Bile acid-mediated induction of cyclooxygenase-2 and Mcl-1 in hepatic stellate cells

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Received 12 February 2006
Available online 21 February 2006

Abstract

In cholestatic liver diseases, bile acids induce hepatocyte apoptosis and thus cause liver injury, but hepatic stellate cells (HSCs) survive in the presence of bile acids. We attempted to analyze anti-apoptotic signaling pathways in HSCs against bile acid-induced apoptosis. In immortalized human HSCs and primarily cultured rat HSCs, bile acid treatment increased the expression levels of cyclooxygenase-2 (COX-2) and Mcl-1. COX-2 induction was found to be due to transcriptional enhancement dependent on p42/44, p38 MAPK, and JNK activation, whereas Mcl-1 induction resulted from bile acid-mediated protein stabilization in a Raf-1-dependent manner. Moreover, the inhibitions of either COX-2 activity by celecoxib or Mcl-1 induction by siRNA transfection rendered HSCs susceptible to bile acid-induced apoptosis. These results imply that the bile acid-mediated inductions of COX-2 and Mcl-1 may lead to HSC survival in cholestatic liver diseases.

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Keywords: Hepatic stellate cell; Bile acid; Cyclooxygenase-2; Mcl-1; Apoptosis

**Hepatic fibrosis is a wound-healing response to liver injury [1], during which the progressive accumulation of extracellular matrix in liver parenchyma results in portal hypertension and hepatocellular dysfunction [2]. Activated hepatic stellate cells (HSCs) are a major source of extracellular matrix, and thus, a main target for preventing hepatic fibrosis [2,3]. The majority of anti-fibrotic strategies are designed to inhibit the activation, proliferation, or synthet-ic products of HSCs [2,3]. More recently, the selective induction of apoptosis of these cells using tumor necrosis

0006-291X/S - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.02.072
COX-2 expression is up-regulated in the cirrhotic liver as compared to normal liver, and its expression is even higher in liver cirrhosis than in hepatocellular carcinoma [15]. Moreover, in a rat experimental hepatic fibrosis model, selective COX-2 inhibition significantly attenuated hepatic fibrosis [16]. These findings collectively suggest that COX-2 may participate in hepatic fibrosis, and more specifically that COX-2 may play an essential role in regulating HSC survival or growth.

Mcl-1 is a potent anti-apoptotic Bcl-2 family protein and can be induced by a variety of stimuli [17]. Specifically, it blocks cytochrome c release, which is required for the intrinsic apoptosis pathway [18,19]. Moreover, it has recently been demonstrated that Mcl-1 is crucial for regulating cholangiocytic survival [11]. Since both cholangiocyes and HSCs are resistant to bile acid-induced apoptosis, it is likely that Mcl-1 may also participate in the regulation of HSC survival.

Therefore, we hypothesized that bile acids may induce COX-2 and Mcl-1 protein in HSCs, and that these inductions may function as anti-apoptotic signals in these cells. To test this hypothesis, we formulated the following questions: (i) Does bile acid increase COX-2 and Mcl-1 protein levels in HSCs? (ii) If so, what is the mechanism of this induction? And finally (iii) Does this induction exert an anti-apoptotic signal in these cells in the presence of bile acid? Collectively, the results of the current study demonstrate that bile acid induces COX-2 and Mcl-1 protein expression in HSCs in a kinase-specific manner, and that these signaling pathways attenuate bile acid-induced HSC apoptosis. Thus, the selective interruption of these signaling cascades in HSCs may provide an efficient anti-fibrotic strategy in cholestatic liver diseases.

Materials and methods

Cell culture. The immortalized human stellate cells, LX-2 cells [20], and primarily cultured rat HSCs were used in this study. LX-2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100,000 U/L penicillin, 100 mg/L streptomycin, and 100 nM insulin. Cells were serum-starved over 12 h before bile acid, chenodocholate (CDC), treatment to avoid the confounding variable of serum-induced signaling. Rat HSCs were isolated from male Sprague–Dawley rats by enzymatic digestion and density gradient centrifugation, as previously described [21,22]. Following isolation, the cells were suspended in metabolically active, proliferating cells. Following each treatment, 20 μL of dye solution was added to each well of a 96-well plate and incubated for 3 h. Subsequently, absorbance was recorded using an ELISA plate reader at 490 nm (Molecular Devices, Sunnyvale, CA).

