Chenodeoxycholic acid suppresses the activation of acetyl-coenzyme A carboxylase-α gene transcription by the liver X receptor agonist T0-901317

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Abstract **The therapeutic utility of liver X receptor (LXR) agonists in treating atherosclerosis is limited by an undesired accumulation of triglycerides in the blood and liver. This effect is caused by an increase in the transcription of genes involved in fatty acid synthesis. Here, we show that the primary bile acid, chenodeoxycholic acid (CDCA), antagonizes the stimulatory effect of the synthetic LXR agonist, T0-901317, on the expression of acetyl-coenzyme A carboxylase-α (ACCα) and other lipogenic enzymes in chick embryo hepatocyte cultures. CDCA inhibits T0-901317-induced ACCα transcription by suppressing the enhancer activity of a LXR response unit (−101 to −71 bp) that binds LXR and sterol-regulatory element binding protein-1 (SREBP-1). We also demonstrate that CDCA decreases the expression of SREBP-1 in the nucleus and the acetylation of histone H3 and H4 at the ACCα LXR response unit. The CDCA-mediated reduction in ACCα expression is associated with a decrease in the expression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and small heterodimer partner and an increase in the expression of fibroblast growth factor-19 (FGF-19). Ectopic expression of FGF-19 decreases T0-901317-induced ACCα expression. Inhibition of p38 mitogen-activated protein kinase (MAPK) and/or extracellular signal-regulated kinase (ERK) suppresses the effects of CDCA on the expression of ACCα, SREBP-1, PGC-1α, and FGF-19. These results demonstrate that CDCA inhibits T0-901317-induced ACCα transcription by suppressing the activity of LXR and SREBP-1. We postulate that p38 MAPK, ERK, PGC-1α, and FGF-19 are components of the signaling pathway(s) mediating the regulation of ACCα gene transcription by CDCA.—**Talukdar, S., S. Bhatnagar, S. Dridi, and F. B. Hillgartner. **Chenodeoxycholic acid suppresses the activation of acetyl-coenzyme A carboxylase-α gene transcription by the liver X receptor agonist T0-901317. *J. Lipid Res.* 2007.** 48: **2647–2663.**

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The liver X receptors (LXRs) have emerged as important regulators of genes involved in lipid and lipoprotein metabolism in higher vertebrates. LXRs were initially identified as orphan members of the nuclear receptor superfamily (1, 2). Two isoforms exist with different expression patterns. LXRα is expressed at high levels in liver, adipose tissue, and macrophages, whereas LXRβ is expressed ubiquitously (2, 3). The majority of the LXR proteins are localized in the nucleus and require heterodimerization with the retinoid X receptor (RXR) to bind DNA and regulate transcription (2). LXR•RXR heterodimers bind *cis*-acting sequences that are composed of hexameric half-sites arranged as direct repeats with a 4 bp spacer separating the half-sites (20. These sequences are referred to as liver X receptor response elements (LXREs). Ligand-bound nuclear receptors activate transcription by recruiting auxiliary transcriptional regulatory proteins referred to as coactivators (4). Examples of coactivators of LXR include steroid receptor coactivator-1 (5) and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) (6). Coactivators facilitate the ability of LXR to activate transcription by interacting directly with the basal transcriptional machinery, by modulating interactions between LXR and the basal transcriptional machinery, and by modifying chromatin structure.

The screening of organic tissue extracts using a cell-based reporter assay has led to the discovery that oxysterols are the endogenous ligands that bind and activate LXRα and LXRβ (7, 8). The most potent endogenous LXR ligands are 24(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol, and 24(*S*)-epoxycholesterol. In avians and mammals, LXR agonists activate a battery of genes involved in cholesterol efflux (ABCA1, ABCG1, ABCG5, and ABCG8), cholesterol clearance (cholesteryl ester transfer protein and apolipoprotein E), and cholesterol catabolism (cholesterol 7α-hydroxylase) (reviewed in Refs. 9, 10). Because oxysterols are produced in proportion to cellular cholesterol content, LXRs have been proposed to function as

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sensors in a feed-forward pathway that stimulates reverse cholesterol transport and cholesterol excretion in response to high cholesterol levels in the diet. Consistent with this proposal, mice lacking the LXRα and/or LXRβ gene exhibit diminished cholesterol excretion and increased cholesterol levels in the blood and liver when fed a high-cholesterol diet (11, 12).

In avians and mammals, LXR agonists also activate the transcription of genes involved in triglyceride synthesis, including ATP-citrate lyase, FAS, stearoyl-coenzyme A desaturase-1 (SCD1), and acetyl-coenzyme A carboxylase-α (ACCα) (13–15). In the case of ACCα, we have shown that this effect is mediated by the activation of LXR•RXR heterodimers bound to the ACCα gene and by the increased expression of sterol-regulatory element binding protein-1 (SREBP-1) that binds a site adjacent to the ACCα LXRE and enhances the ability of LXR•RXR to activate ACCα transcription (15). LXR agonists increase the expression of SREBP-1 by activating a LXRE on the SREBP-1 gene (16).

HMG-CoA reductase inhibitors (statins) are currently the first-line agents to treat and prevent atherosclerosis in humans. Unfortunately, statins are not effective at reducing circulating cholesterol and LDL levels in a significant fraction of patients with dyslipidemia (17). This has triggered a strong interest in the development of new pharmacological approaches to achieve atheroprotection. LXR agonists represent one such approach, because these compounds stimulate reverse cholesterol transport and cholesterol excretion. Several laboratories have identified nonsteroidal, synthetic compounds that are more effective than endogenous oxysterols at stimulating LXR activity (14, 18). The atheroprotective properties of two of these synthetic LXR agonists, designated T0-901317 {*N*-(2,2,2-trifluoro-ethyl)-*N*-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]benzenesulfonamide} and GW3965 {2-(3-{3-[[2-chloro-3-(trifluoromethyl)benzyl](2,2-diphenylethyl)amino]propoxy}-phenyl)acetic acid}, have been evaluated in murine models of atherosclerosis. Oral administration of T0-901317 or GW3965 to mice lacking the LDL receptor or apolipoprotein E stimulates an increase in blood HDL levels and reverses the formation of atherosclerotic lesions in the aorta (19, 20). LXR agonists also improve insulin sensitivity in murine models of type 2 diabetes (21, 22). These exciting findings are tempered by the observation that treatment with T0-901317 or GW3965 also causes hypertriglyceridemia and the development of a fatty liver (10, 14, 23). These undesired effects of T0-901317 and GW3965 are attributable to an increase in hepatic fatty acid synthesis. One approach to overcome this problem is to activate another signaling pathway that selectively inhibits the effect of LXR agonists on lipogenic gene transcription without altering their ability to stimulate genes involved in reverse cholesterol transport.

In the present study, we report that chenodeoxycholic acid (CDCA) inhibits the ability of T0-901317 to increase the expression of ACCα and other lipogenic enzymes in primary cultures of chick embryo hepatocytes. Interestingly, CDCA does not alter the stimulatory effect of T0-901317 on the expression of ABCA1, a key protein controlling reverse cholesterol transport. We also demonstrate that CDCA decreases ACCα gene transcription by inhibiting the activity of LXR•RXR and SREBP-1 on the ACCα gene and that p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) are components of the signaling pathway(s) mediating this response.

EXPERIMENTAL PROCEDURES

Cell culture

Chick embryo hepatocytes were isolated as described previously (24). These cells were incubated in serum-free Waymouth's medium MD752/1 containing penicillin (60 μg/ml) and streptomycin (100 μg/ml) on untreated Petri dishes at 40°C in a humidified atmosphere of 5% CO_2 and 95% air. Hormone and other additions were as described in the figure legends. Concentrated stocks of T0-901317 and CDCA were dissolved in ethanol and water, respectively.

Rat hepatocytes were isolated by a modification of the technique of Seglen (25) as described by Stabile et al. (26). These cells were incubated in serum-free Waymouth's medium MD752/1 containing 20 mM HEPES, pH 7.4, 0.5 mM serine, 0.5 mM alanine, penicillin (100 μg/ml), streptomycin (100 μg/ml), and gentamicin (50 mg/ml) on 60 mm Primaria dishes at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The medium containing treatments [T0-901317 (6 μM) in the absence or presence of CDCA (75 μM)] was added after 18 h of culture.

Measurement of fatty acid synthesis

The rate of de novo fatty acid synthesis was measured in chick embryo hepatocyte cultures using the tritiated water method (27). Cells were incubated with 0.2 mCi/ml ^3H_2O during the last 3 h of a 24 h treatment period with T0-901317 in the absence or presence of CDCA. After removal of the incubation medium, the cells were harvested in 8 N KOH and transferred to screw-cap tubes. An equal volume of ethanol was added, and the tubes were heated in a boiling-water bath for 2 h. Nonsaponifiable lipids were extracted three times with petroleum ether and discarded. The aqueous phase was acidified with 0.5 volume of 12 N HCl, and saponifiable lipids were extracted three times with petroleum ether. The pooled petroleum extracts were washed once with 0.5% acetic acid and dried under a stream of N_2. ^3H radioactivity was determined by scintillation counting. Fatty acid synthesis rates were calculated as described (27).

Isolation of RNA and quantitation of mRNA levels

Medium was removed and total RNA was extracted from hepatocytes by the guanidinium thiocyanate-phenol-chloroform method (28). In some experiments, the abundance of mRNA encoding ACCα, FAS, SCD1, ATP-citrate lyase, ABCA1, SREBP-1, SREBP cleavage-activating protein (SCAP), insulin-induced gene-1 (Insig-1), and insulin-induced gene-2 (Insig-2) was measured by Northern analysis as described previously (15). In other experiments, the abundance of mRNA encoding ACCα, ABCA1, fibroblast growth factor-19 (FGF-19), LXRα, PGC-1α, PGC-1β, nuclear T3 receptor-α (TRα), and small heterodimer partner (SHP) was measured by quantitative real-time PCR analysis using the Qiagen Quantitect SYBR green RT-PCR system. Samples of DNase I-treated RNA (100 ng) were analyzed in triplicate according to the manufacturer's instructions. PCR was performed on 96-well plates using a Bio-Rad iCycler iQ. The relative amount of mRNA was calculated using the comparative threshold cycle method. 18S rRNA was used as a reference gene.

Amplification of specific transcripts was confirmed by analyzing the melting curve profile at the end of each run and by determining the size of the PCR products using agarose electrophoresis and ethidium bromide staining.

