Farnesoid X Receptor Activation by Chenodeoxycholic Acid Induces Detoxifying Enzymes through AMP-Activated Protein Kinase and Extracellular Signal-Regulated Kinase 1/2-Mediated Phosphorylation of CCAAT/Enhancer Binding Protein β

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ABSTRACT:

Farnesoid X receptor (FXR) regulates redox homeostasis and elicits a cytoprotective effect. CCAAT/Enhancer binding protein-β (C/EBPβ) plays a role in regulating the expression of hepatocyte-specific genes and contributes to hepatocyte protection and liver regeneration. In view of the role of FXR in xenobiotic metabolism and hepatocyte survival, this study investigated the potential of FXR to activate C/EBPβ for the induction of detoxifying enzymes and the responsible regulatory pathway. Chenodeoxycholic acid (CDCA), a major component in bile acids, activates FXR. In HepG2 cells, CDCA treatment activated C/EBPβ, as shown by increases in its phosphorylation, nuclear accumulation, and expression. 3-(2,8-Dichlorophenyl)-4-(3’-carboxy-2-chlorostilben-4-yl)-oxymethyl-5-isopropyl-isoxazole (GW4064), a synthetic FXR ligand, had similar effects. In addition, CDCA enhanced luciferase gene transcription from the construct containing −1.65-kb GSTA2 promoter, which contained C/EBP response element (pGL-1651). Moreover, CDCA treatment activated AMP-activated protein kinase (AMPK), which led to extracellular signal-regulated kinase 1/2 (ERK1/2) activation, as evidenced by the results of experiments using a dominant-negative mutant of AMPKα and chemical inhibitor. The activation of ERK1/2 was responsible for the activating phosphorylation of C/EBPβ. FXR knockdown attenuated the ability of CDCA to activate AMPK and ERK1/2 and phosphorylate C/EBPβ. Consistently, enforced expression of FXR promoted the phosphorylation of AMPKα and ERK1/2, and C/EBPβ, verifying that C/EBPβ phosphorylation elicited by CDCA results from the activation of AMPK and ERK1/2 by FXR. In mice, CDCA treatment activated C/EBPβ with the induction of detoxifying enzymes in the liver. Our results demonstrate that CDCA induces antioxidant and xenobiotic-metabolizing enzymes by activating C/EBPβ through AMPK-dependent ERK1/2 pathway downstream of FXR.

Introduction

Farnesoid X receptor (FXR; NR1H4), a member of the nuclear hormone receptor superfamily, is highly expressed in major organs

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ABBREVIATIONS: FXR, farnesoid X receptor; CDCA, chenodeoxycholic acid; C/EBP, CCAAT/Enhancer binding proteins; GCS, γ-glutamylcysteine synthetase; MnSOD, manganese superoxide dismutase; UGT, UDP-glucuronosyl transferase; Mrp2, multidrug resistance protein 2; GST, glutathione S-transferase; AMPK, AMP-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; phospho, phosphorylated; ACC, acetyl-CoA carboxylase; HO-1, heme oxygenase-1; AICAR, 5-aminooimidazole-4-carboxamide-1-β-D-ribofuranoside; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenyl)butadiene; siRNA, small interfering RNA; GW4064, 3-(2,8-dichlorophenyl)-4-(3’-carboxy-2-chlorostilben-4-yl)-oxymethyl-5-isopropyl-isoxazole; PBS, phosphate-buffered saline; DN-AMPKα, dominant-negative mutant form of AMPKα; DN-MKK1, dominant-negative mutant of mitogen-activated protein kinase kinase 1; PCR, polymerase chain reaction; LAP, liver-enriched activator protein; LIP, liver-enriched inhibitory protein; C/EBP-RE, CCAAT/Enhancer binding protein response element; p90RSK, p90 ribosomal S6 kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; JNK, c-jun amino-terminal-kinase; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; SP600125, 1,9-pyrazoloanthranthrene; Nrf2, nuclear factor erythroid-2-related factor 2.
The CCAAT/enhancer binding proteins (C/EBPs) belong to the basic region/leucine zipper class transcription factors. In particular, C/EBPβ is associated with the differentiation of certain cell types, including hepatocytes, adipocytes, macrophages, and granulocytes (Schroeder-Gloeckler et al., 2007). In the resting state, the unphosphorylated form of C/EBPβ is mostly located in the cytoplasm. The activation of C/EBPβ requires phosphorylation at specific residues by cellular kinases, which promotes the process of its nuclear translocation and binding to the C/EBP response element (Buck and Chojkier, 2003). The N-terminal transactivation domain of C/EBPβ that binds to the DNA binding element interacts with p300/Creb binding protein and enhances target gene transactivation (Mink et al., 1997). Activated C/EBPβ induces detoxifying enzymes through gene transcription (Kang et al., 2003). The putative C/EBP binding sites are found in the promoter regions of certain antioxidant genes, including γ-glutamylcysteine synthetase (GCS) and manganese superoxide dismutase (MnSOD) (Maehara et al., 1999; Kang et al., 2003). The induction of phase II enzymes and transporters [e.g., UDP-glucuronosyl transferase (UGT) and multidrug resistance protein 2 (Mrp2)] may contribute to the activation of C/EBPβ in the context of xenobiotics (Kang et al., 2003).

