Inducible nitric oxide synthase upregulates cyclooxygenase-2
in mouse cholangiocytes promoting cell growth

Norihisa Ishimura, Steven F. Bronk, and Gregory J. Gores

Division of Gastroenterology and Hepatology, Mayo Clinic, College of Medicine, Rochester, Minnesota 55905

Submitted 30 December 2003; accepted in final form 14 February 2004

Ishimura, Norihisa, Steven F. Bronk, and Gregory J. Gores. Inducible nitric oxide synthase upregulates cyclooxygenase-2 in mouse cholangiocytes promoting cell growth. Am J Physiol Gastrointest Liver Physiol 287: G88–G95, 2004. First published February 19, 2004; 10.1152/ajpgi.00539.2003.—Both inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 (COX-2) have been implicated in the biliary tract carcinogenesis. However, it is not known whether these inflammatory mediators are induced by interdependent or parallel pathways. Because iNOS activity has been associated with diverse gene expression, the aim of this study was to determine whether iNOS induces COX-2. To address this objective, immortalized, but nonmalignant, murine cholangiocytes, 603B cells were employed for these studies. Both iNOS and COX-2 protein and mRNA were expressed in these cells. However, iNOS inhibition with either N-[3-(aminomethyl) benzyl]acetamide or stable transfection with an iNOS antisense construct inhibited COX-2 mRNA and protein expression, an effect that was reversed by NO donors. COX-2 mRNA expression in 603B cells was reduced by pharmacological inhibitors of the p38 MAPK and JNK1/2 pathways. In contrast, neither inhibitors of the soluble guanylyl cyclase inhibitor/protein kinase G nor p42/44 MAPK pathways attenuated COX-2 mRNA expression. Finally, 603B cells grew at a rate threefold greater than 603B-iNOS antisense cells. The low growth rate of 603B-iNOS antisense cells could be restored to near that of the parent cell line with exogenous PGE2. In conclusion, iNOS induces COX-2 expression in cholangiocytes, which promotes cell growth. COX-2 induction may contribute to iNOS-associated carcinogenesis.

Cholangiocarcinoma is a highly malignant disease originating from cholangiocytes, the epithelial cells lining the intra- and extrahepatic biliary tract (37). Chronic inflammatory disorders of the biliary tract predispose to the development of cholangiocarcinoma. For example, cholangiocarcinoma develops in ~10% of patients with primary sclerosing cholangitis, a chronic inflammatory strictureing disease of the biliary tract (3, 5, 6). The mechanisms by which inflammatory biliary disorders predispose to the development of cholangiocarcinoma are unfortunately obscure. Elucidation of these mechanisms may help provide rationale chemopreventive strategies (45).

Insight into the mechanisms linking inflammation to carcinogenesis can be obtained by determining which inflammatory mediators are expressed in both inflammatory and malignant biliary tract diseases. Two proteins associated with inflammation highly expressed in both inflammatory and malignant biliary tract diseases include inducible nitric oxide (NO) synthase (iNOS) (21, 23) and cyclooxygenase-2 (COX-2) (7, 10, 18). Both proteins may be induced by a variety of proinflammatory stimuli, have been implicated in carcinogenesis, and are retained in the malignant tissue, presumably because they provide a proliferative, survival, and/or angiogenic advantage for the cancer (24, 42). Although both iNOS and COX-2 may be induced independently, recent studies have suggested iNOS, via NO generation, may lead to COX-2 expression (34, 39, 43).

An understanding of the relationship between iNOS and COX-2 by cholangiocytes may provide insight into carcinogenesis of the biliary tree in chronic inflammatory disorders. Thus the mechanistic link between these two proteins is scientifically and clinically important.