Primer sets for each gene were designed using PrimerQuest software from Integrated DNA Technologies. PCR primers used were as follows: chicken ACCα, sense, 5′-CACTTCGAGGCGAAAAACTC-3′; antisense, 5′-GGAGCAAATCCATGACCACT-3′; chicken FGF-19, sense, 5′-TGGGAATTCAGCATGTGGGTAGGA-3′; antisense, 5′-TTTCAAACGGTGCAGGATGAAGCC-3′; chicken LXRα, sense, 5′-ACTCAACTCAGCACACAGGACCAT-3′; antisense, 5′-AGCTTCTTCAGCCGAATCTGCTCT-3′; chicken PGC-1α, sense, 5′-TCAGCATGAAAGGCTGAAGAGGGA-3′; antisense, 5′-TAGCTGTCTCCATCATCCCGCAAA-3′; chicken PGC-1β, sense, 5′-TCAGAAAGCCATGAAGAAGCACCC-3′; antisense, 5′-TGATGCCATCCTTCCACACCATCT-3′; chicken TRα, sense, 5′-TTCTCAGCAGGAGTGCTCGC-3′; antisense, 5′-GGCTTCTGTTCCATTCACCGCAAT-3′; chicken SHP, sense, 5′-ACACCTTCTGGAGCCTGGATTTGA-3′; antisense, 5′-TGAGCTCAACAATGTCTGCGTTGC-3′; chicken 18S, sense, 5′-CGGAGAGGGAGCCTGAGAAA-3′; antisense, 5′-CGCCAGCTCGATCCCAAGA-3′; rat ACCα, sense, 5′-AGGGCAAAGGGACTGGTGTTCAGAT-3′; antisense, 5′-GCCAACGGAGATGGTTCATCCATTA-3′; rat ABCA1, sense, 5′-CCCAGAGCAAAAAGCGACTC-3′; antisense, 5′-GGTCATCATCACTTTGGTCCTTG-3′; rat FAS, sense, 5′-TGCAACTGTGCGTTAGCCACC-3′; antisense, 5′-TGTTTCAGGGGAGAAGAGACC-3′; and rat SCD1, sense, 5′-AGCTCAGCCAAATGCTGTGTTGTC-3′; antisense, 5′-TGCCTTGATCAGTCACAGACACCT-3′.

Plasmids

Reporter plasmids are named by designating the 5′ and 3′ ends of the ACCα DNA fragment relative to the transcription start site of promoter 2. A series of 5′ deletions and 3′ deletions of ACCα promoter 2 in the context of p[ACC−2054/+274] chloramphenicol acetyltransferase (CAT) have been described previously (29). An ACCα promoter construct containing a mutation of the SRE between −79 and −72 bp in the context of p[ACC−108/+274]CAT has been described (30). p[ACC−108/−66]TKCAT, p[ACC−84/−66]TKCAT, and pTKCAT constructs containing mutations in the −108 to −66 bp ACCα fragment have been described (30). To construct the adenoviral expression vector pAdEasy-FGF-19, the coding sequence of chicken FGF-19 was subcloned into the shuttle vector pShuttle-CMV to form pShuttle-CMV-FGF-19. The CMV promoter-FGF-19 transcription unit was inserted into pAdEasy-1 via homologous recombination in the bacterial strain BJ5183-AD-1. Adenoviruses were propagated in HEK-293 cells and purified by CsCl gradient centrifugation.

Transient transfection

Chick embryo hepatocytes were transfected as described by Zhang, Yin, and Hillgartner (29). Briefly, cells were isolated and incubated on 60 mm Petri dishes. At 6 h of incubation, the medium was replaced with one containing 10 μg of Lipofectin (Invitrogen), 1.5 μg of p[ACC−2054/+274]CAT or an equimolar amount of another reporter plasmid, and pBluescript KS(+) to bring the total amount of transfected DNA to 1.5 μg per plate. At 18 h of incubation, the transfection medium was replaced with fresh medium containing T0-901317 (6 μM) with or without CDCA (75 μM). At 66 h of incubation, chick embryo hepatocytes were harvested, and cell extracts were prepared as described (31). CAT activity (32) and protein (33) were assayed by the indicated methods.

Western blot analysis

Nuclear extracts, membrane extracts, and total cell lysates were prepared from chick embryo hepatocytes as described (15, 30). Equal amounts of denatured protein were subjected to electrophoresis on 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Millipore) using an electroblotting apparatus (Bio-Rad Laboratories, Hercules, CA). The blots were blocked in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween) containing 5% nonfat dry milk for 1 h at room temperature and then incubated with primary antibody diluted 1:2,000 in TBST containing 5% BSA. After incubation with primary antibody for 12 h at 4°C, the blots were washed in TBST. Next, the blots were incubated with secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch) diluted 1:5,000 in TBST and 5% nonfat dry milk for 1 h at room temperature. After washing with TBST, antibody-protein complexes on blots were detected using enhanced chemiluminescence (Amersham Biosciences). Chemiluminescence on the blots was visualized using a FluorChem 8000 imager (Alpha Innotech Corp.), and signals were quantified using FluorChem V200 software. A mouse monoclonal antibody against SREBP-1 (IgG-2A4) was obtained from the American Type Culture Collection (Manassas, VA). Antibodies against phosphorylated p38 MAPK (Thr^{180}/Tyr^{182}), phosphorylated ERK ($Tyr^{183/185}$), phosphorylated c-Jun N-terminal kinase (JNK) (Thr^{183}/Tyr^{185}), phosphorylated Raf (Ser^{259}), phosphorylated MAPK kinase 3/6 (MKK3/6) ($Ser^{189/207}$), phosphorylated MAPK/ERK kinase (MEK1/2) (Ser^{221}), total p38 MAPK, total ERK, total JNK, and ACCα were obtained from Cell Signaling Technology. The antibody against ABCA1 was obtained from Novus.

Gel mobility shift analysis

Nuclear extracts were prepared from hepatocytes incubated with or without CDCA in the presence of T0-901317 (30). A double-stranded oligonucleotide containing the ACCα LXRE/T3 response element (T3RE) (−108 to −82 bp relative to the transcription initiation site of ACCα promoter 2) was labeled by filling in overhanging 5′ ends using the Klenow fragment of *Escherichia coli* DNA polymerase in the presence of [α-^{32}P]dCTP. Binding reactions were carried out as described previously (29). DNA and DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gels at 4°C in 50 mM Tris (pH 8.8) and 50 mM glycine. After electrophoresis, the gels were dried and subjected to storage phosphor autoradiography.

Histone acetylation

The extent of histone acetylation on ACCα promoter 2 was measured using a chromatin immunoprecipitation (ChIP) assay. The procedure for this assay was the same as that described by Yin et al. (34). ChIP was carried out with antibodies against acetyl-histone H3 (06-599) and acetyl-histone H4 (06-866) (Upstate Biotechnology). Precipitated DNA was analyzed by PCR using Taq DNA polymerase (New England Biolabs) and primers specific for the ACCα and SCD1 promoters (15). Amplified products were subjected to electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

RESULTS

CDCA inhibits the activation of lipogenic enzyme expression by the LXR agonist T0-901317

The development of hyperlipidemia and fatty liver in avians and mammals treated with LXR agonists is mediated by an increase in the expression of lipogenic enzymes in liver (14, 15, 23, 35). Accordingly, we wanted to develop a combination strategy that suppressed the

undesired effect of LXR agonists on lipogenic genes without altering the beneficial effect of LXR agonists on genes controlling reverse cholesterol transport. We initially tested the ability of cAMP and hexanoate to suppress the stimulatory effect of T0-901317 on lipogenic enzyme expression, as previous studies showed that these compounds inhibited the increase in ACCα and FAS gene transcription caused by nuclear triiodothyronine (T3) receptor activation (36–39). Of these compounds, hexanoate (1 mM) was effective at suppressing T0-901317-induced expression of ACCα mRNA and FAS mRNA in chick embryo hepatocyte cultures (data not shown). However, treatment with hexanoate also inhibited T0-901317-induced expression of ABCA1, thus negating the usefulness of this compound in developing a combination strategy.

We next investigated the ability of CDCA to suppress the effect of T0-901317 on lipogenic enzyme expression, because previous studies showed that oral administration of CDCA reduced hypertriglyceridemia in diabetic hamsters and humans with cholesterol gallstones (40, 41). Treatment with CDCA suppressed the stimulatory effect of T0-901317 on the abundance of mRNAs encoding ACCα, FAS, and SCD1 (**Fig. 1A**). The effect of CDCA on T0-901317-induced expression of ACCα, FAS, and SCD1 was dose-dependent, with a maximal inhibition (61–68%) observed at 50 μM. The CDCA-mediated reduction in ACCα mRNA abundance was associated with a decrease (55%) in ACCα protein concentration (Fig. 1B). In contrast to the results for lipogenic enzymes, CDCA enhanced the stimulatory effect of T0-901317 on the abundance of the mRNA encoding ABCA1, a key gene controlling reverse cholesterol transport (Fig. 1A). CDCA treatment did not alter the stimulatory effect of T0-901317 on ABCA1 protein concentration (Fig. 1B). In the absence of T0-901317, CDCA treatment had no effect on the abundance of mRNAs encoding ACCα, FAS, SCD1, and ABCA1. Thus, activation of a CDCA signaling pathway(s) selectively inhibits the effect of T0-901317 on the expression of lipogenic enzymes in avian hepatocytes.

We next asked whether CDCA altered LXR signaling activity in a mammalian cell culture system. Treatment of rat hepatocyte cultures with CDCA (75 μM) suppressed the stimulatory effect of T0-901317 on the abundance of mRNAs encoding ACCα, FAS, and SCD1 by 33–56% (data not shown). In contrast, CDCA treatment had no effect on T0-901317-induced expression of ABCA1 mRNA. Thus, CDCA inhibition of lipogenic enzyme expression is conserved across different classes of animals.

Oral administration of bile acids causes a reduction in serum triglyceride levels in animals treated with T0-901317 (42). To investigate whether this effect is mediated by a decrease in hepatic lipogenesis, the rate of fatty acid synthesis was measured in chick embryo hepatocytes incubated with T0-901317 in the absence and presence of CDCA. Treatment of hepatocytes with T0-901317 for 24 h caused a 4.3-fold increase in the rate of fatty acid synthesis (**Fig. 2A**), and the addition of CDCA (75 μM) blocked this effect. We also measured hepatic triglyceride production in chick embryo hepatocytes by monitoring the triglycer-

