Induction of cyclooxygenase-2 by tumor promoters in transformed and cytochrome P450 2E1-expressing hepatocytes

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Introduction

Cyclooxygenase (COX) is a rate-limiting enzyme in the cellular production of prostaglandins and thromboxanes from arachidonic acid (1). There are two distinct forms of COX. The COX-1 isoform is a constitutively expressed housekeeping gene that fulfills a variety of homeostatic functions (1). In contrast, COX-2 is undetectable in most normal tissues, but strongly induced by tumor promoters, carcinogens, inflammatory mediators and growth factors (2). COX-2 is, therefore, expressed during cellular processes such as carcinogenesis, inflammation and cell growth. In particular, increased COX-2 expression has been described in a number of non-hepatic cancers (3–5). COX-2 has been implicated as a causative factor in carcinogenesis because genetic deletion or pharmacological inhibition of COX-2 suppresses tumorogenesis (6–8).

Similar to findings in other organs, normal liver has a low level of COX-2 (9), but increased hepatic COX-2 expression has been reported in two pathophysiological conditions. Recent studies have demonstrated increased COX-2 expression in human hepatocellular carcinoma (HCC) (10–12), suggesting a possible role for COX-2 in the initiation or promotion of hepatic carcinogenesis. Induction of COX-2 has also been demonstrated in animal models of alcoholic liver disease (ALD) (13–16). COX-2 expression may be induced in this disease by Kupffer cell-generated inflammatory mediators such as tumor necrosis factor-α (TNF-α), or reactive oxygen species (ROS). Alternatively, ALD in humans and animals is associated with increased expression of the cytochrome P450 isofrom 2E1 (CYP2E1) (17). This enzyme metabolizes a number of hepatotoxins and carcinogens including ethanol (18,19). CYP2E1 also has uncoupled NADPH oxidase activity that in the absence of substrate results in increased production of ROS (20). Overexpression of CYP2E1 may promote hepatic carcinogenesis by acting to induce COX-2 through the production of ROS.

Little is known about which specific liver cell type(s) expresses COX-2 in HCC or ALD. Immunohistochemical analysis of COX-2 in HCC has demonstrated expression in malignant cells and normal hepatocytes as well as in non-parenchymal cells (10). PCR analysis of isolated cell populations from rats with ALD revealed that COX-2 mRNA was induced predominantly in Kupffer cells (13). In vitro studies have demonstrated COX-2 expression in fetal primary hepatocytes (21), but not in normal adult primary rat or mouse hepatocytes (21–23). C/EBPα has been reported to be involved in the repression of COX-2 expression in adult rat hepatocytes following challenge with proinflammatory stimuli (22). The precise conditions under which hepatocytes may express COX-2, therefore, remain unclear. The aim of the present study was to determine whether hepatocellular transformation and/or CYP2E1 expression regulate COX-2 induction in hepatocytes, and to define the signaling cascades that regulate this expression.

Abbreviations: ALD, alcoholic liver disease; CD, chenodeoxycholic acid; COX, cyclooxygenase; CYP2E1, cytochrome P450 2E1; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; MAPK, mitogen-activated protein kinase; PGE2, prostaglandin E2; PI 3-K, phosphatidylinositol 3-kinase; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α.
Materials and methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM), glutamine and antibiotics were from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was from Gemini BioProducts (Calabasas, CA). Phorbol 12-myristate 13-acetate (PMA), sodium arachidonate, chenodeoxycholic acid (CD), L294002, [2-(4-methylphosphoryl)-8-phenyl-1H-l-benzopyran-4-one], hydrogen peroxide, Lowry protein assay kits and secondary antibody to IgG conjugated to horseradish peroxidase were from Sigma Chemical Company (St Louis, MO). Goat polyclonal anti-rat COX-2 antibody, anti-C/EBP, B and C/EBP consensus oligonucleotides were from Santa Cruz Biotechnology (San Diego, CA). PD98059 (2'-H10323) from BIOMOL Research Laborotory (PhosphoLyn Meeting, PA). SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole] was from Calbiochem (San Diego, CA). Nicotilcellose membrane was from Schleicher & Schuell (Keene, NH), Western blotting detection reagents (ECL) were from Amersham Pharmacia Biotech (Piscataway, NJ).

