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ABSTRACT

Analysis of the Mechanisms Mediating the Regulation of Acetyl-CoA Carboxylase Transcription by the Liver X Receptor and Chenodeoxycholic Acid

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Agonists of the liver X receptor (LXR) prevent and decrease arterial plaque formation in experimental models of atherosclerosis. The anti-atherosclerotic effects of LXR agonists are mediated by an increase in expression of genes involved in cholesterol export. Currently, the therapeutic utility of LXR agonists is limited by the fact that these agents increase triglyceride levels in the blood and liver. These undesired effects are caused by an increase in transcription of genes controlling triglyceride synthesis, such as acetyl CoA carboxylaseα (ACCα). We have demonstrated that a synthetic LXR agonist, T0-901317 increases ACCα via both a direct mechanism involving the activation of LXR/retinoid X receptor (RXR) heterodimers on the ACCα gene and by an indirect mechanism involving the increased expression of sterol regulatory element binding protein-1 (SREBP-1). SREBP-1 binds a site adjacent to the ACCα LXRE and enhances the ability of LXR/RXR to activate ACCα transcription. We screened a number of compounds for their ability to inhibit the stimulatory effects of T0-901317 on expression of lipogenic genes in primary cultures of hepatocytes. We found that the bile acid, chenodeoxycholic acid (CDCA), inhibited the T0-901317-induced increase in mRNA abundance encoding ACCα, fatty acid synthase, and stearoyl CoA desaturase-1. CDCA also blocked the stimulatory effects of T0-901317 on triglyceride secretion into the culture medium. Results from transient transfection analyses identified two cis-acting elements that mediated the inhibitory effects of CDCA on T0-901317-induced ACCα transcription. One element bound LXR/RXR heterodimers and the other element bound SREBP-1. Treatment with CDCA decreased the expression of mature, active SREBP-1 and decreased the binding of LXR/RXR heterodimers to the ACCα promoter. Further studies demonstrated that the CDCA-mediated inhibition of ACCα transcription was associated with an activation of extracellular signal-related kinase-1/2 (ERK1/2) and p38 mitogen activated protein kinase (p38MAPK) and that inhibitors of ERK1/2 and p38MAPK abolished or substantially attenuated the inhibitory effect of CDCA on ACCα expression. These results indicate that CDCA inhibits T0-901317-induced ACCα expression by decreasing the transcriptional activity of LXR and SREBP-1 and that ERK1/2 and p38MAPK are components of the signaling pathway mediating the inhibitory effects of CDCA on ACCα expression and triglyceride secretion.

"Whatever we understand and enjoy in human products instantly becomes ours, wherever they might have their origin."

- Rabindranath Tagore (1861-1941)

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List of abbreviations

ABCA1 – ATP-binding cassette protein A1

ACCα – Acetyl CoA carboxylase

CA – Cholic acid

CAT – Chloramphenicol acetyl transferase

CBP – CREB-binding protein

CDCA – Chenodeoxycholic acid

CEH – Chicken embryo hepatocytes

ChIP – Chromatin immunoprecipitation

CYP7A – Cholesterol 7-alpha hydroxylase

DCA – Deoxycholic acid

ER – Endoplasmic reticulum

ERK – Extracellular regulated kinase

FAS – Fatty acid synthase

FGF-19 – Fibroblast growth factor-19

FXR – Farnesoid X receptor

GK – Glucokinase

HAT – Histone acetyl transferase

HDAC – Histone deacetylase

HDCA – Hyodeoxycholic acid

HDL – High density lipoprotein

JNK – c-Jun N terminal kinase

LDL – Low density lipoprotein

LDLr – Low density lipoprotein receptor

LXR – Liver X receptor

LXRE – Liver X receptor element

MAPK – Mitogen activated protein kinase

ME – Malic enzyme

PCR – Polymerase chain reaction

PEPCK - phosphoenolpyruvate carboxykinase

PGC-1α – Peroxisome proliferator activated receptor gamma coactivator-1α

PGC-1β - Peroxisome proliferator activated receptor gamma coactivator-1β

PKC – Protein kinase C

RXR – Retinoid X receptor

SCD – Stearoyl CoA desaturase

SHP – Short heterodimeric partner

SRE – Sterol regulatory element

SREBP-1 – Sterol regulatory element binding protein-1

T3 – Thyroid hormone

TCA – Taurocholic acid

TDCA – Taurodeoxycholic acid

TK – Thymidine kinase

UDCA – Ursodeoxycholic acid

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Chapter 1

I) Introduction

Liver X receptors are ligand dependent transcription factors that belong to the nuclear hormone receptor superfamily. LXRs modulate multiple cellular pathways making it a very attractive drug target to prevent and cure a wide range of diseases. For example, agonists of LXR prevent and decrease arterial plaque formation in experimental models of cardiovascular disease and atherosclerosis. The anti-atherosclerotic effects of LXR agonists are mediated by an increase in expression of genes involved in cholesterol export and high density lipoprotein (HDL) formation. In rodent models of diabetes, an LXR agonist, T0-901317, lowered plasma glucose levels and significantly improved insulin sensitivity. Activation of LXR in mouse liver inhibits gluconeogenesis by inhibiting the expression of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase. In contrast, LXR agonists promote hepatic glucose utilization by increased expression of glucokinase. Recently there is compelling evidence showing an important link between cholesterol and Alzheimer's disease. High plasma levels of HDL have been shown to have an inverse effect on Alzheimer's disease. This effect is primarily due to the increase in ABCA1 by LXRs. LXR agonists applied to a variety of in vitro models, including immortalized fibroblasts from Tangier patients, and primary embryonic mouse neurons and Alzheimer disease mouse model (APP23 transgenic mice) caused a T0-901317-dose-dependent decrease in amyloid beta secretion.

Currently, the therapeutic utility of LXR agonists is limited by the fact that these agents also increase triglyceride levels in the blood and liver. These undesired effects are caused by an increase in transcription of genes controlling triglyceride synthesis. I have developed data demonstrating that the LXR agonist, T0-901317, increases the expression of ACCα in chicken embryo hepatocytes (CEH). This result is consistent with previous work from our laboratory that ACCα is a LXR target gene. Results from transient transfection analyses indicated that the T0-901317 induction of ACCα mRNA is mediated by two cis-acting elements on the ACCα promoter.

We screened a number of compounds for their ability to inhibit the stimulatory effects of the synthetic LXR agonist, T0-901317, on expression of lipogenic genes in primary cultures of hepatocytes. We found that the primary bile acid, chenodeoxycholic acid (CDCA), inhibited the T0-901317-induced increase in abundance of mRNAs encoding acetyl CoA carboxylaseα (ACCα), fatty acid synthase, and stearoyl CoA desaturase-1. Interestingly, CDCA enhanced the T0-901317-induced increase in abundance of mRNA encoding ABCA1, a key protein involved in HDL formation. Thus, CDCA may enhance the ability of T0-901317 to stimulate HDL formation while reducing the effects of T0-901317 on triglyceride accumulation in the blood and liver.

Results from transient transfection analyses identified two cis-acting elements that mediated the inhibitory effects of CDCA on T0-901317-induced ACCα transcription. One element bound LXR/retinoid X receptor (RXR) heterodimers and the other element bound sterol regulatory element binding protein-1 (SREBP-1). Treatment with CDCA decreased the expression of mature, active SREBP-1, via a post translational mechanism. Further studies demonstrated that the CDCA-mediated inhibition of ACCα transcription was associated with an activation of extracellular signal-related kinase-1/2 (ERK1/2) and p38 mitogen activated protein kinase (p38 MAPK). Specific inhibitors of ERK1/2 and p38MAPK abolished or substantially attenuated the inhibitory effect of CDCA on ACCα expression. These results indicate that CDCA inhibits ACCα transcription by decreasing the activity of LXR and SREBP-1, and that ERK1/2 and p38MAPK are involved in mediating this effect.

II) BACKGROUND

A) Acetyl CoA Carboxylase

1) Structure and function

Acetyl CoA carboxylase (ACC) catalyzes the ATP-dependent carboxylation of acetyl CoA to malonyl CoA. This reaction is the first committed step in the fatty acid synthesis pathway (67, 94). There are two isoforms of ACC that are encoded by different genes. ACCα (260 kDa) is the principal isoform that is expressed in tissues that exhibit high rates of fatty acid synthesis such as liver, adipose tissue and mammary gland.

ACCβ (280 kDa) is the major isoform found in heart and skeletal muscle where it is thought to primarily function in the regulation of β-oxidation of fatty acids (173).

2) Regulation of ACC

The levels of ACCα in the liver are dependent on nutritional and hormonal regulation (13, 67). The effects of nutritional regulation on the ACCα concentration are mediated primarily by changes in the rate of transcription of the ACCα gene (65). In livers of starved chickens the rate of ACCα transcription is low. Consumption of a high carbohydrate, low fat diet stimulates an 11-fold increase in ACCα transcription (65). Several signaling pathways are involved in mediating the stimulatory effects of dietary carbohydrate on ACC transcription in liver. Increased insulin secretion and glucose metabolism are two important signals mediating this response. Another factor signaling changes in carbohydrate status is the active form of thyroid hormone, 3, 5, 3'-triiodothyronine (T3). Ingestion of a high carbohydrate meal stimulates a rapid increase in the secretion of thyroxine from the thyroid gland and the conversion of thyroxine to T3 in extrathyroidal tissues (66). The resulting increase in T3 concentration in liver activates the transcription of the genes for ACC. Ingestion of carbohydrate also increases the levels of nuclear T3 receptors (TRs) in liver (23). This phenomenon may also contribute to the stimulation of lipogenic gene transcription by dietary carbohydrate.

Diet-induced changes in ACCα transcription are mimicked in primary cultures of chick embryo hepatocytes by manipulating the concentration of hormones and nutrients in the culture medium. The addition of T3 to the culture medium stimulates a 7-fold increase in ACCα transcription (66). Insulin has no effect on the transcription of ACCα, but amplifies the increase in ACCα transcription caused by T3. Glucagon acting through cAMP suppresses the induction of ACCα transcription caused by T3 and insulin (225).

The ACC gene is transcribed from two promoters, PI and PII, which result in alternatively spliced mRNAs containing different 5' noncoding regions (94). In rodents, PI is active in white adipose tissue and regulated by dietary manipulation (121, 122). In contrast PII is active in all tissues at a low level. PII promoter for ACCα is specifically regulated by sterols through the action of the transcription factor Sp1 and the sterol regulatory element binding proteins (SREBPs) (127). In contrast to the rodents, our laboratory has shown that both PI and PII are regulated by nutrients and hormones in

chick hepatocytes and that the activity of PII accounts for a greater proportion of the changes in total ACCα mRNA abundance than activity of PI (236).

Our laboratory has identified a unique regulatory element between -108 and -82bp in the ACCα gene that confers T3 regulation on ACCα promoter 2 in chicken embryo hepatocytes (CEH). This element stimulates transcription both in the absence and presence of T3, with a greater stimulation observed in the presence of T3. The T3-independent enhancer activity of this regulatory element was mediated by protein complexes containing LXR•RXR heterodimers and the increase in enhancer activity in the presence of T3 was mediated by protein complexes containing thyroid hormone receptor (TR)•RXR heterodimers and LXR•RXR heterodimers (236). This regulatory element is referred to as a composite thyroid hormone response element/liver X receptor response element (T3RE/LXRE). Immediately downstream of the ACCα T3RE/LXRE is a sterol regulatory element (SRE)
(-80 to -71 bp), that increased the ability of the ACCα-T3RE/LXRE to stimulate ACCα transcription in the presence of T3. The stimulatory effect of the SRE-1 on ACCα transcription was mediated by a direct interaction between SREBP-1 and TR. This interaction facilitated the formation of a tetrameric complex comprised of SREBP-1•SREBP-1/TR•RXR on the ACCα gene. The formation of this tetrameric complex stabilized the binding of SREBP-1 to ACCα SRE-1. The binding of T3 to thyroid hormone receptor (TR) enhanced tetrameric complex formation. T3 was also shown to stimulate the production of the mature, transcriptionally active form of SREBP-1 in hepatocytes. Thus multiple interactions between the TR and SREBP-1 signaling pathways contribute to the stimulatory effects of T3 on ACCα transcription (226).

B) Sterol Regulatory Element Binding Protein

1) Structure and function

Sterol regulatory element binding proteins (SREBPs) belong to a large class of transcription factors that contain a basic helix-loop-helix-leucine zipper domain (bHLH-ZIP). SREBPs are synthesized as 125 kDa membrane-bound precursors of ~1150 amino

acids that require cleavage by a two-step proteolytic process in order to release an N-terminal fragment that binds DNA and is transcriptionally active (22). Each SREBP precursor is organized into three domains. The first domain is the N-terminal segment, comprised of about 480 amino acids that contains the bHLH-ZIP and which binds DNA. The second domain is composed of two hydrophobic transmembrane-spanning segments interrupted by a short loop of about 30 amino acids that projects into the lumen of the endoplasmic reticulum (ER). The third domain of 590 amino acids is the C-terminal segment of the protein and plays a role in the regulation of cleavage (22, 149).

To date, three isoforms of SREBP have been identified. SREBP-1a and SREBP-1c are produced from the same gene. They contain differences in their N-terminal region due to the use of alternative promoters and first exons (73). SREBP-2 is derived from a gene distinct from SREBP-1 (74). Most cells in culture express both SREBP-1a and SREBP-2. SREBP-1c is the predominant form of SREBP-1 expressed in liver and adipose tissue (182). In chickens, only one form of SREBP-1 has been identified and this form more closely resembles the mammalian SREBP-1a (7).

2) Activation of SREBP via proteolytic processing

The precursor form of SREBP is anchored to the endoplasmic reticulum (ER). In order for SREBP to enter the nucleus and function as a transcription factor, it must undergo a cleavage resulting in the release of the N-terminal fragment from the endoplasmic reticulum. Three proteins are required for SREBP processing. One is an escort protein designated SREBP cleavage-activating protein (SCAP). The other two are proteases, designated Site-1 protease (S1P) and Site-2 protease (S2P). SREBP is synthesized and inserted into the membrane of the endoplasmic reticulum, where its C-terminal regulatory domain binds to the C-terminal domain of SCAP. SCAP is both an escort for SREBPs and a sensor of sterols. When cells become depleted in cholesterol, SCAP escorts SREBP from the ER to the Golgi, where S1P and S2P are present (41, 177, 178). In the Golgi, S1P, a membrane-bound serine protease, cleaves the SREBP in the luminal loop between its two membrane-spanning segments, dividing the SREBP molecule roughly in half. The N-terminal bHLH-Zip domain is then released from the

membrane after a second cleavage by S2P, a membrane-bound zinc metalloproteinase. The N-terminal fragment, referred to as mature SREBP, is transported into the nucleus, where it activates transcription by binding nonpalindromic sterol response elements (SREs) (93, 128) in the promoter/enhancer regions of genes involved in cholesterol and triacylglycerol synthesis (41, 176).

When the cholesterol content of cells rises, SCAP senses the excess cholesterol through its membranous sterol-sensing domain, causing a conformational change such that the SCAP/SREBP complex is no longer incorporated into endoplasmic reticulum transport vesicles. This results in SREBPs losing their access to S1P and S2P in the Golgi. The active, N-terminal fragment is not released from the ER membrane, and transcription of target genes is inhibited (70).

Recently, an endoplasmic reticulum protein was identified that binds to the sterol-sensing domain of SCAP only in the presence of sterols. This protein, referred to as insulin induced gene-1 (INSIG-1), is required for retention of the SCAP/SREBP complex in the ER in the presence of sterols. Sterols induce binding of SCAP to INSIG-1, and this interaction is correlated with the inhibition of SCAP exit from the ER. When sterol levels in the cell are high, the sterols bind to SCAP and facilitate the binding of SCAP to INSIG-1. The SCAP-INSIG-1 complex then binds to SREBP and facilitates retention of SREBP in the ER. Interestingly, expression of INSIG-1 is dependent on the presence of mature SREBP, and thus constitutes an autoregulatory loop in the regulation of mature SREBP production (45, 80, 223). When sterol levels are high, mature SREBP levels are decreased and hence INSIG-1 levels decrease. On the other hand, when sterol levels are low, mature SREBP levels are increased which in turn increase the INSIG-1 gene expression which in turn inhibit mature SREBP production.

