Kupffer cell-derived cyclooxygenase-2 regulates hepatocyte Bcl-2 expression in choledocho-venous fistula rats

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HEPATOCYTE APOPTOSIS IS A common histopathological feature in a wide variety of liver diseases and contributes to liver injury in these disease states (7, 14, 22). Hepatocytes might be especially prone to apoptosis because this cell type expresses multiple death receptors, i.e., Fas, tumor necrosis factor receptor type 1, and TRAIL-R1 and -R2 (5), but does not express Bcl-2, a 25-kDa protein that is a potent repressor of apoptosis (4). This precarious balance (expression of death receptors in the absence of Bcl-2 expression) may render the hepatocyte uniquely prone to apoptosis. Indeed, Bcl-2 transgenic mice are resistant to hepatocyte apoptosis and liver injury during treatment with agonistic anti-Fas antisera and tumor necrosis factor-α (2, 14). Moreover, transduction of hepatocytes with Bcl-2 expressing adenovirus vectors also protects hepatocytes from ischemia-reperfusion injury (1). These data suggest that strategies to induce endogenous expression of Bcl-2 in hepatocytes could potentially prove therapeutically useful in the treatment of human liver diseases.

We (13) have previously demonstrated de novo hepatocyte expression of Bcl-2 in the bile duct-ligated rat, a model of bile flow impairment or cholestasis. Koga and co-workers (11) have also demonstrated Bcl-2 expression in hepatocytes of patients with primary biliary cirrhosis, a chronic cholestatic liver disease. These observations suggest that factors involved in cholestasis may induce hepatocyte Bcl-2 expression.

Prostanoids, especially PGE2, have been shown to modulate apoptosis and induce expression of Bcl-2 in non-hepatic cell types and are known to be secreted into bile (18, 23). Alterations in prostanoid metabolism during cholestasis are therefore a potential mechanism mediating Bcl-2 expression. Prostanoids are generated by cyclooxygenase (COX), also referred to as PG endoperoxide synthase. COX has been identified in two isoforms: COX-1 and COX-2 (8, 20). The COX-active site in both COX isoforms is formed by a main channel and a side pocket. In the COX-1 isoform, this side pocket contains an isoleucine that prevents the selective COX-2 inhibitor from binding. In contrast, both nonselective nonsteroidal anti-inflammatory drugs and selective inhibitors of COX-2 enter into the side pocket of the COX-2 isoform, causing inhibition of this enzyme (17). COX-1 is constitutively expressed in most cell types whereas COX-2 is induced by cytokines, growth factors, and tumor promoters (12). COX-2 expression with generation of prostanoids has been mechanistically linked to Bcl-2 expression (18). Despite its potential importance, information is lacking on COX-2 expression in the liver during perturbations found in the pathophysiological events observed in cholestasis.

The overall objective of this study was to determine if COX-2 was expressed in the liver and if the induction of COX-2 was associated with the modulation of Bcl-2

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expression during conditions resulting in high concentrations of biliary constituents. We chose the choledocho-venous fistula (CVF) rat model for these experiments, because it duplicates the high serum and hepatic concentrations of biliary compounds observed in cholestasis in the absence of inflammation or the pressure phenomenon observed in bile duct-ligated rats. Our specific goals were to answer the following questions using the CVF rat model:

1) Does hepatocellular expression of Bcl-2 occur?
2) Is COX-2 induced in the liver?
3) Does COX-2 regulate hepatocellular expression of Bcl-2?

Collectively, the results of the current study demonstrate that COX-2 is induced in Kupffer cells within the CVF rat and is associated with hepatocyte Bcl-2 expression. Inhibition of COX-2 catalytic activity reduces Bcl-2 expression. These data suggest Kupffer cell-derived prostanoids may regulate Bcl-2 expression in the hepatocyte. Modulation of liver prostanoid metabolism maybe a strategy to induce hepatocellular expression of Bcl-2 to reduce apoptosis in disease states.

