Biphasic regulation by bile acids of dermal fibroblast proliferation through regulation of cAMP production and COX-2 expression level

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Meng, Jian Ping, Susan Ceryak, Zaheer Aratsu, Loren Jones, Lauren Epstein, and Bernard Bouscarel. Biphasic regulation by bile acids of dermal fibroblast proliferation through regulation of cAMP production and COX-2 expression level. Am J Physiol Cell Physiol 291: C546–C554, 2006. First published May 10, 2006; doi:10.1152/ajpcell.00011.2006.—We have previously reported that the bile acids chenodeoxycholate (CDCA) and ursodeoxycholate (UDCA) decreased PGE1-induced cAMP production in a time- and dose-dependent manner not only in hepatocytes but also in nonhepatic cells, including dermal fibroblasts. In the present study, we investigated the physiological relevance of this cAMP modulatory action of bile acids. PGE1-induced cAMP production in a time- and dose-dependent manner. Moreover, PGE1 (1 μM), forskolin (1–10 μM), and the membrane-permeable cAMP analog CPT-cAMP (0.1–10 μM) decreased dermal fibroblast proliferation in a dose-dependent manner with a maximum inhibition of ~80%. CDCA alone had no significant effect on cell proliferation at a concentration up to 25 μM. However, CDCA significantly reduced PGE1-induced cAMP production by 80–90% with an EC50 of ~20 μM. Furthermore, at concentrations ≤25 μM, CDCA significantly attenuated the PGE1-induced decreased cell proliferation. However, at concentrations of 50 μM and above, while still able to almost completely inhibit PGE1-induced cAMP production, CDCA, at least in part through an increased cyclooxygenase-2 (COX-2) expression level and PGE2 synthesis, produced a direct and significant decrease in cell proliferation. Indeed, the CDCA effect was partially blocked by ~50–70% by both indomethacin and dexamethasone. In addition, overexpression of COX-2 cDNA wild type resulted in an increased efficacy of CDCA to block cell proliferation. The effects of CDCA on both cAMP production and cell proliferation were similar to those of UDCA and under the same conditions cholate had no effect. Results of the present study underline pathophysiological consequences of cholestatic hepatobiliary disorders, in which cells outside of the enterohepatic circulation can be exposed to elevated bile acid concentrations. Under these conditions, low bile acid concentrations can attenuate the negative hormonal control on cell proliferation, resulting in the stimulation of cell growth, while at high concentrations these bile acids provide for a profound and prolonged inhibition of cell proliferation.

Bile acids are known to be co-carcinogenic agents. They are synthesized in the liver and secreted into bile conjugated mainly to either glycine (G) or taurine (T). Under physiological conditions, while chiefly confined to the enterohepatic circulation, bile acid concentration in the systemic circulation can increase postprandially two- to threefold from a fasting level of ~1–3 μM (2, 44). In the serum, bile acids are mainly in the amidated form. The level of unconjugated bile acids exhibits a diurnal variation, attaining a maximum concentration of 30–40% of the total serum bile acids after breakfast (44). In several pathological conditions, tissues outside of the enterohepatic circulation can come in contact with high concentrations of both unconjugated and conjugated bile acids. For example, in cholestatic hepatobiliary disorders, bile acids accumulate in the systemic circulation, resulting in a 20- to 100-fold increase in serum bile acid concentration (29). Under these conditions, serum levels of unconjugated bile acids can increase dramatically, particularly if portal cirrhosis is present (29). Finally, in patients with stagnant loop syndrome, serum unconjugated bile acid levels increase due to bacterial overgrowth in the small intestine (27).

Sodium-dependent bile acid transporters have been reported to be functionally present mainly in the liver, ileum, and kidney

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(1, 46). However, bile acids can enter cells by a sodium-independent transporter, as well as by passive diffusion, which is a function of their respective hydrophobicity (11, 53). Thus these observations underline the relevance that increases of either or both conjugated and unconjugated bile acids in the systemic circulation could have in their accumulation in extrahepatic tissues (12, 19, 34). This is in light of considerable evidence both in human and in animal models that cholestasis is associated with increased deposition of bile acids in extrahepatic tissues, with the skin being one of the predominant ones (5, 12, 16, 43).