Recently, a second ER protein, referred to as INSIG-2, has been identified that functions in a manner similar to that of INSIG-1 except for two differences. First, expression of INSIG-2 is not dependent on the presence of mature SREBP. Second, INSIG-2 requires the presence of sterols in order to retain the SCAP-SREBP complex in the ER (219, 220). Three point mutations in the sterol sensing domain of SCAP, (L315F), (D443N) and (Y298C), prevent sterol-induced binding of SCAP to INSIG-1 and -2 and abolish feedback inhibition of SREBP processing by sterols (221). The

combined actions of INSIG-1 and INSIG-2 may allow for fine-tuning of SREBP processing under conditions of widely varying sterol demand and supply.

3) Regulation of transcription by SREBP

The SREBP target genes include enzymes of cholesterol biosynthesis such as HMG-CoA reductase, HMG-CoA synthase, farnesyl diphosphate synthase, squalene synthase, and SREBP-2. Each of these genes contains a SRE or SRE-like sequence in its promoter. SREBPs also bind to regulatory sequences in the promoters of the genes involved in the biosynthesis of triacylglycerols such as acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase (41, 93, 128, 176).

Results of studies employing transgenic mice indicate SREBP-2 is more effective in regulating genes involved in cholesterol synthesis, whereas SREBP-1 is more effective in controlling genes involved in triacylglycerol synthesis. The mechanism for the specificity of the two forms of SREBP is currently unknown. SREBP-1a is more effective than SREBP-1c in modulating transcription due to the shorter acidic transactivation domain of the latter protein (146, 184). In liver and adipose tissue SREBP-1c binds to the promoters of several lipogenic enzyme genes and increases their expression. In transgenic mice that lack the SREBP-1 gene, the effects of high carbohydrate feeding on lipogenic enzyme expression are abolished (117, 184). Also, in transgenic mice overexpressing SREBP-1c in the liver, rate of lipogenesis and lipogenic enzyme expression are dramatically increased (71, 183).

4) Regulation of SREBP activity

a) Activation of SREBP transcription

Transcription of SREBP-1 but not SREBP-2, is dramatically increased by high carbohydrate feeding. In rat and chick hepatocyte cultures, diet induced changes in SREBP-1 transcription are mimicked by altering the concentration of insulin, T3 and glucagon in the culture medium. Insulin and T3 increase SREBP-1 expression, whereas glucagon has the opposite effect (48, 235).

LXR agonists have also been shown to stimulate SREBP-1 transcription. This effect is mediated by a LXRE in the SREBP promoter. LXR agonist-induced activation

of SREBP-1c transcription is associated with an increase in mature SREBP-1c levels and lipogenic enzyme expression (150, 228). A similar observation has been made in chick hepatocytes. Thus, LXR agonists can stimulate lipogenic genes, at least in part, by altering the expression of SREBP-1.

b) Inhibition of SREBP-1 expression

Long chain polyunsaturated fatty acids have been shown to suppress the insulin-induced SREBP-1 expression in rat hepatocytes. This effect is mediated by a decrease in transcription of SREBP-1 gene, as well as an enhanced turnover of the SREBP-1 mRNA (145, 218). Previous work in our laboratory has shown hexanoate inhibits ACCα transcription, in part, by repressing the ability of insulin and T3 to increase SREBP-1 mRNA abundance in chicken embryo hepatocytes. This effect disrupts the positive interaction between SREBP-1 and T3 bound TR on the ACCα gene previously identified by our laboratory (235). Data from our preliminary studies show that hexanoate decreases the mRNA expression of SREBP-1. These data suggest that fatty acid inhibition of SREBP-1 expression in rat and chicken hepatocytes may occur through a similar mechanism. cAMP inhibits SREBP-1 expression in chicken embryo hepatocytes via a mechanism not involving changes in SREBP-1 mRNA abundance (235).

5) Post translational regulation of SREBP activity

Sumoylation of SREBP-1 has been shown to control SREBP-1 activity. SUMO-1 is a protein responsible for posttranslational modification of many proteins and has a remarkably similar secondary structure to ubiquitin. Most proteins are sumoylated by a multi-step process. SUMO-1 target proteins include several transcription factors and regulate protein function through changes in protein-protein interactions and by stabilizing the target proteins. Recent work has shown that SREBP-1 and -2 have two and one major sumoylation sites respectively. Sumoylation decreases the transcriptional activity of both SREBP-1 and –2 resulting in the decrease of expression of their target genes (68).

SREBP-1 activity is also regulated by phosphorylation. Previous work has shown that SREBPs are substrates for Erk1 and Erk2 in vitro. Phosphorylation of serine 117 of SREBP-1a by ERK induces a conformational change resulting in an increase in transcriptional activity of SREBP-1a (170). SREBP-1a, -1c, and -2 are ubiquitinated and degraded by the 26S proteasome pathway. Phosphorylation of Thr426 and Ser430 in the 424TLTTPPPSD motif in SREBP-1a corresponds to Thr393 and Ser399 in SREBP-1c. Phosphorylation of the TLTTPPPSD motif by Gsk3β promotes binding of the ubiquitin ligase, SCFFbw7, which targets SREBP for 26S proteasomal degradation. Inhibition of Gsk3β activity by LiCl or insulin promotes the accumulation of SREBP. Insulin inhibits Gsk3β activity by increasing the Akt-mediated phosphorylation of Gsk3β at Ser9 (191), (95). n-3 polyunsaturated fatty acids (PUFAs) decrease the mature, active form of SREBP-1 in primary rat hepatocytes through 26S proteasome and Erk-dependent pathways. Specific inhibitors of these pathways attenuate the inhibitory effect of PUFA on mature SREBP-1 levels (15). The mechanisms by which Erk phosphorylation, and 26S proteasomal mediate degradation of mature SREBP-1 are yet to be identified.

C) Liver X Receptors

1) Structure and Function

Liver X receptors (LXRs) were first identified as orphan members of the nuclear receptor superfamily (212). Two members of the LXR family have been identified: LXRα (also known as RD-1) and LXRβ (also known as UR, NER, OR-1 and RIP15) (196, 212). Both LXRα and LXRβ are involved in the control of cholesterol and fatty acid metabolism (25),(126),(169),(166),(34),(106). The expression patterns of the two LXR proteins differ significantly. LXRα in adult animals is predominantly expressed in tissues that are known to play important roles in lipid metabolism. The highest levels are found in the liver (hence the name liver X receptor), kidney, small intestine, spleen, adipose tissue, pituitary and adrenals. In contrast, expression of LXRβ is much more widespread; and it is found in almost every tissue examined, including liver and brain (167),(33). As is the case with other non-steroid nuclear receptors, the majority of LXR

proteins are localized in the nucleus and require heterodimerization with the retinoid X receptor (RXR) in order to bind DNA and regulate transcription (82, 212).

Three structural domains comprise LXRs. An N-terminal domain contains a strong transcriptional activation function (AF1) that is important for cell and target gene specificity. A highly conserved DNA binding domain (DBD) is contained in the more central region of the receptor and is characterized by two C4-type zinc fingers. The C-terminal region of the receptor contains the ligand binding domain (LBD), and a ligand-regulated transcriptional activation function (AF2). This LXR domain also mediates the recruitment of transcriptional co-regulatory proteins. The structural domains of LXR are observed in other members of the nuclear hormone receptor superfamily (52, 130, 192).

2) Identification of ligands of LXR

A major breakthrough in elucidating the physiological role of the LXR was the finding that oxysterols serve as their ligands. The best studied and the most potent activators to date are a specific group of mono-oxidized derivatives of cholesterol that include, 24(S)-hydroxycholesterol, 22(R)-hydroxycholesterol and 24(S) epoxycholesterol (82, 212). All of the activators analyzed thus far are able to activate both LXRα and LXRβ. The compounds elicit a response at concentrations observed in LXR target tissues. 24(S), 25-epoxycholestrol, one of the most potent LXR ligands to be characterized, is known to be present at concentrations of 1-5 μM in human and murine liver where LXRα expression is the highest. 22(R)-hydroxycholesterol is found at micromolar concentrations in the placenta, where LXRβ expression is high. Levels of 24(S)-hydroxycholesterol are high in the brain, adrenals and liver. The binding affinities of these oxysterols (Kd = 70-900 nM) correlates with their ability to activate LXR-mediated transcription in vivo (81-83, 112).

Fluorescence polarization-based screening assays have led to the identification of non-steroidal LXR ligands that may be used as drugs in preventing and treating atherosclerosis. In this assay, the recruitment of a rhodamine-labeled coactivator peptide fragment to LXR is used to assess the binding of ligand to LXR. This coactivator peptide fragment contains a LXXLL motif, (where L is leucine and X is any amino acid) that has

been shown to interact with LXR in an agonist-dependent manner. Screening a chemical library using this assay has led to the identification of T0-314407 (*N*-methyl-*N*-[4-(2, 2, 2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl] benzene sulfonamide). A derivative of T0-314407 that exhibited enhanced selectivity was developed by structure-activity relationship studies. This compound was referred to as T0-901317 (*N*-(2,2,2-trifluoro-ethyl)-*N*-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide). T0-901317 also exhibited a greater potency than endogenous oxysterol ligands in stimulating transcription (49, 82, 112, 179).

3) Biological Actions of LXR

LXR•RXR heterodimers regulate target genes by binding to specific DNA sequences referred to as LXR response elements (LXREs). LXREs are usually comprised of a direct repeat of two hexanucleotide half sites separated by a 4 bp spacer (212). In addition to being activated by oxysterols, LXR•RXR heterodimers have been shown to be activated by ligands of RXR such as 9-cis retinoic acid (9-cis RA) (6, 107).

LXR agonists stimulate multiple processes controlling cholesterol export from the body. For example, LXR agonists cause an increase in expression of genes encoding apolipoprotein E (apo E) and cholesterol transporters ABCA1 and ABCG1 (125). Apo E, ABCA1 and ABCG1 are involved in cholesterol efflux to HDL, a critical component of the reverse cholesterol transport pathway (54, 111, 123, 169). In liver, LXR agonists also activate the transcription of cholesterol 7 alpha hydroxylase (CYP7A1), which encodes the pace setting enzyme catalyzing the conversion of cholesterol to bile acids (150). Bile acid formation is the primary pathway of cholesterol excretion from the body. LXR activation also increases expression of hepatic transporters involved in biliary cholesterol excretion such as ABCG5 and ABCG8. The oxysterol mediated increase in expression of ABCA1, ABCG5 and ABCG8 in the small intestine reduces the efficiency of cholesterol absorption by accelerating cholesterol efflux into the intestinal lumen (12). Another protein induced by LXR agonists is cholesterol ester transfer protein (CETP). CETP catalyzes the transfer of cholesterol ester from HDL to chylomicrons and very low density lipoproteins (VLDL), thus facilitating cholesterol transport to the liver. The mechanism for the effects of LXR agonists on transcription of genes involved in cholesterol export is mediated by the activation of LXR•RXR heterodimers bound to the

promoter/regulatory region (133). The effects of LXR agonists on processes controlling cholesterol export result in a reduction in blood cholesterol levels and increase in HDL formation. Interestingly, administration of GW3965, another synthetic LXR agonist, has been shown to not only prevent but also reverse atherosclerotic lesions in the aorta of mice lacking the LDL receptor and apo E gene (87).

In addition to controlling cholesterol metabolism, LXR regulates the de novo synthesis of fatty acids. LXR agonists increase the expression of SREBP-1c (38, 228), a transcription factor that stimulates transcription of various lipogenic genes including ACCα, FAS and stearoyl CoA desaturase (SCD) (42, 90, 144). Thus LXR agonists may stimulate transcription of lipogenic genes via an indirect mechanism involving the activation of SREBP-1 expression. LXR agonists may also stimulate lipogenesis via a direct mechanism as LXREs have also been identified in the genes for ACCα, FAS and SCD. Increased lipogenesis caused by LXR agonists results in the development of fatty liver. This phenomenon has limited the use of these agents for the treatment and prevention of atherosclerosis.

A second potent and selective LXR agonist identified by fluorescence polarization screening is GW3965. Oral administration of the synthetic LXR agonist, T0901317, 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 (194), (87). Oral administration of GW3965 to mice increased plasma HDL concentration (87). Recently, another synthetic ligand for LXR, termed LN6500, has been identified, that will be discussed later in this section (2). As T0-901317 is commercially available, we are using this compound in our studies analyzing the regulation of ACCα by the LXR pathway.

The role of LXR agonists in glucose homeostasis has been studied in rodent models of diabetes. T0-901317 lowered plasma glucose level in both db/db and Zucker diabetic fatty (ZDF) rat models in a dose dependent manner. In the fa/fa insulin-resistant rat model, T0-901317 significantly improved insulin sensitivity. The low plasma glucose levels were due to a dramatic inhibition of PEPCK mRNA transcription. Further studies in primary hepatocytes have shown that hepatic activation of LXRs is sufficient to mediate the inhibition of gluconeogenic pathway (26).

Moreover, GW3965 improves glucose tolerance in a murine model of diet-induced obesity and insulin resistance. Activation of LXR in mouse liver inhibits gluconeogenic pathway by inhibiting the expression of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase. In contrast, LXR agonists promote hepatic glucose utilization by increased expression of glucokinase. Activation of LXR in adipose tissue, increases transcription of the insulin-sensitive glucose transporter, GLUT4, which is a direct transcriptional target for the LXR•RXR heterodimers. In LXR null cells and intact animals, the ability of LXR agonists to induce GLUT4 expression is abolished (105). These results suggest a major role for LXRs in the coordination of lipid and glucose metabolism.

Recently there is compelling evidence showing an important link between cholesterol and Alzheimer's disease. Vascular risk factors such as high total plasma cholesterol, particularly low density lipoprotein (LDL) cholesterol, influence the progression or the incidence of Alzheimer's disease (21, 96, 97, 114, 213). In contrast, plasma high density lipoprotein (HDL) cholesterol are inversely associated with cardiovascular disease (92) and Alzheimer's disease (14, 135). Oxysterols decrease amyloid beta (A beta) secretion in vitro. This effect is primarily due to the increase in ABCA1 by LXRs. T0-901317 applied to a variety of in vitro models, including immortalized fibroblasts from Tangier patients, and primary embryonic mouse neurons and Alzheimer disease mouse model (APP23 transgenic mice) caused a concentration-dependent decrease in A beta secretion (101).

4) Regulation of LXR activity

Several distinct pathways have been identified that modulate LXR transcription activity. One such pathway involves small heterodimer partner (SHP), an unusual member of the nuclear hormone receptor superfamily. SHP lacks the typical DNA binding domain observed in most nuclear hormone receptors. SHP has been shown to interact with LXR and other nuclear hormone receptors. In transient transfection assays, overexpression of SHP inhibits LXR activation of ABCA1 transcription. Bile acids have

been shown to stimulate SHP expression in human intestinal cell lines. This phenomenon has been proposed as a mechanism for the inhibitory effects of bile acids on the expression of the LXR target, ABCG1 (20).

Another mechanism by which LXR activity is inhibited is by competition for the binding of oxysterols to the receptor. Many compounds antagonize the binding of LXR agonists. For example, polyunsaturated fatty acids (PUFAs) antagonize the binding of oxysterols to LXRα in hepatoma cells with arachidonic acid being the most potent, followed by linoleic and oleic acids, whereas saturated fatty acids have no effect. PUFAs also interfere with LXR•RXR binding to LXREs. PUFAs are natural agonists for peroxisome proliferator activated nuclear receptors (PPARs). Ligand-activated PPAR induces transcription of LXRα gene through a PPAR response element (145, 229). Thus PUFAs may potentially induce LXRα levels in cells while inhibiting LXRα binding of activating ligands such as oxysterols. Evidence that PUFAs are physiological regulators of LXR signaling is currently lacking.