**EXPERIMENTAL PROCEDURES**

**CVF rats.** Animals were cared for using protocols approved by the Mayo Clinic Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (200–300 g) were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg body wt). The peritoneal cavity was opened through a midline incision, and the common bile duct, the right kidney, and its renal vein were all identified. The common bile duct was then cut and the distal end ligated. A 10-cm-long polyethylene tubing catheter (ID, 0.58 mm; OD, 0.965 mm; Becton Dickinson) was placed in the proximal bile duct and secured with 5-0 silk (Ethicon) sutures. The distal end was used to cannulate the right renal vein, and its tip was advanced distally into the inferior vena cava, aimed at the superior vena cava. A right nephrectomy was then performed. Controls underwent a sham nephrectomy operation that consisted of exposure of common bile duct, but not cannulation or ligation, and right nephrectomy. After 1 wk, the animals were killed and liver tissue and blood samples obtained.

**Hepatocyte and Kupffer cell isolation.** Hepatocytes and Kupffer cells were isolated from CVF and sham-operated
rats. Hepatocytes were isolated as we (21) previously described in detail. Kupffer cells were isolated by in situ digestion and cell separation on a discontinuous gradient of arabino-galactan (Larcoll, Sigma Chemical, St. Louis, MO) followed by selective adherence as described previously by Friedman and Roll (6). The viability of isolated hepatocytes was ≥90% and that of Kupffer cells ≥95%. Cell lysates from hepatocytes were obtained immediately after isolation and from Kupffer cells 12 h after plating.

**Measurement of serum bile acids, alanine aminotransferase, and creatinine.** Serum bile acids, alanine aminotransferase (ALT), and creatinine levels were measured enzymatically using a commercially available kit according to the manufacturer’s suggested protocol (Sigma Diagnostics kit no. 450-A for bile acids, no. 555-A for creatinine, and no. 505-A for ALT).

**RNA isolation and RNase protection assay for Bcl-2.** Total cellular RNA was isolated using Tri-Reagent-RNA isolation reagent TR-118 (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s suggested protocol. Bcl-2 mRNA expression was determined by a multiprobe RNase protection assay using the Riboquant kit (Pharmigen, San Diego, CA) with the rAPO-1 multiprobe template.

**Immunoblot analysis for Bcl-2 and Cox-2.** The liver tissue was homogenized on ice in a buffer containing 10 mmol/l HEPES, 150 mmol/l NaCl, 1% Nonidet P-40, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenates were submitted to two cycles of freezing, thawing, and vortexing and then centrifuged at 12,000 rpm for 15 min at 4°C. After centrifugation, the supernatants were collected, and the protein concentration was measured using the Bradford assay (Sigma Chemical). Proteins were separated by electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Nitrobind, Micron Separation, Westboro, MA) by electroblotting overnight at 4°C. The membranes were blocked with 5% (wt/vol) skim milk in 20 mmol/l Tris base-0.5 mol/l sodium chloride, plus 0.05% Tween 20, pH 7.0 (TTBS), for 1 h to prevent nonspecific binding, then incubated for 1 h using a 1:1,000 dilution of mouse monoclonal anti-Bcl-2, 1:1,000 dilution of goat polyclonal anti-Cox-2 antisera, or 1:1,000 dilution of a goat polyclonal anti-β-actin antisera (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then washed three times in TTBS for 15 min each and incubated with a 1:4,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Biosource International, Camarillo, CA) or a 1:5,000 dilution of swine anti-goat IgG for 1 h. After three washes in TTBS for 10 min each, blots were developed by the enhanced chemiluminescence system (Amersham, Arlington Heights, IL) and exposed to imaging film (X-OMAT, Kodak, Rochester, NY). The images were analyzed using a densitometer (model GS-700; Bio-Rad Laboratories, Hercules, CA) and Molecular Analyst software (Bio-Rad).