Enzyme immunoassay reagents for prostaglandin E2 (PGE2) assays and rat COX-2, DNA was from Cayman (Ann Arbor, MI). TNF-α was from R&D Systems (Minneapolis, MN).

Cell and culture conditions

Studies were performed in the adult, rat hepatocyte line RALA255-10G (24). RALA hepatocytes are conditionally transformed with an SV40 virus containing a temperature-sensitive, mutant T antigen (24). At the permissive temperature of 33 °C, cells express T antigen, remain undifferentiated and proliferate. Culture of the cells at the restrictive temperature of 37°C supresses T-antigen expression, markedly slows growth and allows differentiated hepatocyte gene expression (24). Cell lines were maintained in DMEM supplemented with 4% FBS, 2 mM glutamine and antibiotics in a 5% CO2/water saturated incubator at 33 °C.

Cells were grown to confluence at 33°C, trypsinized and replated at 0.65×106 cells/dish on 35 mm plastic dishes. Transformed cells were maintained at 33°C in DMEM supplemented with 4% FBS for the length of the experiment. For non-transformed cells, the medium was changed after 24 h to DMEM with 2% FBS, glutamine, antibiotics and 1 µM dexamethasone to optimize hepatocyte differentiation (24), and the cells were placed at 37°C. After 3 days of culture (at which point cell density was ~2.5×106 cells/dish), these cells received fresh serum-free medium. Transformed cells received fresh DMEM with 4% FBS at the same time. Some cells were treated with PMA (50 ng/ml), sodium arachidonate (10 µM), CD (100–400 µM), TNF-α (10 ng/ml) or H2O2 (1.0 mM), 10 µM) for 6 h. In addition, cells were cultured in the transformed conditions as described in Materials and methods, and treated with PMA (50 ng/ml) for 6 h. In addition, cells were cultured in the absence or presence of dexamethasone (10−6 M) for the 24 h prior to isolation as indicated. Aliquots of cell lysates were subjected to SDS–PAGE and immunoblotting was performed with an anti-COX-2 antibody.

PGE2 assay

Cells were cultured as described above, and levels of PGE2 were measured on aliquots of cell medium by enzyme immunoassay (31). PGE2 production was normalized to the cell protein concentration.

RNA isolation and northern blot hybridization

RNA was extracted from cells as described previously (32). Steady-state mRNA levels were determined by northern blot hybridizations using samples of 20 µg of total RNA. The membranes were hybridized with [32P]CTP-labeled cDNA clones for COX-2 and 18S ribosomal RNA. The hybridized filters were washed under stringent conditions (32).

Transient transfections and reporter gene assays

Cells were transiently transfected and assayed for luciferase activity at 6 h after PMA treatment as described previously (33). Cells were cotransfected with the −1475/−59 COX-2 promoter-driven firefly luciferase reporter construct (34), and pRL-TK (Promega, Madison, WI). pRL-TK is a Renilla luciferase vector driven by a Herpes simplex virus thymidine kinase promoter. Firefly luciferase was normalized to Renilla luciferase to control for transfection efficiency.

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were isolated by the method of Schreiber et al. (35), with slight modification as described previously (36). EMSA were performed on 5 µg of protein with [32P]end-labeled oligonucleotide for the NF-κB or C/EBP consensus sequence. The DNA-binding reaction was performed as described previously (36). For supershift assays, 4 µg of anti-C/EBPα, anti-C/EBPβ, anti-C/EBPδ or anti-Stat 3 antibody were added to the reaction mixture, and the incubation time was extended for an additional 20 min. After incubation, the samples were resolved on a 4% polyacrylamide gel, dried and subjected to autoradiography.