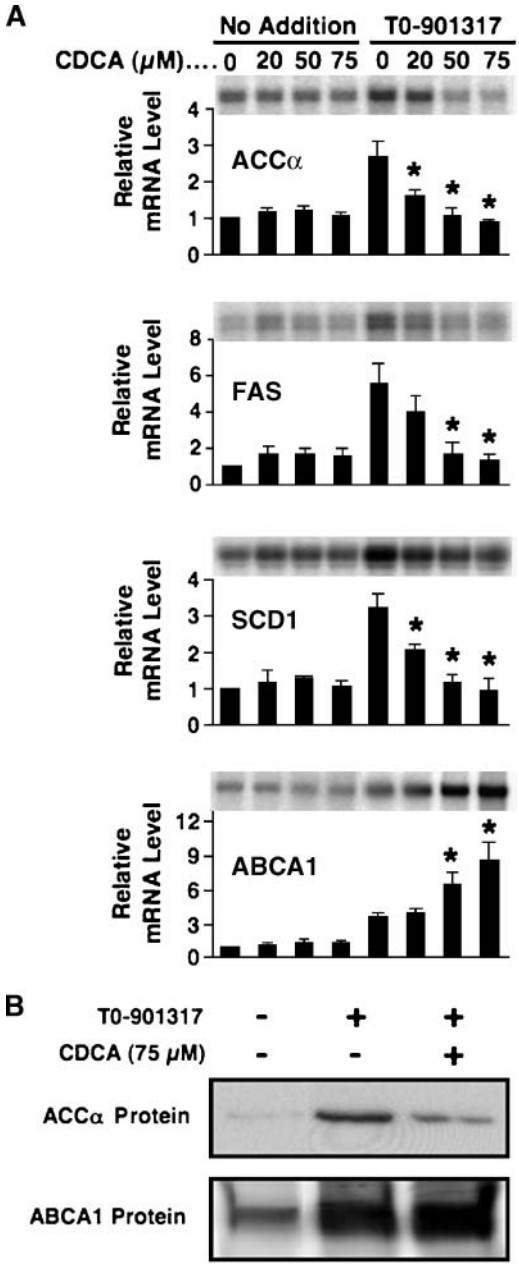

Fig. 1. Chenodeoxycholic acid (CDCA) suppresses the activation of lipogenic enzyme expression by the liver X receptor (LXR) agonist T0-901317. Chick embryo hepatocytes were isolated and incubated in serum-free Waymouth's medium containing insulin (50 nM). At 18 h of incubation, the medium was changed to one of the same composition supplemented with or without T0-901317 (6 μM) in the absence or presence of CDCA (0–75 μM). Total RNA and cell lysates were prepared after 28 and 48 h of treatment, respectively. A: The abundance of mRNA encoding acetyl-coenzyme A carboxylase-α (ACCα), FAS, stearoyl-coenzyme A desaturase-1 (SCD1), and ABCA1 was measured by Northern analysis. Levels of mRNA in cells treated without T0-901317 and CDCA were set at 1, and the other values were adjusted proportionately. Values are means ± SEM of four experiments. Hybridization signals from a representative experiment are shown for each mRNA. Asterisks indicate that the mean is significantly ($P < 0.05$) different from that of cells treated with T0-901317 in the absence of CDCA. B: The abundance of ACCα protein and ABCA1 protein in total cell lysates was measured by Western analysis. The data are representative of three independent experiments.

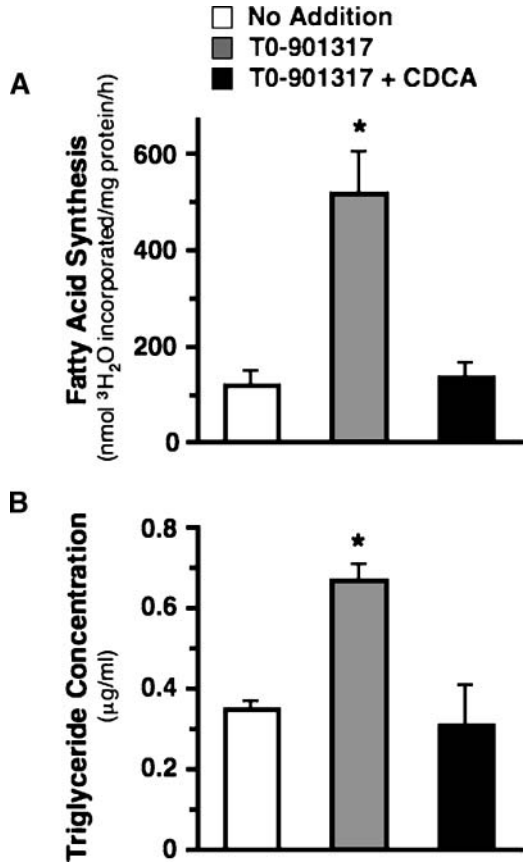

Fig. 2. CDCA suppresses the stimulatory effect of T0-901317 on fatty acid synthesis and triglyceride production. Chick embryo hepatocytes were plated on 90 mm Petri dishes (1×10^7 cells/dish) in Waymouth's medium containing insulin. At 18 h of incubation, the medium was changed to one of the same composition supplemented with or without T0-901317 in the absence and presence of CDCA (75 μM). A: Between 39 and 42 h of incubation, cells were incubated with ^3H_2O and the incorporation of tritium into fatty acids was measured as described in Experimental Procedures. B: At 42 h of incubation, the concentration of triglyceride in the culture medium was measured using a spectrophotometric assay. Data are means ± SEM of three experiments. Asterisks indicate that the mean is significantly ($P < 0.05$) higher than that of cells incubated with T0-901317 and CDCA.

ide concentration of the culture medium. In accordance with the results from the fatty acid synthesis experiments, treatment with CDCA blocked the ability of T0-901317 to increase the triglyceride concentration of the culture medium (Fig. 2B). These findings suggest that alterations in hepatic lipogenic enzyme expression and triglyceride secretion play a role in mediating the inhibitory effect of CDCA on serum triglyceride levels in intact animals.

Identification of *cis*-acting sequences that mediate the effect of CDCA on T0-901317-induced ACCα transcription

To determine the mechanism by which CDCA suppressed the increase in lipogenic enzyme expression caused by T0-901317, transient transfection experiments were performed to identify *cis*-acting elements conferring CDCA regulation of gene transcription. In conducting these studies, we focused on the chicken ACCα gene, as the mechanism mediating the stimulatory effect of T0-901317 on the transcription of this gene is well characterized. T0-901317 increases ACCα transcription by activating LXR•RXR complexes bound to a composite LXRE/T3RE (−101 to −86 bp) and by increasing the binding of SREBP-1 to an adjacent sterol regulatory element (SRE) (−82 to −71 bp) in the more downstream promoter (promoter 2) of the ACCα gene (15). SREBP-1 enhances the ability of ligand-bound LXR•RXR complexes to activate ACCα transcription. To determine the role of the ACCα LXRE, ACCα SRE, and other sequences in the ACCα gene in mediating the inhibitory effect of CDCA on T0-901317-induced ACCα transcription, a series of reporter constructs containing 5′ deletions of ACCα promoter 2 were transfected into chick embryo hepatocytes. In cells transfected with a reporter construct containing 2,054 bp of 5′ flanking DNA, CDCA treatment decreased T0-901317-induced promoter activity by 58% (**Fig. 3A**). 5′ deletion of ACCα sequences to −391, −136, and −108 bp had no effect on CDCA responsiveness. Deletion of ACCα sequences containing the LXRE/T3RE (−108 to −84 bp) abolished the CDCA-mediated inhibition of ACCα transcription. This deletion also decreased T0-901317-induced ACCα promoter activity by 54%. Further deletion to −41 bp had no effect on CDCA responsiveness. Mutation of the SRE (−80 to −71 bp) in the context of 108 bp of 5′ flanking DNA decreased CDCA responsiveness by 61%. These results suggest that the ACCα LXRE is required for CDCA-mediated inhibition of ACCα transcription and that the ACCα SRE enhances the ability of the LXRE to confer inhibition of ACCα transcription by CDCA.

To further investigate the role of the ACCα LXRE and SRE in mediating the effect of CDCA on ACCα transcription, transfection analyses were carried out using constructs containing fragments of the ACCα gene linked to a heterologous promoter. The minimal promoter of the thymidine kinase (TK) gene was unresponsive to CDCA (Fig. 3B). When a DNA fragment containing both the ACCα LXRE and ACCα SRE (−108 to −66 bp) was linked to the TK promoter, treatment with CDCA caused a 60% decrease in promoter activity. Mutation of the ACCα SRE in the context of the ACCα −108 to −66 bp fragment caused a 38% decrease in CDCA responsiveness. When a DNA fragment containing the ACCα SRE alone (−84 to −66 bp) was appended to the TK promoter, CDCA treatment had no effect on promoter activity. These results confirm that the ACCα LXRE alone is effective at conferring the inhibitory effect of CDCA on ACCα promoter activity and that the presence of the ACCα SRE enhances the ability of the ACCα LXRE to confer CDCA regulation. Thus, CDCA inhibits T0-901317-induced ACCα transcription by suppressing the ability of the LXRE and SRE to activate transcription.

CDCA suppresses the stimulatory effect of T0-901317 on the abundance of mature SREBP-1

SREBP-1 is synthesized as a 125 kDa precursor protein that is anchored to the endoplasmic reticulum (ER) (43).

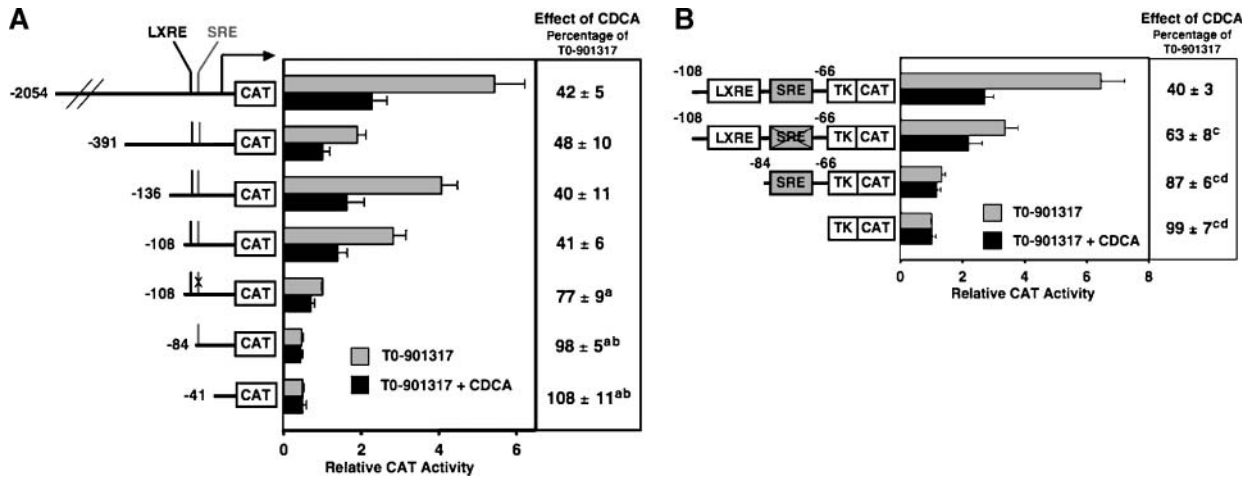

Fig. 3. A LXR response unit composed of a liver X receptor response element (LXRE) and a sterol-regulatory element (SRE) confers the inhibitory effect of CDCA on T0-901317-induced ACCα gene transcription. Chick embryo hepatocytes were transiently transfected with reporter constructs containing portions of ACCα promoter 2 linked to the chloramphenicol acetyltransferase (CAT) gene. After transfection, cells were treated with or without T0-901317 in the absence and presence of CDCA for 48 h. Cells were then harvested, extracts prepared, and chloramphenicol acetyltransferase (CAT) assays performed. A: Effect of mutations of the 5′ flanking region of ACCα promoter 2 on the CDCA regulation of transcriptional activity. The number at the left of each construct is the 5′ end of ACCα DNA in nucleotides relative to the transcription initiation site of promoter 2. The 3′ end of each construct is +274 bp. The locations of the LXRE (−101 to −86 bp) and the SRE (−80 to −71 bp) are indicated by vertical lines. A block mutation of the SRE is indicated by an X through the vertical line. The CAT activity of cells transfected with p[ACC−108/+274]CAT and treated with T0-901317 was set at 1, and the other activities were adjusted proportionately. B: Ability of the ACCα LXRE and SRE to confer CDCA regulation on the minimal thymidine kinase (TK) promoter in TKCAT. A block mutation of the SRE is indicated by an X across the box representing the SRE. CAT activity of cells transfected with TKCAT and treated with T0-901317 was set at 1, and the other activities were adjusted proportionately. The effect of CDCA is the CAT activity of cells treated with T0-901317 and CDCA expressed as a percentage of that in cells treated with T0-901317. The effect of CDCA was calculated for individual experiments and then averaged. The results are means ± SEM of five experiments. Significant difference between means within a column ($P < 0.05$) are as follows: [a] versus p[ACC−108/+274]CAT; [b] versus p[ACC−108/+274]CAT containing a block mutation of the SRE; [c] versus p[ACC−108/−66]TKCAT; [d] versus p[ACC−108/−66]TKCAT containing a block mutation of the SRE.