The overall objective of this study was to determine whether iNOS induces COX-2 in cholangiocytes. To address this objective, the following questions were formulated: Is COX-2 expression iNOS dependent? If so, how does iNOS induce COX-2? Are alterations in growth iNOS and/or COX-2 dependent? To address these questions, immortalized, but nonmalignant, murine cholangiocytes were employed for these studies to permit genetic manipulation of iNOS. The results suggest iNOS induces COX-2 and cell growth by a p38 MAPK- and JNK1/2-dependent mechanism. These studies mechanistically link iNOS to COX-2 in biliary tract carcinogenesis by suggesting iNOS is upstream of COX-2. iNOS inhibition would appear to be the more proximal to target in chemoprevention of biliary tract carcinogenesis.

MATERIALS AND METHODS

Cell lines and culture condition. 603B cells, immortalized mouse cholangiocytes (17, 44), were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 5% CO2 incubator at 37°C. A 603B cell line genetically engineered to prevent iNOS expression was generated. The human iNOS cDNA sequence (L09210) was obtained from GenBank. The cDNA sequence between bp 207 and 641 was chosen, and forward and reverse primers were designed for this sequence with an iNOS antisense construct. After PCR, amplicons were cloned into a modified pBluescript plasmid vector and sequenced. A human iNOS-expressing construct was then engineered to prevent iNOS expression was generated. The results suggest iNOS induces COX-2 and cell growth by a p38 MAPK- and JNK1/2-dependent mechanism. These studies mechanistically link iNOS to COX-2 in biliary tract carcinogenesis by suggesting iNOS is upstream of COX-2. iNOS inhibition would appear to be the more proximal to target in chemoprevention of biliary tract carcinogenesis.

Address for reprint requests and other correspondence: G. J. Gores, Professor of Medicine, Mayo Clinic, College of Medicine, 200 First St. SW, Rochester, MN 55905 (E-mail: gores.gregory@mayo.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
trated, and the DNA was subjected to EcoRI digest. The fragment band was gel purified and ligated into a pLPX retroviral plasmid. TOP 10 cells were again transformed with the pLPX/iNOS DNA, cells propagated, and plasmid DNA was extracted. The plasmid DNA was digested with EcoRI, and the fragment’s presence was verified on a 1% agarose gel. DNA from positive clones was transfected into the viral packaging cell line GPE264.7 obtained by the calculation of total PGE2 production and quantity of cell 

**PGE2 assay.** Cells were plated at a density of 1 X 106 cells/well on six-well plates. After 24 h, medium was replaced in the absence or presence of reagents for 24 h. Supernatant (200 µl) PGE2 concentrations were measured using a PGE2 enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions. The amount of BrdU was spectrophotometrically quantified by immunoblot and nitrite analysis. This cell line was termed 603B-iNOS antisense (AS). The mouse macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in DMEM supplemented with 10% FBS.

**Immunoblot analysis.** Cells were lysed for 20 min on ice with lysis buffer (50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40; 0.25 mM sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 1 mg/ml aprotinin, leupeptin, and pepstatin; 1 mM Na3VO4; and 1 mM NaF). After a 15-min spin at 14,000 g, the supernatant was collected. Protein concentration was determined using the Bradford reagent (Sigma, St. Louis, MO) with BSA as the standard (4). Samples were resolved by 7.5 or 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and blotted with appropriate primary antibodies at a dilution of 1:1000. Horseradish peroxidase-conjugated secondary antibodies (Biosource International, Camarillo, CA) were used as a control for the Bradford reagent (Sigma, St. Louis, MO). Bound antibodies were visualized using chemiluminescent substrate (ECL, Amersham, Arlington Heights, IL) and exposed to Kodak X-OMAT film (Kodak, New Haven, CT). The primary antibodies include goat anti-COX-2 and goat anti-actin obtained from Santa Cruz Biotechnology (Santa Cruz, CA); mouse anti-iNOS obtained from Transduction Laboratories (Lexington, KY); rabbit anti-ACTIVE p38, anti-ACTIVE JNK1/2, and anti-ACTIVE p42/44 MAPK specific for the phosphorylated forms of p38 MAPK, JNK1/2, and p44/42 MAPK, respectively, were obtained from Promega (Madison, WI). Rabbit anti-p38 MAPK, anti-JNK1/2, and anti-p44/42 MAPK, were obtained from Cell Signaling Technology (Beverly, MA).