Statistical analysis

All numerical results are reported as mean ± SE and represent data from a minimum of three independent experiments performed in duplicate.

Results

COX-2 is inducible by PMA in transformed but not non-transformed hepatocytes

To examine the regulation of COX-2 in hepatocytes, COX-2 expression was examined in RALA hepatocytes by western blotting. This cell model allowed investigations into COX-2 expression in both transformed (cells cultured at 33 °C), and non-transformed (cells cultured at 37°C) hepatocytes, because of the temperature-sensitive expression of T antigen in this cell line (24). COX-2 was undetectable by western blotting in both untreated transformed and non-transformed cells (Figure 1). Treatment with the tumor promoter PMA for 6 h markedly induced COX-2 expression in transformed cells, while COX-2 protein was barely detectable in non-transformed cells (Figure 1). Non-transformed RALA hepatocytes are routinely cultured in dexamethasone to promote differentiation (24), while transformed cells are cultured in the absence of dexamethasone. Dexamethasone has been reported to inhibit COX-2 expression in non-hepatic cells (37,38), suggesting the
The possibility that COX-2 expression may be affected by the inclusion of dexamethasone in non-transformed cell medium. The addition of dexamethasone markedly inhibited PMA-induced COX-2 expression in transformed cells (Figure 1). However, COX-2 was not significantly induced in non-transformed cells by PMA in either the absence or presence of dexamethasone (Figure 1).

PMA induces COX-2 enzyme activity

To ensure that PMA-induced COX-2 expression was associated with increased COX-2 enzyme activity, the effect of PMA on PGE2 production was determined. The addition of PMA to transformed cells resulted in a small increase in spontaneous PGE2 synthesis (data not shown). The effect of PMA on PGE2 production was then determined in the presence of excess exogenous arachidonic acid. PGE2 production is affected by the activity of phospholipase A2 which provides the substrate for COX-2, and the addition of excess arachidonic acid minimizes the effect of variable phospholipase A2 activity on PGE2 production. In the presence of exogenous arachidonic acid, the treatment of transformed hepatocytes with PMA induced a 40-fold increase in PGE2 synthesis relative to untreated cells (26.8 ± 9.7 versus 0.7 ± 0.1 pg/mg protein), PMA therefore induced COX-2 activity as well as protein expression in transformed hepatocytes.

COX-2 is induced by chenodeoxycholic acid but not by inflammatory mediators

To examine whether other known inducers of COX-2 in non-hepatic cells regulate this enzyme in transformed RALA hepatocytes, cells were treated with CD, TNF-α and H2O2. CD induced COX-2 expression in transformed cells in a dose-dependent fashion with significant induction occurring at a concentration of 200 µM (Figure 2A). In contrast, TNF-α and H2O2 failed to induce COX-2 expression within 6 h (Figure 2B), or at 24 h (data not shown). TNF-α and H2O2 were used at concentrations known to induce maximal physiological responses in RALA hepatocytes (39,40). COX-2 was therefore inducible in transformed RALA hepatocytes by tumor promoters (PMA and CD), but not inflammatory mediators (TNF-α and H2O2). CD, TNF-α and H2O2 all failed to induce COX-2 in non-transformed cells (data not shown).