To become transcriptionally active, precursor SREBP-1 is translocated to the Golgi, where it is cleaved by two proteases, resulting in the release of the N-terminal segment of SREBP-1, referred to as mature SREBP-1. Mature SREBP-1 is transported into the nucleus, where it binds to target genes and activates transcription. T0-901317 increases ACCα transcription, in part, by increasing the expression of mature SREBP-1 (15). This observation prompted us to investigate whether the CDCA inhibited ACCα SRE activity by suppressing the stimulatory effect of T0-901317 on the concentration of mature SREBP-1. In chick embryo hepatocytes previously incubated with T0-901317 for 24 h, the addition of CDCA caused a rapid decrease (≤2 h) in the concentration of mature SREBP-1, with a maximal inhibition (36%) observed after 12 h of treatment (**Fig. 4**). These changes in mature SREBP-1 concentration were closely associated with alterations in ACCα mRNA abundance. These results suggest that changes in mature SREBP-1 concentration play a role in mediating the decrease in ACCα SRE activity caused by CDCA.

To investigate the mechanism mediating the reduction in mature SREBP-1 levels caused by CDCA, the effect of CDCA on the concentration of precursor SREBP-1 protein and SREBP-1 mRNA was determined in hepatocytes previously treated with T0-901317 for 24 h. Addition of CDCA caused a rapid (≤6 h) and sustained (≥24 h) increase in the abundance of precursor SREBP-1 (Fig. 4). Thus, the decrease in the concentration of mature SREBP-1 caused by CDCA is not associated with a reduction in the concentration of precursor SREBP-1, suggesting that a posttranslational process is involved in mediating the effect of CDCA on mature SREBP-1 levels. Further evidence that a posttranslational process plays a role in mediating the actions of CDCA on mature SREBP-1 levels is provided by the observation that treatment with CDCA has no effect on the abundance of SREBP-1 mRNA (Fig. 4).

One posttranslational process that controls the abundance of mature SREBP-1 is the proteolytic cleavage of precursor SREBP-1 to mature SREBP-1 (43). This process is controlled by two ER proteins, SCAP and Insig. SCAP binds to SREBP-1 and escorts it from the ER to the Golgi. Insig binds to SCAP and retains the SCAP-SREBP-1 complex in the ER, thus preventing the processing of SREBP-1. There are two isoforms of Insig (Insig-1 and Insig-2) that are encoded by separate genes. Insulin decreases the expression of Insig-2 in liver, and this effect has been proposed to play a role in mediating the stimulatory effect of insulin on SREBP-1 processing (44). To investigate whether changes in Insig expression play a role in mediating the inhibitory effect of CDCA on mature SREBP-1 levels, the effect of CDCA on the abundance of Insig-1 mRNA and Insig-2 mRNA was determined. In hepatocytes incubated with T0-901317 for 24 h, the addition of CDCA for 2 h caused a 1.9-fold increase in Insig-1 mRNA

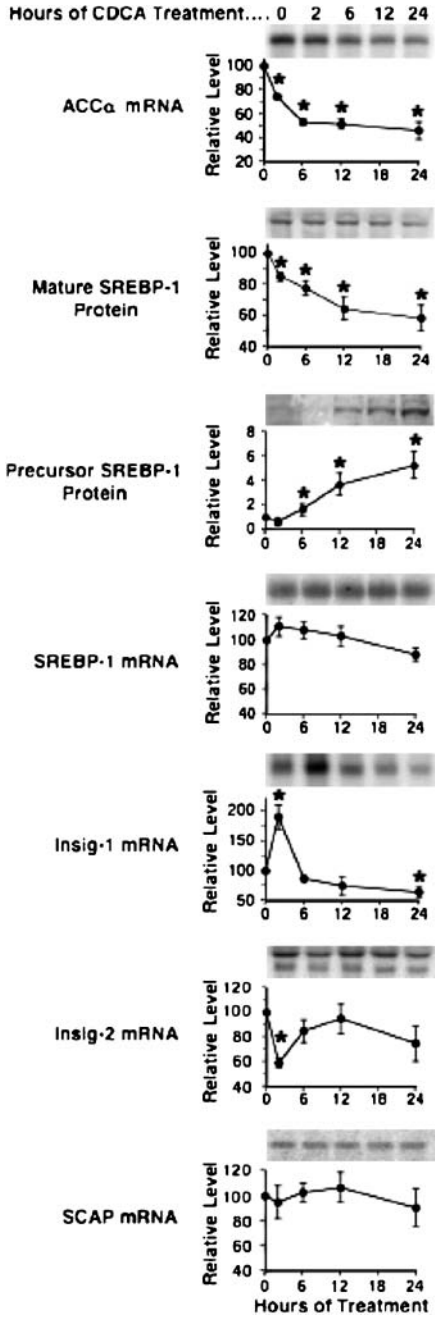

Fig. 4. CDCA suppresses the stimulatory effect of T0-901317 on the concentration of mature sterol-regulatory element binding protein-1 (SREBP-1). Chick embryo hepatocytes were isolated and incubated in serum-free Waymouth's medium containing insulin. At 18 h of incubation, the medium was changed to one containing insulin and T0-901317. At 36 h of incubation, the medium was replaced with one of the same composition. CDCA was added to the medium at 42 h of incubation. After 2, 6, 12, and 24 h of CDCA treatment, cellular extracts or total RNA were prepared. The abundance of mature SREBP-1 in nuclear extracts and precursor SREBP-1 in membrane extracts was measured by Western analyses. The abundance of mRNA encoding SREBP-1, insulin-induced gene-1 (Insig-1), insulin-induced gene-2 (Insig-2), SREBP cleavage-activating protein (SCAP), and ACCα was measured by Northern analysis. Levels of mRNA or protein in cells treated with CDCA for 0 h were set at 100, and the other values were adjusted proportionately. Values are means ± SEM of five experiments. Asterisks indicate that the mean is significantly different ($P < 0.05$) from that of cells incubated with CDCA for 0 h.

levels, whereas Insig-2 mRNA levels were decreased by 41% (Fig. 4). CDCA had no effect on the abundance of Insig-1 mRNA and Insig-2 mRNA after 6 and 12 h of treatment. CDCA did not alter the abundance of SCAP mRNA at any of the time points. The transient and opposing actions of CDCA on Insig-1 and Insig-2 expression suggest that alterations in Insig expression play a minimal role in mediating the effect of CDCA on mature SREBP-1 levels. Previous work has shown that bile acids increase hepatic cholesterol levels (42, 45). This phenomenon may mediate the inhibitory effect of CDCA on SREBP-1 processing by promoting the interaction between Insig and SCAP-SREBP-1.

Effect of CDCA on the binding of nuclear hormone receptor complexes to the ACCα LXRE/T3RE

On the basis of the observation that the ACCα LXRE/T3RE was required for optimal inhibition of ACCα transcription by CDCA, we hypothesized that the effect of CDCA on ACCα transcription was mediated by alterations in the binding of nuclear proteins to the ACCα LXRE/T3RE. To investigate this possibility, gel mobility shift analyses were performed using nuclear extracts from chick embryo hepatocytes incubated with T0-901317 for 24 h followed by the addition of CDCA for various time periods. We previously reported that the ACCα LXRE/T3RE (−101 to −86 bp) bound to four protein complexes in nuclear extracts from chick embryo hepatocytes (29). Three of these complexes (designated complexes 1, 2, and 3) contained LXR•RXR heterodimers, whereas the fourth complex (designated complex 4) contained TR•RXR heterodimers. In **Fig. 5**, we show that the ACCα LXRE/T3RE bound to complex 1, complex 2, and complex 4 in nuclear extracts from hepatocytes incubated with T0-901317 for 24 h. Complex 3 binding activity was not observed in hepatocytes incubated under these conditions. Addition of CDCA had no effect on the binding of complexes 1, 2, and 3, suggesting that the inhibitory effect of CDCA on ACCα LXRE activity was not attributable to changes in the binding of LXR•RXR complexes to the ACCα LXRE/T3RE. In support of this conclusion, CDCA had no effect on the abundance of LXRα mRNA in chick embryo hepatocytes (**Fig. 6**). In contrast to the results for complexes 1, 2, and 3, addition of CDCA decreased the binding of complex 4 after 6, 12, and 24 h of treatment (Fig. 5). This effect was associated with a reduction in the expression of TRα mRNA in chick embryo hepatocytes (Fig. 6). Because TR•RXR heterodimers repress transcription in the absence of T3 (46), it is unlikely that alterations in complex 4 binding activity mediate the inhibitory effect of CDCA on T0-901317-induced ACCα LXRE activity.

Effect of CDCA on histone acetylation of the ACCα gene and the expression of SHP, PGC-1α, and FGF-19 in avian hepatocytes

CDCA is a ligand for the nuclear hormone receptor farnesoid X receptor (FXR) (47). FXR forms heterodimers with RXR and binds DNA sequences [referred to

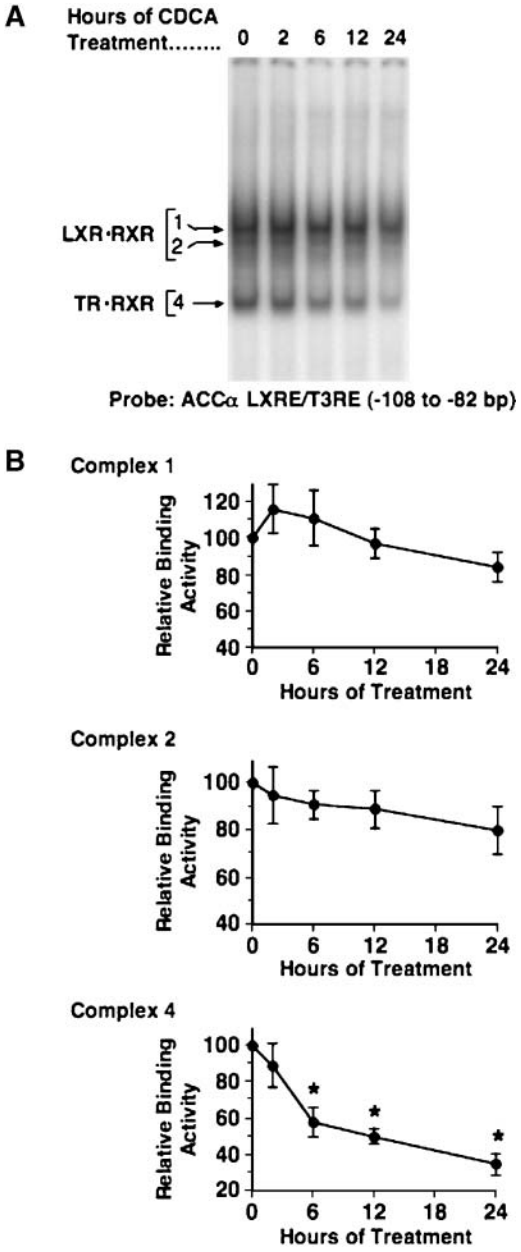

Fig. 5. Effects of CDCA on the binding of nuclear receptor complexes to the ACCα LXRE/T3 response element (T3RE). Chick embryo hepatocytes were isolated and incubated in serum-free Waymouth's medium containing insulin. At 18 h of incubation, the medium was changed to one containing insulin and T0-901317. At 36 h of incubation, the medium was replaced with one of the same composition. CDCA was added to the medium at 42 h of incubation. After 2, 6, 12, and 24 h of CDCA treatment, nuclear extracts were prepared as described in Experimental Procedures. Nuclear extracts were subjected to gel mobility shift analyses using an oligonucleotide probe containing the ACCα LXRE/T3RE (−108 to −82 bp). Specific protein-DNA complexes are indicated by arrows. Previous studies have shown that complexes 1 and 2 contain LXR•retinoid X receptor (RXR) heterodimers, whereas complex 4 contains TR•RXR heterodimers (29). A: Data from a representative experiment. B: Signals for complex 1, complex 2, and complex 4 were quantitated. The binding activities of complex 1, complex 2, and complex 4 in hepatocytes treated with CDCA for 0 h were set at 100, and the other activities were adjusted proportionately. Values are means ± SEM of four experiments. Asterisks indicate that the mean is significantly different ($P < 0.05$) from that of cells incubated with CDCA for 0 h.