Recently, the hypolipidemic agent, fenofibrate, has been shown to repress LXR-activation of lipogenic gene expression in hepatocytes. Fenofibrate binds directly to the LXR ligand binding domain, and displaces a naturally occurring LXR ligand. Interestingly, the antagonistic effects of fenofibrate on LXR signaling are gene specific. Fenofibrate inhibits LXR activation of lipogenic genes (ACCα, FAS, SREBP), but has no effect on LXR activation of ABCA1. Fenofibrate could induce distinct structural changes in LXR that influence its ability to interact with other proteins, such as transcription factors residing at selective target gene promoters or with coactivators or corepressors that are critical for target gene regulation (47, 147, 197).

Data from our laboratory have shown that bile acids (chenodeoxycholic acid) inhibit the T0-901317-induced expression of lipogenic genes. Interestingly, chenodeoxycholic acid enhances T0-901317-induced expression of ABCA1. Thus, the action of CDCA on LXR-induced gene expression is similar to that of fenofibrate in that it is gene-specific. Currently, there is no evidence that CDCA or metabolites of CDCA directly interact with LXR.

Another protein, PGC-1α has been shown to act as a coactivator for the LXRα. More will be discussed about PGC-1α and LXR interactions later in this Chapter.

Previous work has shown that the selective, potent and orally active LXR agonist, GW4064, enhances the recruitment of SRC-1 to human LXRα in vitro (51, 76). Studies have shown that LXRs interact with corepressors such as N-CoR (nuclear receptor corepressor) and SMRT (silent mediator of retinoic acid receptor and thyroid receptor), in the absence of LXR agonists and that ectopic expression of N-CoR represses transcription directed by LXR. Chromatin immunoprecipitation (ChIP) experiments have demonstrated that N-CoR is recruited to endogenous LXR target genes and that addition of LXR agonists releases N-CoR (72, 203). Thus, to develop LXR ligands as drugs for the treatment of atherosclerosis, partial, selective activators of LXRs are needed that induce cholesterol efflux in macrophages but do not induce fatty acid synthesis in liver.

Among the currently known ligands for LXRs, some compounds, such as the T0901317, have a purely agonistic activity, whereas others, such as GW3965, have been reported to be more selective in their activation of LXR function (162, 179). Based on structural data, it has been predicted that cofactor interactions induced by T0901317 and a natural ligand would differ from each other (211). For drug development, partial and selective agonists are desired, which activate the target receptors in a tissue-specific manner. For example, tamoxifen and raloxifen have been shown to activate the estrogen receptor in a tissue-specific manner (134). Responses to raloxifen and tamoxifen are sensitive to the amount of coactivators and corepressors in a cell. Thus availability or changes of coactivators or corepressors are responsible for variable degrees of agonism or antagonism of these agents. These studies have led to the hypothesis that relative availability of corepressors and coactivators in a cell determines the agonistic or antagonistic behavior of partial agonists (186). According to the model, a variety of conformations can be adopted by the receptor between the two extremes of a purely antagonistic state and a purely agonistic state (187). Partial agonists would induce conformations that are to be placed in intermediate positions between the two extreme states.

The different conformations that LXRα can adopt goes beyond a linear scale. For example, GW3965 increases binding of LXRα to coactivators comparably to T0901317, but there is a dramatic difference on corepressor binding. On the other hand, GW3965 and LN6500 have similar effects on corepressor binding, but LN6500 increases

coactivator binding more than that of GW3965. Thus, the hypothesis that availability of corepressors and coactivators in a cell determines the agonistic or antagonistic behavior of partial agonists, can be modified as follows. In addition to the two extremes on the linear scale of pure agonists and pure antagonists the following conformations are also possible (a) induction of corepressor and coactivator binding simultaneously; (b) partial induction of coactivator binding without induction of corepressor binding; (c) partial induction of both coactivator and repressor binding; and (d) inhibition of the binding of any cofactor, that results in derepression. This phenomenon described in (d) has been demonstrated in LXR-knockout mice (203).

The fundamental problem for using LXR pathway as drug targets is that in addition to preventing the formation of atherosclerotic plaque by increasing HDL, LXR agonists increase serum and liver triglyceride levels. Recently, GW3965 has been shown to have a weaker effect on increase in triglyceride levels in mice than T0901317 (136). This provides a good example of partial agonism as described above. Moreover, LN6500 has a weaker agonistic property than GW3965 (2). These observations suggest alterations in coactivator or corepressor recruitment to LXR is a possible mechanism controlling LXR transcriptional activity. T0-901317 and bile acids may regulate ACCα transcription in hepatocytes by modulating the recruitment of coactivators and corepressors to the ACCα gene.

D) Regulation of gene expression by bile acids

Bile acids, derived from cholesterol, are physiologically important amphipathic molecules that perform several functions in lipid physiology. First, their synthesis provides a disposal mechanism to counterbalance cholesterol synthesis and allow homeostasis to be achieved. Second, their detergent actions are essential in the intestine for the uptake of hydrophobic nutrients such as triacylglycerols and fat-soluble vitamins and in the liver for the solubilization of metabolites such as bilirubin. Third, intermediates and end products of the bile acid pathway regulate the expression of genes involved in the synthesis of cholesterol, fatty acids, and bile acids themselves. Cholesterol is oxidized to oxysterols that, in turn, increase expression of bile acid

synthesis via LXR-mediated stimulation of cholesterol 7 alpha hydroxylase (CYP7A1), which catalyzes the first and rate-limiting step in the classical bile acid synthetic pathway, transcription in liver. To prevent the accumulation of potentially harmful levels of bile acids, a feedback regulatory system that controls expression of CYP7A1 has evolved. This system is initiated by the binding of bile acids to the farnesoid X receptor (FXR), a member of the nuclear hormone receptor superfamily (56). Chenodeoxycholic acid (CDCA) is the most potent natural ligand of FXR (19).

1) Structure and function of bile acids

There are several bile acids that function differently in a physiological context. This strongly suggests that diversity of the side chains that lead to minor structural differences of bile acids may lead to their specificity of action. For example, CDCA is the most potent physiological ligand of FXR, but its 7β-epimer, ursodeoxycholic acid (UDCA), has no effect on CYP7A1 in vivo and does not activate FXR (129, 148, 189).

Relative hydrophobicities of bile acids are a primary determinant of the biological properties (175). As bile acids function as detergents to solubilize fat, it has been reported that hydrophobic bile acids have a greater capacity to perturb the structure and partly digest cell membranes (175, 202). Powell et. al. have determined the hydrophobicity of different bile acids arranged in decreasing order of hydrophobicity as follows; DCA>CDCA>HDCA>UDCA>CA (154). DCA and CDCA induce apoptosis very rapidly in colon cancer cell lines via induction of protein kinase-C (PKC) and activator protein-1 (AP-1) (159). However, moderately hydrophobic bile acids such as HDCA also induce apoptosis, although at later time points (154). In contrast, ursodeoxycholic acid (UDCA), a less hydrophobic stereoisomer of CDCA, inhibits proliferation in colon cancer cell lines (64, 77). Hydrophobicity of bile acids facilitates solubilization and crossing the cell membrane. Hepatocytes contain transmembrane bile acid transport proteins and bile acid nuclear receptors, thus elevated bile acid levels result in the activation of signaling pathways by binding directly to intracellular components (129, 148). These examples suggest that one of the major determinants of bile acid action and specificity is a measure of its hydrophobicity.

2) Bile acids and gene regulation

Bile acids decrease expression of the CYP7A1 gene through multiple pathways. One pathway involves the induction of the repressor, short heterodimeric partner (SHP), by ligand-activated FXR (56, 124). Increased SHP levels inhibit the CYP7A1 promoter via interaction with the positive acting transcription factor, LRH. In support of this regulatory pathway, FXR knockout mice exhibit markedly reduced levels of SHP and bile acid regulation of CYP7A1. Recent studies have shown that bile acids also inhibit transcription by causing dissociation of coactivators from the promoter of genes (89).

For a long time bile acids have been shown to have an inverse correlation with triglyceride levels. For example, humans with cholesterol gallstones treated with the bile acid chenodeoxycholic acid (CDCA) has been shown to reduce hypertriglyceridemia (5, 10, 28). Disruption in the sterol 27-hydroxylase gene in mice leads to a decrease in hepatic bile acid pool size that is associated with an increase in CYP7A activity and an increase in SREBP-1 and SREBP-2 expression that is associated with an increase in cholesterol synthesis and lipogenic gene expression (168). Mice lacking FXR exhibit elevated levels of serum and hepatic triacylglycerol and cholesterol suggesting a role for FXR in maintaining lipid homeostasis (185, 207). Disruption of the CYP7A1 gene in mice causes a reduction in bile acid synthesis and bile acid pool size that is associated with hypertriglyceridemia and treatment of these mice with a synthetic FXR agonist causes a reduction of plasma triacylglycerols (158). Treatment of wild type mice with a synthetic FXR agonist causes a reduction in hepatic SREBP-1 levels (207).

Recently, a novel G-protein coupled receptor (GPCR) has been identified that is referred to as TGR5. TGR5 functions as a cell surface receptor that binds bile acids as agonists. The primary structure of the TGR5 receptors and their responsiveness to bile acids are highly conserved in human, bovine, rabbit, rat, and mouse. TGR5 mRNA is present in the placenta, spleen, and monocytes/macrophages, whereas the nuclear receptors are mainly expressed in the liver, kidney, and intestine (91, 129, 148). Treatment of CHO cells that express TGR5 increased ERK and cAMP production (91). An increase in cAMP levels as a second messenger is a classic response of GPCRs.

Moreover, NIH 3T3 murine fibroblasts expressing human muscarinic acetylcholine receptors GPCRs increase the activity of ERK5, p38α, p38γ, and p38δ, that results in the activation of the c-*jun* promoter (131).

Another pathway involves Kupffer cells that act as sensors of concentration of bile acids in the liver via enterohepatic circulation (138). Bile acids increase the expression of inflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin (IL-1β) that are subsequently secreted into the sinusoids as a result of interaction with macrophages. These cytokines are recognized by high affinity receptors on hepatic parenchymal cells, that activate protein kinase-C (PKC) (152) and c-jun N-terminal kinase (JNK) to inhibit expression of CYP7A1 (36, 138). Activation of PKC has been shown to increase Mg^{2+} accumulation in hepatocytes via ERK and p38 MAPK (199).

In addition to activating PKC, bile acids modulate gene expression signaling cascades such as p53 (160), ERK and p38 (159, 161), phosphatidylinositol 3-kinase (PI3K) (174), and the activator protein-1 (AP-1) transcription factor (159). CDCA increases low density lipoprotein receptor (LDLr) gene expression via ERK activation-mediated stabilization of LDLr mRNA via activating SHP (140). These authors reported that ursodeoxycholic acid (UDCA) had no effect on LDLr and SHP mRNA. These results suggested that the structural difference between CDCA and UDCA, specifically the 7β-hydroxy epimer of CDCA, is critical for the activation of ERK and binding to FXR (129, 148). Lithocholic acid (LCA), a weak activator of FXR, is a potent inducer of LDLr via a robust activation of ERK. However, UDCA had no effect on the activation of ERK (18). Octyl β-D-glucopyranoside, a non-ionic and non-cytolytic membrane detergent had no effect on ERK activity (132). These results provide further evidence that bile acids activate MAP kinases in a specific manner, not by their detergent effects, but by specific side chain residues and three-dimensional structure.

Administration of bile acids increases energy expenditure in brown adipose tissue, preventing obesity and insulin resistance in mice. This effect is mediated by an induction of the cyclic-AMP-dependent thyroid hormone activating enzyme type 2 iodothyronine deiodinase (D2). Treatment of brown adipocytes and human skeletal myocytes with bile acids increases D2 activity and oxygen consumption. These effects are independent of

FXR, and are mediated by increased cAMP production from the binding of bile acids with TGR5 (206).

Recently, it has been shown that increased bile acid levels accelerate liver regeneration, and decreased bile acid levels inhibit liver regrowth. This finding is supported by the fact that FXR null mice inhibit liver regeneration. Therefore FXR, and possibly other nuclear receptors, may promote homeostasis not only by regulating expression of appropriate metabolic target genes but also by driving homeotrophic liver growth (75). The effects of bile acids on liver regeneration can be further accounted for by the fact that taurochenodeoxycholic acid (TCDCA), but not glycochenodeoxycholic acid (GCDCA), activates phosphatidylinositol 3-kinase (PI3K)-mediated survival pathway in isolated, perfused rat livers. TCDCA moderately induced hepatic injury by stimulating apoptosis as opposed to GCDCA, that causes a more severe liver injury. It has been shown that TCDCA increases the PI3K pathway more robustly than GCDCA, thus accounting for the relative moderate effects of TCDCA on liver injury. Inhibitors of the PI3 kinase pathway, such as wortmannin, reversed the effects of TCDCA and GCDCA on liver injury, in that TCDCA resulted in a more severe liver injury and GCDCA had a far lesser injury. These results showed that TCDCA block its toxic effect in intact liver, by activating the PI3K dependent survival pathway (174). Another recent report showed that a relatively hydrophilic bile acid UDCA, protects reperfusion injury of the heart by activating the PI3K pathway (164). These findings suggest a protective, or growth promoting role of bile acids. Physiologically, the growth promoting effects of some bile acids may act to oppose the pro-apoptotic effects of more hydrophobic bile acids.

3) Bile acids and fibroblast growth factor-19 (FGF-19)

Another pathway mediating bile acid regulation of CYP7A1 expression involves the fibroblast growth factor 19 (FGF-19). FGF-19 is a member of the fibroblast growth factor (FGF) family of secreted signaling molecules. FGF-19 is a high affinity ligand for its receptor, fibroblast growth factor receptor 4 (FGFR4) and is the first member of the FGF family to show exclusive binding to FGFR4. FGFR4 belongs to the receptor

tyrosine kinase family of receptors. Bile acids increase the expression of FGF-19 in human hepatocytes by binding FXR on the FGF-19 gene promoter. Increased FGF-19 levels, in turn, inhibit transcription of CYP7A1 via a mechanism involving the activation of Jun-N terminal kinase (JNK) signaling pathway (60, 69, 217, 233).

In support of a role of FGF-19 in bile acid regulation of CYP7A1, FGFR4 knockout mice have an elevated bile acid excretion and expression of CYP7A1 (232). In contrast, transgenic mice expressing a constitutively active form of FGFR4 have increased JNK activity, decreased CYP7A1 expression, and a reduced bile acid pool size (231). Transgenic mice overexpressing FGF-19 showed a decrease in fat mass and increased energy expenditure. These animals overexpressing FGF-19 were resistant to high fat diet, in that they did not develop obesity or diabetes. Expression of acetyl CoA carboxylase 2, malic enzyme and stearoyl CoA desaturase in the liver were decreased along with a decrease in liver triglyceride levels in these animals (198). In humans Paraoxonase-1 (PON1), an enzyme that metabolizes organophosphate insecticides is secreted by the liver and transported in the blood complexed to HDL. Bile acids inhibit the expression of PON1 via increase of FGF-19 (181).

Recently, it has been demonstrated that expression of FGF15, the mouse ortholog of human FGF19, is induced by FXR agonists in small intestine. FGF15 mediated inhibition of hepatic bile acid synthesis involves both FGFR4 and SHP (78). We have shown that bile acids increase FGF-19 mRNA expression in chicken embryo hepatocytes. Work from our lab by Sushant Bhatnagar has shown that recombinant human FGF-19 inhibits insulin-induced expression of glucokinase and lipogenic genes such as ACC1 and SCD in primary rat hepatocytes. We hypothesize that inhibition of T0-901317-induced lipogenic gene expression in chick hepatocytes is mediated by increased FGF-19 expression.