PMA induces COX-2 expression in cells over-expressing CYP2E1

In addition to the known association of COX-2 expression with carcinogenesis, COX-2 expression occurs in inflammatory conditions. In the liver, COX-2 is induced in the setting of liver injury and inflammation secondary to alcohol (13–16). Because of the association of increased expression of the pro-oxidant enzyme CYP2E1 in ALD (17), and the ability of oxidant stress to induce COX-2 in non-hepatic systems (41), levels of CYP2E1 expression may regulate hepatocyte COX-2 expression. To test this hypothesis, levels of COX-2 expression were examined in RALA hepatocytes with differential levels of CYP2E1. S-CYP15 cells and AN-CYP10 cells, which over and under express CYP2E1, respectively, were employed for these studies. Similar to wild-type, non-transformed RALA hepatocytes, untreated S-CYP15 and AN-CYP10 cells did not express COX-2 by western blotting (Figure 3A). However, following PMA stimulation COX-2 was induced in S-CYP15 but not AN-CYP10 cells (Figure 3A). PMA induced a functional COX-2 in S-CYP15 cells as indicated by the marked increase in PGE2 synthesis in these cells as compared with untreated cells (28.2 ± 7.0 versus 0.3 ± 0.1 pg/mg protein). In contrast to transformed cells, COX-2 was not induced by CD in S-CYP15 cells (Figure 3B). TNF-α and H2O2 also failed to induce COX-2 in S-CYP15 cells (data not shown). COX-2 was not expressed in AN-CYP10 cells following CD, TNF-α or H2O2 treatment (data not shown).

PMA induces COX-2 mRNA in transformed and S-CYP15 cells

To further elucidate the mechanisms responsible for the induction of COX-2 protein expression, steady-state levels of COX-2 mRNA were determined by northern blot analysis. COX-2 mRNA was not detected in untreated transformed or non-transformed cells (Figure 4A). PMA significantly increased the expression of COX-2 mRNA in transformed, but not non-transformed cells (Figure 4A). Similarly, COX-2 mRNA was undetectable in untreated S-CYP15 and AN-CYP10 cells, and markedly increased with PMA treatment only in S-CYP15 cells (Figure 4B). PMA induction of COX-2 in transformed or CYP2E1-expressing RALA hepatocytes, therefore, occurred at the level of transcription or mRNA stabilization.

PMA-induced COX-2 expression is dependent on ERK, p38 MAPK and PI-3K signaling

The signaling pathways mediated by ERK, p38 MAPK and PI-3K have been implicated in the regulation of COX-2 expression in non-hepatic cells (4,41–43). To determine whether any of these signaling cascades regulate hepatocyte COX-2 expression, the effects of chemical inhibition of each pathway on PMA induction of COX-2 were examined. In transformed cells the induction of COX-2 by PMA was significantly decreased by PD98059, SB202190 and LY294002.
Fig. 3. PMA but not CD induces COX-2 in CYP2E1-expressing cells. (A) S-CYP15 and AN-CYP10 cells were treated with PMA for 6 h. (B) S-CYP15 cells were untreated or treated for 6 h with PMA or CD at 100, 200, 300 and 400 µM concentrations as shown. In both panels, immunoblotting was performed with an anti-COX-2 antibody.

Fig. 4. PMA induces COX-2 mRNA expression in transformed and S-CYP15 cells. Cells were untreated or treated with PMA for 4 h. Total RNA was isolated and used in northern blot hybridizations with COX-2 and 18S ribosomal cDNA probes as indicated. Shown are autoradiograms of northern blot hybridizations with RNA from transformed and non-transformed cells (A), and S-CYP15 and AN-CYP10 cells (B).

Fig. 5. PMA-induced COX-2 expression in transformed and S-CYP15 cells is dependent on ERK, p38 MAPK and PI-3K. Cells were pre-treated with PD98059 (PD, 50 µM), SB202190 (SB, 10 µM) or LY294002 (LY, 10 µM) for 2 h, followed by PMA for 6 h. The immunoblots were probed with antibodies specific for COX-2, and constitutively expressed protein disulfide isomerase (PDI). Shown are immunoblots of transformed cells (A), and S-CYP15 cells (B).

Similarly in S-CYP15 cells PMA induction of COX-2 was markedly decreased by all three inhibitors (Figure 5B). Both transformed and S-CYP15 cells relied on the ERK, p38 MAPK and PI-3K pathways for PMA-mediated COX-2 induction.