Both the physiological and the pathophysiological effects of bile acids have been well documented for the liver and intestine; while they are less understood for tissues outside of the enterohepatic circulation, despite their exposure to bile acids, which is particularly significant in hepatobiliary diseases. Therefore, the present study was designed to investigate the chronic effect of bile acids on stimulated cAMP synthesis in human dermal fibroblasts. Moreover, the functional consequence of bile acid-induced inhibition of stimulated cAMP on human dermal fibroblasts. Inhibition is particularly significant in hepatobiliary diseases.

METHODS

Materials. Ursodeoxycholic acid (UDCA) was supplied by Tanabe (Tokyo, Japan), and CDCA was supplied by Dr. Falk (Pharma, Freiburg, Germany). Deoxycholic acid (DCA) and cholic acid (CA) were purchased from Steraloids (Wilton, NH). All bile acids used were 98–99% pure, as judged by gas-liquid chromatography. Prostaglandin (PG) E1, forskolin (FK), and 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) were 98–99% pure, as judged by gas-liquid chromatography. Prostaglandin E1, forskolin (FK), and 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) were purchased from Sigma (St. Louis, MO). [125I]cAMP was purchased from Linco (St. Louis, MO). 3-isobutyl-1-methylxanthine (IBMX) was purchased from Research Products (St. Charles, MO), and [methyl-3H]thymidine of 50 Ci/mmol was purchased from NEN Life Science Products (Boston, MA). RPMI and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Life Technologies (Grand Island, NY). Deoxycholic acid (DCA) and cholic acid (CA) were purchased from Steraloids (Wilton, NH). All bile acids used were 98–99% pure, as judged by gas-liquid chromatography. Prostaglandin (PG) E1, forskolin (FK), and 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) were purchased from Sigma (St. Louis, MO). [125I]cAMP was purchased from Linco Research Products (St. Charles, MO), and [methyl-3H]thymidine (specific activity, 80 Ci/mmol) was purchased from Amersham (Piscataway, NJ). Dulbecco’s modified Eagle’s minimum essential medium (DMEM; CellGro) was purchased from Fisher Scientific (Pittsburgh, PA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Other chemicals were from either Sigma or Fisher Scientific and were of the highest purity available.

Culture of human skin fibroblasts. Human skin fibroblasts obtained from forearm skin biopsy were purchased from Coriell Institute for Medical Research (Camden, NJ). The cells were cultured, as previously described (8), in DMEM with 1% l-glutamine, 2% essential and nonessential amino acids, 1% penicillin and streptomycin, and 10% FBS. The cells were plated at a density of 3–5 × 10^4 cells·mL⁻¹ in 12-well plates. Four to eight hours before an experiment, the cells were cultured in DMEM, containing either 0.1% FBS for cAMP determination or 1% FBS to measure cell proliferation.

cAMP determination. Cells were either incubated alone or with the sodium salt of the respective bile acid for the designated period of time. cAMP synthesis was stimulated either simultaneously, or following bile acid preincubation, with PGE1 in the presence or absence of 50 μM IBMX, for the indicated period of time. Following incubation with the designated agents, cAMP was measured in cellular HClO₄ extracts, as well as in the medium, by radioimmunoassay as previously described (8), using the method of Gettys et al. (15). The results were expressed either per milligram of total cell protein as determined by the BCA assay (Pierce, Rockford, IL) or as percentage of the maximum obtained by incubating the cells with PGE1 + IBMX alone.

Cell proliferation assessment. Cells were seeded at 3–5 × 10^4 cells/well in 12-well plates. After 24 h, the culture medium was replaced with medium containing 1% FBS 4 h before preincubation with the respective bile acid or tested agents. Cells were incubated for an additional 24 h after PGE1 addition, and the uptake of [methyl-3H]thymidine was determined as an index of cell proliferation. Briefly, 1 μCi [methyl-3H]thymidine was added to each well, and the cells were incubated for an additional 4 h. Cells were then washed, harvested with trypsin, and pipetted onto glass fiber filters (Whatman, GF/B). Filters were washed four times with Tris-buffered saline (20 mM Tris·HCl and 150 mM NaCl, pH 7.4), and cellular [3H]thymidine incorporation was measured by scintillation counting (model SL6000; Beckman, Palo Alto, CA).