E) Peroxisome gamma coactivator -1 alpha (PGC-1α)

1) Structure and function

PGC-1α belongs to a small family of transcriptional coactivators that includes PGC-1β and PGC-1-related coactivator. PGC-1α was first identified as a protein that interacts with the nuclear hormone receptor PPARγ, that is the regulator of adipocyte differentiation (157). From structure-function analysis of PGC-1α it has been demonstrated that the N terminus of the protein consists of a transcriptional activation domain that includes the nuclear hormone receptor-interacting motif (LXXLL). This motif mediates ligand-dependent interaction of coactivators with nuclear hormone receptors. The C-terminal region of PGC-1α consists of an RNA-binding motif (RMM) and a serine-arginine-rich (RS) domain (4, 62, 102, 119). Transcriptional coactivators are recruited to particular DNA sequences in promoters of genes through direct interaction with transcription factors. The LXXLL motif of PGC-1α has been shown to mediate ligand-dependent interaction with nuclear receptors such as estrogen receptor (ER) (195), PPARα (200), RXRα (39), and glucocorticoid receptor (GR) (98).

2) PGC-1α and gene regulation

PGC-1α has been shown to be a coactivator for LXRα. In transient transfection studies, PGC-1α potentiates the LXR-mediated autoregulation and transactivation of the LXRα promoter via the LXRE on the cholesteryl ester transfer-protein (CETP) gene promoter in a ligand-dependent manner. As described above, the LXXLL motif of PGC-1α is located in the vicinity of the binding region for a putative repressor that will be described later in this section. The repressor sequesters PGC-1α from PPARα and the glucocorticoid receptor. However, this repressor does not interfere with PGC-1α-mediated coactivation of LXR-dependent gene transcription (142). Previous reports have shown that p38 MAPK phosphorylates PGC-1α on Thr262, Ser265, and Thr298. Phosphorylation of PGC-1α on these residues results in increased stability and half-life of the protein (156). Inhibition of p38 MAP kinase activity had only a moderate inhibitory effect on LXR coactivation by PGC-1α. Thus PGC-1α has been established as a bona fide LXR coactivator and suggests the involvement of distinct motifs of PGC-1α and/or

additional cofactors in the modulation of LXR and PPARα transcriptional activities (142). Interestingly, HNF4 also interacts with this motif of PGC-1α in the absence of an agonist, suggesting that this nuclear hormone receptor is in an active conformation even without the addition of exogenous ligand (227).

Non-LXXLL motifs of PGC-1α also mediate interaction with transcription factors. For example, a region between amino acids 200 and 400 interacts with PPARγ (157) and NRF-1 (216) and a region between amino acids 400 to 500 interacts with MEF2-C (137). Most coactivators increase transcriptional activity by either intrinsic chromatin remodeling activity, or by recruiting other proteins that have intrinsic chromatin remodeling activity (53, 139, 190). PGC-1α lacks significant amino acid sequence homology to other transcriptional coactivator families and it does not possess intrinsic histone acetyl transferase (HAT) activity. However, the N-terminal domain of PGC-1α recruits proteins that contain HAT activity, such as SRC-1 and CREB binding protein CBP/p300 (155). In addition, PGC-1α is also present in a complex that contains RNA polymerase II.

Interestingly, the binding of SRC-1 and CBP/p300 to the N-terminus of PGC-1α is dependent upon docking of transcription factors such PPARγ and NRF-1 to the amino acids 200-400 region of PGC-1α (155). This suggests that PGC-1α is in a relatively inactive state when not bound to a transcription factor. However, when a transcription factor binds to PGC-1α, it is activated, and results in a conformation change that recruits SRC-1 and CBP/p300 into the complex. Thus, transcription factor docking switches on the activity of PGC-1α. Whether this phenomenon of transcriptional activation via a transcription factor docking event is a common feature of coactivators remains to be determined. Transcription factors are targets of multiple signal transduction pathways, but there is evidence to suggest that coactivators can also be targets of these signaling pathways in response to hormones, growth factors and ligands. For example, transcriptional activity of CBP is increased in response to calcium signaling through calmodulin kinase IV (29). In addition, SRC-1 has been shown to be a target of cAMP signaling through protein kinase A PKA (172). Therefore, regulation of transcriptional coactivator function provides a target to integrate different responses to specific signals across multiple transcription factors.

As discussed previously, p38 MAPK phosphorylates PGC-1α at three residues Thr262, Ser265, and Thr298 (156). Phosphorylation of PGC-1α on those residues leads to increased stability and half-life of PGC-1α. It is interesting that these phosphorylations occur in a region previously shown to play a regulatory role in PGC-1α binding to transcription factors. It is unclear whether the p38 MAPK-mediated phosphorylations affect transcription factor docking or the recruitment of other coactivator proteins to the PGC-1α complex. It is also unclear as to how these phospho-amino acids change protein stability.

Activation of p38 MAPK leads to an increase in transcriptional activity of PGC-1α. In the absence of nuclear hormone receptor, PGC-1α associates with a strong transcriptional repressor, referred to as p160 myb binding protein (p160MYB), that keeps PGC-1α in an inactive state. It is unclear whether p160myb by itself is a transcriptional repressor, or whether it recruits the binding of another repressor on the promoter. Activation of p38 MAPK phosphorylates PGC-1α that removes p160myb and facilitates the association of PGC-1α with the nuclear hormone receptor at the transcription site (43, 99). Binding to the nuclear hormone receptor causes a conformational change on PGC-1α that helps to stabilize the active state (155). Interestingly, transcriptional activity of PPARα associated with PGC-1α can be activated via the p38 MAPK pathway (9).

Hepatic nuclear factor-4α (HNF-4α) and PGC-1α are key activators of hepatic gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and Glucose-6-Phosphatase (G6Pase). These genes are activated during fasting and inhibited during the fed state. SREBP-1a and -1c inhibited PEPCK promoter activity that was induced by HNF-4α. Electrophoretic mobility shift assays showed that SREBP-1 did not bind to the PEPCK promoter, and that the inhibitory effect was more potent in SREBP-1a than SREBP-1c. This inhibitory effect was abolished by deletion of the amino-terminal transactivation domain of SREBP-1. SREBP-1 competitively inhibits PGC-1α recruitment, that is a fundamental requirement for HNF-4α activation. Transgenic mice overexpressing SREBP-1a and -1c showed an inhibition of hepatic PEPCK and G6Pase mRNA abundance. These results identify a novel role of SREBP-1 as an inhibitor of gluconeogenic genes by inhibiting PGC-1α recruitment to HNF-4α (222).

F) Peroxisome proliferator activated receptor gamma coactivator -1 beta (PGC-1β)

PGC-1β is a transcriptional coactivator that has been recently identified as a close member of PGC-1α. The biological activities of PGC-1β are currently unknown (102, 118). The tissue distribution of PGC-1β is similar to that of PGC-1α. However, they are differentially regulated during development and in response to changes in nutritional status (88, 118). Recently it has been shown that high fat feeding increases PGC-1β and SREBP-1a and -1c. PGC-1β has been shown to be a coactivator of SREBP and LXR. This increase in SREBP-1 and LXR activity increases de novo lipogenesis and lipoprotein secretion. PGC-1β coactivation of SREBP and LXR by high fat leads to hypertriglyceridemia and hypercholesterolemia (120).

G) Mitogen activated protein kinases

Mitogen activated protein kinases (MAPKs) consist of a family of protein kinases that are conserved across species from unicellular organisms to complex organisms including humans. Protein kinases are enzymes that covalently attach phosphate to Ser, Thr and Tyr residues on other proteins in the cell. MAPKs phosphorylate Ser and Thr residues on target proteins that are substrates, and modulate multiple cellular processes such as gene regulation, mitosis, metabolism and apoptosis. The phosphorylation of substrate proteins by MAPKs functions as a molecular switch to turn on or turn off the activity of the target protein that ultimately alter cellular processes. There are several substrates of MAPKs that modulate multiple functions such as other protein kinases, transcription factors and phospholipases. Protein phophatases remove the phosphate attached by MAPKs to inactivate the substrate proteins (165, 209).

MAPKs are part of a signaling cascade comprised of three kinases that are regulated by phosphorylation. MAPKs are substrates for MAPK kinases (MKKs) that phosphorylate and activate MAPKs. Specific phosphatases of the MAPKs, remove the phosphate from the MAPKs and render them inactive. MKKs in turn, are phosphorylated and activated by MAPK kinase kinases (MKKKs). In multicellular organisms, there are three conserved and well characterized MAPK families. These include the extracellular signal-regulated kinases ERK1 and ERK2 (16, 17), the c-Jun N-terminal kinases JNK-1, -

2 and -3 (63, 104), and p38 MAPKs p38α, p38β, p38γ and p38δ (84, 85, 108, 116, 188). Recently a fourth MAPK, ERK5 has been identified and is being studied (237). Additional proteins kinases have been identified during sequencing the human and mouse genomes that may function as MAPKs, one of them being ERK7, but very little is known about this protein (1).

1) ERK

Mammalian ERK1/2 MAPKs are referred to as the classical mitogen kinase cascade, consisting of the MAPKKKs A-Raf, B-Raf, and Raf-1, the MAPKKs MEK1 and MEK2, and the MAPKs ERK1 and ERK2. ERK1 (44 kD) and -2 (42 kD) have 85% amino acid identity and are expressed in almost all tissues; however, their relative distribution in tissues differ widely (31). Both ERK1/2 are activated by a vast number of ligands and cellular processes with some cell type specificity. Activators of ERK1/2 include serum, growth factors, phorbol esters and to a lesser extent by ligands of the heterotrimeric G protein-coupled receptors, cytokines and osmotic stress (115). These agents and events activate ERK pathway by activating receptor tyrosine kinases (RTKs) on the cell surface and G protein-coupled receptors. Activated RTKs in turn, activate the Raf/MEK/ERK cascade through different isoforms of the small GTP-binding protein Ras (24, 214).

ERK1/2 are distributed in almost all cells in the inactive, unphosphorylated state, but upon activation, a significant population of ERK1/2 accumulates in the nucleus (30, 55, 113). The mechanism of nuclear accumulation of ERK1/2 are unclear; however, phosphorylation, dimerization, nuclear retention and release from cytoplasmic anchors are suggested in mediating the effect (153). Since ERK1/2 pathway has been shown to be a key regulator of cellular proliferation, chemical inhibitors of the ERK pathway are being tested in clinical trials as potential anticancer agents (100). Two structurally unrelated compounds, U0126 (44) and PD98059 (3, 40), are commonly used to specifically inhibit the ERK1/2 pathway in cell culture. The use of these compounds has been widely validated in the literature in various cell culture systems. Both these

compounds are noncompetitive inhibitors of MEK1/2/5 and inhibit phosphorylation and activation of ERK1/2/5 (8).

2) Transcriptional regulation by ERK

ERK1/2 increases the phosphorylation and activation of several downstream MAPK-activated protein kinases referred to as p90 ribosomal S6 kinases (RSKs) proteins. The RSK family consists of four human isoforms that share 70% amino acid similarity. RSK like proteins have also been identified in other species such as Drosophila, Xenopus, chicken and yeast. These proteins are Ser/Thr kinases that modulate the activity of target proteins (46, 86, 204).

Activation of the ERK pathway facilitates the interaction between RSK1 and the transcriptional coactivator CREB-binding protein (CBP) (141). CBP and its paralog p300 facilitate complex formation between different components of the basal transcriptional machinery. RSK1 interaction with CBP was found to modulate its function, but the exact outcome of this interaction remains to be determined. Binding of phospho-p90RSK (P-p90RSK) to CBP mediated transcription inhibition of cAMP-responsive genes via the cAMP-inducible factor CREB. In contrast, formation of the P-p90RSK-CBP complex was required for increase in transcription of Ras-responsive genes (141, 205). Interestingly, CBP and p300 have been shown to associate with several transcription factors also known to be RSK1 and RSK2 substrates, such as c-Fos, c-Jun, ER81, and NF-κB, suggesting that RSK1 binding to CBP may provide a second mechanism of transcriptional control (171). It is interesting to note that although both ERK and RSK accumulate in the nucleus upon activation, it is unclear whether ERK1/2 directly modulates the activity of proteins. All data so far suggest that RSK proteins mediate the effects of ERK on target proteins.

Deoxycholic acid (DCA), lithocholic acid (LCA) and CDCA, but not ursodeoxycholic acid (UDCA), increase the mRNA abundance of the LDL receptor, even in the presence of a potent inhibitor of the LDL receptor, 25-hydroxycholesterol. Surprisingly, this increase of the LDL receptor mRNA is not mediated by FXR. CDCA increased the phosphorylated, active form of ERK, and inhibition of ERK activity by

U0126 abolished the CDCA-mediated increase of LDL receptor. Thus, CDCA-mediated increase of ERK stabilizes LDL receptor mRNA and the ERK inhibitor accelerates its turnover (140).

As discussed previously, ERK has been shown to mediate lipogenic gene inhibition by inhibiting the mature, transcriptionally active form of SREBP-1 (15). This is another example by which ERK can inhibit gene transcription. Our studies have shown that activation of ERK mediates inhibitory effects of CDCA on ACCα mRNA expression.

3) p38 MAP kinase

The p38 MAPK module consists of the four known p38 isoforms (α, β, γ, and δ), several MAPKKKs, including MEKKs 1 to 4, the MAPKKs MKK3 and MKK6 (103). Most stimuli or agents that stimulate JNK pathway also stimulate p38 MAPK. The functional role of p38 MAPK has largely been determined by using the anti-inflammatory drug SB203580 (110). SB203580 inhibits p38α, β and β2 (not γ and δ) by competing with the substrate, ATP (57, 230). While SB203580 inhibits p38 activity, it does not significantly affect the activation of p38. SB203580 does not inhibit PKA, PKC, MEKs, MEKKs or ERK and JNK MAPKs (35, 50, 230).

p38 is present in both the nucleus and cytoplasm of cells. The subcellular localization of p38 MAPK kinase upon cellular stimuli is unclear. On one hand, upon receiving activation stimuli, p38 MAPK translocates from the cytoplasm to the nucleus (163), and on the other, active p38 MAPK is also present in the cytoplasm of stimulated cells (11). p38 MAPK activation in macrophages, neutrophils, and T cells by numerous extracellular modulators of inflammation mediates normal immune and inflammatory responses (143). p38 MAPK phosphorylates several cellular targets, including the transcription factors ATF1 and -2, MEF2A, Sap-1, Elk-1, NF-κB, Ets-1, and p53 (103).

4) Transcriptional regulation by p38 MAPK

Mitogen- and stress-activated kinases 1 and 2 (MSK1 and MSK2) are downstream substrates of both ERK1/2 and p38α and p38β MAPK. MSKs are

predominantly located in the nucleus in the inactive state as they contain a C-terminal nuclear localization signal (NLS). MSK1 and MSK2 phosphorylate and activate CREB on ser133 in vitro with a significantly lower K_m than that of RSK1 and MK2 (37, 151). This data has been confirmed by data that showed the involvement of MSK1 and -2 in CREB phosphorylation with different inhibitors (58, 109). MSK1 has been shown to mediate NF-κB-dependent transcription by phosphorylating the NF-κB isoform p65 on Ser276 (201). MSK1 has also been shown to phosphorylate and activate other transcription factors such as ER81 (79) and STAT3 (210, 234).