**Real time PCR.** Total RNA was obtained from whole cell lysate using the TRIzol Reagent (Invitrogen). For each RNA sample, a 10 µg- aliquot of cDNA was synthesized using oligo(dT) random primers and Moloney murine leukemia virus reverse transcriptase. After the reverse transcription reaction, the cDNA template was amplified by PCR with Taq polymerase (Invitrogen). COX-2, transforming growth factor-α (TGF-α), EGF, and heparin binding EGF-like growth factor (HB-EGF) mRNA were quantified using real-time PCR technology. Primers are as follows: COX-2, forward 5’-ATCCTGATGGGTTGATGAG-3’, reverse 5’-CCAGCACTTGCTGTTGAA-3’ (yielding a 337 bp-product), TGF-α, forward 5’-CTAGCGCTGGAATCTGGT-3’, reverse 5’-CGAGTGATGGTTGTCCTT-3’ (272 bp), EGF, forward 5’-GACCCCGAATCTCTAAACA-3’, reverse 5’-GGCTATACCGGAAAGCAA-3’ (466 bp), HB-EGF, forward 5’-TTTCTCCCAAAGCCCAAG-3’, reverse 5’-CAGATGAGCAAGGGACCA-3’ (315 bp). Universal 18S primers (Ambion, Austin, TX) were used as a control for RNA integrity. All PCR products were confirmed by gel electrophoresis. Real-time PCR was performed using the LightCycler (Roche Diagnostics, Mannheim, Germany) and SYBRgreen as the fluorophore (Molecular Probes, Eugene, OR). The result was expressed as a ratio of product copies per microliter to copies per microliter of housekeeping gene 18S from the same RNA (respective cDNA) sample and PCR run.

**Cell proliferation and DNA synthesis assays.** Proliferation was assessed by the 3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium tetrazolium assay (MTS; Promega), which measures the number of viable cells. Cells were plated at a density of 2.5 105 cells/well on 96-well plates. After 24 h, medium was replaced in the absence or presence of reagents for 3 more days. Twenty microtiter of MTS solution reagent were added to 100 µl of culture medium of each well. After incubation of 4 h at 37°C, the plates were read in a microplate autoreader at a wavelength of 490 nm. The result was expressed as a mean optical density (OD) of replicate wells. DNA synthesis was determined by measuring the incorporation of 5-bromo-2-deoxyuridine (BrdU) into DNA using a cell proliferation kit (BrdU cell proliferation assay; Exalpa Biological, Watertown, MA) according to the manufacturer’s instructions. The amount of BrdU was spectrophotometrically quantified with a microplate spectrophotometer at a wavelength of 450 nm with reference at a wavelength of 570 nm.

**Statistical analysis.** All data represent at least three independent experiments and are expressed as the means ± SD unless otherwise indicated. Differences between groups were compared using ANOVA for repeated measures and a post hoc Bonferroni test to correct for multiple comparisons. Differences with P < 0.05 were considered significant.

**Reagents.** The selective iNOS inhibitor N-[3-(aminomethyl)benzyl]acetamide (1400W), NO donors S-nitroso-N-acetyl-d,l-penicillamidine (SNAP), diethylenetriamine-nonoate (DETA-NONO), and S-nitroso-L-glutathione (GSNO), soluble guanylyl cyclase (sGC) inhibitor 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ), and COX-2 inhibitor NS398 were purchased from Cayman Chemical (Ann Arbor, MI), PKG inhibitor KT5823, p38 MAPK inhibitor SB203580, and COX-2 inhibitor SP600125 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). LPS, TNF-α, and PGE2 were purchased from Sigma-Aldrich (St Louis, MO).