In view of the role of FXR in xenobiotic metabolism and detoxification, this study investigated the effect of FXR activation by CDCA on C/EBPβ and the expression of its target detoxifying enzymes. Because FXR promotes hepatocyte survival (Lee et al., 2010a), we further explored the role of AMP-activated protein kinase (AMPK) and extracellular signal-regulated kinase 1/2 (ERK1/2) activation by FXR in the phosphorylation of C/EBPβ and its target gene induction. In this study, we report that the activation of FXR by CDCA contributes to the activating phosphorylation of C/EBPβ, which may be mediated by AMPK-dependent ERK1/2 activation.

Materials and Methods

Materials. Antibodies directed against phosphorylated (phospho) acetyl-CoA carboxylase (ACC), phospho-AMPK, phospho-C/EBPβ, ERK1/2, phospho-ERK1/2, and lamin were purchased from Cell Signaling Technology (Beverly, MA). Antibodies recognizing C/EBPα, C/EBPβ, UGT1A, and Mrp2 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-heme oxygenase-1 (HO-1) and anti–γ-GCS antibodies were purchased from Alexis Biochemicals (San Diego, CA) and NeoMarkers (Fremont, CA), respectively. Anti-GSTA2 antibody was a gift from Detroit Aldrich (Beverly, MA). Antibodies recognizing C/EBPβ, C/EBPα, and 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) were obtained from Dharmacon (Chicago, IL). Anti-β-actin antibody, CDCA, 3-(2,6-dichlorophenyl)-4-(3′-carboxy-2-chlorostilben-4-yl)-oxymethyl-5-isopropyl-isoxazole (GW4064), and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Culture. HepG2 cells, a human hepatocyte-derived cell line, were purchased from American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in humidified atmosphere with 5% CO₂. The cells were plated at a density of 5 × 10⁵ cells/10-cm diameter dish and preincubated for 24 h at 37°C. For all experiments, HepG2 cells were grown from 70 to 80% confluency and were subjected to no more than 20 cell passages.

Primary hepatocytes were isolated from Sprague-Dawley rats as previously described with a minor modification (Kang et al., 2002). In brief, livers of rats were perfused with Ca²⁺-free Hank’s balanced saline solution at 37°C for 5 min. Livers were then perfused for 20 min with 0.05% collagenase buffer (collagenase from Clostridium histolyticum, type IV; Sigma-Aldrich) at a perfusion flow rate of 10 ml/min. After perfusion, the livers were minced gently with scissors and suspended with sterilized phosphate-buffered saline (PBS). The cell suspension was filtered through sterilized gauze and centrifuged at 500g for 10 min to separate parenchymal and nonparenchymal cells. The pellet was resuspended in PBS and further centrifuged at 500g for 5 min. The cells were collected from the pellet and cultured on plastic dishes. They were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in humidified atmosphere with 5% CO₂. The cells were plated at a density of 5 × 10⁶ cells/10-cm diameter dish and preincubated for 24 h at 37°C.

Preparation of Nuclear Extracts. Nuclear extracts were prepared according to the previously published method (Kang et al., 2003). In brief, HepG2 cells (1 × 10⁶) in dishes were washed twice with ice-cold PBS and then scrapped from the dishes with 1 ml of PBS and transferred to microfuge tubes. Cells were then centrifuged at 2000g for 5 min. The supernatant was discarded, and the cells were allowed to swell after the addition of 100 μl of hypotonic buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. The lysates were incubated for 10 min on ice and then centrifuged at 7200g for 5 min at 4°C. Pellets containing crude nuclei were resuspended in 50 μl of extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, and then were incubated for 30 min on ice. The samples were then centrifuged at 15,800g for 10 min to obtain supernatants containing nuclear fractions. Nuclear fractions were stored at −70°C until use.