RNA extraction and reverse transcription. Total RNA was extracted from fibroblasts using RNA Bee (Tel-Test, Friendswood, TX). Oligo dT and Superscript III were used for reverse transcription containing 1–2 μg of RNA. Reverse transcription was conducted at 50°C for 60 min after RNase H treatment for 30 min. DNA (5–10 ng) was used for PCR reaction using Taq DNA polymerase (GIBCO-BRL). PCR reactions were conducted at 94°C for 3 min for denaturation, followed by 94°C for 45 s, 55°C for 30 s, 72°C for 40–60 s, for either 24–27 cycles for GAPDH or 27–32 cycles for human COX-2, followed by a final extension at 72°C for 1 min. The reaction products were analyzed by electrophoresis on 1.5% agarose gels. The gels were analyzed by densitometric scanning with the use of photo imaging (Molecular Dynamics, Sunnyvale, CA). The densities of the COX-2 bands were normalized with the respective GAPDH bands.

Immunoblot analysis and PGE2 synthesis. Total cellular homogenates were prepared from cultured fibroblasts in modified RIPA buffer (0.5 M Tris·HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% Nonidet P-40, and 10 mM EDTA) containing one tablet of protease inhibitor (Roche, Indianapolis, IN). In addition, 1 mM of NaVO₃ and 1 mM of NaF were used in all the buffer preparations when phosphorylation studies were performed. Protein samples (20–30 μg) of the respective cellular fractions were separated by SDS-PAGE, using a mini-gel apparatus (Invitrogen, Carlsbad, CA) and transferred to Hybond-P Amersham Biosciences (Arlington Heights, IL) membranes using the Invitrogen transfer apparatus according to the manufacturer’s directions. The protein-containing membranes were blocked in 5% casein and 0.1% Tween (pH 7.4) and further incubated overnight at 4°C with either goat anti-human COX-2 (1:500), rabbit anti-human PKCα (1:1,000), anti-phospho P38 (1:500), anti-P38 (1:1,000), or anti-β-actin (1:5,000) antibodies. The protein content was visualized using horseradish peroxidase-conjugated corresponding secondary antibodies, followed by enhanced chemiluminescence (Amersham), and analyzed densitometrically. The immunoreactive signals were normalized against that of β-actin. The production of PGE2 was determined spectrometrically (Spectra-MAX, Molecular Dynamics) using an ELISA assay kit according to the manufacturer’s protocol (Cayman).

Statistical analysis. Except as otherwise indicated, the results were expressed as means ± SE. The statistical significance was determined by either Student’s t-test or ANOVA when more than two groups were compared.

RESULTS

Comparative effect of various agents on cAMP production. Several hormones and agents were investigated for their ability to significantly stimulate cAMP synthesis in human dermal fibroblasts. Histamine, PGE1, 1OH-PGE1, and PGE2 were found to stimulate cAMP synthesis in these cells as previously
were able to significantly inhibit human dermal fibroblast
production, including IBMX, PGE1, 1OH-PGE1, FK, and the
nonhydrolyzable, cell-permeable cAMP analog cpt-cAMP,
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Comparative effect of bile acids on PGE1-induced cAMP
production. Two-hour incubation of human skin fibroblasts
with the dihydroxy bile acids DCA, CDCA, and UDCA had no
effect on the basal cellular level of cAMP synthesis (data not
shown). However, DCA, CDCA, and UDCA dramatically
inhibited PGE1-induced cAMP production after 2-h incubation
(Fig. 1A). The approximate 80-fold increase in cAMP induced
by 1 µM PGE1 plus 50 µM IBMX exposure was inhibited by
50% at a concentration (EC50) of 4.6 µM by DCA and of 20 to
25 µM by CDCA and UDCA, respectively (Table 1). Under
these conditions, 50–100 µM of either DCA or CDCA almost
completely (96%-98%) inhibited the maximum production of
cAMP. Furthermore, as previously reported (8, 9), bile acids
inhibited PGE1-induced cAMP production to a similar extent
in the presence and absence of IBMX following incubation
with the fibroblasts for 2 h (data not shown). Thus the effect
of the bile acid is not at the level of the phosphodiesterase and
cAMP breakdown. Therefore, IBMX is not required for the
bile acid to induce this inhibitory effect.