**RESULTS**

**603B, but not 603B-iNOS AS, express iNOS.** The two cell lines were examined for iNOS expression by immunoblot analysis. The parent 603B cells constitutively expressed iNOS (Fig. 1A), although cellular protein levels were reduced compared with the macrophage cell line RAW264.7. However, iNOS protein expression could be substantially upregulated by treating the 603B cells with the inflammation mediators LPS (10 µg/ml) plus TNF-α (10 ng/ml) for 24 h (Fig. 1B). After stimulation, cellular iNOS protein levels were actually greater than those observed in the untreated RAW 264.7 cells. In contrast, iNOS expression was not identified by immunoblot analysis in the 603B-iNOS AS cells either under basal or stimulated conditions (Fig. 1, A and B). Nitrate and nitrite

http://ajpgi.physiology.org/
levels in the culture medium in the 603B-iNOS AS cells also remained at very low levels compared with those in the 603B cells (0.14 vs. 0.45 μM, respectively). Thus the stably transfected iNOS antisense construct effectively inhibits iNOS expression allowing us to selectively compare and contrast the effects of iNOS expression on cholangiocyte biology, especially COX-2 induction.

**COX-2 expression in cholangiocytes is iNOS-dependent.**

Next, COX-2 expression in the 603B and 603B-iNOS AS cells was examined by immunoblot analysis. COX-2 protein was expressed constitutively by the 603B cells but was only minimally expressed by the 603B-iNOS AS cells as assessed by immunoblot analysis (Fig. 2A). Indeed, COX-2 protein levels were fourfold greater in 603B vs. 603B-iNOS AS cells. The results of the immunoblot analysis were confirmed by quantitating COX-2 mRNA by real-time PCR and measuring the PGE2 production. COX-2 mRNA expression was sevenfold greater in 603B vs. 603B-iNOS AS cells. The amount of PGE2 production was 40-fold higher in 603B vs. 603B-iNOS AS cells (Fig. 2C). Importantly, treatment with the NO donor GSNO 1 mM for 14 h, increased COX-2 mRNA expression fourfold in the

---

**Fig. 1.** Immunoblot analysis of inducible nitric oxide synthase (iNOS) in 603B and 603B-iNOS antisense (AS) cells. Mouse macrophage cell line RAW264.7 cells were used as a positive control of iNOS protein. iNOS protein (130 kDa) was detected in 603B cells and upregulated by LPS + TNF-α stimulation. In contrast, iNOS expression was not identified in 603B-iNOS AS cells under the basal (A) or stimulated condition (B); treated with 10 μg/ml of LPS and 10 ng/ml of TNF-α for 24 h.

**Fig. 2.** A: immunoblot analysis of cyclooxygenase-2 (COX-2) in 603B and 603B-iNOS AS cells. COX-2 protein (72 kDa) was expressed constitutively by the 603B cells but was only minimally expressed by the 603B-iNOS AS cells. COX-2 protein was quantified relative to β-actin by densitometry. COX-2 protein levels were 4-fold greater in 603B vs. 603B-iNOS AS cells (*P < 0.05; n = 4 for each group). B: COX-2 mRNA expression in 603B and 603B-iNOS AS cells. COX-2 mRNA was quantitated by real-time PCR. The expression was normalized as a ratio using 18S as a housekeeping gene. A value of 1 for this ratio was arbitrarily assigned to the data obtained from 603B-iNOS AS. COX-2 mRNA expression was 7-fold greater in 603B vs. 603B-iNOS AS cells (*P < 0.01, compared with 603B activity, n = 3 for each group). Although stimulation with LPS/TNF-α induced COX-2 mRNA expression in 603B cells (**P < 0.05, compared with basal activity, n = 3 for each group), COX-2 mRNA was not increased even under stimulated conditions in 603B-iNOS AS cells. C: PGE2 production in 603B and 603B-iNOS AS cells. PGE2 concentrations in the supernatant were measured using a PGE2 enzyme immunoassay kit. The data are expressed as picograms of PGE2 per microgram of protein. The amount of PGE2 production was 40-fold greater in 603B vs. 603B-iNOS AS cells. (*P < 0.01; n = 3 for each group). D: NO donor-induced COX-2 protein in 603B-iNOS AS cells. COX-2 protein expression was greatly increased by S-nitroso-L-glutathione (GSNO; 500 μM) in the 603B-iNOS AS cells in a time-dependent manner.
603B-iNOS AS cells (data not shown). COX-2 protein expression was also greatly increased by GSNO in the 603B-iNOS AS cells in a time-dependent manner (Fig. 2D). To further explore the relationship between iNOS and COX-2 expression, iNOS inhibitors and NO donors were employed with the 603B cells. The selective iNOS inhibitor 1400W decreased COX-2 protein and mRNA levels in 603B cells (Fig. 3, A and B). Consistent with these observations, the NO donors SNAP or DETA-NO upregulated COX-2 mRNA and protein expression, respectively. Likewise, the amount of PGE2 was significantly lower in the 1400W-treated group than in controls, and SNAP increased PGE2 production (Fig. 3C). The COX-2 inhibitor NS398 partially reduced SNAP-mediated PGE2 generation, indicating that NO-stimulated PGE2 formation was, in part, COX-2 dependent. Thus iNOS expression and activity enhance COX-2 expression and function in cholangiocytes.