Immunoblot Analyses. SDS-polyacrylamide gel electrophoresis and immunoblot analyses were performed according to previously published procedures (Kang et al., 2003). Proteins were resolved by gel electrophoresis and electrophotically transferred to nitrocellulose paper. The nitrocellulose paper was incubated with antibodies overnight and reacted with horseradish peroxidase-conjugated secondary antibody. Bands were developed using an ECL chemiluminescence detection kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Equal loading of proteins was verified by immunoblotting for β-actin or lamin. Scanning densitometry of the immunoblots was performed with Image Scan and Analysis System (Alpha Innotech Corp., San Leandro, CA). The area of each lane was integrated using the software AlphaEase version 5.5, followed by background subtraction.

Transient Transfection. HepG2 cells were transfected with the plasmid encoding dominant-negative mutant form of AMPKα (DN-AMPKα) or dominant-negative mutant of mitogen-activated protein kinase kinase 1 (DN-MKK1) using FuGENE HD (Roche, Indianapolis, IN). The plasmids encoding for FXR and AMPKα were kindly provided by Drs. Bart Staelens (Institut Pasteur de Lille, Lille, France) (Claudel et al., 2002) and J. Ha (Kyunghee University, Seoul, Korea), respectively. DN-MKK1 was a gift from Dr. N. G. Ahn (Howard Hughes Medical Institute, University of Colorado, Boulder, CO).

GSTA2 Luciferase Assay. To determine the promoter activities of pGL-1651, pGL-1651-ΔCGBP, and pGL-1651-ΔARE constructs, the dual-luciferase reporter assay system was used (Promega, Madison, WI), as described previously (Kang et al., 2003). The pGL-1651 reporter gene construct was generated by ligating the region −1.65 kb upstream of the transcription start site of the GSTA2 gene to the firefly luciferase reporter gene coding sequences. The mutants of GSTA2 promoter-lucerase plasmid, pGL-1651-ΔCGBP and pGL-1651-ΔARE, in which the C/EBP response element (−905 to −898) and ARE (−696 to −687) were deleted and replaced with 5′-CTCGAG-3′, respectively, were constructed as described previously (Kang et al., 2003; Park et al., 2004).

Real-Time Polymerase Chain Reaction Assays. Total RNA was extracted using TRIZol (Invitrogen) according to the manufacturer’s instructions. Total RNA (1 μg) was reverse-transcribed using an oligo(dT)₁₂₁₈ primer to obtain cDNA. The cDNA was amplified by polymerase chain reaction (PCR). Real-time PCR was performed with a Light Cycler 1.5 apparatus (Roche, Mann-
heim, Germany) using Light Cycler DNA master SYBR Green-1 kit according to the manufacturer’s instructions. The relative mRNA levels were normalized to those of glycerinaldehyde-3-phosphate dehydrogenase using Lightcycler software 4.0 (Roche). After PCR amplification, a melting curve of each amplification was determined to verify its accuracy. The sequences of the primers used were as follows: human GCLC (sense, 5′-TGGAATGGTGATGGGATTTC-3′ and antisense, 5′-AACACACCTCTCTCCATGGA-3′); human GCLM (sense, 5′-GATCCAAAAAGACTCTCTGTAAG-3′ and antisense, 5′-CCTCATTGTTTCAATGACCGAAT-3′); human MnSOD (sense, 5′-GAGAATGGTGATGGGATTTC-3′ and antisense, 5′-GGAAACCATACTCGCAATACA-3′); human FXR (sense, 5′-GAGAATGGTGATGGGATTTC-3′ and antisense, 5′-GATCCAAAAGAACTGCTTTCTGAAG-3′); human GCLM (sense, 5′-GAGAATGGTGATGGGATTTC-3′ and antisense, 5′-GAAGTCGGAGTCGGAGTA-3′); human glyceraldehyde-3-phosphate dehydrogenase (sense, 5′-GAAGATGGTGATGGGATTTC-3′ and antisense, 5′-GGAAACCATACTCGCAATACA-3′); and human glyceraldehyde-3-phosphate dehydrogenase (sense, 5′-TACCTTCCTACATATCCATCA-3′ and antisense, 5′-TTACTTTTCACAATGACCGAAT-3′).

Animal Experiments. The experiments using animals were conducted under the guidelines of the Institutional Animal Care and Use Committee at Seoul National University. Male ICR mice at 6 weeks of age (25–30 g) were purchased from Orient (Osan, Korea) and housed under filtered pathogen-free condition in a controlled environment with 12-h light/dark cycles and relative humidity of 50%. Mice were treated with CDCA (30 mg/kg i.p., as dissolved in dimethyl sulfoxide) or vehicle once daily for 3 days. Six hours after administration of CDCA, liver samples were excised.

