DMD Fast Forward. Published on May 19, 2011 as doi:10.1124/dmd.111.038414

DMD #38414

FXR Activation by Chenodeoxycholic Acid Induces Detoxifying Enzymes through AMPK and ERK1/2-mediated Phosphorylation of C/EBPβ

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DMD Fast Forward. Published on May 19, 2011 as DOI: 10.1124/dmd.111.038414
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Running Title: C/EBPβ activation by FXR

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The information of manuscript statistics:

Number of Text Pages: 31

Number of Tables: 0

Number of Figures: 6

Number of References: 41

Words in Abstract: 249

Words in Introduction: 474

Words in Materials and methods: 1156

Words in Results: 1484

Words in Discussion: 1117

Abbreviations AICAR, 5-Aminoimidazole-4-carboxamide-1-β-d-ribofuranoside; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; CDCA, chenodeoxycholic acid; C/EBPβ, CCAAT/enhancer binding protein β; C/EBP-RE, CCAAT/enhancer binding protein response element; ERK1/2, extracellular signal-regulated kinase 1/2; FXR, farnesoid X receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; γ-GCS, glutamylcysteine synthetase; GST, glutathione *S*-transferase; HO-1, heme oxygenase-1; LAP, liver-enriched activator protein; LIP, liver-enriched inhibitory protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MKK1, mitogen-activated protein kinase kinase 1; MnSOD, manganese superoxide dismutase; Mrp2, multidrug resistance protein 2; Nrf2, nuclear factor erythroid-2-related factor 2; p90RSK, p90 ribosomal S6 kinase; UGT, UDP-glucuronosyl transferase

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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, contributing 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. GW4064, a synthetic FXR ligand, had similar effects. Also, CDCA enhanced luciferase gene transcription from the construct containing −1.65 kb GSTA2 promoter containing 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, 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.

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Introduction

Farnesoid X receptor (FXR, NR1H4), a member of the nuclear hormone receptor superfamily, is highly expressed in major organs including liver, intestine, kidney, and adrenal gland. FXR functions as a ligand-mediated transcription factor by binding to specific DNA motifs in the promoter regions of target genes, and regulates the expression of various genes involved in bile acid, lipid, and glucose metabolism (Wang et al., 1999). FXR plays a role in the induction of genes encoding for proteins involved in xenobiotic metabolism and detoxification (Urquhart et al., 2007; Lee et al., 2010a). Bile acids are synthesized from cholesterol by a series of enzymatic reactions in hepatocytes and work as a digestive surfactant, promoting lipid absorption (Lefebvre et al., 2009). Chenodeoxycholic acid (CDCA), a major component in bile acids, serves as a potent ligand of FXR and exerts a hepatoprotective effect (Makishima et al., 1999; Parks et al., 1999).

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

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transporters [e.g., UDP-glucuronosyl transferase (UGT) and multidrug resistance protein 2 (Mrp2)] may also depend on C/EBPβ (Emi et al., 1996; Tanaka et al., 1999). Previously, we reported the role of C/EBPβ activation in the induction of glutathione *S*-transferase (GST) A2 by a chemopreventive agent (Kang et al., 2003; Ko et al., 2006). Hence, C/EBPβ activation is necessary for detoxification, and may contribute to hepatocyte protection against the challenge 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 (ERK) 1/2 activation by FXR in the phosphorylation of C/EBPβ and its target gene induction. Here, we report that the activation of FXR by CDCA contributes to the activating phosphorylation of C/EBPβ which may be mediated with AMPK-dependent ERK1/2 activation.

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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, C/EBPδ, UGT1A, and Mrp2 were provided from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-heme oxygenase-1 (HO-1) and anti-γ-GCS antibodies were purchased from Alexis Biochemicals Stressgen (San Diego, CA) and Neomarkers (Fremont, CA), respectively. Anti-GSTA2 antibody was a gift from Detroit R&D (Detroit, MI). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgGs were obtained from Zymed Laboratories (San Francisco, CA). 5-Aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) was purchased from Toronto Research Chemicals (Ontario, Canada). Compound C and U0126 were supplied from Calbiochem (San Diego, CA). A non-targeting control siRNA pool and a pool of siRNA duplexes directed against FXR were supplied from Dharmacon (Chicago, IL). Anti-β-actin antibody, CDCA, GW4064, and all other reagents were provided from Sigma (St. Louis, MO).

Cell culture

HepG2 cells, a human hepatocyte-derived cell line, were purchased from ATCC (Rockville, MD). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum, 50 units/mL penicillin, and 50 μg/mL streptomycin at 37^{o}C in humidified atmosphere with 5% CO_2. The cells were plated at a density of 5×10^6 cells/10 cm diameter dish and preincubated for 24 h at 37^{o}C. For all experiments, HepG2 cells were grown to 70-80% 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). Briefly, livers of rats were perfused with Ca^{2+}-free

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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 PBS. The cell suspension was filtered through sterilized gauze and centrifuged at 500 *g* for 10 min to separate parenchymal and nonparenchymal cells. The pellet was resuspended in PBS and further centrifuged at 500 *g* for 5 min. The cells were collected from the pellet and cultured on plastic dishes. They were maintained in DMEM containing 10% fetal bovine serum, 50 units/mL penicillin, and 50 μg/mL streptomycin at 37°C in humidified atmosphere with 5% CO_2. The cells were plated at a density of 5×10^6 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). Briefly, HepG2 cells (1×10^7) in dishes were washed twice with ice-cold PBS and then scraped from the dishes with 1 mL of PBS and transferred to microfuge tubes. Cells were then centrifuged at 2000 *g* 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 dithiothreitiol, and 0.5 mM phenylmethylsulfonyl fluoride. The lysates were incubated for 10 min on ice and then centrifuged at 7200 *g* 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 dithiothreitiol, and 1 mM phenylmethylsulfonyl fluoride, and then were incubated for 30 min on ice. The samples were then centrifuged at 15800 *g* for 10 min to obtain supernatants containing nuclear fractions. Nuclear fractions were stored at –70°C until use.