After 20 h of exposure of the fibroblasts to DCA, CDCA, or
UDCA, and in the presence of 1 µM PGE1 and 50 µM IBMX,
the cellular cAMP formation was inhibited to the same extent
as that measured after 2 h (Fig. 1B). However, as previously
observed in acute studies (8, 9), CA remained without effect on
PGE-1-induced cAMP formation. Furthermore, the basal level
of cAMP at ~45 pmol/mg protein, was not affected by pro-
longed exposure of the cells to any of the bile acids studied
(data not shown). The maximum cAMP formation, in the
presence of PGE1 and IBMX, at 7,500 pmol/mg protein, was
inhibited by 50% at similar respective EC50 concentration that
was observed after 2 h of exposure (Table 1). The respective
level of inhibition at a bile acid concentration of 100 µM was
also similar as that observed at 2 h, suggesting that the bile acid
inhibitory effect was maximum by 2 h and persisted for at least
20 h. Under the same 20 h conditions, the increased cAMP
production induced by 1 µM 1OH-PGE1 was decreased by
CDCA in a dose-dependent manner with an almost complete
inhibition with CDCA concentrations >25 µM (Fig. 2).

To determine whether the bile acid effect to inhibit cAMP
production was dependent on the initial presence of PGE1, the
fibroblasts were incubated first with CDCA for 20–24 h and
then with PGE1 + IBMX for 8 min before the cellular cAMP
production was assessed. Under these conditions, CDCA de-
creased PGE1-induced cAMP production by 70% compared
with >90% when both CDCA and PGE1 are chronically
incubated together (data not shown).

Effect of cAMP synthesis-stimulating agents on cell prol-
eration. The various agents tested, which stimulate cAMP
production, including IBMX, PGE1, 1OH-PGE1, FK, and the
nonhydrolyzable, cell-permeable cAMP analog cpt-cAMP,
were able to significantly inhibit human dermal fibroblast
proliferation, as measured by thymidine incorporation. As
shown in Fig. 3A, 20–24 h incubation with 50 µM IBMX, a
phosphodiesterase inhibitor that prevents cAMP breakdown,
inhibited cell growth by 40%. Furthermore, this inhibitory
effect was mimicked by 1 µM of both PGE1 and 1-OH-PGE
after 20–24 h incubation, and the PGE1 effect was potentiated
by IBMX. Although not shown, little inhibition of cell prolif-
eration was observed when sulprostone, an EP1/EP3 agonist,
as well as in the presence of increasing concentrations of the
indicated bile acids. At the end of this period, the total cellular cAMP level was
determined by radioimmunoassay. Results are expressed as a percentage of the
maximum level of cAMP (~2,200 pmol/mg protein at 2 h, and 7,500 pmol/mg
protein at 20 h), corrected for the basal level (28–45 pmol/mg protein), and
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dehydroxy acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic
acid.
Table 1. Bile acid concentrations eliciting 50% inhibition of the maximum PGE1-induced cAMP synthesis

<table>
<thead>
<tr>
<th>Bile Acid</th>
<th>EC50, μM</th>
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<tr>
<td>UDCA</td>
<td>20.1 ± 10.3</td>
</tr>
<tr>
<td>CDCA</td>
<td>25.6 ± 10.5</td>
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<tr>
<td>DCA</td>
<td>4.6 ± 1.7</td>
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Values are means ± SE. IBMX, 3-isobutyl-1-methylxanthine; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid. Cultured human skin fibroblasts were incubated at 37°C for either 2 or 20 h with 1 μM PGE1 and 50 μM IBMX (maximum), as well as in the presence of increasing concentrations of the indicated bile acids, and the total cAMP level was determined. The respective EC50 was determined from the equations of the regression lines depicted in Fig. 1. A and B. *P < 0.01, significantly different vs. UDCA.

dermal fibroblasts. However, under the same conditions, 1 μM of either 15ΔPGJ2 or PJD2 did not significantly affect fibroblast proliferation (data not shown).