Data Analyses. One-way analysis of variance procedures were used to assess significant differences among treatment groups. For each significant treatment effect, the Newman-Keuls test was used to compare multiple group means.

Results

C/EBPβ-LAP Activation by FXR Agonists. To determine whether activation of FXR affects the expression of C/EBP isoforms, we first examined the effects of CDCA on C/EBPβ, C/EBPβ-LAP, C/EBPβ-liver-enriched inhibitory protein (C/EBPβ-LIP), and C/EBPδ levels. Treatment of HepG2 cells with 100 μM CDCA increased the levels of C/EBPβ-LAP1/2 in a time-dependent manner (Fig. 1A). C/EBPβ-LAP expression levels were significantly increased 6 h after treatment with a maximal increase being noted at 12 h (Fig. 1A). C/EBPβ-LIP, a form expressed by alternative translation from C/EBPβ mRNA, dimerizes with C/EBPβ-LAP, and this complex functions as a dominant-negative transcription factor (Descombes and Schibler, 1991; Luedde et al., 2004). Moreover, the LAP/LIP ratio is important for the transcriptional regulation of the C/EBPβ target genes (Descombes and Schibler, 1991). CDCA treatment increased the C/EBPβ-LIP level slightly (e.g., ∼1.4-fold at 24 h), whereas it increased C/EBPβ-LAP level to a much greater extent (∼4-fold) (Fig. 1A). So, the ratio of LAP to LIP was significantly increased, beginning at 6 h after CDCA treatment, and reached the maximum at 12 h (Fig. 1A, inset).

The protein levels of other C/EBP isoforms, C/EBPα and C/EBPδ, were minimally changed, which suggests the specific effect of CDCA on C/EBPβ (Fig. 1A). We next examined the concentration-response effect of CDCA on C/EBPβ isoforms. The isoforms of C/EBPβ were immunoblotted on the lysates of HepG2 cells treated with 100 μM CDCA for the indicated times. Inset shows the ratio of C/EBPβ-LAP to LIP. B, the concentration-response effect of CDCA on C/EBPβ expression. Cells were treated with CDCA for 12 h. C, increase in the nuclear C/EBPβ-LAP/LIP level by CDCA. Nuclear fractions were prepared from cells treated with 100 μM CDCA for the indicated time periods (left) and those treated with CDCA for 6 h (right). Immunoblotting for lamin verified equal loading of the nuclear proteins. D, luciferase reporter activity. Induction of luciferase activity by CDCA in HepG2 cells transfected with pGL-1651 that contains the C/EBP response element of the GSTA2 promoter. Luciferase reporter assays were performed on the lysates of HepG2 cells that had been transfected with pGL-1651 or pGL-1651ΔC/EBP and exposed to vehicle or 100 μM CDCA for 12 h. For A–C, the relative C/EBP protein levels were assessed by scanning densitometry of the immunoblots. The level of C/EBPβ was normalized to that of β-actin or lamin. The ratio of C/EBPβ-LAP to LIP was obtained from scanning densitometry of the bands. The data represent the means ± S.E. of at least three separate experiments (significant compared with control, *p < 0.05 or **p < 0.001; N.S., not significant).
effect of CDCA on the expression of C/EBPβ; treatment of CDCA at 50 μM or higher for 12 h increased the levels of C/EBPβ (Fig. 1B).

In the resting state, C/EBPβ is largely present in the cytoplasm as an unphosphorylated form. After activating phosphorylation, C/EBPβ is translocated into the nucleus and is capable of binding to its cognate DNA sequence (Buck and Chojkier, 2003). Next, we measured the levels of nuclear C/EBPβ. As expected, treatment with 50 or 100 μM CDCA notably elevated nuclear C/EBPβ content (Fig. 1C). In the subsequent experiments, we used 100 μM CDCA to explore target gene transcription and the associated signaling pathway. To further assess the functional role of C/EBPβ activation by CDCA for target gene induction, reporter gene assays were performed using pGL-1651 plasmid that has the luciferase gene downstream of the −1.65-kb GSTA2 promoter region containing the C/EBPβ response element (C/EBPβ-RE). Treatment of pGL-1651-transfected cells with CDCA for 12 h resulted in a 3-fold increase in luciferase activity. CDCA treatment failed to increase lucerase activity from pGL-1651ΔC/EBPβ, a reporter with the C/EBP-RE deleted (Fig. 1D), confirming that the induction of the reporter gene results from increased binding of C/EBPβ to the gene promoter.