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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 electrophoretically 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 (Amersham Biosciences, 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 & 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 DN-AMPKα were kindly provided from Drs. Bart Staels (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-ΔCEBP, and pGL-1651-ΔARE constructs, the dual-luciferase reporter assay system was used (Promega, Madison, WI), as previously described (Kang et al., 2003). The pGL-1651 reporter gene construct was

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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-luciferase plasmid, pGL-1651-ΔC/EBP and pGL-1651-ΔARE, in which the C/EBP response element (-905~-898) and ARE (-696~-687) were deleted and replaced with 5′-CTCGAG-3′, respectively, were constructed as previously described (Kang et al., 2003; Park et al., 2004).

Real-time PCR assays

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed using an oligo$(dT)_{16}$ primer to obtain cDNA. The cDNA was amplified by PCR. Real-time PCR was performed with a Light Cycler 1.5 apparatus (Roche, Mannheim, Germany) using Light Cycler DNA master SYBR green-I kit according to the manufacturer's instructions. The relative mRNA levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using Lightcycler software 4.0 (Roche, Mannheim, Germany). After PCR amplification, a melting curve of each amplicon was determined to verify its accuracy. The sequences of the primers used were as follows; human GCLC (sense: 5'-TGGCAATGCAGTGGTGGAT-3', antisense: 5'-AACACACCTTCCTTCCCATTGA-3'); human GCLM (sense: 5'-GATCCAAAAGAACTGCTTTCTGAAG-3', antisense: 5'-CCTCTACTTTTCACAATGACCGAAT-3'); human MnSOD (sense: 5'-CGACCTGCCCTACGACTACG-3', antisense: 5'-TGACCACCACCATTGAACTT-3'); human FXR (sense: 5'-GGAACCATACTCGCAATACA-3', antisense: 5'-TCGCATGTACATATCCATCA-3'); and human GAPDH (sense: 5'-GAAGATGGTGATGGGATTTC -3', antisense: 5'- GAAGGTGAAGGTCGGAGTC -3')

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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 the supply of filtered pathogen-free air, commercial chow (Purina, Korea) and water *ad libitum* at a temperature between 20 and 23^{o}C with 12 h light and dark cycles and relative humidity of 50%. Mice were treated with CDCA (30 mg/kg, i.p., as dissolved in DMSO) or vehicle once daily for three 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 utilized to compare multiple group means.

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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/EBPα, C/EBPβ-liver-enriched activator protein (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 C/EBPβ-LIP level slightly (e.g., ~1.4-fold at 24 h), whereas it did C/EBPβ-LAP level to a much greater extent (~4-fold) (Fig. 1A). So, the ratio of LAP to LIP was significantly increased, beginning from 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, suggesting the specific effect of CDCA on C/EBPβ (Fig. 1A). We next examined the concentration-response 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

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nuclear C/EBPβ content (Fig. 1C). In the subsequent experiments, we used 100 μM of 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/EBP response element (C/EBP-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 luciferase activity from pGL-1651-ΔCEBP, 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 up to 24 h with the maximal increase 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 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

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(Thr235 residue is phosphorylated by ERK in human, Nakajima et al., 1993; Lee et al., 2006) in a time-dependent manner (Fig. 3A). The levels of phosphorylated C/EBPβ peaked 12 or 24 h after CDCA treatment. Total C/EBPβ content was also increased by CDCA since the activated form of C/EBPβ transcriptionally induces C/EBPβ through its binding to the UF1 and UF2 sites in the gene promoter region (Chang et al., 1995). So, the ratio of phospho-C/EBPβ to total C/EBPβ was increased at early times after CDCA treatment (i.e., 1-6 h), but not at later times due to the increase in total C/EBPβ level (Fig. 3A inset). CDCA treatment enhanced the phosphorylation of ERK1/2 (Fig. 3B). The strong increase in phospho-C/EBPβ level at later times could be due to not only the increase in total C/EBPβ, but 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 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

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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). In consistence 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 et al., 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). Similarly, GW4064 treatment enhanced the phosphorylation of AMPKα, ACC, ERK1/2 and C/EBPβ, which was inhibited by FXR knockdown (Fig. 5C). The total

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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 causes cell death when the stress persists or exceeds the range of cellular anti-oxidative capacity (Shaw et al., 2004). In a continuing effort to define the functional outcome of CDCA treatment in terms of anti-oxidant capacity, we measured the effect of CDCA treatment on the levels of glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit (GCLM), 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 by CDCA (Fig. 6B). These results demonstrate that FXR agonists transcriptionally induce antioxidant enzymes.

Finally**,** we determined the effect of CDCA on C/EBPβ *in vivo*. Administration of CDCA to mice (i.p., 30 mg/kg/day, for 3 days) notably increased phosphorylated and total C/EBPβ 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. Collectively, CDCA induces antioxidant and xenobiotic-metabolizing enzymes by activating C/EBPβ through AMPK-dependent ERK1/2 pathway downstream of FXR, which may contribute to maintaining overall homeostasis in hepatocytes.

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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; 1) phase II enzymes function to protect the body from environmental insults by conjugating xenobiotics with endogenous compounds; 2) transporters in the liver mediate the efflux of transformed metabolites across the hepatocyte canalicular membrane to the bile. They increase the capacity of antioxidant defense as well as metabolic detoxification, contributing 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 antioxidant defense system. CDCA, a major component in bile acids, exerts a hepatoprotective effect. C/EBPβ is a key transcription factor necessary for the expression of genes involved in maintaining normal liver physiology (Rastegar et al., 2000). Here, we found for the first time that CDCA activates C/EBPβ in hepatocytes. This finding together with the results of luciferase reporter gene assay supports the role of C/EBPβ activation by CDCA in the transcriptional induction of detoxifying enzymes. The necessity of C/EBPβ 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). So, 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

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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/EBPδ (Fig. 1A). Hence, it is apparent that CDCA 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 hepatoprotective effect by activating FXR. The importance of FXR activation in C/EBPβ-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, explaining the cytoprotective and adaptive response elicited by bile acid.