Quantitation of apoptosis. Apoptosis was quantitated by assessing the characteristic nuclear changes of apoptosis (i.e., chromatin condensation and nuclear fragmentation) using the nuclear binding dye 4′,6-diamidino-2-phenylindole dihydrochloride (Sigma, St. Louis, MO) and a fluorescence microscope [24].

Results

Bile acid increased the expression levels of COX-2 and Mcl-1 protein in HSCs

We first examined if bile acid treatment induces COX-2 and Mcl-1 protein expression in LX-2 cells and in primarily secondary antibodies (BioSource International, Camarillo, CA), were incubated at a dilution of 1:10,000, and 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 used were: rabbit anti-Mcl-1, goat anti-COX-2, and goat anti-actin from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Real-time reverse transcription-PCR. Total RNA was extracted from the cells using the Trizol Reagent (Invitrogen, Carlsbad, CA). The cDNA template was prepared using oligo(dT) random primers and Moloney Murine Leukemia Virus reverse transcriptase, as described in detail [6]. After the reverse transcription reaction, the cDNA template was amplified by PCR using Taq polymerase (Invitrogen). COX-2 and Mcl-1 mRNA was quantitated using real-time PCR and the following primers: COX-2 forward, 5′-TGAATACCCACTACCAAA-3′, reverse, 5′-CCATGGGGCATTCAATAAC-3′; Mcl-1 forward, 5′-ATGCTTCCGG AAACGGACAT-3′, reverse, 5′-TCTTGATGCACCTTCTAGG-3′. For quantification, we used real-time PCR (LightCycler; Roche Molecular Biochemicals, Mannheim, Germany) using SYBR green as the fluorescent probe (Molecular Probes, Eugene, OR). After electrophoresis in 1% agarose gel, the portion of gel containing the expected PCR product of COX-2 or Mcl-1 was excised, and the product was eluted into Tris–HCl using a DNA elution kit (Gel extraction kit; Qiagen, Valencia, CA). The eluted and purified PCR product was quantitated using a spectrophotometer (Beckman DU 7400) at 260 nm. An inverse linear relationship was obtained between copy number and cycle number, and the resulting standard curve was used to calculate the copy numbers/mL in experimental samples. Results were expressed as ratios of COX-2 or Mcl-1 mRNA copies/mL in samples stimulated with CDC to those in control samples.

Mcl-1 siRNA transfection. A specific double-stranded 21-nucleotide RNA sequence homologous to the target message was used to silence Mcl-1 in LX-2 cells, as previously described [23]. Cells grown to subconfluence for 3–7 days were transfected with 40 nM siRNA for human Mcl-1 (Dharmacon, Lafayette, CO.) using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions.

Cell proliferation. Cell proliferations were measured using CellTitre 96 Aqueous One Solution cell proliferation assays (Promega, Madison, WI), based on the cellular conversion of colorimetric reagent MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt] into soluble formazan by dehydrogenase enzymes found only in metabolically active, proliferating cells. Following each treatment, 20 μL of dye solution was added to each well of a 96-well plate and incubated for 3 h. Subsequently, absorbance was recorded using an ELISA plate reader at 490 nm (Molecular Devices, Sunnyvale, CA).

Inmunoblot 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% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 1 μg/mL aprotinin, leupeptin, and pepstatin; 1 mM Na3VO4; and 1 mM NaF) and centrifuged at 14,000g for 10 min at 4°C. 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. Peroxidase-conjugated secondary antibodies (BioSource International, Camarillo, CA), were incubated at a dilution of 1:10,000, and 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 used were: rabbit anti-Mcl-1, goat anti-COX-2, and goat anti-actin from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Statistical analysis. Statistical analysis was performed using SAS version 8.01 (SAS Institute, Cary, NC). Differences between two groups were analyzed using the two-tailed Student’s t tests or Wilcoxon rank sum tests. p values <0.05 were taken to indicate statistically significant differences.
cultured rat HSCs. Treatment of LX2 cells with CDC promptly increased Mcl-1 protein expression levels within 30 min, while COX-2 induction occurred relatively later as compared to Mcl-1 induction (Fig. 1A). The CDC-induced induction of these proteins was also observed in rat HSCs (Fig. 1B). These findings indicate that bile acid increases COX-2 and Mcl-1 protein expression levels in HSCs.