To verify the functional role of FXR in C/EBPβ activation, we further determined the effect of GW4064, a synthetic FXR ligand, on C/EBPβ levels. GW4064 treatment (5 μM) caused the induction of C/EBPβ-LAP1/2 notably from 6 h (at least) to 24 h, and the maximal increase was noted at 12 h (Fig. 2A). GW4064 at 3 or 5 μM induced C/EBPβ-LAP1/2 (Fig. 2B). Consistently, it also promoted the nuclear accumulation of C/EBPβ (Fig. 2C).

**ERK1/2-Dependent C/EBPβ Phosphorylation by CDCA.** Kinases including Ras-ERK-p90 ribosomal S6 kinase (p90RSK), calcium/calmodulin-dependent protein kinase, protein kinase C, and protein kinase A activate C/EBPβ through phosphorylation (Wegner et al., 1992; Trautwein et al., 1994). Bile acids stimulate the Ras/Raf-1/mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK)-1/ERK pathway (Lee et al., 2010b). Given the regulatory role of ERK1/2 in C/EBPβ phosphorylation and the known effect of bile acids on ERK1/2 activation (Lee et al., 2010b), we assessed the phosphorylation of C/EBPβ and ERK1/2 in cells treated with CDCA. Immunoblot analyses showed that CDCA treatment gradually increased the phosphorylation of C/EBPβ at Thr residue (Thr235 residue is phosphorylated by ERK in human; Nakajima et al., 1993; Lee and Kim, 2006) in a time-dependent manner (Fig. 3A, inset). CDCA treatment enhanced the phosphorylation of ERK1/2 (Fig. 3B). The strong increase in phospho-C/EBPβ level at later times may not only be due to the increase in total C/EBPβ but also to the persistent activation of ERK1/2. To determine whether ERK1/2 activation affects C/EBPβ phosphorylation, we monitored the effects of DN-MKK1 and a chemical inhibitor, which inhibit the MEK1/2 necessary for ERK1/2 phosphorylation, on the phosphorylation of C/EBPβ by CDCA. As expected, either DN-MKK1 transfection or U0126 treatment (an MEK1/2 inhibitor) (Duncia et al., 1998) inhibited the ability of CDCA to promote phosphorylation of C/EBPβ (Fig. 3C).

**AMPK Activation That Leads to ERK1/2 Phosphorylation.** AMPK serves as a key regulator of cell survival or death in response to pathological stress (e.g., endoplasmic reticulum stress, oxidative stress, osmotic stress, and hypoxia) and is activated to reserve energy content in cells (Shaw et al., 2004). AMPK activation is involved in the induction of metabolizing enzymes (Rencurel et al., 2006). As an effort to identify the upstream regulator of ERK1/2, we examined the effect of CDCA on AMPK and the role of AMPK in ERK1/2 phosphorylation. CDCA treatment increased AMPK phosphorylation, as shown by increase in the phosphorylation of AMPKα and its substrate ACC (Fig. 4A). Moreover, the extent of ERK1/2 phosphorylation...
elicited by CDCA was attenuated by DN-AMPKα transfection or compound C treatment (a chemical inhibitor of AMPK) (Machrouhi et al., 2010) (Fig. 4B), suggesting that AMPK activation by CDCA may be involved in the ERK1/2 phosphorylation. Consistently, inhibition of AMPK activity reversed the phosphorylation of C/EBPβ by CDCA (Fig. 4C), indicating that the activation of AMPK by CDCA facilitates C/EBPβ phosphorylation via ERK1/2 activation.

To verify the role of AMPK in the activation of C/EBPβ, we determined the effect of AICAR (a chemical activator of AMPK) on C/EBPβ and ERK1/2 phosphorylation. As expected, treatment of HepG2 cells with AICAR (1 mM, 6 h) enhanced the phosphorylation of both C/EBPβ and ERK1/2 (Fig. 4D). Consistent with the results in HepG2 cells, CDCA treatment promoted the phosphorylation of AMPKα and ERK1/2 in rat primary hepatocytes and increased C/EBPβ phosphorylation at Thr (Thr189 residue is phosphorylated by ERK in rats; Nakajima et al., 1993; Lee and Kim, 2006) (Fig. 4E). All of these results provide strong evidence that CDCA has the ability to activate AMPK, which may lead to the phosphorylation of ERK1/2 and ERK1/2-dependent phosphorylation of C/EBPβ.

C/EBPβ Phosphorylation by AMPK-ERK1/2 Pathway Downstream of FXR. In an effort to clarify the role of FXR in the activation of the AMPK-ERK1/2 pathway responsible for C/EBPβ phosphorylation, we assessed whether the effect of CDCA on the AMPK-ERK1/2 axis was altered by modulations of FXR activity. We observed that knockdown of FXR attenuated the increases in the phosphorylation of AMPKα and ERK1/2 by CDCA (Fig. 5A, left).