AMPK monitors energy status by responding to changes in the cellular AMP:ATP ratio, functioning as an energy-sensing molecule (Shaw et al., 2004). AMPK activation exerts an anti-apoptotic effect, whereas its abrogation increases cell injury and apoptosis (Ido et al., 2002). Another important finding of this study is the identification of FXR as a mediator that activates AMPK. Our data demonstrate the role of FXR-dependent AMPK activation in promoting C/EBPβ phosphorylation by CDCA. It seems that FXR gives a signal to activate AMPK through the LKB1-dependent pathway by regulating a specific microRNA expression (Lee et al., unpublished data). Considering the importance of AMPK and C/EBPβ in hepatocyte survival, it is predicted that AMPK-dependent 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

DMD Fast Forward. Published on May 19, 2011 as DOI: 10.1124/dmd.111.038414
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activation induces HO-1 via PPARα 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 et al., 2006). Our additional results showed that CDCA treatment enhanced the phosphorylation of JNK and p38 kinase (Supplemental Fig. 2A). However, only U0126 (an MEK1/2 inhibitor), but not SB203580 (a p38 kinase inhibitor) or SP600125 (a JNK inhibitor), inhibited the phosphorylation of C/EBPβ by CDCA (Supplemental Fig. 2B), supporting the contention that ERK1/2 is predominantly responsible for the phosphorylation of C/EBPβ among the MAPKs.

The results of the present study also confirmed the ability of AMPK to regulate ERK1/2, being consistent with the observation that AICAR activated Raf-1/MEK/ERK/p90RSK signaling cascade (Wang et al., 2007). Our results that FXR knockdown attenuated increases in the phosphorylation of ERK1/2 and C/EBPβ by CDCA, whereas enforced expression of FXR increased them, provided compelling 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. 3A and 3B), as further supported by the result of antioxidant response

element reporter assay (Supplemental Fig. 3C). Our data match 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 clinically used 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 enhanceosome 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: S.G. Kim, K. Noh, Y.M. Kim, and Y.W. Kim

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

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

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

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Footnotes

This work was supported by the National Research Foundation of Korea grant [MEST No. 2011-0001204] funded by the government of Korea.

K.N. and Y.M.K. contributed equally to this work.

DMD Fast Forward. Published on May 19, 2011 as DOI: 10.1124/dmd.111.038414
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Figure Legends

Fig. 1. C/EBPβ activation by CDCA

A) The time-course effect of CDCA on the expression of C/EBP isoforms. The isoforms of C/EBP 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 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-1651-ΔC/EBPβ, and exposed to vehicle or 100 μM CDCA for 12 h.

For panels A-C, the relative C/EBP protein levels were assessed by scanning densitometry of the immunoblots. The level of C/EBP 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 to control, *$p<0.05$ or **$p<0.01$; N.S., not significant).

Fig. 2. C/EBPβ activation by GW4064

A) The time-course effect of GW4064 on the expression of C/EBPβ. C/EBPβ levels were determined on the lysates of HepG2 cells treated with 5 μM GW4064.

B) The concentration-response effect of GW4064 on C/EBPβ expression. Cells were treated with GW4064 for 12 h.

C) Increase in the level of nuclear C/EBPβ by GW4064. C/EBPβ levels were measured in the nuclear fractions of HepG2 cells treated with GW4064 for 6 h. Immunoblottings for lamin verified equal loading of the nuclear proteins.

For panels A-C, the relative C/EBPβ-LAP protein levels were assessed by scanning densitometry of the immunoblots. The level of C/EBP-LAP was normalized to that of β-actin or lamin. The data represent the means ± S.E. of at least three separate experiments (significant compared to control, *$p<0.05$ or **$p<0.01$).

Fig. 3. The role of ERK1/2 in C/EBPβ phosphorylation by CDCA

A) The time-course effect of CDCA on C/EBPβ phosphorylation. Phosphorylated C/EBPβ levels were assessed in the lysates of HepG2 cells treated with 100 μM CDCA. The relative protein levels were measured by scanning densitometry of the immunoblots (lower). The level of p-C/EBPβ was normalized to that of β-actin. Inset shows the relative ratio of p-C/EBPβ to total C/EBPβ.

B) The time-course effect of CDCA (100 μM) on ERK1/2 phosphorylation in HepG2 cells.

C) The role of ERK1/2 in C/EBPβ phosphorylation by CDCA. HepG2 cells that had been transfected with DN-MKK1 for 6 h were treated with 100 μM CDCA for 12 h (left). Cells were exposed to CDCA for 12 h after U0126 treatment (10 μM, for 1 h) (right).

The relative protein levels were measured by scanning densitometry of the immunoblots. The protein level was normalized to that of β-actin or ERK1/2. The data represent the means ± S.E. of at least three separate experiments (significant compared to control, *$p<0.05$ or **$p<0.01$; significant compared to CDCA treatment, $^{\#}p<0.05$).

DMD Fast Forward. Published on May 19, 2011 as DOI: 10.1124/dmd.111.038414
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Fig. 4. AMPK activation by CDCA and its regulation of ERK1/2

A) AMPK activation by CDCA. Immunoblot analyses were performed on the lysates of HepG2 cells treated with 100 μM CDCA for the indicated times (left).

B) Reversal of CDCA-induced ERK1/2 phosphorylation by AMPK inhibition. HepG2 cells that had been transfected with DN-AMPKα for 6 h were treated with 100 μM CDCA for 12 h (left). Cells were exposed to CDCA for 12 h after compound C treatment (5 μM, for 1 h) (right).

C) Reversal of CDCA-induced C/EBPβ phosphorylation by AMPK inhibition. HepG2 cells were treated as described in panel B.

D) The phosphorylation of C/EBPβ and ERK1/2 by AICAR. Immunoblottings were performed on the lysates of HepG2 cells treated with AICAR (1 mM, 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 to control, *$p<0.05$ or **$p<0.01$; significant compared to CDCA , $^{\#}p<0.05$).

Fig. 5. The role of FXR in AMPK-ERK1/2-dependent C/EBPβ 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. Immunoblottings were 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. Immunoblottings were 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 to 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 GCLC, GCLM, 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 to control, *p<0.05 or **p<0.01).

B) Immunoblottings for γ-GCS. γ-GCS was immunoblotted on the lysates of HepG2 cells treated with 100 μM CDCA.