The mechanism of COX-2 and Mcl-1 induction by bile acid

We next investigated whether the transcript levels of these two proteins are increased by CDC treatment in LX2 cells. CDC significantly increased COX-2 mRNA levels, but did not significantly increase Mcl-1 mRNA levels (Fig. 2A), which suggested that COX-2 induction is regulated at the transcriptional level, and that Mcl-1 induction is regulated at the post-transcriptional level. Since Mcl-1 contains a PEST sequence, and thus, has a very short half-life due to rapid proteasome-mediated degradation [25], we next examined if bile acid treatment increases Mcl-1 protein stability. As shown in Fig. 2B, CDC treatment increased Mcl-1 protein stability as shown in cells treated with the proteasome inhibitor, MG-132. These results indicate that bile acid increases COX-2 transcript levels and inhibits Mcl-1 protein degradation.

To further elucidate the signals regulating the bile acid inductions of these proteins, LX2 cells were treated with kinase inhibitors. Treatment of LX2 cells with the kinase inhibitors, U0126 for p42/44 MAPK, SB203580 for p38 MAPK, or JNK inhibitor blocked COX-2 but not Mcl-1 induction by CDC (Fig. 3A), whereas Raf-1 inhibitor blocked Mcl-1 induction by CDC (Fig. 3B). These results indicate that bile acid increases COX-2 transcription in a p42/44, p38 MAPK, and JNK-dependent manner, and that Mcl-1 protein accumulation occurs in a Raf-1 dependent manner in HSCs.

The selective inhibition of COX-2 and Mcl-1 induction and HSC viability

In the presence of CDC, COX-2 inhibition by celecoxib treatment reduced LX-2 cell viability significantly in a
concentration-dependent manner (Fig. 4A), while this reduced viability was not observed in cells not treated with CDC (Fig. 4B). To selectively inhibit Mcl-1 induction, LX-2 cells were transfected with Mcl-1-specific siRNA, and this was found to reduce Mcl-1 expression in transfected cells (Fig. 5A), and these cells showed reduced viability in the presence of CDC (Figs. 5B and C).

Finally, we evaluated whether the selective inhibition of bile acid-mediated COX-2 and Mcl-1 induction renders HSCs susceptible to bile acid-induced apoptosis. As shown in Fig. 6, cells treated with COX-2 inhibitor or transfected with Mcl-1 siRNA showed enhanced CDC-induced apoptosis. These findings collectively indicate that bile acid-mediated COX-2 and Mcl-1 protein induction represents anti-apoptotic signals in HSCs, which inhibit the pro-apoptotic effects of bile acids.

**Discussion**

The principal findings of this study relate to the effect of bile acid on COX-2 and Mcl-1 expression in HSCs. The results obtained demonstrate that bile acid increases COX-2 and Mcl-1 protein expression levels in HSCs, and that these inductions exert anti-apoptotic signals in HSCs, which counter bile acid-induced apoptosis. These results provide new information regarding bile acid signaling in hepatic fibrosis, implicating that enhanced COX-2 and Mcl-1 protein expressions by bile acid may facilitate HSC survival in cholestasis.

It has recently been demonstrated that COX-2 and Mcl-1 proteins are crucial for regulating cholangiocyte survival [10,11]. In the present study, bile acid-mediated COX-2 and Mcl-1 induction was also observed in HSCs.

![Graph A](image1.png)

**Fig. 4.** COX-2 inhibition suppressed HSC proliferation. LX-2 cells were treated with the selective COX-2 inhibitor, celecoxib, at concentrations of 0, 25, or 50 μM in the (A) presence or (B) absence of CDC (200 μM). At each indicated time, MTS assays were performed, as instructed by the manufacturer. Data are expressed as means ± SD of relative ratios of optical densities at each time versus that of control cells at day 0. *p < 0.05 celecoxib 50 μM versus 0 or 25 μM, **p < 0.05 celecoxib 25 μM versus 0 μM.