**Effect of bile acid on the modulation of cell proliferation by PGE1.** As reported in Fig. 3B, only concentrations of CDCA >25 μM were able to directly and significantly inhibit fibroblast proliferation by 35–45%. Under these conditions, 50 and 100 μM UDCA decreased cell proliferation by 50 and 65%, respectively, whereas 25 μM CA had no significant inhibitory effect (Fig. 3B). However, in keeping with its observed effect on PGE1-induced cAMP formation, 10 μM CDCA preincubation was associated with a significant attenuation of PGE1-induced growth arrest (Fig. 4A). Moreover, UDCA, which has a similar effect on PGE1-induced cAMP formation, was also able to significantly attenuate the PGE1 effect, while CA was again without effect (Fig. 4A). Finally, CDCA also induced a dose-dependent reversal of the inhibition of cell proliferation induced by 0.5 μM of the EP2/EP4-specific agonist 1-OH-PGE1 (Fig. 4B). Collectively, these results suggest that bile acids, at physiologically relevant concentrations that reduce PGE1-induced cAMP production, are also able to reverse the cAMP-mediated PGE1-induced inhibition of cell proliferation.

**Role of COX-2 on regulation of cell proliferation by cAMP and bile acid.** To study the role of COX in the regulation of cell proliferation by cAMP and bile acids, the fibroblasts were incubated with 1 μM indomethacin, a nonspecific COX inhibitor. Indomethacin had no effect alone at this concentration but significantly reduced the inhibitory effect of PGE1, FK, and 50 μM CDCA by 40–60% and that of CDCA by ∼42% (Fig. 5A). Moreover, incubation of fibroblasts with 2 μM NS-398, a specific COX-2 inhibitor reduced the effect of PGE1 and FK by 40–60% and that of CDCA by ∼42% (Fig. 5B). Furthermore, preincubation of the cells with 1 μM dexamethasone, which prevents COX-2 protein synthesis, results in an almost complete abrogation of the inhibitory effect of PGE1 and PGE2 on cell proliferation, while

Fig. 2. Dose-dependent effect of CDCA on 1-OH-PGE1-induced cAMP formation. Fibroblasts were incubated for 20 h with 1 μM 10H-PGE1 and 50 μM IBMX, as well as in the presence of increasing concentrations (1–100 μM) of CDCA. See Fig. 1 for details. Result is a representative experiment performed in quadruplicate. *P < 0.01, significantly different from control determined in the absence of bile acid.

Fig. 3. Effect of cAMP-modulating agents and bile acids on cell proliferation. Fibroblasts were incubated in the absence and presence of IBMX, 10H-PGE1, forskolin (FK), a membrane-permeable cAMP analog (cpt-cAMP) and PGE1 (A) or increasing concentrations of CDCA, UDCA, and CA (B). These agents were present for 20 h. One μCi [methyl-3H]thymidine was then added to each well and maintained at 37°C for an additional 4 h. Finally, the cells were harvested, washed, and filtered, and the uptake of [3H]thymidine was determined as an index of cell proliferation and expressed as percentage of control, i.e., in the absence of added agents. Results are means ± SE of 3–6 experiments performed in triplicate or quadruplicate. CTL, control; cpt-cAMP, 8-(4-chlorophenylthio)-cAMP. The concentrations of the various agents tested are in μM. *P < 0.05, significantly different from control; **P < 0.01, significantly different from control.

Fig. 4. Role of COX on regulation of cell proliferation by cAMP and bile acid. To study the role of COX in the regulation of cell proliferation by cAMP and bile acids, the fibroblasts were incubated with 1 μM indomethacin, a nonspecific COX inhibitor. Indomethacin had no effect alone at this concentration but significantly reduced the inhibitory effect of PGE1, FK, and 50 μM CDCA by 25–65% (Fig. 5A). Moreover, incubation of fibroblasts with 2 μM NS-398, a specific COX-2 inhibitor reduced the effect of PGE1 and FK by 40–60% and that of CDCA by ∼42% (Fig. 5B). Furthermore, preincubation of the cells with 1 μM dexamethasone, which prevents COX-2 protein synthesis, results in an almost complete abrogation of the inhibitory effect of PGE1 and PGE2 on cell proliferation, while
it significantly reduces that of 50–100 μM CDCA by >45% (Fig. 5C). Together, these results suggest that at least COX-2 is involved in the inhibition of cell proliferation by PGE and higher concentrations of certain bile acids.