C) Immunoblottings 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/day, i.p.) for 3 days.

D) Immunoblottings 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 panels 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 to control,

*p<0.05 or **p<0.01).

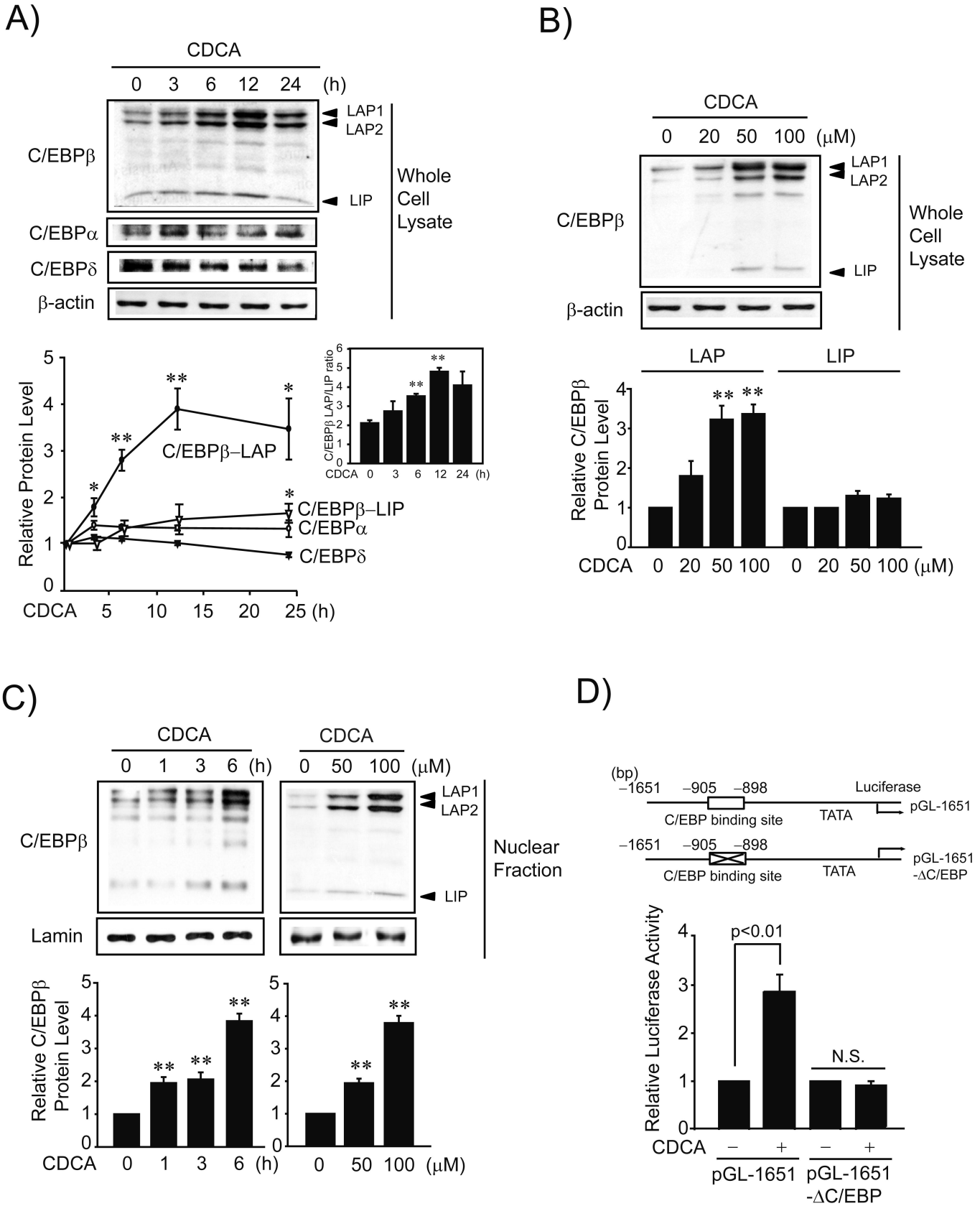
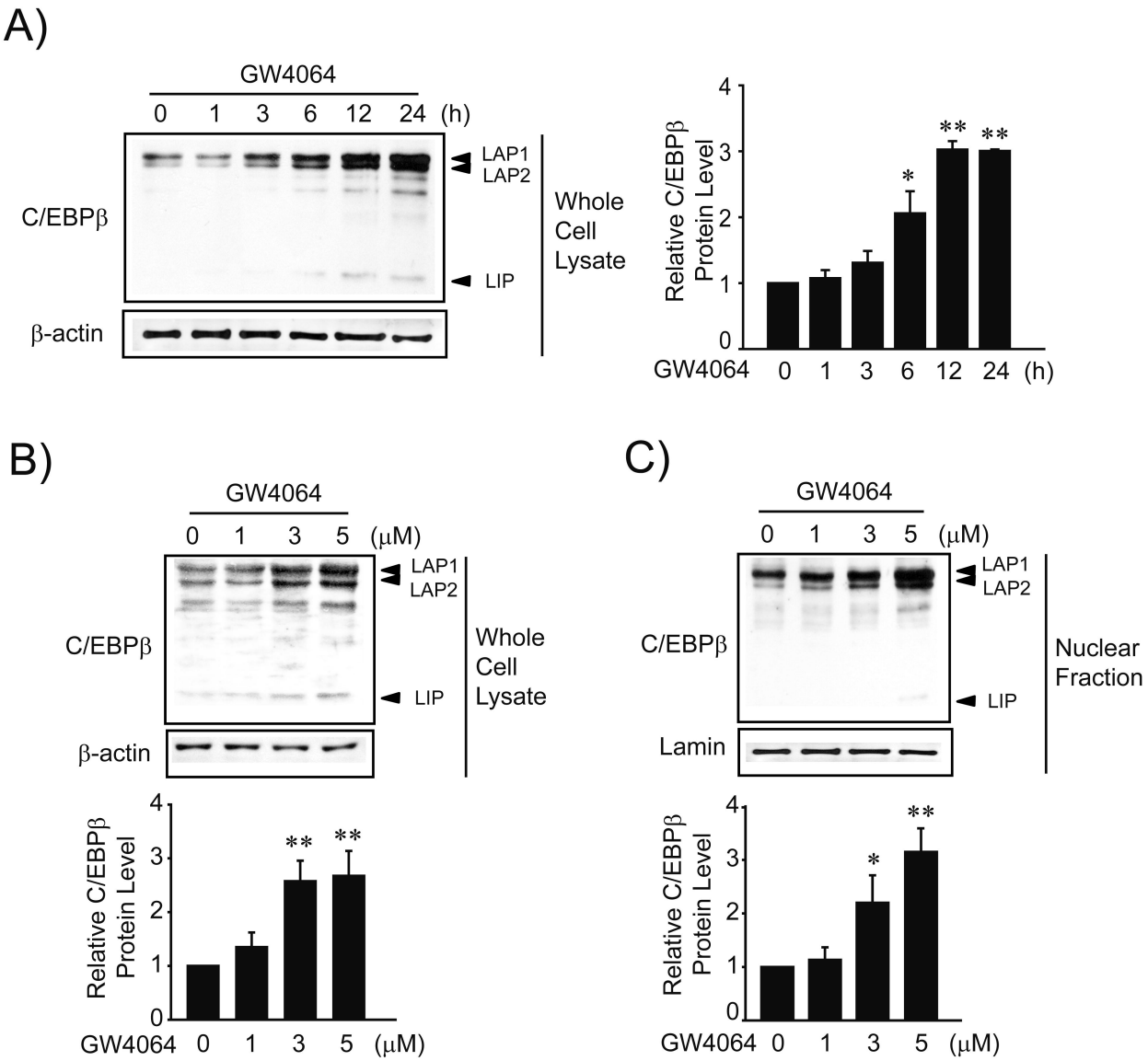