**Immunohistochemistry for Bcl-2.** Liver tissue (5 × 5 mm) was immersion fixed in freshly prepared 4% paraformaldehyde in PBS for 24 h at 4°C. The tissue blocks were embedded in Tissue Path (Curtin Matheson Scientific, Houston, TX). Tissue sections (4 μm) were prepared using a microtome (Reichert Scientific Instruments, Buffalo, NY) and placed on glass slides. The sections were deparaffinized in xylene, rehydrated in ethanol, and washed in tap water and PBS. Subsequently, endogenous peroxidases were blocked with 0.3% hydrogen peroxide in 80% methanol for 20 min at room temperature. The slides were washed two times in PBS for 5 min per wash and blocked for 20 min with 0.2% normal horse serum, 0.3% Triton X-100, and 0.5% BSA in PBS. The horse serum solution was removed, and the slides were incubated for 1 h in a 1:40 dilution of mouse monoclonal anti-Bcl-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS at 4°C. After 1 h incubation, the slides were washed two times in PBS (5 min/wash) and developed using the Vectastain Elite avidin-biotin complex kit (Vector Laboratories, Burlingame, CA). The reaction was visualized using the diaminobenzidine substrate kit for peroxidase (kit SK-4100, Vector Laboratories) according to the manufacturer’s suggested protocol. After treatment with the diaminobenzidine solution, the slides were rinsed in tap water and counterstained with hematoxylin and eosin. The slides were then dehydrated in ethanol, rinsed with xylene, and mounted in Permount (Fisher Scientific, Fair Lawn, NJ).

**Hematoxylin and eosin.** Paraffin slides (4 μm) of liver specimens from sham nephrectomy, CVF, and nimesulide-treated CVF rats were prepared in the same manner as described above. After the slides were deparaffinized with xylene and rehydrated in alcohol, hematoxylin and eosin staining was performed. Slides were mounted in Permount after ethanol dehydration and xylene rinse.

**Statistical analysis.** All data are expressed as means ± SD from at least three separate experiments. Differences be-
between groups were compared using ANOVA for repeated measures and a post hoc Bonferroni test for multiple comparisons. All statistical analyses were performed using Instat Software (GraphPAD, San Diego, CA).

RESULTS

Establishment and validation of CVF rat model. After we developed the CVF procedure, we did not observe operative mortality, and all animals survived until killed on the seventh day. We initially confirmed that CVF rats have high serum concentrations of bile acids without evidence of biliary obstruction. Total serum bile acid levels were 70-fold greater in CVF than nephrectomy rats (708±193 vs. 11±8 μM) (Fig. 1). These serum bile acid concentrations are comparable to those observed in the bile duct-ligated rat (Fig. 1). At the time of animal death, the biliary-venous catheter was cut, and bile flow was evaluated. In all animals, bile flow was observed at the cut end of the catheter, demonstrating its patency. In the CVF rats, serum ALT values were normal and liver histology demonstrated normal liver architecture with no inflammation or increased fibrosis (Fig. 1). Consistent with previous studies (9) using this model, the serum bilirubin values were 15.1±2.2 mg/dl and serum alkaline phosphatase values were 3.5-fold greater than controls (359±16 vs. 103±19 U/l; \( P < 0.05 \)). Because we utilized the COX-2 inhibitor nimesulide in later experiments, we also assessed the effect of this drug on serum bile acid concentrations, serum ALT values, and liver histology. Serum bile acid concentrations were similar in untreated and nimesulide-treated CVF rats (Fig. 1). Nimesulide also had no effect on serum ALT values or liver histology (Fig. 1). Finally, we measured serum creatinine to assess renal function, because our model includes a right nephrectomy. Serum creatinine levels were different and remained <1 mg/dl in controls, animals undergoing a right nephrectomy, and untreated and nimesulide-treated CVF rats (data not shown). Thus as shown by Hardison et al. (9), CVF rats have a high circulating concentration of bile acids without biliary obstruction or alterations in renal function.