NF-κB and C/EBP activation in transformed and non-transformed cells
The induction of COX-2 mRNA by PMA suggested that known transcriptional regulators of COX-2 such as NF-κB and C/EBP may be regulating hepatocyte COX-2 expression. To examine this possibility, levels of NF-κB and C/EBP activation were determined by EMSA in transformed and non-transformed cells. Both cell types had low levels of nuclear NF-κB binding activity under basal conditions (Figure 6A). NF-κB activation occurred in both cell types after PMA treatment, and was even greater in non-transformed than transformed cells (Figure 6A). The failure of non-transformed cells to up-regulate COX-2 in response to PMA was, therefore, not due to an inability to activate NF-κB. NF-κB activation also occurred in both cell types after TNF-α treatment (Figure 6A), demonstrating that a lack of NF-κB activation could not explain the absence of a COX-2 response to TNF-α.
An alternative mechanism of the differential COX-2 expression in the two cell types could be that both express the positive regulator of COX-2 expression (NF-κB), but differ in the expression of a repressor of COX-2 transcription. Recent investigations into COX-2 expression in primary rat hepatocytes have suggested that C/EBPα may act as a COX-2 repressor in these cells (22). On EMSA analysis of nuclear C/EBP levels, untreated non-transformed cells had a marked increase in C/EBP binding as compared with transformed cells (Figure 6B). Levels of C/EBP were unchanged in non-transformed cells after PMA or TNF-α treatment, but increased further in non-transformed cells (Figure 6B). Supershifts performed with antibodies specific for C/EBP isoforms revealed that in PMA-stimulated non-transformed cells the complex was mainly composed of the β isoform, with a small amount of δ and no α (Figure 6C). The mechanism underlying the failure of non-transformed cells to express COX-2 after PMA stimulation therefore does not involve C/EBPα.

**COX-2 induction occurs by a post-transcriptional mechanism**

Given our inability to explain differential COX-2 expression based on differences in known COX-2 transcriptional regulators, the effect of PMA on COX-2 promoter activity was determined. In transformed cells, COX-2-dependent luciferase activity increased only 20.9 ± 9.6% at 6 h after PMA treatment. In S-CYP15 cells activity decreased 18.1 ± 3.8% with PMA treatment. Thus, PMA did not cause a significant increase in COX-2 promoter activity in either cell type. Taken together, these results suggest that PMA induced COX-2 by a post-transcriptional mechanism such as mRNA stabilization.

**Discussion**

Up-regulation of COX-2 has been implicated as a critical event in the process of non-hepatic carcinogenesis based on the findings of COX-2 over-expression in a number of human cancers (3–5), and the ability of COX-2 inhibitors to suppress tumor formation in animals (7,8). Although little is known about the mechanistic role of COX-2 expression in HCC, several recent studies have demonstrated increased COX-2 expression in human HCC (10–12). A delineation of the mechanisms by which COX-2 is induced in hepatocytes may, therefore, promote further understanding of HCC formation, and therapeutic advances directed against HCC.

The present studies capitalized on the temperature-sensitive, adult, rat hepatocyte cell line RALA255-10G to examine the effect of cellular transformation on hepatocyte COX-2 expression. Non-transformed RALA hepatocytes did not express COX-2, and were refractory to COX-2 induction by the tumor promoters PMA and CD, and the inflammatory mediators TNF-α and H₂O₂. These data are consistent with prior investigations in which adult, primary rat and mouse
hepatocytes failed to express COX-2 constitutively, or following treatment with known inducers of COX-2 (21–23). Under culture conditions in which RALA hepatocytes undergo transformation, these cells still did not express COX-2. However, COX-2 was induced in transformed cells by the tumor promoters PMA and CD. Thus, in contrast to some non-hepatic cell systems in which cellular transformation enhances COX-2 expression (31), the transformation of RALA hepatocytes occurred in the absence of COX-2 expression. However, transformation rendered the cells receptive to COX-2 induction by the tumor promoters PMA and CD. In HCC, increased levels of COX-2 may not be a primary mechanism of tumor initiation, but rather a secondary event possibly leading to tumor promotion.

