DCA (300 μM; Table 1). Cell viability was ≥94% after all treatment periods up to 72 h.

DCA induces PGHS-2 mRNA and protein expression. Northern blots were performed to analyze basal expression and upregulation of PGHS-1 and -2 in response to DCA. Expression of PGHS-2 mRNA, barely detectable in cellular RNA from untreated CCD-18Co fibroblasts (Fig. 3A), had obviously increased at 3 h and was maximal by 18 h. PGHS-1 mRNA levels were unaffected by DCA treatment (data not shown).

Western analysis was performed to determine whether exposure of colonic fibroblasts to DCA led to increased expression of PGHS-2 protein. PGHS-2 protein was almost undetectable in untreated CCD-18Co cells (Fig. 3B). Increased expression, first evident at 12 h, was maximal at 24 h. PGHS-1 protein levels were unaffected by DCA treatment.

Increases in PGHS-2 mRNA and protein levels after exposure of CCD-18Co fibroblasts to DCA are depicted quantitatively in Fig. 3C. Maximal increases of mRNA and protein expression, respectively, were 18- and 24-fold greater than control.

Nonsteroidal anti-inflammatory drugs attenuate stimulated and basal PGE2 synthesis. Indomethacin is a nonselective nonsteroidal anti-inflammatory drug (NSAID; see Ref. 7), inhibiting PGHS-1 and -2, and

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<th>DCA Exposure, h</th>
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Results are given as average % of viable cells ± SE. Cells were seeded at the same time from the same parent culture and incubated without (control) or with deoxycholic acid (DCA, 300 μM). Timing of the addition of DCA was staggered, and all cultures were harvested 72 h after seeding. Harvested cells were incubated with trypan blue and counted using a hemacytometer. Four fields each of duplicate preparations from each culture were counted for the number of cells that excluded dye.
celecoxib is a highly selective PGHS-2 inhibitor (12). Exposure of CCD-18Co fibroblasts to either NSAID completely prevented DCA-stimulated PGE2 increases and caused declines to below pretreatment levels (Fig. 4). As with CCD-18Co cells, indomethacin and celecoxib not only blocked the effects of DCA but also led to reductions of PGE2 to below basal levels in cultures of primary colonic fibroblast strains (data not shown).

Intracellular Ca2+ release mediates DCA effects. Bile acids are known to raise [Ca2+]i in a variety of cell types (16, 26, 29). Because agonist-stimulated increases in [Ca2+]i can alter gene expression (14, 42, 44), we determined the effects of DCA and CA on [Ca2+]i in CCD-18Co fibroblasts.

Figure 5 shows the 340- and 380-nm fluorescence ratio measured in an isolated fibroblast as a direct indicator of [Ca2+]i (9). The addition of CA (300 μM) to the solution bathing the fibroblast had no effect on [Ca2+]i. However, the addition of DCA (300 μM) caused an abrupt rise in the fluorescence ratio from the baseline value, which lasted ~3 min. This response to DCA was seen in 21 of the 22 cells tested on five coverslips, and only 1 cell of the 21 that responded to DCA responded to CA (1 of the 22 cells did not respond to DCA). The addition of DCA caused the fluorescence ratio to increase to 0.42 ± 0.03 from an average resting value of 0.20 ± 0.03. These ratio values correspond to a baseline value of [Ca2+]i of 62 nM and peak response to DCA of 200 nM. Responses were transient and lasted from 2 to 4 min. When cells were exposed to DCA before CA, the results were the same (14 cells on 3 coverslips). We demonstrated next that rapid increases of [Ca2+]i are required for induction of PGE2 synthesis by DCA.

Fibroblasts were incubated in Ca2+-deprived medium supplemented with a chelator of intracellular Ca2+, BAPTA-AM (5 μM), before exposure to DCA (Fig. 6). PGE2 increased approximately twofold from 1.8 ± 0.3 to 3.8 ± 0.3 ng/mg protein, compared with a sevenfold increase and maximal level of 11.2 ± 2.5 (Fig. 1) after exposure to DCA in cells not treated with BAPTA-AM.

PKC mediates Ca2+-dependent DCA-stimulated PGE2 synthesis. Isoforms of the PKC superfamily participate in a wide array of cellular responses (30). Activation of conventional PKCs (cPKCs) is Ca2+-dependent, and cPKCs are also targets of the tumor-promoting phorbol ester TPA (28). Incubation of CCD-18Co fibroblasts with TPA (20 ng/ml) caused a 26-fold increase in PGE2 synthesis that was maximal at 12 h (Fig. 7).

Having obtained indirect evidence of PKC involvement in the pathway for stimulation of PGE2 synthe-
sis, we next examined PKC activity directly after exposure of CCD-18Co fibroblast cultures to DCA (300 μM; Fig. 8). PKC activity rose twofold from a baseline of 10.0 pmol/min/mg protein to a maximum 3 min after exposure to DCA (300 μM) of 20.5.