Hepatocytes express Bcl-2 in CVF rats. Expression of hepatic Bcl-2 mRNA and protein was assessed in CVF and sham nephrectomy rats by the RNase protection assay and immunoblot analysis, respectively. Bcl-2 mRNA expression was observed in the livers of CVF animals, but only minimal expression was observed in sham nephrectomy rats (Fig. 2). In accordance with the results of the RNase protection assay data, immunoblot analysis demonstrated a significant increase in Bcl-2 protein expression in the liver of CVF rats compared with sham nephrectomy rats (Fig. 2). To determine which liver cell type(s) expressed Bcl-2 in the CVF rat, we performed 1) immunobots on isolated Kupffer cells and hepatocytes and 2) immunohistochemistry in fixed liver specimens. Bcl-2 was found in hepatocytes but not in Kupffer cells of CVF rats as assessed by both approaches (Fig. 3). In contrast, no
significant Bcl-2 immunoreactivity was detected in the hepatocytes of sham nephrectomy rats by either approach (Fig. 3). With immunohistochemistry, Bcl-2 expression was noted to be predominantly in zone 3 of the hepatic acinus, the region of the liver where Kupffer cells are more phagocytically active (24). Thus our results demonstrate that in CVF rats there is de novo hepatocyte expression of Bcl-2.

**COX-2 is expressed in Kupffer cells of CVF rats.** We assessed hepatic expression of COX-2 in CVF and sham nephrectomy rats by immunoblot analysis. COX-2 protein was strongly induced in the liver of CVF rats but not in sham nephrectomy animals (Fig. 4). In contrast to Bcl-2, COX-2 protein was identified in Kupffer cells but not hepatocytes isolated from CVF rats. These data demonstrate for the first time, to our knowledge, that high circulating concentrations of biliary constituents are associated with COX-2 induction in hepatic Kupffer cells.

**Does COX-2 regulate Bcl-2 expression in CVF rats?** To determine if prostanoids generated by COX-2 were responsible for the increased Bcl-2 expression in CVF animals, rats were treated with the selective COX-2 inhibitor nimesulide. The animals were treated with a calculated dose of 60 mg·kg\(^{-1}\)·day\(^{-1}\) of nimesulide (1 g nimesulide/kg chow). Animals received nimesulide 2 days before surgery to ensure that they would tolerate the diet and throughout the duration of the postoperative period. The diet was well tolerated, and there was no significant weight loss during the treatment period (data not shown). The rats consumed an average of 20 mg chow/day, matching our calculated treatment dose. However, hepatic Bcl-2 protein expression was fourfold lower in the liver of nimesulide-treated rats compared with untreated CVF rats (Fig. 5). Thus inhibition of COX-2 reduces hepatic expression of Bcl-2, suggesting

**Fig. 4.** Cyclooxygenase-2 (COX-2) protein is increased in the liver Kupffer cells of CVF rats. *A:* immunoblot analysis for COX-2, using \(\beta\)-actin as a control for protein loading, was performed using whole liver homogenates. Quantitative comparisons between groups of animals were made by determining COX-2-to-\(\beta\)-actin ratio of the immunoreactive areas by densitometry. *B:* in separate experiments, hepatocytes and Kupffer cells were isolated from CVF animals as described in EXPERIMENTAL PROCEDURES. Immunoblots were performed for COX-2 using cell lysates. COX-2 protein was readily identified in Kupffer cells but not in hepatocytes.

**Fig. 5.** Bcl-2 protein is decreased in CVF rats treated with the COX-2 inhibitor nimesulide. Immunoblot analysis for Bcl-2, using \(\beta\)-actin as a control for protein loading, was performed with liver homogenates of 7-day CVF and nimesulide-treated CVF rats (60 mg·kg\(^{-1}\)·day\(^{-1}\)). Comparisons between groups of animals were made by determining the Bcl-2-to-\(\beta\)-actin ratio of the immunoreactive area by densitometry.
a mechanistic link between prostanoids and Bcl-2 expression in CVF animals.

DISCUSSION

The original observations reported here relate to the mechanisms responsible for Bcl-2 expression in hepatocytes during cholestasis. Our results directly demonstrate the following in the CVF rat: 1) there is expression of Bcl-2 by hepatocytes; 2) there is expression of COX-2 by Kupffer cells; and 3) inhibition of COX-2 catalytic activity with nimesulide reduces Bcl-2 expression. Collectively, these observations suggest prostanoids regulate hepatocyte Bcl-2 expression.