**Effect of bile acids on COX-2 mRNA expression and PG2 synthesis.** A recent report (25) demonstrating that the PGE-2 induced inhibition of fibroblast proliferation was abrogated in COX-2 but not COX-1 knockout mice supports a fundamental role of COX-2 and PG synthesis in the regulation of cell proliferation. Therefore, we undertook experiments to study the COX-1 and COX-2 mRNA expression level by RT-PCR in human dermal fibroblasts after exposure to different agents. The basal COX-1 mRNA expression was not affected by any of the agents tested (data not shown). The basal COX-2 mRNA level was barely detectable (Fig. 6A). Furthermore, while 1 μM PGE1 and 10 μM CDCA induced a slight but statistically insignificant increase in COX-2 mRNA expression, 100 μM of either CDCA, UDCA, or DCA stimulated COX-2 mRNA expression by 8-, 6-, and 4-fold, respectively (Fig. 6, A and B); 200 nM PMA was used as control. Furthermore, although not shown, the increase in COX-2 mRNA expression by the bile acids was time and dose dependent, i.e., a maximum effect observed at 12 h and with 200–400 μM CDCA. CDCA and

**Fig. 4. Combined effect of PGE1 and bile acids on cell proliferation.** Fibroblasts were incubated in the absence and presence of IBMX and either PGE1 (A) or 1OH-PGE1 (B), as well as various concentrations of CDCA, UDCA, or CA for 20 h. One μCi [methyl-3H]thymidine was then added to each well and maintained at 37°C for an additional 4 h. The cells were processed as described in the legend of Fig. 3. In addition, see legend of Fig. 1 for abbreviations. Results are means ± SD of 3–4 determinations. *P < 0.05, significantly different from respective control determined in the absence of bile acid; **P < 0.05, significantly different from control; #P < 0.05, significantly different from 1OH-PGE1 without CDCA.

**Fig. 5. Effect of COX-2 inhibitors on cell proliferation in the presence of cAMP modulating agents and CDCA.** Fibroblasts were incubated in the absence and presence of 1 μM indomethacin (A), 2 μM NS-398 (B), or 1 μM dexamethasone (DEX; C) 2–4 h before the addition of either the cAMP modulating agents (PGE1, PGE2, or FK) and CDCA for 20 h. One μCi [methyl-3H]thymidine was then added to each well and maintained at 37°C for an additional 4 h. The cells were processed as described in Fig. 3. Results are means ± SE of 4 determinations. *P < 0.01, significantly different from control; **P < 0.05, significantly different from respective condition without COX-2 inhibitor.
The reaction products were analyzed by electrophoresis on 1.5% agarose gels at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s. and enzyme activation at 94°C for 3 min, followed by 27 cycles of denaturation and the internal control primers at 1 fold increase over control. The arbitrary value for control was set at 1. The results are representative of at least three different experiments. Results from the mean of the three experiments were analyzed by one-way ANOVA and Bonferroni’s posttest.

**DISCUSSION**

The results from the present study clearly demonstrate that chronic exposure of human dermal fibroblasts to dihydroxy bile acids causes an almost complete inhibition (>90%) of the PGE1-induced cAMP production. This inhibitory effect was maximal after 2 h for CDCA, UDCA, or DCA and plateaued for at least 20 h, suggesting that the bile acid inhibitory effect was not significantly desensitized even after chronic exposure. The loss of responsiveness of the G protein-coupled receptor signal transduction is an integral part of the adaptive receptor regulatory mechanism to prevent either overstimulation or signal termination, as previously reported for the β-adrenergic.

PMA stimulated COX-2 protein expression by ~4- and ~9-fold, respectively, 8 and 12 h after their addition to the cell culture (Fig. 6B). In addition, this COX-2 transcripational stimulation was associated with an increased PGE2 synthesis. Indeed, whereas 10 μM CDCA had no effect on PGE2 synthesis, 10 μM FK and 100 μM CDCA significantly increased prostaglandin synthesis by 20–30% and 80%, respectively (Fig. 7). After 24 h of incubation, 300 μM DCA increased PGE2 synthesis by over fivefold.