Hepatic gluconeogenesis is essential for maintaining blood glucose levels during fasting and is the major contributor to postprandial and fasting hyperglycemia in diabetes. Glucagon, elevated in the blood during fasting and diabetes increases gluconeogenesis. Glucagon activates p38 MAPK in primary hepatocytes. Mice treated with the p38 MAPK inhibitor SB203580 reduced fasting plasma glucose levels. Inhibition of p38 MAPK inhibited gluconeogenesis by decreasing the mRNA abundance of gluconeogenic genes such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. PGC-1α and CREB have been shown to mediate hepatic gluconeogenesis. Inhibition of p38 MAPK decreased PGC-1α and CREB. These results have established a role for p38 MAPK in cAMP-dependent activation of genes involved in gluconeogenesis (27).

Recently, it has been shown that polyunsaturated fatty acids such as arachidonic acid inhibit the insulin-induced abundance of glucose-6-phosphate dehydrogenase (G6PD) mRNA in hepatocytes. Arachidonic acid increases phosphorylation and activation of p38 MAPK that phosphorylates Ser307 residue of insulin receptor substrate (IRS-1) and inhibits the activity. This inhibition of IRS-1 inhibits activation of phosphor-Akt and inhibits insulin signaling (193). As G6PD is a lipogenic gene, this report suggests a link between activation of p38 MAPK and inhibition of lipogenic genes. Our data shows that CDCA inhibits ACCα mRNA abundance at least in part, by increasing the phosphorylation and activation of p38 MAPK.

5) JNK

Three members have been identified that are referred to as JNK1, JNK2, and JNK3 (also known as SAPKγ, SAPKα, and SAPKβ, respectively) that are ubiquitously expressed. Activators of JNK include cytokines, UV irradiation, growth factor deprivation, DNA-damaging agents, and, to a lesser extent, growth factors, serum and some G protein-coupled receptors (103). The MAPKKs that phosphorylates and activates JNK are referred to as MEK4 and MEK7, which are themselves phosphorylated and activated by several MAPKKKs, that include MEKK1-4, MLK2 and -3 (103). JNKs relocalize from the cytoplasm to the nucleus following activation (103). The best known substrate for JNKs is the transcription factor c-Jun. Phosphorylation of c-Jun on ser63 and ser73 by JNK leads to increased c-Jun-dependent transcription (208). Several other transcription factors have been shown to be phosphorylated by the JNKs, such as ATF-2, NF-ATc1, HSF-1, and STAT3 (31, 103). It is interesting to note that although some cytoplasmic targets of JNK are known, the fact that activated JNK does not exhibit exclusive nuclear localization suggests that many other cytoplasmic substrates remain to be identified. Interestingly, JNK-activated MKs are unknown.

As discussed above, FGF-19 activates JNK that results in feedback inhibition of CYP7A. Transcription regulation by JNK has been discussed in Section D 2). Our data shows that CDCA increases the phosphorylation and activation of JNK in primary hepatocytes.

H) AMP-activated protein kinase

When cellular AMP:ATP ratio increases, AMP activates a wide range of cellular processes by binding to AMP-activated protein kinase (AMPK) that activates pathways that generate ATP while inhibiting pathways that utilize ATP (59). AMP causes an allosteric activation of AMPK. Binding of AMP makes AMPK a better substrate for upstream kinases, that phosphorylates AMPK at Thr172. Binding of AMP to AMPK also inhibits dephosphorylation of Thr172 by phosphatases (59). AMPK is phosphorylated and activated by its upstream protein kinase, referred to as LKB1 (61, 180, 215). Phosphorylated and activated AMPK (P-AMPK) inhibits fatty acid synthesis and

cholesterol biosynthesis and activates catabolic pathways such as fatty acid oxidation, glucose uptake and glycolysis (59). P-AMPK inhibits lipogenesis by inhibiting SREBP-1 (238). P-AMPK also inhibits the coactivator p300 by phosphorylating the protein at (Ser89), and inhibiting the binding to nuclear hormone receptors (224). P-AMPK also phosphorylates and inactivates ACC at Ser79. Phosphorylation of ACC at this residue is a direct measure of AMPK activity (32, 59).

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Chapter 2

The Mechanism Mediating the Activation of Acetyl-CoA Carboxylase-α Gene Transcription by the Liver X Receptor

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The Mechanism Mediating the Activation of Acetyl-CoA Carboxylase-α Gene Transcription by the Liver X Receptor

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ABSTRACT

In avians and mammals, agonists of the liver X receptor (LXR) increase the expression of enzymes comprising the fatty acid synthesis pathway. Here, we investigate the mechanism by which the synthetic LXR agonist, T0-901317, increases the transcription of the acetyl-CoA carboxylase-α (ACCα) gene in chick embryo hepatocyte cultures. Transfection analyses demonstrate that activation of ACCα transcription by T0-901317 is mediated by a cis-acting regulatory unit (-101 to -71 bp) that is comprised of a LXR response element (LXRE) and a sterol regulatory element (SRE). The SRE enhances the ability of the LXRE to activate ACCα transcription in the presence of T0-901317. Treating hepatocytes with T0-901317 increases the concentration of mature sterol regulatory element-binding protein-1 (SREBP-1) in the nucleus and the acetylation of histone H3 and histone H4 at the ACCα LXR response unit. These results indicate that T0-901317 increases hepatic ACCα transcription by directly activating LXR• retinoid X receptor (RXR) heterodimers and by increasing the activity of an accessory transcription factor (SREBP-1) that enhances ligand-induced-LXR•RXR activity. We also show that T0-901317 treatment decreases LXR•RXR binding and increases nuclear T3 receptor•RXR binding to the ACCα LXRE. We propose that changes in the binding of nuclear receptor complexes to the ACCα LXRE limit the activation of ACCα transcription by T0-901317.

Supplementary keywords: fatty acid synthesis, liver, sterol regulatory element binding protein, LXR, chicken, histone acetylation

INTRODUCTION

The first committed step of the fatty acid synthesis pathway is the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA. This reaction, catalyzed by acetyl-CoA carboxylase-α (ACCα), constitutes a key control point in the synthesis of long-chain fatty acids from carbohydrate (1, 2). Malonyl-CoA serves as a donor of C_2 units for the synthesis of palmitate catalyzed by fatty acid synthase. Malonyl-CoA is also a substrate of specific elongases involved in the chain elongation of fatty acids to very long-chain fatty acids (3). The essential role of ACCα in lipid biosynthesis has been confirmed by studies demonstrating that knockout of the ACCα gene disrupts embryonic development prior to day 7.5 (4).

In lipogenic tissues of avians and mammals, transcription of the ACCα gene is regulated by nutritional and hormonal factors. For example, ACCα transcription is low in livers of starved chicks, feeding a high-carbohydrate, low-fat diet stimulates an 11-fold increase in ACCα transcription (5). Diet-induced changes in ACCα transcription are mimicked in primary cultures of chick embryo hepatocytes by manipulating the concentrations of hormones and nutrients in the culture medium (6). Incubating chick embryo hepatocytes with the active form of thyroid hormone, 3,5',3-triiodothyronine (T3), stimulates a 5 to 7-fold increase in ACCα transcription. The mechanism by which T3 increases ACCα transcription involves multiple processes. First, T3 interacts with the nuclear T3 receptor (TR) bound to a T3 response element (T3RE) on the more downstream promoter (promoter 2) of the ACCα gene (7). This T3RE (-101 to -86 bp) is comprised of two hexameric half-sites arranged as direct repeats with 4 bp separating the

half-sites (DR-4 element). Second, T3 treatment increases the binding of TR•retinoid X receptor (RXR) heterodimers to the ACCα T3RE. The mechanism for this effect has not yet been defined. Third, T3 treatment increases the binding of sterol regulatory element-binding protein-1 (SREBP-1) to a sterol regulatory element (SRE) (-80 to -71 bp) located immediately downstream of the ACCα T3RE (8). SREBP-1 directly interacts with TR•RXR heterodimers and enhances the ability of this complex to activate ACCα transcription in the presence of T3 (9).

In our studies analyzing the regulation of ACCα transcription by T3, we observed that the ACCα T3RE not only bound protein complexes containing TR•RXR heterodimers but also bound protein complexes containing liver X receptor (LXR)•RXR heterodimers (7). LXRs are nuclear hormone receptors that are bound and activated by naturally occurring oxysterols (10, 11). Two isoforms of LXR, designated LXRα and LXRβ, have been identified in avians and mammals. LXRβ is expressed in a wide variety of tissues, whereas LXRα is selectively expressed in liver, adipose tissue, intestine, and macrophages (12, 13). LXRs play a key role in regulating cholesterol excretion by mediating the stimulatory effects of oxysterols on the transcription of genes involved in reverse cholesterol transport and bile acid synthesis. For example, naturally occurring oxysterols and synthetic, non steroidal LXR agonists activate the transcription of a battery of genes involved in cholesterol efflux (ABCA1, ABCG1, ABCG5, and ABCG8), cholesterol clearance (cholesterol ester transfer protein and apolipoprotein E), and cholesterol catabolism (cholesterol 7α-hydroxylase) (14-16). For each of these genes, regulation of transcription by LXR agonists is conferred by a LXR response element (LXRE) that binds LXR•RXR heterodimers. Because oxysterols are produced in

proportion to cellular cholesterol content, LXRs have been proposed to function as 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 elevated cholesterol levels in the blood and liver when fed a high-cholesterol diet (17, 18).

The ability of LXR agonists to activate genes involved in cholesterol excretion has led to an evaluation of the atheroprotective properties of these compounds in murine models of atherosclerosis. Oral administration of the synthetic LXR ligand/agonist, T0901317, to mice lacking the LDL receptor or apolipoprotein E causes an increase in blood HDL levels and reverses the formation of atherosclerotic lesions in the aorta (19, 20). These exciting findings are tempered by the observation that treatment with T0901317 also causes hypertriglyceridemia and the development of a fatty liver in rodents and chickens (21-24). These undesired effects of T0901317 are caused by alterations in the expression of enzymes comprising the fatty acid synthesis pathway. For example, T0-901317 treatment increases the hepatic expression of the mRNAs encoding ACCα, fatty acid synthase (FAS), ATP-citrate lyase (ATP-CL), and stearoyl-CoA desaturase-1 (SCD1) (23-25). The aim of the present study is to determine the mechanism by which T0-901317 regulates the expression of ACCα in avian liver. We show that T0-901317 acts directly on the liver to increase the expression of ACCα and that the extent of this effect is modulated by the presence of insulin and T3. We further show that T0-901317 increases ACCα transcription by activating LXR•RXR

heterodimers bound to the ACCα gene and that SREBP-1 interacts with LXR•RXR to enhance the stimulatory effects of T0-901317 on ACCα transcription.

EXPERIMENTAL PROCEDURES

Cell culture and analytical assays

Hepatocytes were isolated from livers of 19-day-old chick embryos as previously described (26). 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 legends of figures. The triacylglycerol concentration of the culture medium was measured using an enzymatic kit (Sigma).

Isolation of RNA and quantitation of mRNA levels

Medium was removed and RNA was extracted from hepatocytes by the guanidium thiocyanate/phenol/chloroform method (27). Total RNA (15 μg) was separated by size in 0.9% agarose, 0.7 M formaldehyde gels, and then transferred to a Nytran membrane (Schleicher & Schuell) using a Vacuum blotting apparatus (Pharmacia Biotechnology). The RNA was crosslinked to the membrane by UV and baked at 80°C for 30-60 min. RNA blots were hybridized with ^{32}P-labeled DNA probes labeled by random priming (28). Hybridization and washes were as described (29). Membranes were subjected to storage phosphor autoradiography. Hybridization signals were quantified using ImageQuant software (Molecular Dynamics). cDNAs for chicken

ACCα (5), FAS (30), SCD1 (31), SREBP-1 (32), ATP-CL (33), ABCA1 (23), LXR (34), and malic enzyme (35) have been described.

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]CAT have been previously described (7). 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 previously described in (9). p[ACC-108/-66]TKCAT, p[ACC-84/-66]TKCAT and pTKCAT constructs containing mutations in the -108 to -66 bp ACCα fragment are described in (9).

Transient transfection

Chick embryo hepatocytes were transfected as described in Zhang et al (7). 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 with or without T0-901317 (6 μM). At 66 h of incubation, chick embryo hepatocytes were harvested, and cell extracts were prepared as described in (36). CAT activity (37) and protein (38) were assayed by the indicated methods.

Western blot analysis

Nuclear extracts were prepared from chick embryo hepatocytes as described (9). The proteins of the nuclear extract were subjected to electrophoresis in 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Millipore) using an electroblotting apparatus (Bio-Rad Laboratories, Hercules, CA). Immunoblot analysis was carried using a mouse monoclonal antibody against SREBP-1 (IgG-2A4) (American Type Culture Collection, Manassas, VA). 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 Corporation) and signals for mature form of SREBP-1 were quantified using FluorChem V200 software.

Gel mobility shift analysis

Nuclear extracts were prepared from hepatocytes incubated with or without T0-901317 (9). A double-stranded oligonucleotide containing the ACCα LXRE/T3RE (-108 to -82 bp relative to the transcription initiation site of ACCα promoter 2) was labeled by filling in overhanging 5'-ends using Klenow fragment of *E. coli* DNA polymerase in the presence of [α-^{32}P] dCTP. Binding reactions were carried out as previously described (7). DNA and DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gels at 4 ºC in 50 mM Tris (pH 8.8) and 50 mM glycine. Following 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. (39). Chromatin immunoprecipitations were carried out with antibodies against acetyl-histone H3 (06-599) and acetyl-histone H4 (06-866) (Upstate Biotechnology). Precipitated DNA was analyzed in PCR reactions using Taq DNA polymerase (New England Biolabs) and primers specific for the ACCα and malic enzyme promoters. The cycling parameters were: 1 cycle of 95°C for 4 min, 30 cycles of 95°C for 1 min, 61°C for 1 min, 72°C for 1 min, and 1 cycle of 72°C for 10 min. The forward primer of the ACCα gene was 5'-TCCCCTCCGTCAGCAGCCAATGGG-3'; the reverse primer was 5'-ATCCCCGGTCCCGCCCTCGGCTCC-3'. The forward primer of the SCD1 gene was 5'-AGCGAACAGCAGATTGCGGCAG-3'; the reverse primer was 5'-TCTCGGCGTGCCAGAAGGGAGGT-3'. Amplified products were subjected to electrophoresis in 2% agarose gels and visualized by ethidium bromide staining.

Statistical methods

Data were subjected to analysis of variance, and statistical comparisons were made with Dunnett's test or Student's *t*-test. Statistical significance is defined as $P < 0.05$.

RESULTS

LXR activation increases the expression of ACCα in primary cultures of chick embryo hepatocytes

Oral administration of T0-901317 to chickens and rats causes a 2- to 3-fold increase in hepatic ACCα mRNA levels (23, 24). To investigate whether this phenomenon was due to a direct effect of T0-901317 n the liver, we determined whether T0-901317 regulated the expression of ACCα in primary cultures of chick embryo hepatocytes. Incubating hepatocytes with T0-901317 in the absence of other hormones for 24 h caused a 2.4-fold increase in the abundance of ACCα mRNA (Fig. 1). Treatment with the RXR agonist, 9-cis retinoic acid, had no effect on ACCα mRNA abundance in the absence or presence of T0-901317. Thus, LXR agonists but not RXR agonists regulate ACCα expression in hepatocytes in culture.

In chick embryo hepatocytes, insulin has no effect by itself but amplifies the increase in ACCα transcription caused by T3 (6). This observation prompted us to investigate whether insulin modified the effects of T0-901317 on ACCα expression. Incubating hepatocytes with T0-901317 stimulated a greater increase in ACCα mRNA abundance in the presence of insulin (3.5-fold) than in the absence of insulin (2.4-fold) (Fig. 1). Thus, as observed for T3 regulation of ACCα, insulin enhances the stimulatory effects of T0-901317 on ACCα expression.