Staurosporine is a nonspecific protein kinase inhibitor, and BIM is a specific PKC inhibitor. Addition of staurosporine (20 μM) or BIM (10 μM) at the same time as DCA (300 μM) to CCD-18Co cultures incubated with the agents for up to 72 h completely blocked DCA-stimulated synthesis of PGE2 (Fig. 9) and up-regulation of PGHS-2 protein expression (Fig. 10).

**DISCUSSION**

Empirical and epidemiological evidence of colorectal antineoplastic activity by aspirin and other NSAIDs sparked interest in expression of the PGHS enzymes and synthesis of their products in colorectal tissue. Bile acids, particularly DCA, were first suspected of contributing to the development of colorectal cancer long before upregulation of PGHS-2 was linked causally to neoplastic progression in colorectal tissue. These lines of investigation have converged in recent reports that DCA, in similar concentrations to those that we used, can induce PGHS-2 expression in transformed epithelial cell lines of colonic and other origin (46, 47). However, the relevance of observations in colon cancer cell lines to events in normal or preneoplastic colorectal epithelium is questionable.

Our finding that DCA, but not CA, caused a dramatic induction of PGE2 synthesis in a fibroblast strain, CCD-18Co, initiated from normal fetal human colon endorses other strands of evidence that the major site of inducible PGE2 synthesis in nonmalignant colorectal tissue is in stromal cells of the subepithelial compartment rather than in the epithelium itself (32, 40, 41). Having demonstrated upregulated PGE2 synthesis, we confirmed the expected preceding inductions of

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**Fig. 7.** Effects of a phorbol ester on fibroblast PGE2 synthesis. CCD-18Co cultures were incubated in the presence of 12-O-tetradecanoylphorbol-13-acetate (TPA; 20 ng/ml) for the periods indicated. PGE2 levels in harvested medium were determined by RIA. Columns represent the mean results from 4 experiments ± SE (bars).

**Fig. 8.** Effect of DCA on PKC activity. CCD-18Co cells were cultured in 60-mm dishes for 3 days and exposed to DCA (300 μM) for periods of 1–10 min. Cell homogenates were pelleted, and particulate fractions were resolubilized. PKC activity of these samples was determined from transfer of [γ-32P]ATP to a PKC-specific peptide and were expressed as pmol·min⁻¹·mg protein⁻¹. C, control.

**Fig. 9.** Effects of protein kinase inhibitors on DCA-stimulated PGE2 synthesis. CCD-18Co cells were coincubated with DCA (300 μM) and staurosporine (20 μM) or bisindoylmalemide (BIM; 10 μM) for the time periods indicated. PGE2 levels in harvested medium were determined by RIA. Columns represent the mean results from 4 experiments ± SE (bars).

**Fig. 10.** Effects of protein kinase inhibitors on DCA-stimulated PGHS-2 protein expression. CCD-18Co cells were coincubated with DCA (300 μM) and staurosporine (Sta; 20 μM) or BIM (10 μM) for 24 h. Cell lysates were electrophoresed and transferred to membranes, which were then incubated with monoclonal antibody to PGHS-2. Bound antibodies were detected by chemiluminescence.

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PGHS-2 mRNA and protein expression in response to DCA treatment.

Time course and maximal levels of DCA-stimulated PGE2 synthesis in primary fibroblasts of strains initiated from normal adult colons bore a striking similarity to CCD-18Co cells. In contrast, peak PGE2 production in fibroblast strains initiated from invasive adenocarcinomas was fivefold greater than in the fibroblast strains from normal colons. Interactions between cancer cells and their micro- and macroenvironment are under intense scrutiny (4), and the extracellular matrix and stromal fibroblasts are key contributors to the cancer cell environment. Carcinoma-associated fibroblasts were reported to direct tumor progression of initiated prostate epithelial cells in an in vitro coculture system (31). We propose that persistent stimulation of colorectal fibroblasts by colonic luminal components, such as DCA, to synthesize prostanooids and other compounds may be an important mechanism of colorectal tumorigenesis.

We and others have reported previously that exposure to bile acids can increase [Ca\(^{2+}\)]\(_i\) and activate PKC in gastrointestinal epithelial cells (9, 10, 26, 47). We have now demonstrated that bile acids can stimulate PGE2 synthesis by this pathway in gastrointestinal fibroblasts. Other agents provoke PGE2 synthesis by similar transduction mechanisms in nongastrointestinal cell lines. For example, IL-1\(\beta\) stimulated hyaluronan synthesis in cultured orbital fibroblasts by a Ca\(^{2+}\)-dependent PKC pathway (45). In rabbit corneal epithelium, platelet-activating factor (PAF) induced first Cox-2 mRNA levels, maximal after 4 h, and then protein expression, maximal at 16 h (2). In striking similarity to the pattern of our results with DCA (Fig. 5), there was a transient increase in [Ca\(^{2+}\)]\(_i\) that peaked between 30 and 60 s after exposure to PAF.

In a variety of cell types, bile acids have been shown to activate transcription factors (13), nuclear receptors (33), and genes other than Cox (10). We believe that a broader examination of DCA-mediated effects in colorectal fibroblasts will provide useful insights into the mechanisms of intestinal inflammation and neoplasia, with therapeutic potential.

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