To further confirm the role of COX-2 on the CDCA-induced inhibition of cell proliferation, the fibroblasts were transfected with wild-type COX-2 cDNA using the procedure described by Amaxa (Gaithersburg, MD). Under these conditions, the transfection efficiency measured by fluorescent microscopy using GFP cDNA and expressed as a ratio of the fluorescent cells to the total cell expression was always >50%. Thirty-five to forty-eight hours after COX-2 cDNA transfection, a concentration of 20 μM CDCA, which was without significant effect on cell proliferation, decreased fibroblast proliferation by 80%, while the 68% inhibition observed with 100 μM CDCA was almost complete at 99% after COX-2 overexpression (Fig. 8).
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receptor (see Ref. 22 for review). Furthermore, the fact that the bile acids are able to attenuate PGE1-induced cAMP production even when added before PGE1 stimulation supports a mechanism independent of both increased cAMP production and activated cAMP-dependent protein kinase.

In the present study, the respective EC50 for UDCA and CDCA was similar at both 2 and 20 h, at ~20 μM, whereas that for DCA was just ~5 μM. These EC50 concentrations are clearly attainable in the systemic circulation during cholestasis, and possibly, even postprandially (2, 29, 44), and further underline the relevance of this bile acid effect. Even after 20 h of exposure, CA was without effect on stimulated cAMP formation, which is consistent with our previous reports (8, 9), suggesting that the potency of the bile acid to inhibit stimulated cAMP formation varied with its hydroxylation state. On the other hand, the present results also differ somewhat from our previous report (9). While in isolated hepatocytes the order of potency among the bile acids tested to inhibit stimulated cAMP formation was UDCA > CDCA > DCA, in dermal fibroblasts, the order of potency is the following: DCA > CDCA > UDCA. Different hypotheses can be proposed to explain this discrepancy in bile acid potency between these two cellular models. First, although the PGE1 and glucagon receptors are coupled to cAMP synthesis in fibroblasts and hepatocytes, respectively, either or both the mechanism of desensitization of and the cellular machinery available to desensitize the respective receptor signaling cascade may be different. In addition, the difference in relative potency of the dihydroxy bile acids in fibroblasts and hepatocytes may reflect the respective levels of intracellular bile acid-binding proteins in these two cell types (47).

There is considerable evidence in human and in animal models, that cholestasis is associated with increased deposition of bile acids in the skin (5, 12, 16, 19, 34, 43). In two hamster models of hepatic failure, namely bile duct ligation and functional hepatectomy, we have shown that bile acids were targeted to several tissues outside of the enterohepatic circulation, most notably, the skin (12). Thus, one could imagine that in the event of hepatobiliary disorders, which result in cholestasis, similar effects may be observed in vivo, as those observed in situ, in the present study. Furthermore, under conditions of impaired liver function and decreased bile secretion, as those found in patients with portal cirrhosis (29) and in infants with extrahepatic biliary atresia and neonatal hepatitis (21), serum concentrations of unconjugated CDCA could reach a level ≥20 μM, which is shown in the present study to completely inhibit the PGE1/G protein-coupled receptor signaling response.

Elevated serum bile acid levels under cholestatic conditions have been associated with hepatotoxicity (17, 18), hepatic fibrosis (33), pruritus (39), cardiomyopathy (28), and vasodilation (7). Ligation of the common bile duct in rodents is associated with increased fibroblast proliferation and fibrosis (52). This condition is also associated with a delayed or impaired wound healing of the skin combined with a weaker scar (3, 13). Bile and bile acids in particular have been suggested to be central to the pathologies associated to disorders of hepatic, coagulation, renal, skin, or immune function coincident with deep jaundice, which can be reduced via biliary drainage (36).

Results from the present study are suggesting that bile acids affect fibroblast proliferation at least in part through modulation of the PGE1-induced cAMP production (see Fig. 9). Previously, Okuyama et al. (37) have reported that prostaglandins inhibit cell proliferation through the EP2/EP4 receptor population, which involves cAMP. Furthermore, our data support this inhibitory effect by PGE1, PGE2, and 1-OH-PGE, a EP2/EP4 specific agonist. Although not shown, we observed virtually no inhibition of cell proliferation with sulprostone, an EP1/EP3 agonist, or misoprostol, an EP2/EP3 agonist, thus suggesting a predominant EP4 receptor-dependent growth inhibitory mechanism in these cells. Moreover, the ability of the bile acid to abrogate the PG effect was observed with both PGE1 and 1-OH-PGE1. Finally, the bile acid effect was observed with CDCA and UDCA, but not CA, in keeping with their respective effect on inhibition of cAMP production.