The phosphorylation of C/EBPβ at Thr235 was also inhibited. Decreased levels of FXR protein and mRNA verified the knockdown effect (Fig. 5A, right). Consistently, enforced expression of FXR increased the phosphorylation of AMPKα, ACC, ERK1/2, and C/EBPβ (Fig. 5B, left). FXR overexpression was confirmed by marked increases in FXR protein and mRNA content (Fig. 5B, right). Likewise, GW4064 treatment enhanced the phosphorylation of AMPKα, ACC, ERK1/2, and C/EBPβ, which was inhibited by FXR knockdown (Fig. 5C). The total protein levels of AMPKα and ERK1/2 were not affected by FXR knockdown or overexpression (Supplemental Fig. 1). These results indicate that the activation of FXR by its ligand contributes to the phosphorylation of C/EBPβ through the pathway involving AMPK-dependent ERK1/2 activation.

Effects of FXR Agonists on the Induction of Detoxifying Enzymes. Oxidative stress promotes the progression of various diseases by altering intracellular redox homeostasis, thereby sometimes causing cell death when the stress persists or exceeds the range of cellular antioxidative capacity (Shaw et al., 2004). In a continuing effort to define the functional outcome of CDCA treatment in terms of antioxidative capacity, we measured the effect of CDCA treatment on the levels of glutamate-cysteine ligase catalytic subunit, glutamate-cysteine ligase modifier subunit, and MnSOD transcripts from the genes containing the C/EBP-RE (Maehara et al., 1999; Yang et al., 2001). As expected, CDCA treatment significantly increased all of the transcript levels in a time-dependent manner (Fig. 6A). GW4064 had similar effects. Immunoblot analysis verified the induction of γ-GCS.
These results demonstrate that FXR agonists transcriptionally induce antioxidant enzymes. Finally, we determined the effect of CDCA on C/EBP in vivo. Administration of CDCA to mice (30 mg/kg i.p. per day for 3 days) notably increased phosphorylated and total C/EBP levels in the liver (Fig. 6C). Moreover, CDCA treatment induced UGT1A, HO-1, GSTA2, and Mrp2 (Fig. 6D), protein products from the genes containing C/EBP-RE. Taken together, these data suggest that CDCA induces antioxidant and xenobiotic-metabolizing enzymes by activating C/EBP through the AMPK-dependent ERK1/2 pathway downstream of FXR, which may contribute to maintaining overall homeostasis in hepatocytes.

**Discussion**

The liver plays a major role in eliminating xenobiotics from the body, and this process is accomplished by a series of metabolizing enzymes. Detoxifying enzymes include phase II enzymes and transporters: phase II enzymes function to protect the body from environmental insults by conjugating xenobiotics with endogenous compounds, and transporters in the liver mediate the efflux of transformed metabolites across the hepatocyte canalicular membrane to the bile. Detoxifying enzymes increase the capacity of antioxidant defense as well as metabolic detoxification, which contributes to the maintenance of redox homeostasis and cell viability. Transactivation of the genes encoding for phase II enzymes and transporters are coordinately regulated by transcription factors in response to external stimuli (Emi et al., 1996).

In animal models, feeding diets containing bile acids enhanced the expression of phase II enzymes and transporters (Zollner et al., 2003), suggesting that bile acids modulate the gene expression. It is also likely that bile acids have the ability to protect the liver from oxidative stimuli by inducing an antioxidant defense system. CDCA, a major component in bile acids, exerts a hepatoprotective effect. C/EBP is a key transcription factor necessary for the expression of genes involved in maintaining normal liver physiology (Rastegar et al., 2000). In this study, we found for the first time that CDCA activates C/EBP in hepatocytes. This finding, together with the results of luciferase reporter gene assay, supports the role of C/EBP activation by CDCA in the transcriptional induction of detoxifying enzymes. The necessity of C/EBP binding to the C/EBP-RE in inducing the target genes was strengthened by our experiment using a mutant promoter lacking the C/EBP binding site.