Although wild-type, non-transformed RALA hepatocytes were refractory to COX-2 induction, PMA did induce COX-2 in non-transformed cells over-expressing the pro-oxidant enzyme CYP2E1. Hepatic CYP2E1 is induced by ethanol, and can activate chemical carcinogens (17,19). Hepatic COX-2 expression has been demonstrated in animal models of ALD (13–16), a disease that occurs in association with marked increases in CYP2E1 (17). Similar to findings in transformed cells, CYP2E1 expression by itself did not result in COX-2 expression, but allowed cells to undergo COX-2 induction by PMA. CYP2E1-mediated regulation of COX-2 expression is not unique to hepatocytes as Nieto et al. recently demonstrated that CYP2E1 expression in hepatic stellate cells constitutively induced COX-2 expression (25). The ability of CYP2E1 to affect COX-2 expression in hepatocytes may be an indirect mechanism by which hepatocarcinogens promote HCC formation. Supportive of this possibility are the facts that ethanol-induced CYP2E1 has been shown to increase HCC formation by the chemical carcinogen N-nitrosodimethylamine (44), and CYP2E1 polymorphisms have been linked to human HCC (45).

PMA-mediated COX-2 induction in transformed and CYP2E1-expressing RALA hepatocytes occurred by similar mechanisms. Both cell types had increased levels of COX-2 mRNA while non-transformed and AN-CYP10 cells failed to significantly up-regulate COX-2 mRNA after PMA treatment. The present study demonstrates that PMA-induced COX-2 induction in RALA hepatocytes depends on ERK, p38 MAPK and PI-3K signaling. In both transformed cells and CYP2E1-expressing cells, COX-2 induction was dependent on these same pathways. The involvement of ERK, p38 MAPK and PI-3K in COX-2 induction has been described previously in non-hepatic cells (4,41–43). However, the present study is the first description of COX-2 being a target gene downstream of these signaling cascades in hepatocytes. While these signaling pathways usually affect protein expression at the transcriptional level, the differential induction of COX-2 in transformed and non-transformed cells could not be explained by differences in NF-κB or C/EBP activity. NF-κB has been identified as a positive regulator of COX-2 expression in non-hepatic cells (41). However, NF-κB activation was not a critical regulator of RALA hepatocyte COX-2 expression because: (i) PMA induced NF-κB activation in both transformed and non-transformed cells, yet COX-2 expression was only seen in transformed cells; and (ii) TNF-α failed to induce COX-2 expression despite inducing NF-κB activation. In hepatocytes C/EBPα has been implicated as a transcriptional repressor of COX-2 induction (22). However, differences in responsiveness to PMA cannot be explained by the effects of C/EBPα, because both transformed and non-transformed cells failed to express this C/EBP isoform. Interestingly, non-transformed cells failed to express COX-2 despite high levels of the positive COX-2 regulator C/EBPβ. The failure to explain COX-2 induction by changes in NF-κB or C/EBP suggested that regulation might be post-transcriptional. This possibility was further supported by the failure of PMA to induce COX-2 promoter activity in either transformed or S-CYP15 cells. These findings, together with recent studies in intestinal cells (42), support an important role for ERK and p38 MAPK in the post-transcriptional regulation of COX-2 expression through the mechanism of mRNA stabilization.

These investigations demonstrate conditions under which adult hepatocytes may respond to tumor promoters with up-regulation of COX-2. Evidence of in vitro hepatocyte COX-2 expression strengthens prior in vivo studies suggesting that COX-2 up-regulation may be involved in HCC. Further studies must define the precise effectors of hepatocyte COX-2 gene expression, and determine the effects of COX-2 expression on heptocyte processes such as proliferation and apoptosis.

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