as FXR response elements (FXREs)] that are distinct from LXREs. FXREs are composed of two hexamers (consensus AGGTCA) arranged as inverted repeats with 1 bp separating the repeats. In the presence of CDCA, FXR•RXR heterodimers activate genes involved in bile acid export, bile acid conjugation, lipoprotein metabolism, and blood clotting (reviewed in Ref. 47). In mouse hepatocytes, CDCA-mediated activation of FXR•RXR heterodimers also increases the transcription of SHP, an atypical member of the nuclear receptor superfamily that lacks the ability to bind DNA (42). Alterations in SHP expression play a role in mediating the inhibitory effect of CDCA on the transcription of cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting step in the bile acid synthesis pathway (48, 49). SHP interacts with liver receptor homolog-1 and represses the ability of liver receptor homolog-1 to activate CYP7A1 transcription. Other studies have shown that SHP overexpression inhibits the ability of LXR to activate transcription in transfection assays (50). To investigate the role of SHP in mediating the inhibitory effect of CDCA on LXR activation of ACCα, we determined whether CDCA modulated the expression of SHP in chick embryo hepatocytes. In contrast to the effect of CDCA on SHP expression in mouse hepatocytes, CDCA caused a dose-dependent decrease in the abundance of SHP mRNA in chick embryo hepatocytes incubated with or without T0-901317 (Fig. 6A, B). If SHP is a repressor of LXR activity, then alterations in SHP expression do not mediate CDCA regulation of ACCα transcription in chick embryo hepatocytes.

Another factor that influences LXR activity is the level of expression of the coactivator, PGC-1α (6, 51). Previous studies have shown that activation of FXR causes a reduction in PGC-1α expression in mouse liver (52). This observation led us to investigate whether CDCA modulated the expression of PGC-1α in chick embryo hepatocytes. Addition of CDCA in the presence of T0-901317 caused a rapid (≤2 h), sustained (≥24 h), and dose-dependent decrease in the abundance of PGC-1α mRNA in chick embryo hepatocytes (Fig. 6A, B). CDCA treatment also decreased PGC-1α mRNA abundance in the absence of T0-901317 (Fig. 6B). In contrast, CDCA treatment had no effect on the abundance of the mRNA encoding the related protein, PGC-1β. These observations are consistent with a role of PGC-1α in mediating the effect of CDCA on T0-901317-induced ACCα transcription.

PGC-1α activates transcription by recruiting histone acetyltransferases (i.e., steroid receptor coactivator-1 and CREB binding protein/p300) that modify the chromatin of target promoters (53). To obtain further evidence that alterations in PGC-1α expression play a role in mediating the inhibitory effect of CDCA on ACCα LXRE, the extent of histone acetylation in a region of chromatin encompassing the ACCα LXRE was measured using a ChIP assay. Treatment of hepatocytes with CDCA reduced the acetylation of histone H3 and histone H4 at the ACCα LXRE (**Fig. 7**). This observation is consistent with the inhibitory effect of CDCA on PGC-1α expression. The decrease in histone acetylation caused by CDCA may inhibit ACCα

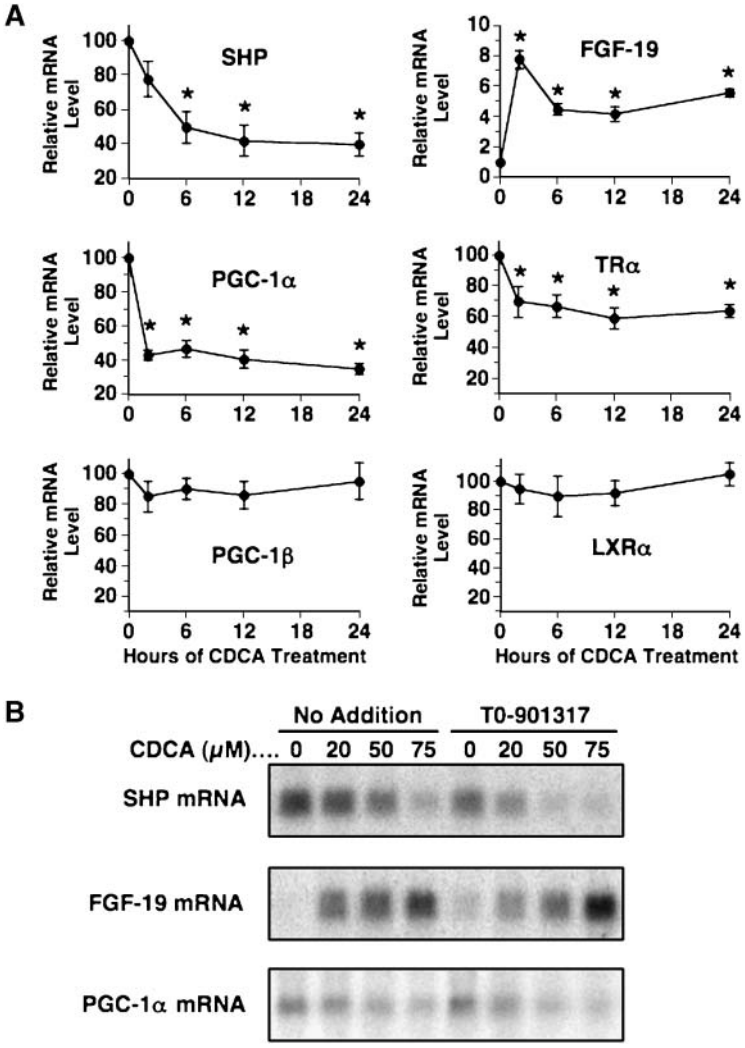

Fig. 6. Effects of CDCA on the expression of small heterodimer partner (SHP), peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), PGC-1β, fibroblast growth factor-19 (FGF-19), LXRα, and nuclear T3 receptor-α (TRα). A: Time course of CDCA action. Chick embryo hepatocytes were isolated and incubated in serum-free Waymouth's medium containing insulin. At 18 h of incubation, the medium was changed to one containing insulin and T0-901317. At 36 h of incubation, the medium was replaced with one of the same composition. CDCA (75 μM) was added to the medium at 42 h of incubation. After 2, 6, 12, and 24 h of CDCA treatment, total RNA was prepared. The abundance of mRNA encoding SHP, PGC-1α, PGC-1β, FGF-19, LXRα, and TRα was measured by quantitative real-time PCR analysis. Levels of mRNA in cells treated with CDCA for 0 h were set at 100, and the other values were adjusted proportionately. Values are means ± SEM of five experiments. Asterisks indicate that the mean is significantly ($P < 0.05$) different from that of cells incubated with CDCA for 0 h. In cells treated with CDCA for 2 h, the mean threshold cycle from quantitative real-time PCR analysis of FGF-19 mRNA and ACCα mRNA (100 ng of total RNA) was 17.2 and 17.8, respectively. The mean threshold cycle for 18S RNA (1 ng of total RNA) was 16.5. B: The effects of CDCA on the expression of SHP, FGF-19, and PGC-1α are dose-dependent. Chick embryo hepatocytes were isolated and incubated in serum-free Waymouth's medium containing insulin. At 18 h of incubation, the medium was changed to one of the same composition supplemented with or without T0-901317 in the absence or presence of different concentrations of CDCA (0–75 μM). After 28 h of treatment, the abundance of SHP mRNA, FGF-19 mRNA, and PGC-1α mRNA was measured by Northern analysis. The data are representative of four experiments.

LXRE activity by promoting chromatin condensation, thereby limiting the access of DNA-bound LXR•RXR complexes to the basal transcriptional machinery and other transcriptional regulatory proteins. The effect of CDCA on histone acetylation was sequence-specific, as CDCA had no effect on histone acetylation at an uncharacterized region of the SCD1 promoter (Fig. 7). Because of a lack of suitable antibodies, ChIP could not be performed to analyze the recruitment of LXRα and PGC-1α to the ACCα gene.

Holt et al. (54) have identified an alternative pathway through which natural and synthetic FXR agonists inhibit CYP7A1 expression in human hepatocytes. In this pathway, treatment with FXR agonists stimulates an increase in the expression and secretion of FGF-19. This effect is mediated by a FXRE in the FGF-19 gene. Secreted FGF-19 binds a cell surface receptor (fibroblast growth factor receptor 4) and initiates a signaling cascade that results in the activation of JNK and repression CYP7A1 transcription. To assess the role of FGF-19 in mediating the inhibitory effect of CDCA on ACCα transcription, we first determined whether CDCA modulated the expression of FGF-19 in chick embryo hepatocytes. Treatment with CDCA for 2 h stimulated a robust increase (7.8-fold) in FGF-19 mRNA abundance (Fig. 6A). The stimulatory effect of CDCA on FGF-19 mRNA abundance was sustained for at least 24 h of treatment and was dose-dependent (Fig. 6A, B). We also determined whether the expression of exogenous FGF-19 mimicked the effect of CDCA on T0-901317-induced ACCα expression. Infection of chick hepatocytes with a recombinant adenovirus containing the chicken FGF-19 gene (AdEasy-FGF-19; 100 plaque-forming units/cell) decreased T0-901317-induced ACCα mRNA abundance by 36% relative to cells infected with adenovirus lacking the chicken FGF-19 gene (AdEasy-Null) (**Fig. 8**). These observations provide support for a role of FGF-19 in mediating the effect of CDCA on ACCα transcription. The exogenous FGF-19 mRNA expression level (AdEasy-FGF-19; 100 plaque-forming units/cell) required to elicit a decrease in ACCα expression was higher than the endogenous FGF-19 mRNA expression level observed in hepatocytes treated with 75 μM CDCA. One interpretation of this finding is that other processes in addition to an increase in FGF-19 expression are involved in mediating the inhibitory action of CDCA on ACCα expression. These processes may increase the sensitivity of hepatocytes to FGF-19.