In previous work, we showed that both TR and LXR bind the ACCα T3RE as heterodimers with RXR (7). This observation raised the possibility that a common cis-acting regulatory sequence is involved in mediating the effects of T0-901317 and T3 on ACCα transcription. As a first step in investigating this possibility, we determined the effects of T0-901317 on the expression of ACCα in the presence of T3. Incubating

hepatocytes with T3 and insulin caused a 4.6-fold increase in the abundance ACCα mRNA abundance (Fig. 1). Addition of T0-901317 in the presence of T3 and insulin caused a further increase in ACCα mRNA abundance, however the magnitude of this effect (39%) was substantially smaller than the effect of T0-901317 on ACCα expression in the presence of insulin alone (350%). Addition of T0-901317 in the presence of T3, insulin, and 9-cis retinoic acid had no effect on ACCα mRNA abundance. The non-additive effects of T0-901317 and T3 on ACCα mRNA abundance support the proposal that a common cis-acting sequence(s) mediates the actions of T0-901317 and T3 on ACCα transcription.

We also investigated the effects of T0-901317 on the expression of other lipogenic enzymes. In hepatocytes incubated in the absence and presence of insulin, addition of T0-901317 to the culture medium increased the abundance of mRNAs encoding FAS, SCD1, and ATP-CL (Fig. 1). T0-901317-induced expression of FAS, SCD1, and ATP-CL was higher in the presence of insulin than in the absence of insulin. In hepatocytes incubated with T3 and insulin, addition of T0-901317 had little or no effect on the abundance of mRNA encoding FAS, SCD1, and ATP-CL. Incubating hepatocytes with 9-cis retinoic acid in the absence or presence of T0-901317 had no effect on FAS, SCD1, and ATP-CL mRNA levels. Thus, regulation of expression of FAS, SCD1, and ATP-CL by agonists of LXR and RXR is similar to that of ACCα.

In contrast to ACCα, FAS, SCD1, and ATP-CL, the ability of T0-901317 to increase the expression of malic enzyme (ME) was substantially lower than that of T3. Treatment with T0-901317 and insulin caused a 2.9-fold increase in ME mRNA abundance, whereas treatment with T3 and insulin caused a 35-fold increase in ME

mRNA abundance (Fig. 1). These observations are consistent with previous work demonstrating that the major T3RE mediating T3 regulation of malic enzyme transcription lacks the ability to bind LXR•RXR heterodimers (7).

To determine whether the T0-901317-induced increase in lipogenic enzyme expression was associated with an elevation in triacylglycerol production, the triacylglycerol concentration in the culture medium was monitored in hepatocytes treated with or with or without T0-901317. The triacylglycerol concentration of the culture medium increased progressively during a 48 h incubation period (Fig. 2). The extent of the increase in triacylglycerol concentration was higher in cells treated with T0-901317 relative to cells not receiving T0-901317. An elevation in hepatic lipogenic enzyme expression and triacylglycerol secretion likely contributes to the hypertriglyceridemia observed in animals treated with T0-901317 (21, 22, 24).

Identification of a LXR response unit that mediates the effects of T0-901317 on ACCα transcription

Previous studies have shown that T3 regulation of ACCα transcription is mediated by a 23 bp region (-101 to -71 bp) in promoter 2 of the ACCα gene (7). This region contains a DR-4 element (-101 to -86 bp) that binds heterodimers comprised of TR•RXR and LXR•RXR and a SRE (-82 to -71 bp) that binds SREBP-1. To determine the role of these sequences and other sequences in ACCα gene in mediating the stimulatory effects of T0-901317 on ACCα transcription, transient transfection experiments were performed using reporter constructs containing portions of ACCα promoter 2 linked to the chloramphenicol acetyltransferase (CAT) gene. In chick embryo

hepatocytes transfected with a reporter construct containing 2054 bp of 5'-flanking DNA, T0-901317 caused a 2.9-fold increase in promoter activity (Fig. 3). 5'-Deletion of ACCα sequences to -391, -136, and -108 bp had no effect on T0-901317 responsiveness. Deletion of ACCα sequences containing the DR-4 element (-108 to -84 bp) abolished the stimulatory effect of T0-901317 on ACCα transcription. Further deletion to -41 bp had no effect on T0-901317 responsiveness. Mutation of the SRE (-80 to -71 bp) in the context of 108 bp of 5'-flanking DNA caused a 49% reduction in T0-901317 responsiveness. These results indicate that the DR-4 element (LXRE) is required for T0-901317 regulation of ACCα transcription and that the SRE enhances the ability of the LXRE to activate ACCα transcription in the presence of T0-901317.

To determine whether the functional interaction between the ACCα LXRE and SRE required the presence of additional cis-acting sequences, hepatocytes were transfected with constructs containing fragments of the ACCα gene linked to the minimal promoter of the herpes simplex virus thymidine kinase (TK) gene. The TK promoter alone was unresponsive to T0-901317 (Fig. 4). When a DNA fragment containing both the ACCα LXRE and ACCα SRE (-108 to -66 bp) was linked to the TK promoter, treatment with T0-901317 caused a 5.5-fold increase in promoter activity. Mutation of the ACCα SRE in the context of the ACCα -108 to -66 bp fragment caused a 49% decrease in T0-901317 responsiveness. When a DNA fragment containing the ACCα SRE alone (-84 to -66 bp) was appended to the TK promoter, T0-901317 treatment had no effect on promoter activity. These data demonstrate that the ACCα SRE can function alone to enhance T0-901317 regulation conferred by the ACCα LXRE. Thus, a region of the ACCα gene containing a LXRE and a SRE is responsible for mediating the effects of

T0-901317 on ACCα promoter 2 activity. We refer to this region as the ACCα liver X receptor response unit (LXRU).

LXR activation increases the abundance of mature SREBP-1 in chick embryo hepatocytes

In our studies analyzing the regulation of the ACCα gene by T3, we showed that T3 treatment increased the abundance of the mature, active form of SREBP-1 in chick embryo hepatocytes and that this effect contributed to the activation of ACCα transcription by T3 (8). This observation prompted us to ask whether T0-901317 regulated SREBP-1 levels in chick embryo hepatocyte cultures. The time course of the effects of T0-901317 on the abundance of mature SREBP-1 protein, SREBP-1 mRNA, and ACCα mRNA was determined in hepatocytes cultured in the presence of insulin. Treatment with T0-901317 for 6 h caused an 1.5-fold increase in mature SREBP-1 concentration (Fig. 5). A larger increase in mature SREBP-1 concentration (2.2-fold) was observed after 24 h of treatment with T0-901317. In contrast to the time course for mature SREBP-1, the T0-901317-induced stimulation of ACCα mRNA levels occurred at a later time point (between 24 and 48 of treatment). This observation is consistent with the proposal that alterations in SREBP-1 levels play a role in mediating the regulation of ACCα transcription by T0-901317. Treatment with T0-901317 increased in the abundance of SREBP-1 mRNA and this effect was maximal (1.9 to 2.1-fold) at or before 2 h of incubation. Thus, T0-901317-induced changes in mature SREBP-1 concentration appear to be mediated by a pretranslational mechanism.

LXR activation modulates the binding of nuclear receptor complexes to the ACCα LXRE/T3RE

In previous work, we showed that the ACCα LXRE/T3RE (-101 to -86 bp) bound four protein complexes in nuclear extracts prepared from chick embryo hepatocytes (7). 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 the absence of nuclear receptor agonists, complexes 1 and 2 were the predominant complexes that bound to the ACCα LXRE/T3RE. T3 treatment caused a marked reduction in the binding of complexes 1 and 2 and an increase in the binding of complexes 3 and 4. These findings have led us to propose that changes in the binding of nuclear receptor complexes to the ACCα T3RE/LXRE play a role in mediating the activation of ACCα transcription by T3. In the present study, we wanted to determine whether treatment with LXR agonists also modulated the binding of nuclear receptor complexes to the ACCα LXRE/T3RE. Gel mobility shift assays were performed using nuclear extracts from chick embryo hepatocytes treated with or without T0-901317. As reported previously, a DNA probe containing the ACCα LXRE/T3RE bound to complex 1, complex 2, and complex 4 in nuclear extracts from hepatocytes incubated in the absence of nuclear receptor agonists (Fig. 6). The binding activity of complex 1 and complex 2 was substantially greater than that of complex 4. Complex 3 binding activity was not detected in the absence of T0-901317 treatment. Incubating hepatocytes with T0-901317 had no effect on the binding activity of complexes 1 and 3. In contrast, T0-901317 treatment decreased the binding activity of complex 2 after 6, 12, and 24 h of treatment. Between 24 and 48 h of T0-901317 treatment, the binding activity of complex

2 increased to a level that was similar to that of cells not treated with T0-901317. Treatment with T0-901317 also regulated the binding activity of complex 4. Incubating hepatocytes with T0-901317 had no effect on complex 4 binding activity after 2, 6, and 12 h of treatment but increased complex 4 binding activity between 24 and 48h of treatment. These data indicate that T0-901317 has opposing effects on the binding of LXR•RXR and TR•RXR complexes to the ACCα LXRE/T3RE.

The effect of LXR activation on histone acetylation of the ACCα gene

The T0-901317-induced increase in ACCα transcription may be mediated in whole or in part by alterations in histone acetylation. Previous studies have shown that the activation of LXR•RXR heterodimers by LXR ligands/agonists triggers the recruitment of coactivator complexes containing histone acetyltransferase (HAT) activity (40, 41). Increased histone acetylation causes a chromatin decondensation that enhances the accessibility of the basal transcriptional machinery and other transcription factors to the target promoter. To investigate the role of histone acetylation in mediating the activation of ACCα transcription by T0-901317, ChIP experiments were performed in chick embryo hepatocytes incubated in the absence or presence of T0-901317. Hepatocytes were treated with 1% formaldehyde to cross-link DNA to associated proteins. Protein-DNA complexes were immunoprecipitated with an antibody against acetylated histone H3 or an antibody against acetylated histone H4. Immunoprecipitated DNA was analyzed by PCR using primers that flanked the ACCα LXRU. In hepatocytes incubated in the absence of T0-901317, acetylation of histone H3 and histone H4 was detected at the ACCα LXRU. Addition of T0-901317 to the culture medium stimulated a

rapid increase (≤ 2 h) in the acetylation of histone H3 and H4 (Fig. 7, Table 1). Histone acetylation remained elevated for 6 h of T0-901317 treatment and then declined between 6 and 24 h of T0-901317 treatment. These results support the proposal that an elevation in histone acetylation plays a role in mediating the effects of T0-901317 on ACCα transcription.

We also used ChIP to assess the effects of T0-901317 treatment on histone acetylation in a uncharacterized region of the SCD1 promoter using a primer set that amplified SCD1 sequences between -369 and -127 bp. In contrast to the data for the ACCα gene, treatment with T0-901317 had no effect on histone acetylation at this region of the SCD1 gene (Fig. 7). This observation indicates that the effects of T0-901317 on histone acetylation are sequence specific.

DISCUSSION

In previous work analyzing the effects of T3 on ACCα transcription in avian hepatocytes, we identified a T3RE that conferred T3 regulation on ACCα promoter 2 (7). Interestingly, this T3RE not only bound protein complexes containing TR•RXR heterodimers but also bound protein complexes containing LXR•RXR heterodimers. In the present report, we provide functional evidence that LXR•RXR heterodimers regulate ACCα transcription. A specific ligand/agonist of LXR (T0-901317) activates ACCα transcription and this effect is mediated by the LXRE/T3RE in ACCα promoter 2. We also demonstrate that SREBP-1 is an accessory factor that enhances the ability of LXR•RXR to increase ACCα transcription in the presence of T0-901317 and that LXR

activation by T0-901317 increases the concentration of mature, active form of SREBP-1 in chick embryo hepatocytes.

How does SREBP-1 enhance the stimulatory effects of T0-901317 on ACCα transcription? One possibility is that SREBP-1 facilitates the recruitment of coactivators to T0-901317-bound LXR•RXR complexes. LXRα, LXRβ, and SREBP-1 interact with several coactivator proteins including CREB-binding protein (CBP) and the TRAP/ARC/DRIP complex (40-45). We postulate that the presence of SREBP-1 on ACCα promoter 2 provides additional coactivator interaction sites that stabilize the binding of CBP, TRAP/ARC/DRIP, and other coactivators to T0-901317-bound LXR•RXR. In support of this model, SREBP-1 and nuclear hormone receptors interact with separate peptide sequences on CBP and separate subunits of the TRAP/ARC/DRIP complex (46-49).

SREBP-1 also enhances the ability of TR•RXR to activate ACCα transcription in the presence of T3 (9). In analyzing the mechanism mediating this effect, we showed that SREBP-1 physically interacted with TR and that binding of T3 to TR enhanced this interaction. We postulated that SREBP-1•SREBP-1 homodimers formed a tetrameric complex with TR•RXR heterodimers and that tetrameric complex formation enhanced the recruitment of coactivators to ACCα promoter 2. We also showed in these studies that LXR•RXR heterodimers do not physically interact with SREBP-1. Thus, in contrast to the mechanism by which SREBP-1 enhances TR•RXR activity, direct interactions between SREBP-1 and LXR•RXR do not play a role in mediating the stimulatory effects of SREBP-1 on LXR•RXR activity.

The mechanism by which LXR agonists regulate transcription has been analyzed for other lipogenic genes. In human hepatoma cells, T0-901317 activation of FAS transcription is mediated by a single LXRE in the FAS promoter (50). In primary rat hepatocyte cultures, the T0-901317-induced increase in SREBP-1c transcription is mediated by two LXREs in the SREBP-1c promoter (51, 52). Both the FAS promoter and the SREBP-1c promoter contain one or more SREs that are located approximately 110 to 540 bp downstream of the LXRE(s). In cells that express physiological levels of LXR, these SREs enhance basal transcription but have little or no effect on the regulation of transcription by T0-901317. In contrast to these findings, the SRE in ACCα promoter 2 enhances T0-901317 regulation of transcription but has no effect on basal transcription (Figs. 3 and 4). These gene-specific differences in SRE activity may be due to variations in the proximity of the SRE relative to the LXRE and other cis-acting regulatory sequences. For example, the close association of the SRE with the LXRE/T3RE in ACCα promoter 2 may facilitate interactions between LXR•RXR and SREBP-1 and the ability of SREBP-1 to enhance T0-901317 responsiveness, whereas the wide separation of the SREs and LXREs in the FAS and SREBP-1c promoters may impede interactions between LXR•RXR and SREBP-1 and the ability of SREBP-1 to enhance T0-901317 responsiveness. Previous studies have shown that the SREs in the FAS and SREBP-1c promoters are closely linked to a binding site for nuclear factor-Y (NF-Y) and that SREBP-1 activity is dependent on interactions between SREBP-1 and NF-Y (51-54). These interactions enhance the ability of SREBP-1 to stimulate basal transcription. In contrast, the SRE in ACCα promoter 2 is not closely associated with binding sites for

NF-Y. The lack of interaction of SREBP-1 with NF-Y on ACCα promoter 2 may explain why the ACCα SRE is not effective in modulating basal transcription.

Another finding of the present study is that LXR plays a permissive role in mediating the actions of insulin on ACCα transcription in chick embryo hepatocytes. Insulin stimulates ACCα expression in the presence of T0-901317 but has no effect on ACCα expression in the absence of T0-901317 (Fig. 1). In rat hepatocytes, insulin enhances the ability of T0-901317 to increase mature SREBP-1 concentration due to a stimulatory effect of insulin on the processing of precursor SREBP-1 to mature SREBP-1 (55). We have confirmed this finding in chick embryo hepatocytes (data not shown). Because SREBP-1 enhances LXR activation of ACCα transcription in chick embryo hepatocytes, we postulate that alterations in mature SREBP-1 abundance mediate the stimulatory effects of insulin on ACCα transcription. In support of this hypothesis, insulin does not increase the expression of ABCA1 in the presence of T0-901317 (Fig. 1). Previous work has shown that the ABCA1 gene is not a target of SREBP-1 (56).