Other isoforms of C/EBP may also be involved in the expression of liver-specific genes. In untreated cells, C/EBPα may be part of the proteins that bind to the C/EBP binding site (Lin et al., 1993). Thus, the biological effect evoked by C/EBP on the transcription of liver-specific genes might depend on the relative activities of C/EBPα and C/EBPβ. It is also possible that an active form of C/EBPβ competes with C/EBPα for the C/EBP binding site. In the present study, we observed that CDCA treatment did not affect the expression levels of C/EBPα and C/EBPβ (Fig. 1A). Hence, it is apparent that CDCA AMPK inhibition. HepG2 cells were treated as described in B, D, the phosphorylation of C/EBPβ and ERK1/2 by AICAR. Immunoblotting was performed on the lysates of HepG2 cells treated with 100 μM CDCA for 6 h. E, the activation of AMPK, ERK1/2, and C/EBPβ by CDCA in rat primary hepatocytes. Immunoblot analyses were performed on the lysates of primary hepatocytes treated with 100 μM CDCA. Values indicate fold changes. The relative protein levels were measured by scanning densitometry of the immunoblots. The protein level was normalized to that of β-actin. The data represent the means ± S.E. of at least three separate experiments (significant compared with control, *p < 0.05 or **p < 0.01; significant compared with CDCA, #p < 0.05).
specifically activates C/EBPβ. Our results are consistent with the previous observation that C/EBPβ plays a role as a transcription factor in regulating the expression of hepatocyte-specific genes (Rastegar et al., 2000).

FXR is expressed in major organs including liver, intestine, kidney, and adrenal gland (Wang et al., 1999). Upon bile acid binding, FXR positively regulates a series of genes responsible for maintaining normal liver physiology, thus protecting the hepatocyte from environmental insults (Huang et al., 2006; Lee et al., 2010a). In particular, CDCA is a major bile acid constituent that exerts a hepatoprotective effect by activating FXR. The importance of FXR activation in CDCA-mediated detoxifying enzyme induction was further supported by our finding that GW4064 also activated C/EBPβ and induced the genes encoding antioxidant enzymes. All of our results lend support to the contention that the activation of C/EBPβ by FXR accounts, at least in part, for the molecular basis of target enzyme induction, which explains the cytoprotective and adaptive response elicited by bile acid.

AMPK monitors energy status by responding to changes in the cellular AMP/ATP ratio, which functions as an energy-sensing molecule (Shaw et al., 2004). AMPK activation exerts an antiapoptotic effect by activating FXR. The receptor-activated signaling pathways regulate the phosphorylation of C/EBPβ in its activation domains. Several kinases including Ras-ERK-p90RSK, calcium/calmodulin-dependent protein kinase, protein kinase C, and protein kinase A are responsible for the phosphorylation of C/EBPβ (Wegner et al., 1992; Trautwein et al., 1994).

It has been shown that deoxycholic acid treatment activated the MEK1/ERK-p90RSK, calcium/calmodulin-dependent protein kinase, and adrenal gland (Wang et al., 1999). Upon bile acid binding, FXR activated receptor-mediated detoxifying enzyme induction was further supported by our finding that GW4064 also activated C/EBPβ and induced the genes encoding antioxidant enzymes. All of our results lend support to the contention that the activation of C/EBPβ by FXR accounts, at least in part, for the molecular basis of target enzyme induction, which explains the cytoprotective and adaptive response elicited by bile acid.

The results of the present study also confirmed the ability of AMPK to regulate the phosphorylation of C/EBPβ in its activation domains. Several kinases including Ras-ERK-p90RSK, calcium/calmodulin-dependent protein kinase, protein kinase C, and protein kinase A are responsible for the phosphorylation of C/EBPβ (Wegner et al., 1992; Trautwein et al., 1994). It has been shown that deoxycholic acid treatment activated the MEK1/ERK pathway (Lee et al., 2010b). In addition, ERK1/2 is responsible for the phosphorylation of the human C/EBPβ form at Thr235 residue (Nakajima et al., 1993; Lee and Kim, 2006). Our additional results showed that CDCA treatment enhanced the phosphorylation of C/EBPβ by FXR accounts, at least in part, for the molecular basis of target enzyme induction, which explains the cytoprotective and adaptive response elicited by bile acid.

C/EBPβ activation may contribute to the ability of CDCA to maintain hepatocyte viability. This concept is in line with the previous findings that AMPK activation induces HO-1 via peroxisome proliferator-activated receptor α activation in the liver and protects cells from oxidative stress (Shin and Kim, 2009; Lin et al., 2010).