iNOS mediates COX-2 expression by p38 MAPK and JNK1/2 signaling pathways. Recently, iNOS and NO have been demonstrated to regulate cellular gene expression by a variety of kinase-signaling pathways (16, 19). In particular, MAPK pathways have been shown to upregulate COX-2 mRNA expression (8, 13, 26). Therefore, we next determined whether NO-dependent COX-2 expression was MAPK related in the 603B-iNOS AS cells. All three MAPK proteins p38 and p44/42 and JNK1/2 were promptly phosphorylated after treatment of the cells with GSNO (Fig. 4A). Inhibition of p38 MAPK with SB203580 or JNK1/2 inhibition with SP600125 reduced COX-2 expression (Fig. 4B and 5); inhibition of MEK1 with PD98059 did not significantly diminish COX-2 mRNA expression. One of the major targets of NO is the sGC/PKG pathway; however, neither the sGC inhibitor ODQ nor the PKG inhibitor KT5823 attenuated COX-2 mRNA expression (Fig. 5) despite using concentrations of the inhibitors that have been established to block their activation (20, 46). Thus iNOS appears to induce COX-2 expression by a p38 MAPK and JNK1/2-mediated pathway.

iNOS stimulation of cell growth is COX-2 mediated. As a physiological endpoint to gauge the importance of iNOS and COX-2 on cholangiocyte biology, we quantitated cell growth and DNA synthesis. 603B cells grew at a rate greater than that of the 603B-iNOS AS cell line (Fig. 6A). This was despite similar expression of growth-stimulating ligands by the two

![Fig. 3. A: effects of NO on COX-2 protein expression in 603B cells. 603B cells were incubated for 12, 24, and 48 h with 1400W (50 μM) or DETA-NO (1 μM). COX-2 protein expression was evaluated by immunoblot analysis. COX-2 protein expression was greatly decreased in a time-dependent manner by 1400W treatment. Meanwhile, COX-2 protein expression was elevated at 24 h after DETA treatment. B: effects of NO on COX-2 mRNA expression in 603B cells. 603B cells were incubated for 8 h with 1400W (50 μM) or SNAP (300 μM). COX-2 mRNA expression was quantitated by real-time PCR. The expression was normalized as a ratio using 18s as a housekeeping gene. A value of one for this ratio was arbitrarily assigned to the data obtained from control. Data were expressed as mean ± SD. COX-2 mRNA expression was significantly decreased by N-[3-(aminomethyl)benzylacetamidine (1400W). This effect was reversed by the addition of 5-nitroso-N-acetyl-d,L-penicillamine (SNAP) (*P < 0.05, **P < 0.01; n = 3 for each group). C: effect of NO on PGE2 production. PGE2 concentrations in the supernatant were measured using a PGE2 enzyme immunoassay kit. The data were expressed as picograms of PGE2 per microgram of protein. The amount of PGE2 production was significantly lower in the 1400W-treated group than in control, and SNAP increased the PGE2 production. NS398 partially reduced SNAP-mediated PGE2 generation. (*P < 0.05, **P < 0.01; n = 2 for each group).]
cell lines. Indeed, expression of TGF-β, EGF, and HB-EGF was virtually identical in 603B vs. 603B-iNOS AS cells (Fig. 6B). DNA synthesis in 603B cells could be reduced by the COX-2 inhibitor NS-398, an effect partially reversed with PGE₂, a COX-2-dependent metabolite (Fig. 7A). Consistent with these data, PGE₂ also stimulated cell growth in the 603B-iNOS AS cells (Fig. 7B). These data suggest that iNOS stimulates cholangiocyte cell growth by a COX-2/PGE₂-mediated pathway.