A)
CDCA
0 3 6 12 24 (h)
C/EBPβ
LAP1
LAP2
LIP
Whole Cell Lysate
C/EBPα
C/EBPδ
β-actin
Relative Protein Level
C/EBPβ–LAP
C/EBPβ–LIP
C/EBPα
C/EBPδ
CDCA 5 10 15 20 25 (h)
C/EBPβ LAP/LIP ratio
CDCA 0 3 6 12 24 (h)
B)
CDCA
0 20 50 100 (μM)
C/EBPβ
LAP1
LAP2
LIP
Whole Cell Lysate
β-actin
LAP
LIP
Relative C/EBPβ Protein Level
CDCA 0 20 50 100 0 20 50 100 (μM)
C)
CDCA
CDCA
0 1 3 6 (h)
0 50 100 (μM)
C/EBPβ
LAP1
LAP2
LIP
Nuclear Fraction
Lamin
Relative C/EBPβ Protein Level
CDCA 0 1 3 6 (h)
0 50 100 (μM)
D)
(bp)
−1651 −905 −898
Luciferase
C/EBP binding site
TATA
pGL-1651
pGL-1651 -ΔC/EBP
Relative Luciferase Activity
p<0.01
N.S.
CDCA
pGL-1651
pGL-1651 -ΔC/EBP

Fig. 1


A)
GW4064
0 1 3 6 12 24 (h)
C/EBPβ
LAP1
LAP2
LIP
β-actin
Whole Cell Lysate
Relative C/EBPβ Protein Level
GW4064 0 1 3 6 12 24 (h)
B)
GW4064
0 1 3 5 (μM)
C/EBPβ
LAP1
LAP2
LIP
β-actin
Whole Cell Lysate
Relative C/EBPβ Protein Level
GW4064 0 1 3 5 (μM)
C)
GW4064
0 1 3 5 (μM)
C/EBPβ
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LAP2
LIP
Lamin
Nuclear Fraction
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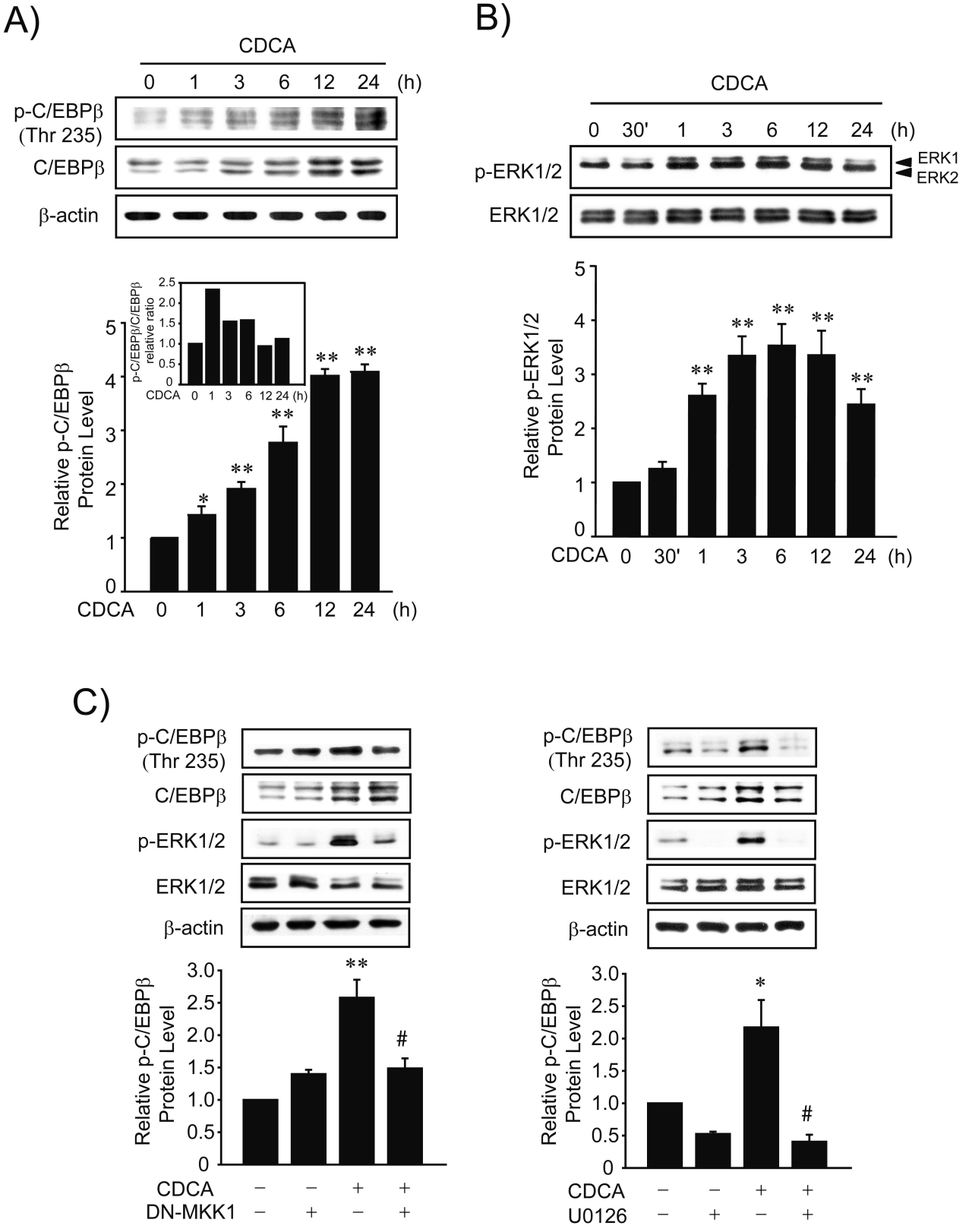
GW4064 0 1 3 5 (μM)