Previous studies performed in rat hepatocytes indicate that insulin induces ACCα expression by increasing the activity of LXR. For example, Tobin et al. (57) have shown that insulin stimulates a 10-fold increase in the expression of LXRα mRNA and that ablation of the LXRα gene abolishes the stimulatory effect of insulin on ACCα expression. Other laboratories have shown that insulin increases the transcription of SREBP-1c and that this effect is mediated by two LXR•RXR binding sites in the SREBP-1c promoter (51, 52). SREBP-1c is a key factor mediating the effects of insulin on ACCα transcription in rat hepatocytes (25, 58). These findings contrast with our data indicating that LXR plays a permissive role in mediating the effects of insulin on ACCα

transcription in chick embryo hepatocytes. The reason for the differences between avians and rodents in the mechanism by which insulin regulates ACCα transcription is not clear. They may reflect subtle class-dependent differences in the role of insulin in the control of lipogenesis and/or other metabolic processes in liver.

Results from DNA binding studies suggest that T0-901317 treatment causes a transient reduction in the binding of a complex containing LXR•RXR (complex 2) (Fig. 6). This change in the ACCα LXRE/T3RE protein binding profile would limit the activation of ACCα transcription by T0-901317. T0-901317 treatment also causes an increase in the binding of a complex containing TR•RXR (complex 4). The latter phenomenon would also suppress the stimulatory effects of T0-901317 on ACCα transcription because unliganded TR•RXR functions as a repressor of gene transcription (59). Evidence that unliganded TR•RXR is associated with a subset of ACCα promoters during LXR activation is provided by the observation that T3 causes an increase in ACCα mRNA abundance in the presence of T0-901317 (Fig. 1). We postulate that T0-901317-induced changes in the binding of nuclear receptor complexes to the ACCα LXRE/T3RE constitutes a mechanism to prevent the over stimulation of ACCα transcription by the LXR signaling pathway. The mechanism by which T0-901317 alters the binding of nuclear receptor complexes to the ACCα LXRE/T3RE is presently unclear but does not involve changes in the expression of LXRα mRNA (Fig. 1), TRα mRNA, and TRβ mRNA (data not shown).

The ACCα LXRE/T3RE enhances ACCα transcription both in the absence and presence T0-901317 and T3 with a greater activation observed in the presence of T0-901317 and T3 (Figs. 3 and 4) (7). We previously hypothesized that the enhancer

activity of the ACCα LXRE/T3RE in the absence of T0-901317 and T3 was mediated by LXR•RXR heterodimers, as the primary protein complexes that bind the ACCα LXRE/T3RE in the absence of T0-901317 and T3 contain LXR•RXR heterodimers (7). Recent studies have shown that unliganded form of LXR•RXR represses gene transcription due to its ability to interact with corepressor proteins and that the addition of LXR ligand increases gene transcription by causing the release of corepressors and the recruitment of coactivators to LXR•RXR (60). In view of these observations, we further hypothesize that LXR•RXR complexes associated with the ACCα LXRE/T3RE in the absence of T0-901317 and T3 are bound by endogenous LXR and/or RXR ligands. Several lines of evidence support this proposal. First, treatment of chick embryo hepatocytes with naturally occurring agonists of LXR [22-(R)-hydroxy cholesterol] and RXR (9-cis retinoic acid) has little or no effect on ACCα mRNA abundance (Fig. 1 and data not shown). Second, the ACCα LXRE/T3RE is not associated with the corepressor protein, nuclear receptor corepressor (NCoR), in the absence of T0-901317 and T3 (39). Last, the ACCα LXRE/T3RE is associated with acetylated histone H3 and acetylated histone H4 in the absence of T0-901317 and T3 (Fig. 7). The ability of T0-901317 to increase the transcriptional activity and histone acetylation of the ACCα LXRE/T3RE is likely due to the fact that this synthetic agonist is more effective than endogenous LXR agonists in stimulating the recruitment of HAT-containing coactivators to LXR•RXR (40).

In summary, we show that T0-901317 activates of ACCα transcription by increasing the activity of LXR•RXR and SREBP-1 and the acetylation of histone H3 and histone H4 on ACCα promoter 2. The identification of small molecules that selectively

inhibit of one or more of these processes will enhance the utility of T0-901317 in the treatment of atherosclerosis.

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FIGURE LEGENDS

Figure 1. The LXR agonist, T0-901317, increases the expression of ACCα and other lipogenic enzymes in primary cultures of chick embryo hepatocytes. Hepatocytes were isolated as described under Material and Methods and incubated in serum-free Waymouth's medium. At 18 h of incubation, the medium was changed to one of the same composition supplemented with or without T0-901317 (10 μM) in the absence or presence of insulin (50 nM), insulin and 9-cis retinoic acid (9-cis RA) (100 nM), insulin and T3 (1.5 μM), or insulin, T3 and 9-cis-RA. After 28 h of treatment, total RNA was isolated and the abundance of mRNA encoding acetyl-CoA carboxylase α (ACCα), fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD1), ATP-citrate lyase (ATP-CL), malic enzyme (ME), ATP-binding cassette transporter A1 (ABCA1), and liver X receptor α (LXRα) was measured by Northern analysis. Levels of mRNA in cells treated without T0-901317, 9-cis-RA, insulin, and T3 were set at 1, and the other values were adjusted proportionately. Values are the means ± SEM of four experiments. Hybridization signals from a representative experiment are shown for each mRNA. Ribosomal subunits (27S and 18S) stained with ethidium bromide are shown as controls for RNA loading. [a]Mean is significantly ($P < 0.05$) different from that of cells treated without T0-901317, 9-cis-RA, insulin, and T3. [b]Mean is significantly ($P < 0.05$) different from that of cells

treated with insulin. [c]Mean is significantly ($P < 0.05$) different from that of cells treated with insulin and T3.

Figure 2. Effect of T0-901317 on the accumulation of triacylglycerols in the culture medium of chick embryo hepatocytes. Hepatocytes were plated on 90 mm petri dishes (1 x 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. After 0, 24, 36, and 48 h of incubation with T0-901317, the concentration of triacylglycerol in the culture medium was measured using a spectrophotometric assay. Data are the means ± SEM of three experiments. *Mean is significantly ($P < 0.05$) different from that of cells incubated with insulin for the same time period.

Figure 3. Effect of mutations of the 5'-flanking region of ACCα promoter 2 on transcriptional activity in the absence and presence of T0-901317. Chick embryo hepatocytes were transiently transfected with p[ACC-2054/+274]CAT or equimolar amounts of other plasmids as described under Materials and Methods. After transfection, cells were treated with or without T0-901317 for 48 h. Cells were then harvested, extracts prepared, and CAT assays performed. Left: The constructs used in these experiments. 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 was +274 bp. The location of the LXR response element (LXRE) (-101 to -86 bp) and the sterol regulatory element (SRE) (-80 to -71 bp) is indicated by vertical lines. A block mutation of the SRE is indicated by a X through the vertical line. Right: 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. The fold stimulation by T0-901317 was calculated by dividing the CAT activity for hepatocytes treated with T0-901317 (+T0-901317) by that for hepatocytes not treated with T0-901317 (-T0-901317). The fold responses were calculated for individual experiments and then averaged. The results are the means ± SEM of six experiments. [a]The fold stimulation by T0-901317 is significantly ($P < 0.05$) lower than that of p[ACC-108/+274]CAT. [b]The fold stimulation by T0-901317 is significantly ($P < 0.05$) lower than that of p[ACC-108/+274]CAT containing a block mutation of the SRE.

Figure 4. The ACCα SRE alone enhances the transcriptional activity of the ACCα LXRE in the presence of T0-901317. Fragments of the ACCα gene containing the LXRE and/or SRE were linked to the minimal TK promoter in TKCAT. Chick embryo hepatocytes were transiently transfected with these constructs and treated with or without T0-901317 as described in the legend of Fig. 3 and under Materials and Methods. Left: The constructs used in these experiments. Numbers indicate the 5' and 3' boundaries of ACCα DNA relative to the transcription initiation site of promoter 2. A block mutation of the SRE is indicated by a X across the box representing the SRE. Right: CAT activity in hepatocytes transfected with TKCAT and treated without T0-901317 was set at 1, and the other activities were adjusted proportionately. The fold stimulation by T0-901317 was calculated as described in the legend to Fig. 3. The results are the means ± SEM of five experiments. [a]The fold stimulation by T0-901317 for p[ACC-108/-66]TKCAT is significantly ($p < 0.05$) higher than any other construct.

Figure 5. T0-901317 increases the concentration of mature SREBP-1 in chick embryo hepatocyte cultures. 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. T0-901317 was added at this time. After 2, 6, 12, 24, and 48 h of T0-901317 treatment, cellular extracts or total RNA were prepared as described under Materials and Methods. A: The abundance of mature SREBP-1 in nuclear extracts was measured by Western analyses. The abundance of SREBP-1 mRNA and ACCα mRNA was measured Northern analysis.. These data are from a representative experiment. B: Signals for mature SREBP-1 protein from Western analyses and SREBP-1 mRNA and ACCα mRNA from Northern analyses were quantitated. Levels of mature SREBP-1 protein, SREBP-1 mRNA, and ACCα mRNA in hepatocytes treated with T0-901317 for 0 h were set at 1. Values are the means ± SEM of four experiments. Asterisks indicate that the mean is significantly ($P < 0.05$) different from that of cells incubated with T0-901317 for 0 h or without T0-901317 for 24 or 48 h.

Figure 6. Effect of T0-901317 treatment on the binding of nuclear receptor complexes to the ACCα LXRE/T3RE in chick embryo hepatocytes. Eighteen hours after being placed in culture, chick embryo hepatocytes were incubated in Waymouth's medium containing insulin with or without T0-901317 for the indicated time periods. Cells were harvested and nuclear extracts were prepared as described in Methods and Materials 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·RXR heterodimers, whereas complex 4 contains TR·RXR heterodimers (5). 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 T0-901317 for 0 h were set at 1, and the other activities were adjusted proportionately. Values are the means ± SEM of four experiments. Asterisks indicate that the mean is significantly ($P < 0.05$) different from that of cells incubated with T0-901317 for 0 h or without T0-901317 for 24 or 48 h.

Figure 7. Treatment of chick embryo hepatocyte cultures with T0-901317 causes a transient increase in histone acetylation at the ACCα liver X receptor 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 of the same composition. T0-901317 was added at this time. After 2, 6, 24, and 48 h of T0-901317 treatment, the association of acetylated histone H3 and acetylated histone H4 with ACCα and SCD1 genomic sequences was measured. ChIP assays were performed as described in Materials and Methods. Immunoprecipitates were analyzed by PCR using primers that flanked the liver X receptor response unit (LXRU) of ACCα promoter 2 and an uncharacterized region of the SCD1 promoter. The region of the ACCα gene and SCD1 gene that was amplified by PCR is indicated at the top of the figure. Chromatin samples that were processed in parallel without the application of primary antibody served as controls. The input lanes show the results of PCR reactions using chromatin

samples taken before the immunoprecipitation step. Results are representative of three independent experiments

TABLE 1.

Effect of T0-901317 treatment on the acetylation of histones at the acetyl-CoA carboxylase-α LXR response unit

	T0-901317 Treatment (h)				
	0	2	6	24	48
Ac-H3	1.0	1.4 ± 0.1*	1.8 ± 0.1*	1.1 ± 0.2	1.1 ± 0.1
Ac-H4	1.0	1.7 ± 0.1*	2.2 ± 0.2*	1.0 ± 0.1	1.1 ± 0.1

Signals from ChIP assays using the indicated antibodies were quantitated. The data are expressed as the fold difference relative to samples from hepatocytes treated with T0-901317 for 0 h. Values are the means ± SEM of three experiments. Results from a representative experiment are shown in Fig. 7. *Significantly different ($P < 0.05$) from cells treated with T0-901317 for 0 h.

FIGURES

Figure 1

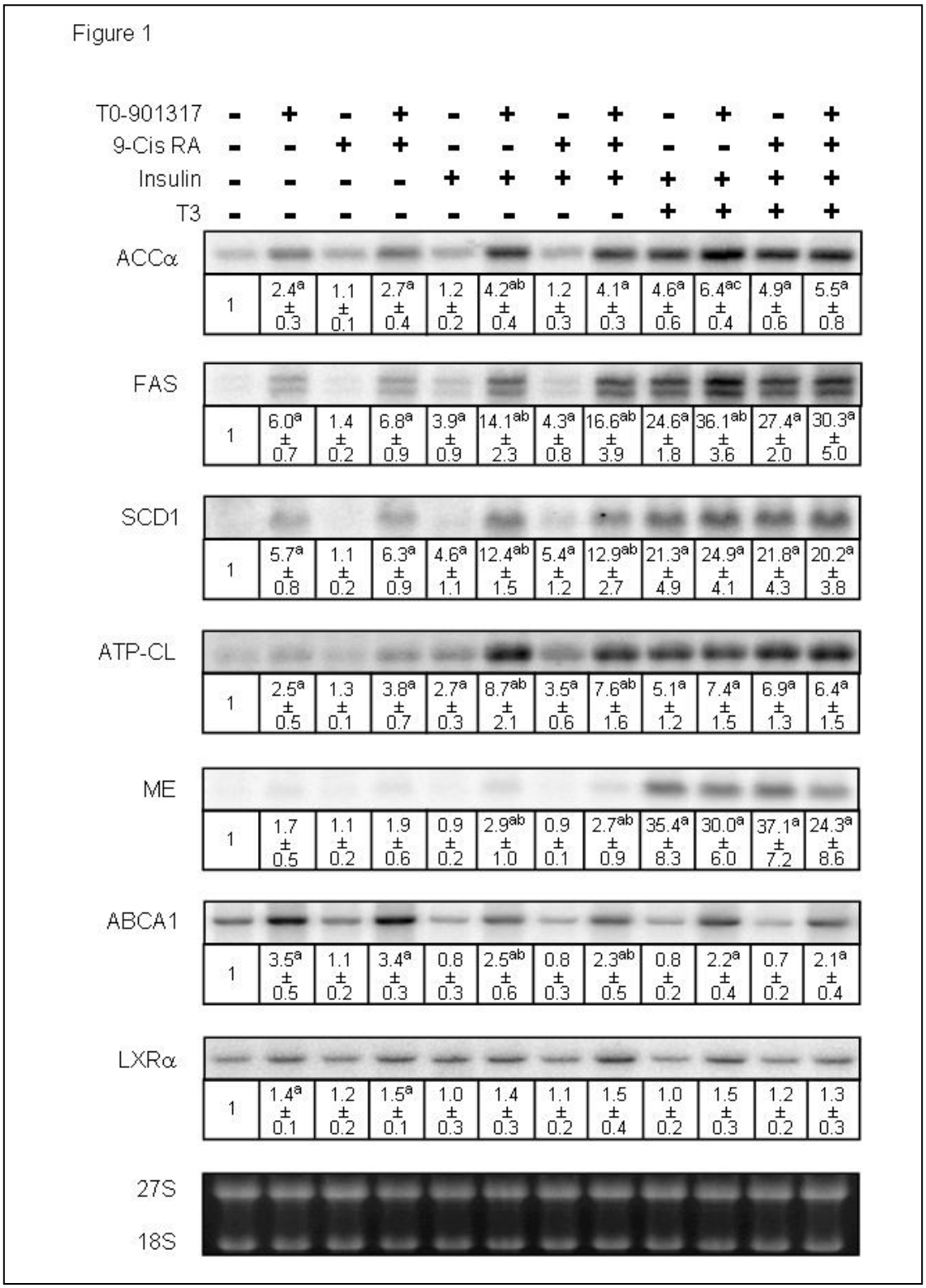


Figure 2

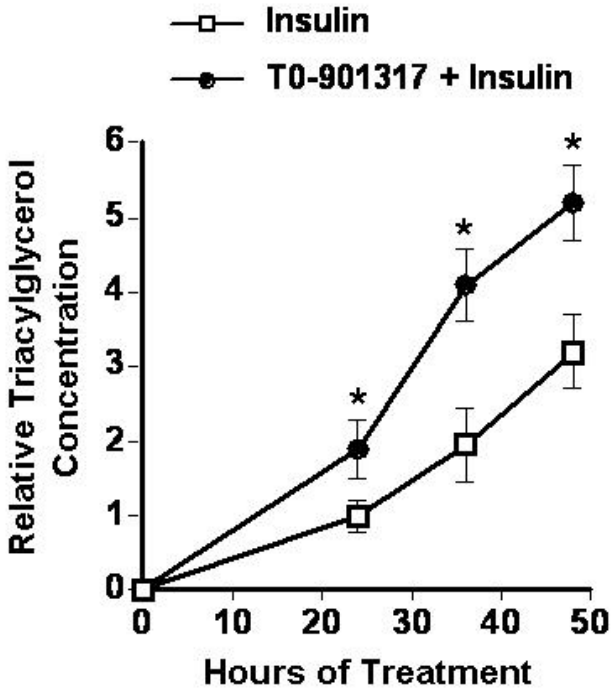

Insulin
T0-901317 + Insulin
Relative Triacylglycerol Concentration
0
1
2
3
4
5
6
*
*
*
0
10
20
30
40
50
Hours of Treatment