DISCUSSION

The principal findings of this study relate to expression of iNOS and COX-2 in biliary epithelia. The observations demonstrate that 1) iNOS via NO generation induces COX-2; 2) iNOS-mediated COX-2 induction is, in part, p38 MAPK and JNK1/2 dependent; and 3) iNOS-mediated cell growth is dependent on COX-2 generation of PGE₂. These results are germane to inflammation-related carcinogenesis of the biliary tract and suggest that iNOS inhibition would be the most proximal and optimal target for chemoprevention.

Both iNOS and COX-2 are expressed in inflammatory diseases of the biliary tract and in the cancers arising from these diseases (7, 10, 18, 21, 23). Because both enzymes have been strongly implicated in carcinogenesis and cancer progression, it is important to understand the relationship between their expressions. Our current data suggest iNOS expression via NO generation induces functional COX-2, implying a strong interrelationship between the expression of these two proteins. This
latter data are consistent with our findings in which neither the sGC inhibitor ODQ nor the PKG inhibitor KT5823 diminished COX-2 mRNA expression. Recently, NO has been shown to effect expression of diverse genes as assessed by transcription profiling employing a microarray hybridization approach (19). cGMP-independent responses were identified involving MAPK, NF-κB, PKC, p53, and phosphatidylinositol-3 kinase pathways (12, 27, 31). We observed that both a p38 MAPK and a JNK1/2 inhibitor reduced COX-2 expression. Consistent with these data, Guan et al. (13) demonstrated that the activation of both p38 MAPK and JNK1/2 signaling cascades were required for IL-1β-induced COX-2 expression. Although the MEK1 inhibitor PD98059 has been reported to reduce COX-2 expression in human umbilical vein endothelial cells by the NO donor SIN-1 (9), this inhibitor did not significantly reduce COX-2 mRNA expression in the cholangiocyte cell lines. Thus, as has been observed with various stimuli in other cell types, the p38 MAPK (8, 14) and JNK1/2 (2, 13) pathways contribute to COX-2 expression by NO in cholangiocytes.

Our data suggest iNOS via COX-2 induction enhances growth of cholangiocytes. Indeed, the parent cell line grew threefold faster than the cells stably transfected with iNOS antisense, which do not express COX-2. Consistent with these data, growth of 603B cells was inhibited by the COX-2 inhibitor NS398 and partially restored in the presence of the inhibitor by exogenous PGE2. Thus the ability of iNOS to modulate growth is largely dependent on COX-2 induction. The ability of COX-2 to enhance cell growth has been amply documented (15). Although our data are at odds with those of Liu et al. (30), who demonstrated that the NO donor GNSO inhibits cell growth in colon cancer cell lines despite COX-2 expression, exogenous NO donors may not always duplicate the concentrations, regulation, and cellular compartmentation conferred by iNOS-mediated NO generation. Collectively, our interpretation of our data is supported by several observations. COX-2 expression under both basal and stimulated (TNF-α + LPS) conditions was considerably reduced in cells stably transfected with an iNOS antisense construct compared with the parent cell line. Likewise, inhibition of iNOS with a selective pharmacological inhibitor, 1400W, also reduced COX-2 expression in the 603B cells. Consistent with these data, the NO donors SNAP or DETA-NO enhanced COX-2 mRNA and protein expression and PGE2 generation. These observations are consistent with several studies demonstrating that NO stimulates prostanooid synthesis via COX-2 activity (33, 36, 38). For example, in colonic epithelial cells, the NO donors SNAP and NOR-1 increased both COX-2 mRNA transcription and protein synthesis (32). In aggregate, these in vitro studies suggest iNOS, whose expression is induced by inflammation, secondarily induces COX-2 in diseased tissue.