Fig. 2


A)
CDCA
0 1 3 6 12 24 (h)
p-C/EBPβ (Thr 235)
C/EBPβ
β-actin
p-C/EBPβ/C/EBPβ relative ratio
Relative p-C/EBPβ Protein Level
B)
CDCA
0 30' 1 3 6 12 24 (h)
p-ERK1/2
ERK1/2
ERK1
ERK2
Relative p-ERK1/2 Protein Level
C)
p-C/EBPβ (Thr 235)
C/EBPβ
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ERK1/2
β-actin
Relative p-C/EBPβ Protein Level
CDCA
DN-MKK1
U0126

Fig. 3

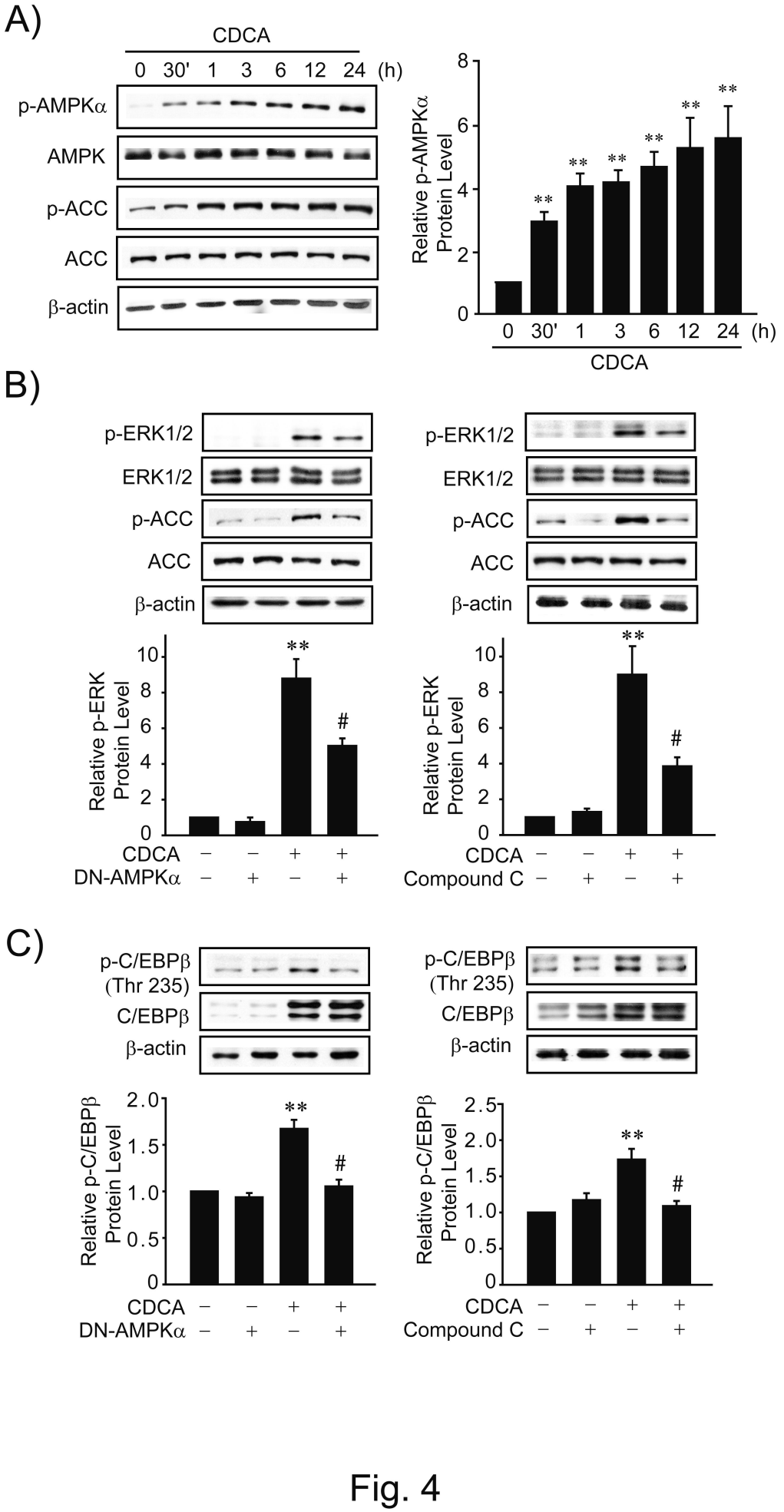
A)
CDCA
0 30' 1 3 6 12 24 (h)
p-AMPKα
AMPK
p-ACC
ACC
β-actin
Relative p-AMPKα Protein Level
0 30' 1 3 6 12 24 (h)
CDCA
B)
p-ERK1/2
ERK1/2
p-ACC
ACC
β-actin
Relative p-ERK Protein Level
CDCA
DN-AMPKα
Compound C
C)
p-C/EBPβ (Thr 235)
C/EBPβ
β-actin
Relative p-C/EBPβ Protein Level