For these studies, we used the CVF rat model. Serum bile acid concentrations, serum bilirubin, urinary bile acid secretion, and bile acid pool size are similar in the CVF animal compared with the bile duct-ligated animal (9). We confirmed that serum bile acids are elevated in this model in the absence of significant changes in liver histology and serum ALT values. Thus the CVF rat is a useful model for evaluating the pathophysiological consequences of high circulating concentrations of biliary constituents without interrupting bile flow, increasing biliary pressure, or inducing the changes in liver histology that occur in the bile duct-ligated rat (9).

Using the CVF rat, we have demonstrated that high concentrations of biliary constituents are associated with hepatocyte induction of Bcl-2 in vivo. The induction of Bcl-2 by high circulating concentrations of biliary constituents required COX-2 activity. Indeed, inhibition of COX-2 activity markedly reduced Bcl-2 protein expression, suggesting the induction of Bcl-2 was regulated by COX-2-mediated prostanoid generation. Furthermore, Bcl-2 expression was greatest in zone 3 where Kupffer cells are known to be more active (24). Prostanoids are known to regulate Bcl-2 expression in nonhepatic cells (15, 18). Growth in a colon cancer cell line was reduced by a COX-2 inhibitor, and this suppression was reversed by PGE2, which concomitantly induced Bcl-2 expression (18). Similarly, a COX-2 inhibitor induced apoptosis in a human prostate cell line, and this induction was associated with decreased expression of Bcl-2 protein (15). Thus now it appears from our data that prostanoids may also be able to induce hepatocyte Bcl-2 expression.

Bcl-2 is a potent antiapoptotic protein not expressed in hepatocytes under physiological conditions (4). The potential importance of inducing Bcl-2 expression in the liver has been shown in Bcl-2 transgenic mice models (1, 2). In these studies, liver cells are resistant to apoptosis, and more importantly, hepatic injury is reduced. Although transgenic mice greatly overexpress Bcl-2, causing uncertainty about the physiological relevance of these studies, we (13) have shown previously that hepatocyte expression of Bcl-2 in bile duct-ligated rats renders the hepatocytes resistant to bile salt-induced apoptosis. Thus physiological induction of Bcl-2 appears to be sufficient to inhibit apoptosis in hepatocytes and may represent an adaptive response to limit injury in diseases states such as cholestasis.

We observed that hepatic induction of COX-2 expression in the CVF rat occurred in Kupffer cells but not in hepatocytes. The failure of hepatocytes to express COX-2 has been previously reported (3) and attributed to the high levels of C/enhancer binding protein-α in this cell type. COX-2 expression in Kupffer cells has previously been identified (16) in alcoholic liver disease in the rat. Furthermore, additional studies (10) have also found COX activity in Kupffer cells. The induction of COX-2 by Kupffer cells is consistent with their macrophage lineage of COX-2 expression in proinflammatory states (25). The mechanism for COX-2 induction in the CVF rat remains unclear. Although serum concentrations of bile acids are elevated in the CVF rat and dihydroxy bile acids have been shown to activate the transcription of COX-2 (26), Kupffer cells are not known to transport bile acids. Furthermore, we were unable to induce COX-2 in Kupffer cells by exposing them directly to bile acids (data not shown). Perhaps other biliary constituents or additional inflammatory signals stimulated by bile acids result in COX-2 induction by Kupffer cells.

The data in our current study suggest that induction of Bcl-2 in hepatocytes may be modulated by hepatic prostanoids. Prostanoids have been shown (19) to be hepatoprotective in liver injury in animal models and humans. Our data suggest the cytoprotective effects of prostanoids may be beneficial, in part, by inducing expression of Bcl-2. Further study of the effects of prostanoids on Bcl-2 expression in hepatocytes will not only help provide a more complete picture of the role of prostanoids in modulating Bcl-2 expression, but also help evaluate prostanoids as potential pharmacological agents for reducing cholestatic liver injury.

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