NO has been suggested to mediate transcriptional changes in gene expression via both cyclic GMP-dependent and independent pathways (9, 39, 43). In squamous carcinoma cells, NO increases COX-2 activity, and this effect correlates with cellular cGMP levels (11). However, Salvemini et al. (39) demonstrated that NO enhances COX-2 activity through a cGMP-independent pathway in a mouse macrophage cell line.

Fig. 7. A: 5-bromo-2′-deoxyuridine (BrdU) uptake in 603B cells in the presence of NS398, NS398 + PGE2, and control groups. DNA synthesis was assessed after 2 days by a BrdU assay kit. The amount of BrdU was spectrophotometrically quantified with a microplate spectrophotometer at a wavelength of 450 nm with reference at a wavelength of 570 nm. DNA synthesis was inhibited by NS398 (100 μM), and the inhibitory effect of NS398 was abolished by pretreated PGE2 (10 μM; *P < 0.05; n = 3 for each group). B: BrdU uptake in 603B-iNOS AS cells in the presence of NS398, NS398 + PGE2, and control groups. DNA synthesis was assessed after 4 days by a BrdU assay kit. In contrast to 603B cells, DNA synthesis was not inhibited by NS398 (100 μM) in 603B-iNOS AS cells. Moreover, PGE2 (10 μM) stimulated DNA synthesis (*P < 0.01; n = 3 for each group).

Fig. 8. Proposed model of signaling pathways for iNOS-mediated COX-2 induction in mouse cholangiocytes. iNOS appears to induce COX-2 expression by the p38 MAPK and JNK1/2-mediated pathway. NO inhibitor can diminish COX-2 expression through the reduction of NO. COX-2 might play a central role in production of PGE2, and the specific inhibition of COX-2 inhibits proliferation of cholangiocytes via suppression of PGE2 production. In these processes, the continuous upregulation of COX-2 by iNOS may facilitate the tumorgenesis of nontumorigenic cells.
data suggest that by inducing COX-2, iNOS indirectly mediates cell growth of cholangiocytes.

In summary, we have shown that iNOS induces COX-2 expression in mouse cholangiocytes likely through a p38 MAPK and JNK1/2 pathway (Fig. 8). These findings lend biological plausibility to the hypothesis that iNOS-derived NO may play an important role in the tumorigenesis of cholangiocytes. Not only would NO promote carcinogenesis by allowing accumulation of DNA mutations by inhibiting DNA repair (22, 23, 25), but it would also induce COX-2. Induction of COX-2 has been strongly implicated in epithelial cell carcinogenesis by promoting cell growth, blocking apoptosis, and in promoting angiogenesis (28, 29, 41). Although both proteins could independently promote carcinogenesis, given that iNOS induces COX-2, iNOS inhibition would appear to be the most proximal and optimal target for chemoprevention of inflammation-related cholangiocarcinoma. Consistent with this concept, genetic and pharmacological inactivation of iNOS is chemopreventive in animal models of colon and lung cancer (35, 40). Inhibition of iNOS as a chemopreventive strategy in biliary tract diseases, therefore, merits further consideration.

ACKNOWLEDGMENTS

The secretarial assistance of Erin Bungum is gratefully acknowledged. We thank Dr. Vijay Shah for performing the media NO determinations and Nate Wenreb for superb technical guidance.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-59427 and the Mayo Foundation.

REFERENCES