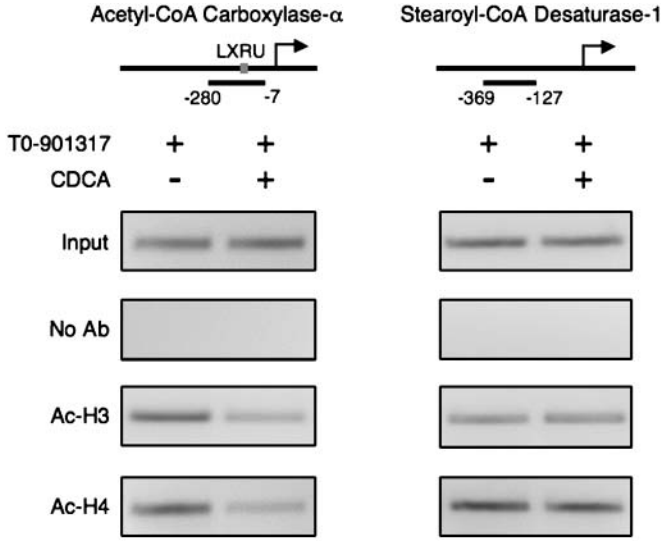

Fig. 7. CDCA decreases histone acetylation at the ACCα LXR response unit. Chick embryo hepatocytes were isolated and incubated in serum-free Waymouth's medium containing insulin. At 18 h of incubation, the medium was changed to one containing insulin and T0-901317. At 36 h of incubation, the medium was replaced with one of the same composition. CDCA was added to the medium at 42 h of incubation. After 6 h of CDCA treatment, the association of acetylated (Ac) histone H3 and acetylated histone H4 with ACCα and SCD1 genomic sequences was measured. Chromatin immunoprecipitation (ChIP) assays were performed as described in Experimental Procedures. Immunoprecipitates were analyzed by PCR using primers that flanked the LXR response unit (LXRU) of ACCα promoter 2 and an uncharacterized region of the SCD1 promoter. The regions of the ACCα gene and the SCD1 gene that were amplified by PCR are indicated at the top. Chromatin samples that were processed in parallel without the application of primary antibody (No Ab) served as controls. The input lanes show the results of PCR using chromatin samples taken before the immunoprecipitation step. Results are representative of four independent experiments.

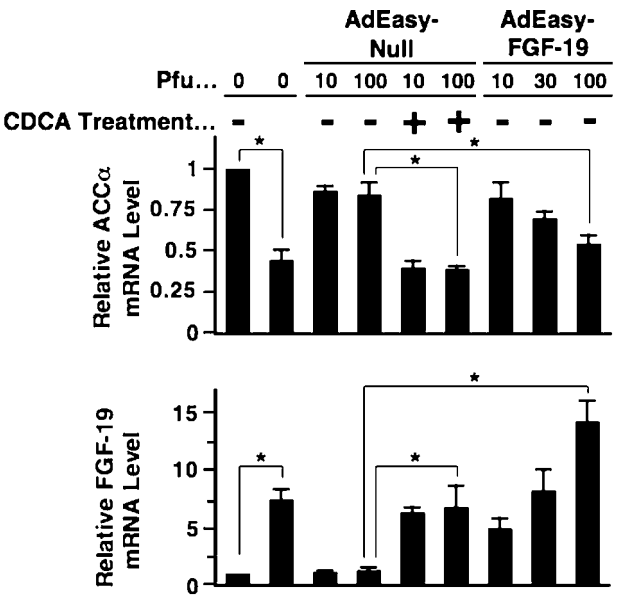

Fig. 8. FGF-19 mimics the inhibitory effect of CDCA on ACCα expression. Chick embryo hepatocytes were isolated and incubated in Waymouth's medium lacking hormones. At 4 h of incubation, the medium was changed to one containing T0-901317 and insulin, and recombinant adenovirus [10–100 plaque-forming units (Pfu)/cell] was added at this time. At 24 h of incubation, the medium was replaced with one containing T0-901317 and insulin with or without CDCA. At 48 h of incubation, the cells were harvested and total RNA was isolated. The abundance of ACCα mRNA and FGF-19 mRNA was measured by quantitative real-time PCR analysis. The level of mRNA in cells treated without adenovirus and CDCA was set at 1, and the other values were adjusted proportionately. Values are means ± SEM of four experiments. Significant differences ($P < 0.05$) between means are indicated by the asterisks.

Role of ERK and p38 MAPK in mediating the regulation of ACCα transcription by CDCA

In addition to JNK, there is evidence that other MAPK signaling pathways are involved in mediating the actions of bile acids on gene expression and cellular processes. For example, bile acid induction of hepatic apoptosis and LDL receptor expression is mediated by the activation of p38 MAPK and ERK, respectively (55, 56). To investigate the role of MAPKs in mediating the effect of CDCA on T0-901317-induced ACCα transcription, we first determined whether CDCA modulated the phosphorylation of amino acid residues that control the activity of ERK, JNK, and p38 MAPK. Treatment of chick embryo hepatocytes with CDCA in the presence of T0-901317 (**Fig. 9**) or the absence of T0-901317 (data not shown) caused a rapid (≤5 min) and sustained (≥24 h) increase in the phosphorylation of ERK ($Tyr^{183/185}$), JNK (Thr^{183}/Tyr^{185}), and p38 MAPK (Thr^{180}/Tyr^{182}). CDCA had a similar effect on the phosphorylation of the upstream kinases for ERK (MEK1/2) and p38 MAPK (MKK3/6) (Fig. 9). CDCA had no effect on the concentration of total ERK, JNK, and p38 MAPK. These findings indicate that CDCA stimulates the activity of ERK, JNK, and p38 MAPK in chick embryo hepatocytes.

We next determined the effects of cell-permeable inhibitors of MEK1/2 (U0126) and p38 MAPK (SB203580) on the regulation of ACCα, PGC-1α, FGF-19, and SREBP-1 by CDCA. Treatment of hepatocytes with U0126 or SB203580 suppressed the inhibitory effect of CDCA on T0-901317-induced ACCα mRNA levels (**Fig. 10A**). This observation suggests that the regulation of ACCα by CDCA requires the presence of ERK and p38 MAPK. Treatment of hepatocytes with SB203580 also suppressed the effects of CDCA on the abundance of PGC-1α mRNA, FGF-19 mRNA, and mature SREBP-1 protein (Fig. 10A, B). These findings are consistent with the suggestion that p38 MAPK mediates the effect of CDCA on ACCα transcription by modulating the expression of PGC-1α, FGF-19, and/or mature SREBP-1. Treatment with U0126 also suppressed the effect of CDCA on FGF-19 mRNA abundance but had no effect on CDCA-induced changes in PGC-1α mRNA concentration and mature SREBP-1 levels. These observations are consistent with the suggestion that ERK acts through the FGF-19 signaling pathway in mediating CDCA regulation of ACCα gene transcription. Results of experiments using a selective inhibitor of JNK (SP600126) were inconclusive, as this compound inhibited the induction of ACCα mRNA levels caused by T0-901317 (data not shown).

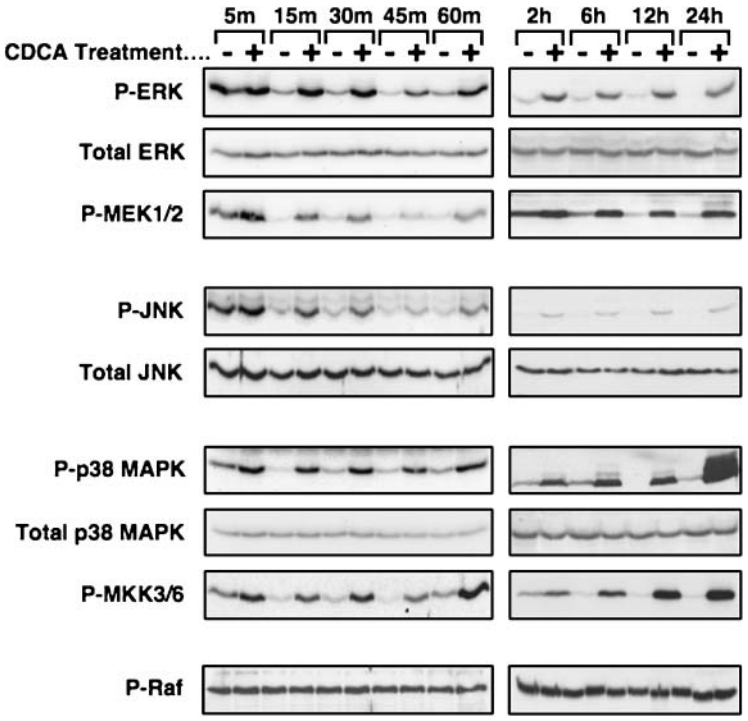

Fig. 9. CDCA increases the phosphorylation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) in chick embryo hepatocytes incubated with T0-901317. Chick embryo hepatocytes were isolated and incubated in serum-free Waymouth's medium containing insulin. At 18 h of incubation, the medium was changed to one containing insulin and T0-901317. At 36 h of incubation, the medium was replaced with one of the same composition. CDCA was added to the medium at 42 h of incubation. After 5, 15, 30, 45, and 60 min and 2, 6, 12, and 24 h of CDCA treatment, total cell lysates were prepared. Western analyses were performed using antibodies against phosphorylated p38 MAPK (Thr^{180}/Tyr^{182}), phosphorylated ERK ($Tyr^{183/185}$), phosphorylated JNK (Thr^{183}/Tyr^{185}), phosphorylated Raf (Ser^{259}), phosphorylated MAPK kinase 3/6 (MKK3/6) ($Ser^{189/207}$), phosphorylated MAPK/ERK kinase (MEK1/2) (Ser^{221}), total p38 MAPK, total ERK, and total JNK. The data shown are from a representative experiment. This experiment was repeated two times with similar results.

To investigate whether the CDCA-mediated activation of p38 MAPK was dependent on ERK, we determined the effect of U0126 on the phosphorylation of p38 MAPK. Inhibition of ERK by U0126 had no effect on the ability of CDCA to stimulate the phosphorylation of p38 MAPK (Fig. 10C). We also demonstrated that inhibition of p38 MAPK by SB203580 had no effect on the ability of CDCA to stimulate the phosphorylation of ERK. These results indicate that activation of p38 MAPK by CDCA is not linked to the activation ERK and vice versa. This finding is consistent with the observation that CDCA-induced changes in the expression of PGC-1α and mature SREBP-1 are sensitive to p38 MAPK inhibition but not to ERK inhibition.