Fig. 4

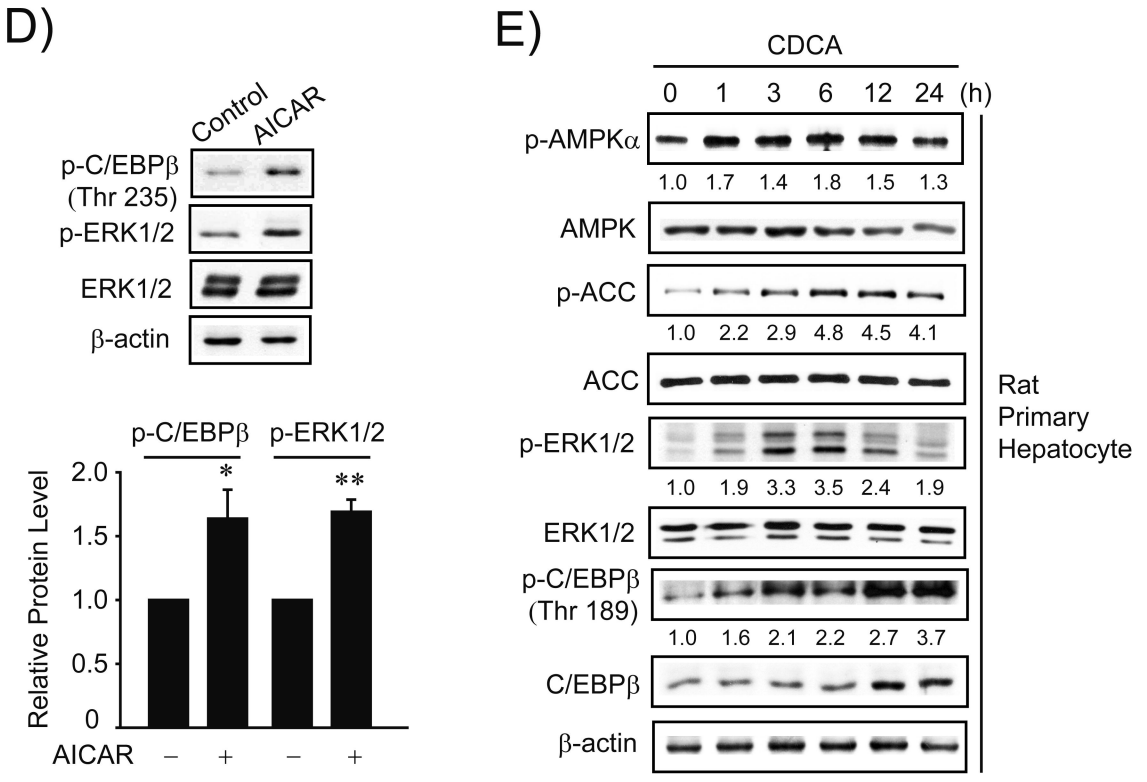

Fig. 4 (Continued)

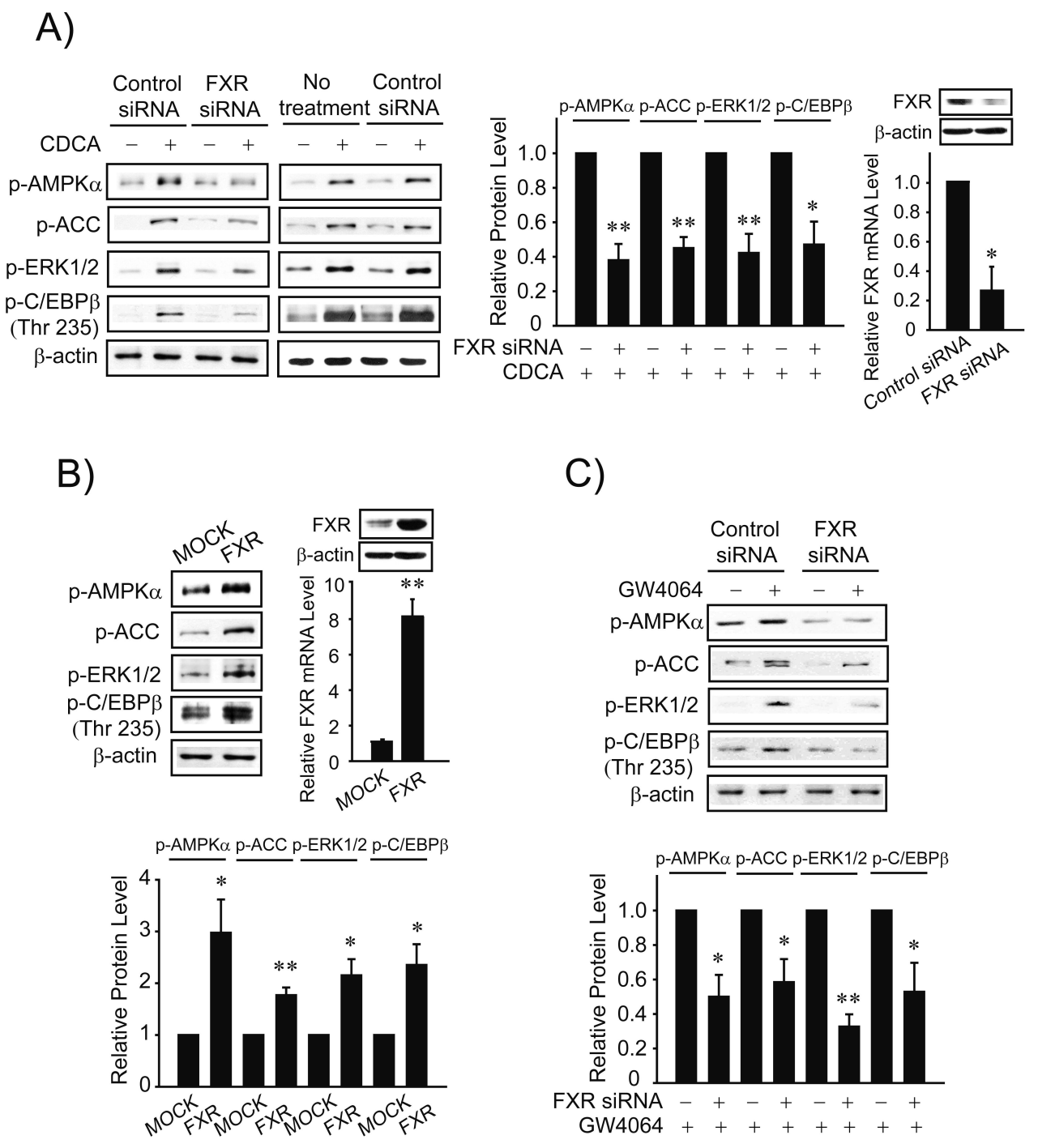
A)
Control siRNA
FXR siRNA
No treatment
Control siRNA
CDCA
p-AMPKα
p-ACC
p-ERK1/2
p-C/EBPβ (Thr 235)
β-actin
p-AMPKα p-ACC p-ERK1/2 p-C/EBPβ
Relative Protein Level
FXR siRNA
CDCA
FXR
β-actin
Relative FXR mRNA Level
Control siRNA
FXR siRNA
B)
MOCK
FXR
p-AMPKα
p-ACC
p-ERK1/2
p-C/EBPβ (Thr 235)
β-actin
Relative FXR mRNA Level
p-AMPKα p-ACC p-ERK1/2 p-C/EBPβ
Relative Protein Level
C)
Control siRNA
FXR siRNA
GW4064
p-AMPKα
p-ACC
p-ERK1/2
p-C/EBPβ (Thr 235)
β-actin
p-AMPKα p-ACC p-ERK1/2 p-C/EBPβ
Relative Protein Level
FXR siRNA
GW4064