The receptor-activated signaling pathways regulate the phosphorylation of C/EBPβ in its activation domains. Several kinases including Ras-ERK-p90RSK, calcium/calmodulin-dependent protein kinase, protein kinase C, and protein kinase A are responsible for the phosphorylation of C/EBPβ (Wegner et al., 1992; Trautwein et al., 1994). It has been shown that deoxycholic acid treatment activated the MEK1/ERK pathway (Lee et al., 2010b). In addition, ERK1/2 is responsible for the phosphorylation of the human C/EBPβ form at Thr235 residue (Nakajima et al., 1993; Lee and Kim, 2006). Our additional results showed that CDCA treatment enhanced the phosphorylation of c-jun amino-terminal-kinase (JNK) and p38 kinase phosphorylation. A, the effect of siRNA knockdown of FXR. HepG2 cells that had been transfected with control siRNA or FXR siRNA for 48 h were treated with 100 μM CDCA for 12 h (left). No difference was noted between control siRNA and “no” treatment. Real-time PCR and immunoblot assays confirmed the knockdown of FXR (right). B, the effect of enforced expression of FXR. Immunoblotting was performed on the lysates of cells that had been transfected with the plasmid encoding for FXR (1 μg) (left). Real-time PCR and immunoblot assays confirmed the overexpression of FXR (right). C, the effect of GW4064. Immunoblotting was performed on the lysates of HepG2 cells transfected with control siRNA or FXR siRNA for 48 h and subsequently treated with GW4064 (5 μM for 12 h). The relative protein levels were measured by scanning densitometry of the immunoblots. The protein level was normalized to that of β-actin. The data represent the means ± S.E. of at least three separate experiments (significant compared with control, *, p < 0.05 or **, p < 0.01).
Fig. 6. The induction of detoxifying enzymes by FXR agonists. A, real-time PCR assays. The levels of glutamate-cysteine ligase catalytic subunit, glutamate-cysteine ligase modifier subunit, and MnSOD transcripts were determined in HepG2 cells treated with 100 μM CDCA or 5 μM GW4064 for the indicated times. Data represent the mean ± S.E. of at least three separate experiments (significant compared with control, *, p < 0.05 or **, p < 0.01). B, immunoblot assays for γ-GCS. γ-GCS was immunoblotted on the lysates of HepG2 cells treated with 100 μM CDCA. C, immunoblot assays for phosphorylated and total C/EBPβ in the liver. Proteins of interest were immunoblotted on the liver homogenates of mice treated with CDCA (30 mg/kg, i.p. per day) for 3 days. D, immunoblot assays for hepatic UGT1A, HO-1, GSTA2, and Mrp2. E, a schematic diagram illustrating the proposed mechanism by which FXR agonists induce detoxifying enzymes in hepatocytes. For B–D, the relative protein levels were assessed by scanning densitometry of the immunoblots. The protein level was normalized to that of β-actin. The data represent the means ± S.E. of at least three separate experiments or four mice (significant compared with control, *, p < 0.05 or **, p < 0.01).
pawning evidence that FXR plays a role in activating C/EBPβ through ERK1/2. Overall, our finding that AMPK inhibition reversed CDCA-induced ERK1/2 and C/EBPβ phosphorylation supports the conclusion that AMPK-mediated ERK1/2 activation downstream of FXR contributes to CDCA-induced C/EBPβ phosphorylation, which leads to the transcriptional induction of C/EBPβ target genes.

CDCA treatment also activated nuclear factor erythroid-2-related factor 2 (Nrf2) in HepG2 cells (Supplemental Fig. 3, A and B), as further supported by the result of antioxidant response element reporter assay (Supplemental Fig. 3C). Our data are in agreement with the observation that CDCA treatment activated Nrf2 and induced its target genes in the liver and intestinal cells (Tan et al., 2007). Ursodeoxycholic acid, another bile acid, has been used in clinical studies to treat liver diseases including primary biliary cirrhosis, and a large part of its beneficial aspect may be attributable to its property of Nrf2 activation (Okada et al., 2008; Kawata et al., 2010). Hence, it is presumed that an enhanced complex containing C/EBPβ and Nrf2 may work together in inducing target genes by CDCA.

In summary, the results of the present study demonstrate that CDCA activates C/EBPβ by phosphorylating C/EBPβ through the AMPK-ERK1/2 pathway downstream of FXR activation (Fig. 6E), contributing to the cooperative assembly of an activated transcription complex at the target gene promoters. Consequently, activation of C/EBPβ by CDCA may be necessary for the induction of detoxifying enzymes.

Authorship Contributions

Participated in research design: Noh, Y.M. Kim, Y.W. Kim, and S.G. Kim.

Conducted experiments: Noh, Y.M. Kim, and Y.W. Kim.

Performed data analysis: Noh, Y.M. Kim, Y.W. Kim, and S.G. Kim.

Wrote or contributed to the writing of the manuscript: Noh, Y.M. Kim, and S.G. Kim.

References