We next compared the effects of different bile acids on the phosphorylation of p38 MAPK and ERK and the expression of ACCα mRNA, SREBP-1 mRNA, mature SREBP-1 protein, PGC-1α mRNA, and FGF-19 mRNA in chick embryo hepatocytes incubated with T0-901317. As observed above, incubating hepatocytes with CDCA in the presence of T0-901317 for 24 h stimulated a 5.5- to 7.1-fold increase in the phosphorylation of ERK and p38 MAPK, respectively (**Fig. 11A**). Treatment with cholic acid also increased the phosphorylation of ERK and p38 MAPK, but the extent of this effect (2.3- to 2.8-fold) was less than that observed for CDCA. Treatment with deoxycholic acid also caused a small increase (1.8-fold) in the phosphorylation of p38 MAPK but not ERK. Treatment with ursodeoxycholic acid, taurocholic acid, hyodeoxycholic acid, and taurodeoxycholic acid had no effect on the phosphorylation of ERK and p38 MAPK. Of the bile acids tested, only CDCA was effective at inhibiting the expression of ACCα mRNA, PGC-1α mRNA, and mature SREBP-1 protein and increasing the expression of FGF-19 mRNA (Fig. 11B). Thus, bile acids that were not effective at stimulating the activity of p38 MAPK and ERK were also not effective at regulating the expression of ACCα mRNA, FGF-19 mRNA, PGC-1α mRNA, and mature SREBP-1.

This observation provides further support for the proposal that the CDCA-mediated changes in the expression of ACCα, FGF-19, and PGC-1α require the activation of ERK and/or p38 MAPK. The inability of cholic acid and deoxycholic acid to modulate the expression of ACCα mRNA, FGF-19 mRNA, PGC-1α mRNA, and mature SREBP-1 protein may be attributable to the fact that cholic acid and deoxycholic acid stimulate a smaller increase in the phosphorylation of ERK and p38 MAPK than CDCA or that activation of ERK and p38 MAPK is necessary but not sufficient to mediate changes in the expression of ACCα mRNA, FGF-19 mRNA, PGC-1α mRNA, and mature SREBP-1 protein caused by CDCA. Why CDCA is more effective than other bile acids at modulating ERK and p38 MAPK phosphorylation is not known. One possible mechanism may involve variations in the transport of different bile acids across the cell membrane. The results of this experiment comparing the effects of different bile acids also demonstrate that alterations in ACCα expression are tightly correlated with changes in the expression of mature SREBP-1 protein, FGF-19 mRNA, and PGC-1α mRNA. This observation provides further support for a role of SREBP-1, FGF-19, and PGC-1α in mediating CDCA regulation of ACCα gene transcription.

DISCUSSION

Previous studies have shown that activation of the CDCA/FXR signaling pathway modulates triglyceride synthesis in intact animals. For example, oral administration of CDCA

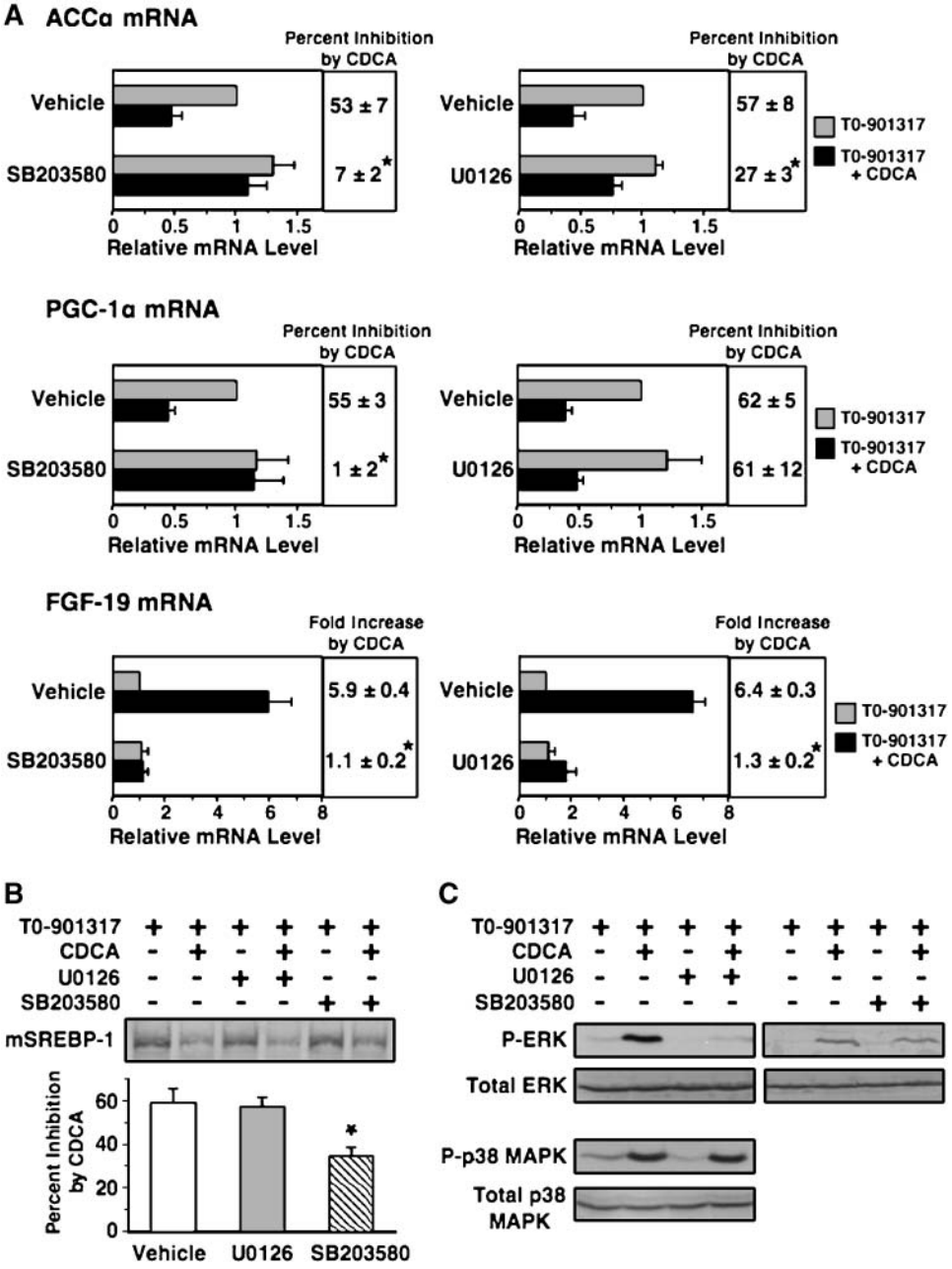

Fig. 10. Inhibition of ERK and/or p38 MAPK suppresses the effects of CDCA on the expression of ACCα, SREBP-1, PGC-1α, and FGF-19. Chick embryo hepatocytes were isolated and incubated in serum-free Waymouth's medium containing insulin. At 18 h of incubation, the medium was changed to one containing insulin and T0-901317. At 30 h of incubation, the medium was replaced with one of the same composition. At 42 h of incubation, SB203580 (20 μM), U0126 (20 μM), or DMSO (vehicle) was added to the culture medium. CDCA (75 μM) was added to the culture medium at 43 h of incubation, and the incubation was continued for 6 h. Cells were harvested and total RNA and cellular extracts were prepared. A: ACCα mRNA, PGC-1α mRNA, and FGF-19 mRNA were measured by quantitative real-time PCR. The level of mRNA in cells treated with T0-901317 and vehicle was set at 1, and the other values were adjusted proportionately. Percentage inhibition or fold increase by CDCA was calculated for cells treated with vehicle, SB203580, or U0126. Values were calculated for individual experiments and then averaged. The results are means ± SEM of five experiments. Asterisks indicate that the mean is significantly different ($P < 0.05$) from that of cells treated with vehicle. B: The abundance of mature SREBP-1 in nuclear extracts was measured by Western analysis. Top: A representative immunoblot. Bottom: Signals for mature SREBP-1 were quantitated, and the percentage inhibition by CDCA was calculated for cells treated with vehicle, SB203580, or U0126. The results are means ± SEM of four experiments. The asterisk indicates that the mean is significantly different ($P < 0.05$) from that of cells treated with vehicle. C: The abundance of phosphorylated ERK, phosphorylated p38 MAPK, total ERK, and total p38 MAPK in total cell lysates was measured by Western analysis. These data are representative of three independent experiments.

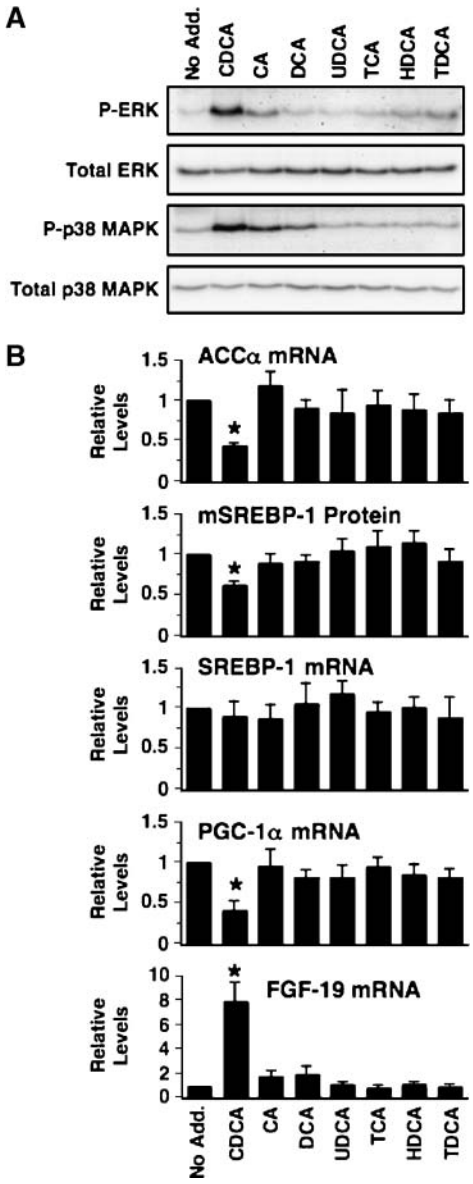

Fig. 11. Effects of different bile acids on the phosphorylation of ERK and p38 MAPK and the expression of ACCα, SREBP-1, PGC-1α, and FGF-19. Chick embryo hepatocytes were isolated and incubated in serum-free Waymouth's medium containing insulin. At 18 h of incubation, the medium was changed to one containing insulin and T0-901317. At 30 h of incubation, the medium was replaced with one of the same composition. At 42 h of incubation, CDCA, cholic acid (CA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), taurocholic acid (TCA), hyodeoxycholic acid (HDCA), or taurodeoxycholic acid (TDCA) was added to the culture medium, and the incubation was continued for 6 h. The concentration of all bile acids was 75 μM. Cells were harvested, and total RNA and cell lysates were prepared. A: The abundance of phosphorylated ERK, phosphorylated p38 MAPK, total ERK, and total p38 MAPK in total cell lysates was measured by Western analysis. These data are representative of three independent experiments. B: ACCα mRNA, SREBP-1 mRNA, PGC-1α mRNA, and FGF-19 mRNA were measured by quantitative real-time PCR. Mature SREBP-1 protein was measured by Western analysis. The level of mRNA or protein in cells treated in the absence of bile acids was set at 1, and the other values were adjusted proportionately. The results are means ± SEM of three experiments. Asterisks indicate that the mean is significantly different ($P < 0.05$) from that of any other treatment.

reduces hypertriglyceridemia and hepatic triglyceride secretion in hamsters fed a high-fructose diet (41). Treatment with CDCA also attenuates hypertriglyceridemia in patients with gallstone disease (40). Further support for an inhibitory effect of CDCA/FXR signaling on triglyceride production is derived from knockout mouse studies demonstrating that disruption of the FXR gene causes an increase in triglyceride levels in the blood and liver (45). The results of the present study indicate that CDCA inhibits triglyceride production, at least in part, by acting directly on the liver to decrease the expression of enzymes that constitute the de novo fatty acid synthesis pathway.