Fig. 5

DMD Fast Forward. Published on May 19, 2011 as DOI: 10.1124/dmd.111.038414

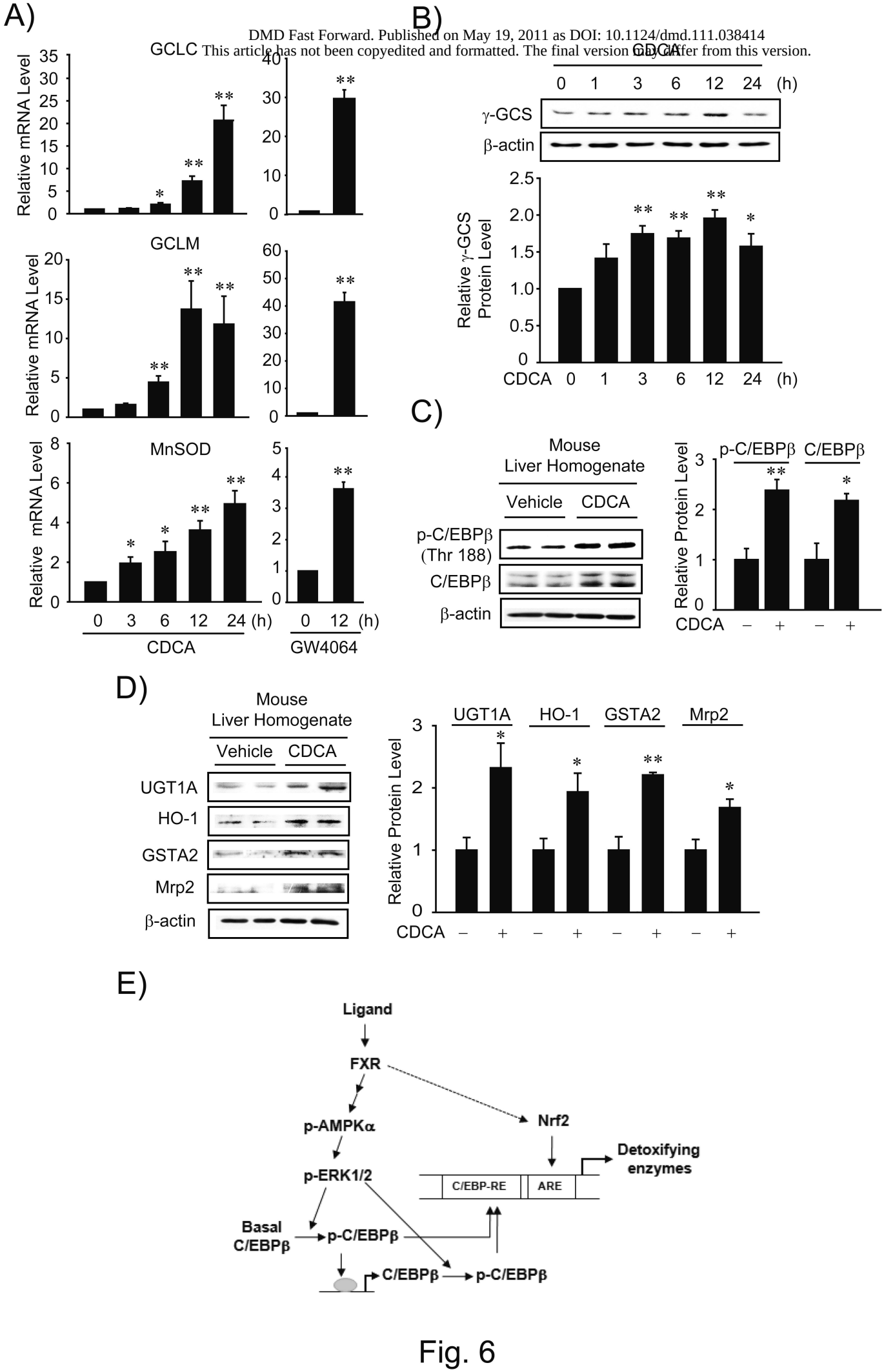

Fig. 6