The implications for bile acid-induced modulation of PGE1-induced cAMP formation and the consequent effects on PGE-1 induced cell proliferation are significant. We hypothesize that an increased deposition of bile acids not only in the liver but also in the skin could lead to tissue injury and the alteration of the balance between cell proliferation and cell death induced by various hormones, cytokines, and growth factors. In addition, PGE1 has been suggested to play an important role in tissue regeneration, due to its ability to regulate hepatocyte growth factor expression in human dermal fibroblasts in a cAMP-dependent fashion (31). Finally, PGE1 has been shown to inhibit collagenase gene expression in human skin fibroblasts (14).

It is tempting to speculate that the bile acid-induced modulation of stimulated cAMP formation and cell proliferation could similarly occur in mesenchyme-derived cells of the liver under certain liver disease conditions.
Most studies have found COX-2 to be primarily, if not exclusively, located in the stromal compartment in human and rodent tissues (45). In the skin, COX-2 is mostly expressed in keratinocytes (26). Topical PGE2 application to the skin results in an increased expression of COX-2 (41). Furthermore, co-culture of dermal fibroblasts with keratinocytes resulted in an increased COX-2 activity in fibroblasts (42), supporting cross-talk between the dermis and epidermis. The results of the present study suggest that COX-2 expression can also be induced in fibroblasts. Indeed, the direct incubation of fibroblasts with concentrations >20 μM CDCA results in a significant increase in COX-2 mRNA and protein level. This supports reports showing that bile acid-induced increased COX-2 expression and PGE2 synthesis in various cell lines, including human pancreatic cancer cell lines (51), human pharyngeal cells (48), and colonic cell lines (57, 58). In the skin, the stimulation of PGE2 by bile acids could have not only an autocrine but also a paracrine effect. This increased COX-2 expression and PGE2 synthesis could in turn influence the cellular cAMP production as previously demonstrated in fibroblasts (8) and the phenotype not only of the fibroblasts but of the keratinocytes, as well by affecting proliferation, apoptosis, intercellular adhesion, and extracellular matrix production, which are key steps in the pathology of fibrosis.

Furthermore, in support of the findings from the present study, it is worthwhile to mention that several other reports associate COX-2 overexpression to suppression of tumor development. Indeed, Wilson and Potten (56) have reported that topically applied PGE2 inhibited tumor promotion in Min/+ mice. Furthermore, Lama et al. (25) have shown that fibroblasts from COX-1 knockout but not from COX-2 knockout mice were able to synthesize PGE2 and to inhibit cell proliferation. Finally, Bol et al. (6) have reported that COX-2 overexpression conferred resistance to skin tumor development to the K14.COX-2 transgenic mice. Therefore, these studies would support a biphasic effect of bile acid, i.e., a high concentration will decrease cell proliferation and potentially tumor formation, whereas low doses that can be reached under mild cholestatic conditions in the skin could perturb the local hormonal balance leading to increased cell proliferation. Furthermore, pathological conditions would be consequential to the loss by the cells of a normal response of COX-2 gene expression to extracellular agents, including prostanoids.

The biphasic effect of CDCA on cell proliferation is of interest in light of recent findings by Nishioka et al. (35). These authors suggest that CDCA stimulated proliferation of squamous cell carcinomas at low concentrations, while having a profound effect on the inhibition of cell proliferation at concentrations >30 μM. Those results are in agreement with the present findings as far as the regulation of cell proliferation is concerned. However, these authors have suggested that the bile acid effect on cell proliferation was independent of the bile acid-induced increase COX-2 expression. Those results are in contrast to those of the present study in normal diploid dermal fibroblasts, which demonstrates that the bile acid effect on cell proliferation was at least partially inhibited by both indomethacin and NS-398 while enhanced after COX-2 overexpression, supporting at least a partial COX-2 mediated inhibition of cell proliferation.

In summary, these findings underline the importance of potential pathophysiological mechanisms by which significant increase in particular systemic bile acids may not only result in cytotoxicity but also lead to changes in the response of extrahepatic tissues to hormones and transmitters, which are regulated by second messengers.

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