![Graph B](image2.png)

**Fig. 5.** Inhibition of Mcl-1 induction suppressed HSC proliferation. (A) Twenty-four hours after the transfection of Mcl-1 siRNA, LX2 cells were treated with CDC (200 μM) for 6 h. Cells were then lysed and immunoblotted for Mcl-1 and β-actin. (B) LX2 cells, transfected or not with Mcl-1 siRNA, were cultured in the (left panel) presence or (right panel) absence of CDC (200 μM). At the indicated times, MTS assays were performed, as instructed by the manufacturer. Data are expressed as means ± SD of relative ratio of optical densities at each time versus that of control cells at day 0. *p < 0.05 Mcl-1 siRNA versus control cells.
HSCs are resistant to bile acid-induced apoptosis, and this is attributed to epidermal growth factor receptor (EGFR) transactivation in these cells [12]. Since bile acid-induced EGFR transactivation in cholangiocytes is responsible for COX-2 and Mcl-1 induction [10,11], it is most likely that this receptor-dependent signaling is also active in maintaining HSC survival in cholestasis.

COX-2 expression can be regulated by MAPK signaling pathways, which are activated by bile acids [10]. In the present study, bile acid increased COX-2 transcript levels, and this induction was inhibited in the presence of p42/44, p38 MAPK or JNK inhibitors. It is known that COX-2 expression can be regulated by MAPKs via transcriptional processes [26]. Activated p42/44 MAPK can directly phosphorylate transcription factors, such as Elk-1 and Sap-1, which then bind to the serum-responsive element of the c-fos promoter. This increases the expression of c-fos and c-jun and the binding activity of AP-1 (a transcription factor which is comprised of c-fos and c-jun protein complexes) [27,28]. The JNK signal transduction cascade is also known to result in transcriptionally active AP-1 complexes [29]. The AP-1 complex transactivates COX-2 promoter, increasing the expressions of COX-2 mRNA and protein [26]. Moreover, the 3′ untranslated region of COX-2 has been reported to contain AU-rich motifs important for its mRNA stability [30]. In addition, sequences that determine the stability of its mRNA may also reside in the 5′ untranslated or coding region in addition to its 3′ untranslated region [31]. COX-2 mRNA stability has also been reported to be increased p38 MAPK-dependently in oxysterol-treated cholangiocytes [32]. Therefore, it appears plausible that bile acid may induce COX-2 expression in HSCs via multiple mechanisms that include p42/44, p38 MAPK, and JNK signaling pathways.

In this study, MAPK inhibition did not block bile acid-stimulated increases in Mcl-1 protein levels. In contrast, Raf-1 inhibition effectively prevented increases in cellular Mcl-1 levels. Raf-1 is activated by bile acids [33] and is most commonly known as an upstream kinase that regulates MEK leading to p42/44 MAPK activation [34]. However, in addition to MEK regulation, Raf-1 may have other signal regulatory functions. Thus, Raf-1 signals, independent of MAPK activation, are likely to participate in bile acid-mediated Mcl-1 induction in HSCs. The maintenance of cellular protein levels is complex and includes interplay between transcriptional, translational, and post-translational regulatory processes. In the present study, real-time RT-PCR showed that bile acid did not increase Mcl-1 mRNA levels, thus implying that a translational or post-translational process is involved in Mcl-1 induction. Indeed, the present study demonstrated that bile acid and the proteasome inhibitor, MG-132, inhibited Mcl-1 protein turnover. Since Raf-1-dependent inhibition of Mcl-1 protein degradation was previously demonstrated in cholangiocytes [11], this analogy is likely to be applied in HSCs during cholestasis.

In this study, we demonstrate that bile acid induces HSC apoptosis when either COX-2 activity or Mcl-1 induction is inhibited. In particular, bile acid treatment or COX-2 inhibition alone did not suppress HSC growth, whereas cellular growth was significantly attenuated when cells were co-cultured with COX-2 inhibitor and bile acid. The present study also shows that reducing Mcl-1 protein expression itself has a pro-apoptotic effect and that this potentiates bile acid-induced apoptosis, which indicates that this protein has an essential role in maintaining HSC viability. Therefore, our findings implicate that the inhibition of COX-2 or Mcl-1 signaling may induce HSC apoptosis during cholestasis.

In conclusion, the present study demonstrates that bile acid increases COX-2 and Mcl-1 protein expression levels in HSCs, and that these inductions function as anti-apoptotic signals in these cells against bile acid-induced apoptosis. Since COX-2 and Mcl-1 induction by bile acid may perpetuate HSC survival during cholestasis, the selective interruption of these signaling pathways may present a useful anti-fibrotic strategy in a variety of cholestatic liver diseases.
References