One of the key findings of this study is that treatment with CDCA inhibits the activation of lipogenic enzyme expression caused by the LXR agonist T0-901317 (Figs. 1, 2) and that the mechanism mediating the effect of CDCA on ACCα transcription involves a decrease in the activity of the LXRE in promoter 2 of the ACCα gene (Fig. 3). What is the mechanism by which CDCA inhibits the activity of the ACCα LXRE? Results from DNA binding studies indicate that the CDCA-mediated decrease in ACCα LXRE activity is not attributable to changes in the binding of protein complexes to the ACCα LXRE (Fig. 5). The reduction in ACCα LXRE activity is also not the result of an increase in expression of the LXR repressor SHP, as CDCA decreases the abundance of SHP mRNA in chick embryo hepatocytes (Fig. 6). We propose that CDCA inhibits ACCα LXRE activity, at least in part, by decreasing the expression of the LXR coactivator PGC-1α. In support of this proposal, the CDCA-mediated reduction in ACCα mRNA abundance in chick embryo hepatocytes is preceded or paralleled by a decrease in the concentration of PGC-1α mRNA and the acetylation of histone H3 and histone H4 at the ACCα LXRE (Figs. 6, 7). Further support for this proposal is derived from the observation that the pattern of regulation of PGC-1α mRNA by different bile acids is similar to that of ACCα mRNA (Fig. 11). The observation that FXR activation decreases PGC-1α expression in mice (52) raises the possibility that alterations in PGC-1α expression also play a role in mediating the effects of CDCA on hepatic lipogenic enzyme expression in mammalian species.

In addition to the ACCα LXRE, a SRE located immediately downstream of the ACCα LXRE plays a role in mediating the inhibitory effects of CDCA on T0-901317-induced ACCα transcription in chick embryo hepatocytes. Previous work has shown that this SRE enhances the ability of the ACCα LXRE to activate ACCα transcription in the presence of T0-901317 (15). In the present study, we show that CDCA inhibits the LXR accessory function of the ACCα SRE and that the mechanism mediating this effect involves a decrease in mature SREBP-1 concentration (Figs. 3, 4). We also demonstrate that CDCA regulates the concentration of mature SREBP-1 at a posttranslational step. Previous work has shown that CDCA decreases the expression of SREBP-1c in mouse hepatocytes (42). Thus, alterations in SREBP-1 activity appear to play a role in mediating the effects of CDCA on lipogenic enzyme expression in both rodents and avians.

As in avian hepatocytes, CDCA inhibits the stimulatory effects of LXR agonists on lipogenic enzyme expression in mouse hepatocytes (42, 57) and rat hepatocytes (S. Bhatnagar and F. B. Hillgartner, unpublished data). In mouse and rat hepatocytes, CDCA increases the expression of SHP mRNA (42, 58). Based on this finding and the observation that overexpression of SHP in cell lines represses the LXR activation of transfected target promoters, Watanabe et al. (42) have proposed that alterations in SHP expression mediate the inhibitory effects of CDCA on hepatic lipogenic gene transcription. In agreement with this proposal, Watanabe et al. (42) have shown that ablation of the SHP gene in mice abolishes the inhibitory effect of FXR agonists on hepatic SREBP-1c expression. Although this observation clearly demonstrates that SHP is required for CDCA regulation of lipogenic enzyme expression, data from a study by Boulias et al. (59) indicate that an increase in SHP expression does not initiate the CDCA response. Boulias et al. (59) have shown that transgenic expression of SHP at physiological levels stimulates the hepatic accumulation of triglycerides and the expression of mRNAs encoding ACCα, FAS, SCD1, and SREBP-1c. Such a finding suggests that an increase in SHP expression promotes an increase rather than a decrease in hepatic lipogenesis. Furthermore, based on ChIP analyses, Boulias et al. (59) have shown that SHP is not associated with the promoter/regulatory region of the SREBP-1c gene and the FAS gene in livers of wild-type mice and transgenic mice expressing SHP. This observation indicates that the mechanism by which SHP controls hepatic lipogenic gene transcription does not involve interactions of SHP with LXR or other regulatory proteins on lipogenic gene promoters. The results of the present study demonstrating that CDCA inhibits ACCα transcription in the absence of an increase in SHP expression provide further evidence that an increase in SHP expression is not involved in mediating the effects of CDCA on lipogenic enzyme expression. Further experimentation is needed to determine how SHP participates in the CDCA regulation of lipogenic genes.

FGF-19 and its mouse ortholog, FGF-15, are potent inhibitors of CYP7A1 gene transcription in liver (54, 60). Results from experiments using FGF-15 knockout mice indicate that FGF-15 plays a critical role in mediating the inhibitory effect of FXR agonists on hepatic CYP7A1 expression (60). In human hepatocyte cultures, CDCA activates the expression of FGF-19 via a FXR-dependent mechanism (54). Accordingly, FGF-19 secreted by the liver has been proposed to function in an autocrine or paracrine manner to mediate the inhibitory effect of CDCA on hepatic CYP7A1 transcription. In intact mice, oral administration of FXR agonists induces the expression of FGF-15 in the small intestine but has no effect on the expression of FGF-15 in liver (60). Thus, FGF-15 secreted by the small intestine has been proposed to function in an endocrine manner to mediate the feedback regulation of hepatic CYP7A expression by FXR agonists. In the present study, we show that CDCA induces the expression of FGF-19 in chick embryo hepatocytes and that ectopic expression of chicken FGF-19 in chick embryo hepatocytes mimics the inhibitory effect of CDCA on T0-901317-induced expression of ACCα. We postulate that FGF-19 plays a role in mediating the effect of CDCA on the expression of ACCα and other lipogenic enzymes. In support of this proposal, Tomlinson et al. (61) and Fu et al. (62) have shown that administration of recombinant human FGF-19 or transgenic expression of the human FGF-19 gene in obese/diabetic mice causes a reduction in serum and hepatic triglyceride levels. Treatment of obese/diabetic mice with FGF-19 also decreases fat accumulation in adipose tissue and enhances insulin sensitivity. These effects of FGF-19 on adipose tissue and insulin sensitivity may be attributed, in whole or in part, to a reduction in hepatic lipogenic enzyme expression, as increased rates of hepatic fatty acid synthesis contribute to enhanced adipose stores and insulin resistance in obese/diabetic animal models (63). Interestingly, activation of FXR with CDCA or knockdown of hepatic ACCα expression with antisense oligonucleotides modulates hepatic triglyceride levels, adiposity, and insulin sensitivity in a manner similar to that observed with FGF-19 treatment (41, 64, 65). This observation provides further support for a role of FGF-19 in mediating the regulation of lipogenic enzyme expression by CDCA.

In contrast to the inhibitory effect of CDCA on the T0-901317-induced expression of lipogenic enzyme mRNAs in avian and rodent hepatocytes, CDCA has no effect or enhances T0-901317-induced expression ABCA1 mRNA (Fig. 1) (S. Bhatnagar and F. B. Hillgartner, unpublished data) (42). Previous studies have shown that SREBP binds to an E-box element in the ABCA1 promoter and suppresses ABCA1 gene transcription via an undefined mechanism (66). We postulate that the lack of an inhibitory effect of CDCA on ABCA1 transcription is attributable to the CDCA-mediated reduction in mature SREBP-1 levels, resulting in a derepression of the ABCA1 promoter. Such an effect would counteract the inhibitory action of CDCA on ligand-activated LXR•RXR complexes bound to the ABCA1 promoter.

Another finding of the present study is that p38 MAPK and ERK play a role in mediating the inhibitory action of CDCA on ACCα expression. CDCA activates p38 MAPK and ERK, and pharmacological inhibition of p38 MAPK and ERK suppresses the ability of CDCA to inhibit T0-901317-induced ACCα expression (Figs. 9, 10). Inhibition of p38 MAPK and/or ERK also suppresses the effects of CDCA on the expression of potential mediators of CDCA action, such as mature SREBP-1, PGC-1α, and FGF-19. Thus, p38 MAPK and ERK may regulate lipogenic enzyme expression by controlling the expression of these regulatory factors. In support of a role of p38 MAPK in mediating the effect of CDCA on hepatic lipogenic gene expression, Xiong et al. (67) recently showed that activation of p38 MAPK with a constitutively active form of MKK6 inhibits the expression of FAS and SREBP-1c in rat hepatocytes. Interestingly, glucagon and polyunsaturated fatty acids also inhibit the hepatic expression of glucose-6-phosphate dehydrogenase, FAS, and SREBP-1c via a mechanism that is dependent on the presence of p38 MAPK and/or ERK (67–69). Thus, p38 MAPK and ERK appear to have a

general role in inhibitory pathways controlling lipogenic gene expression.

In addition to changes in gene expression, the activity of PGC-1α is controlled at the posttranslational level. Fan et al. (70) have shown that p160 Myb binding protein (p160 MBP) binds to PGC-1α, causing a repression PGC-1α activity, and that the phosphorylation of PGC-1α by p38 MAPK disrupts this interaction, resulting in a de-repression/activation of PGC-1α activity. Phosphorylation of PGC-1α by p38 MAPK mediates the stimulatory effects of β-adrenergic agonists on uncoupling protein-1 gene transcription and does so by increasing the ability of PGC-1α to coactivate the peroxisome proliferator-activated receptor α (PPARα) on the uncoupling protein-1 promoter (71, 72). These findings are inconsistent with our data demonstrating that p38 MAPK mediates the inhibitory effect of CDCA on LXR agonist-induced ACCα expression. One explanation for this discrepancy is that the effect of p160 MBP binding on PGC-1α activity varies depending on the nature of the nuclear receptor that interacts with PGC-1α. In support of this possibility, Oberkofler and colleagues (6, 72) have shown that the binding of p160 MBP to PGC-1α inhibits the ability of PGC-1α to coactivate PPARα but has no effect on the ability of PGC-1α to co-activate LXR and PPARγ. The molecular basis for the different effects of p160 MBP on PGC-1α coactivation of PPARα, PPARγ, and LXR may be attributable to differences among these nuclear receptors in their site of interaction on the PGC-1α molecule (6, 72).

In summary, we show that CDCA suppresses the stimulatory effects of T0-901317 on the secretion of triglycerides and the expression of lipogenic enzymes in hepatocytes. Thus, activation of the CDCA signaling pathway represents a potential therapeutic approach to counteract the undesired effects of LXR agonists on triglyceride levels in the blood and liver. As the stimulatory effect of T0-901317 on hepatic ABCA1 expression is not inhibited by CDCA, we postulate that the antiatherosclerotic action of LXR agonists is not diminished by CDCA. Additional studies are needed to assess the effects of CDCA and other FXR agonists on the development of atherosclerosis in animal models of obesity and diabetes.

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