Figure 3

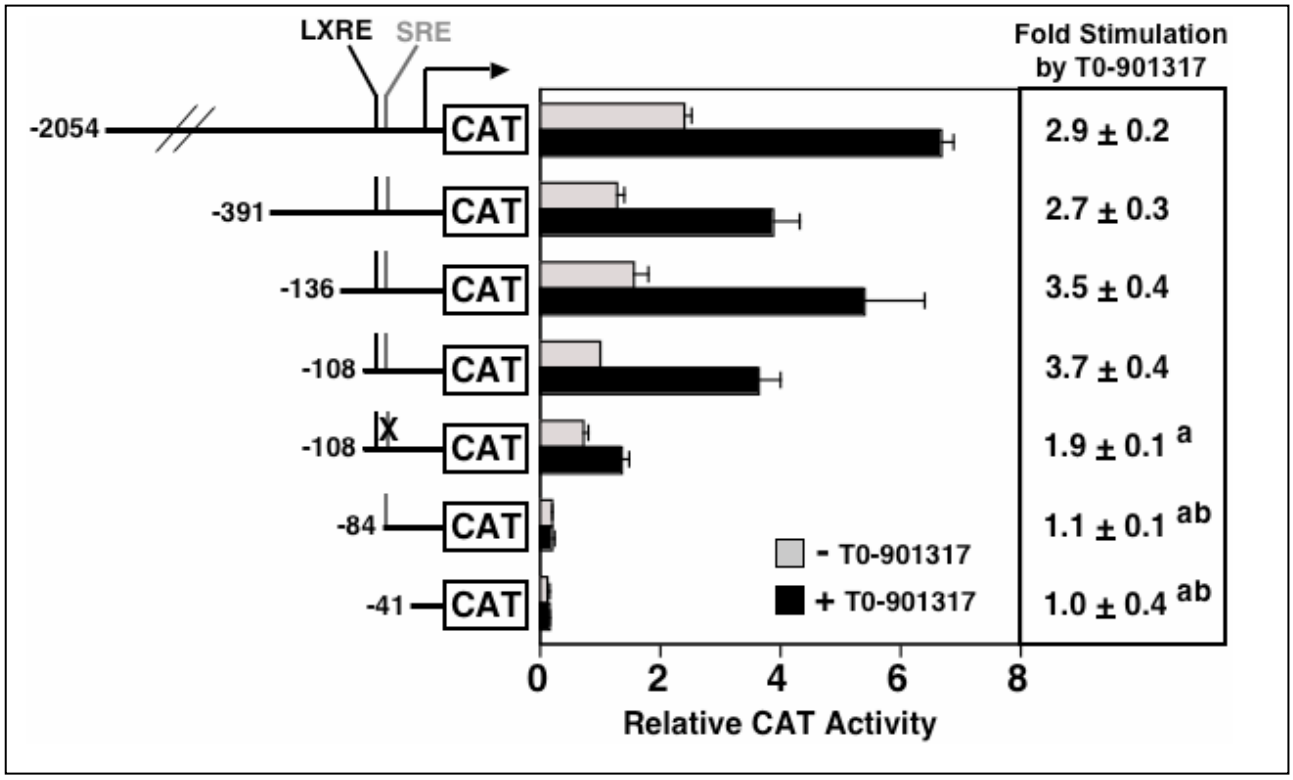

LXRE
SRE
Fold Stimulation by T0-901317
-2054
CAT
2.9 ± 0.2
-391
CAT
2.7 ± 0.3
-136
CAT
3.5 ± 0.4
-108
CAT
3.7 ± 0.4
-108
CAT
1.9 ± 0.1 a
-84
CAT
1.1 ± 0.1 ab
-41
CAT
1.0 ± 0.4 ab
- T0-901317
+ T0-901317
0
2
4
6
8
Relative CAT Activity

Figure 4

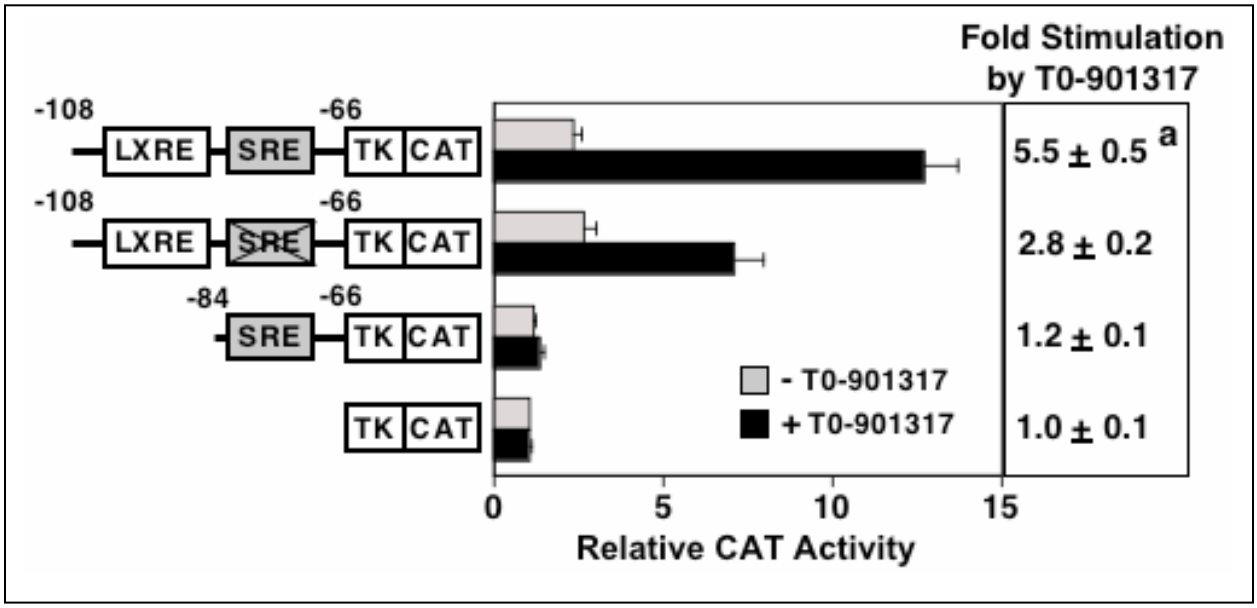

Fold Stimulation by T0-901317
-108
-66
LXRE
SRE
TK
CAT
5.5 ± 0.5 a
-108
-66
LXRE
SRE
TK
CAT
2.8 ± 0.2
-84
-66
SRE
TK
CAT
1.2 ± 0.1
TK
CAT
1.0 ± 0.1
- T0-901317
+ T0-901317
0
5
10
15
Relative CAT Activity

Figure 5

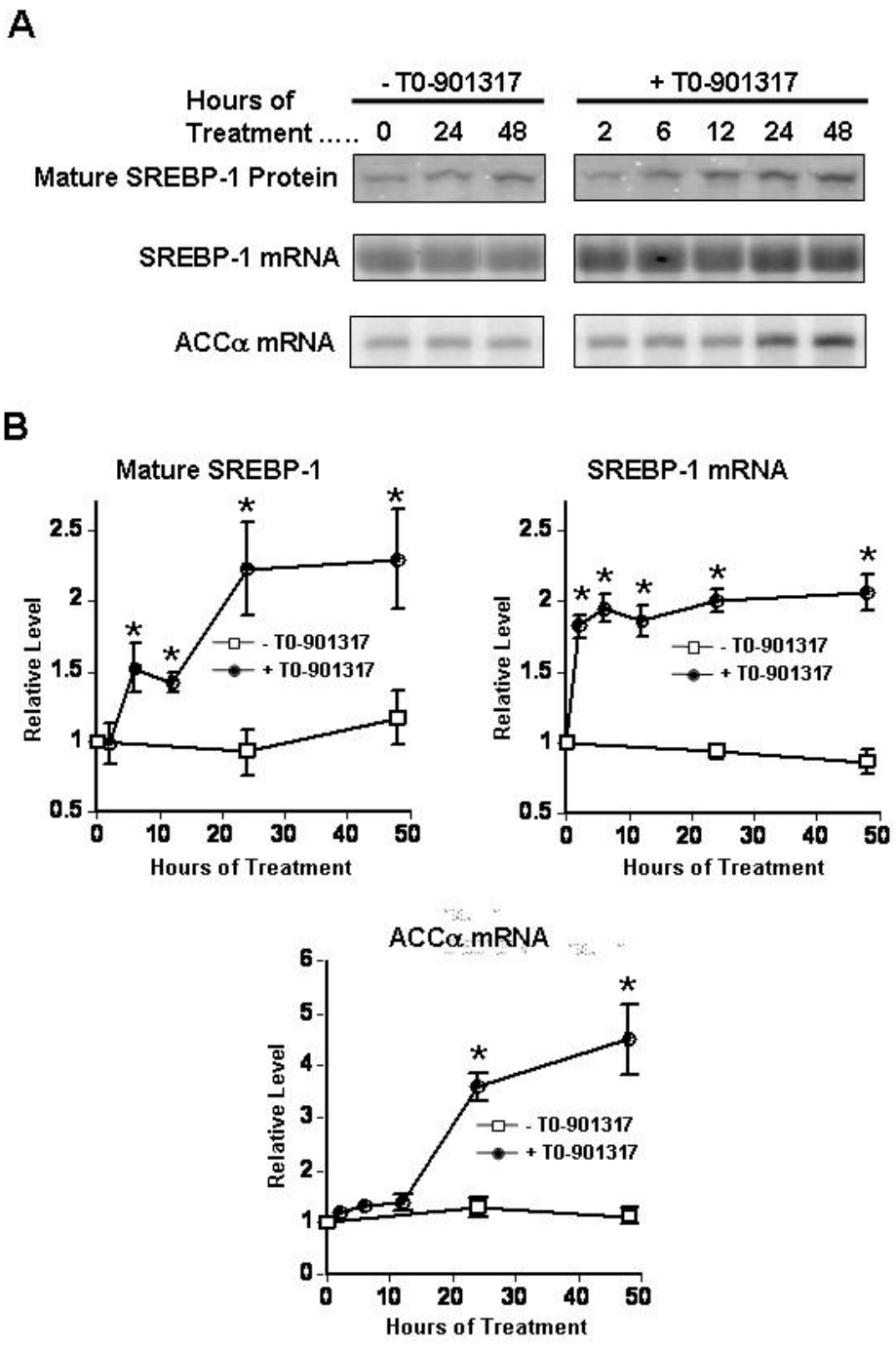

A
Hours of Treatment .....
- T0-901317
+ T0-901317
0 24 48
2 6 12 24 48
Mature SREBP-1 Protein
SREBP-1 mRNA
ACCα mRNA
B
Mature SREBP-1
SREBP-1 mRNA
ACCα mRNA
Relative Level
Hours of Treatment
- T0-901317
+ T0-901317

Figure 6

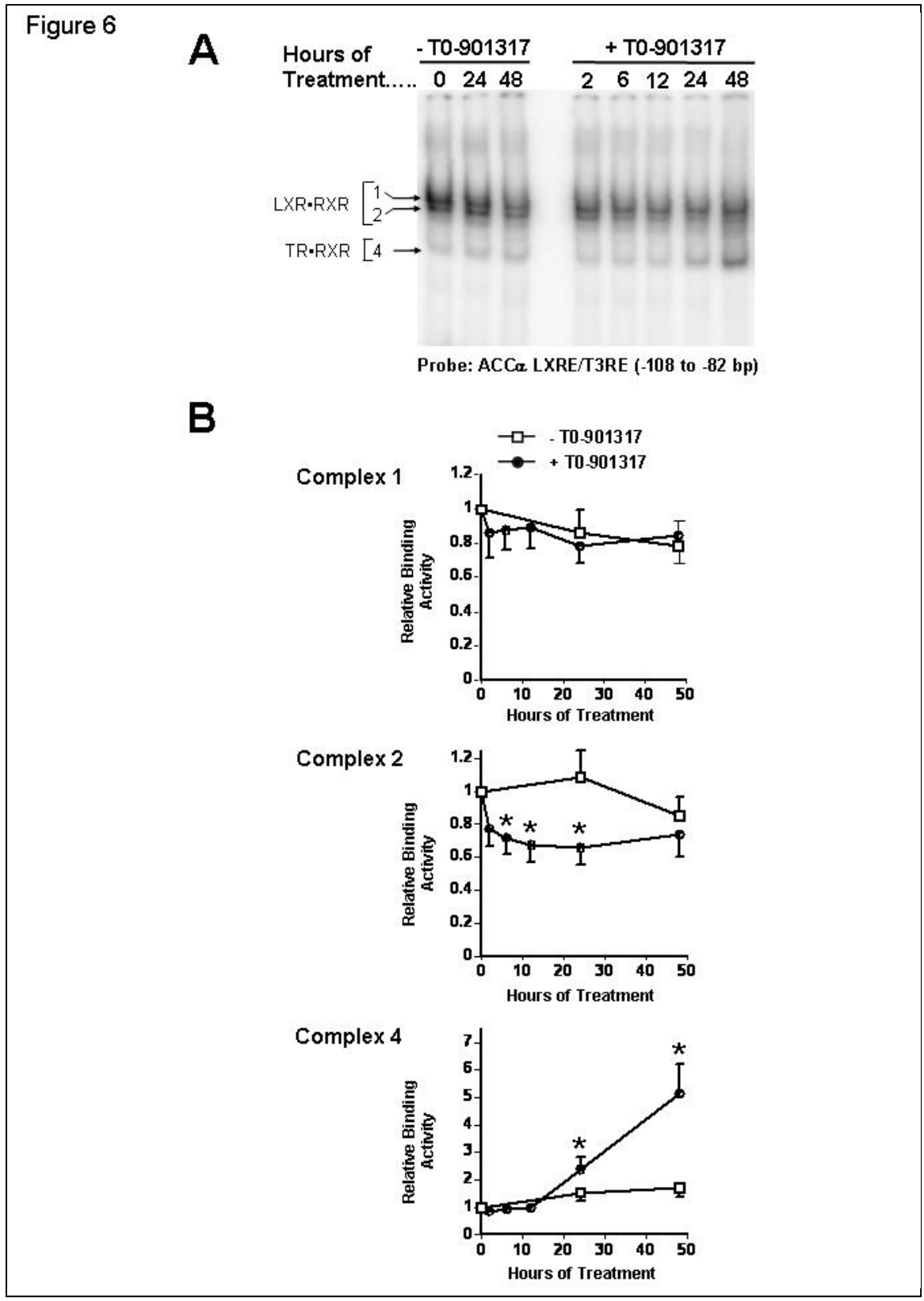
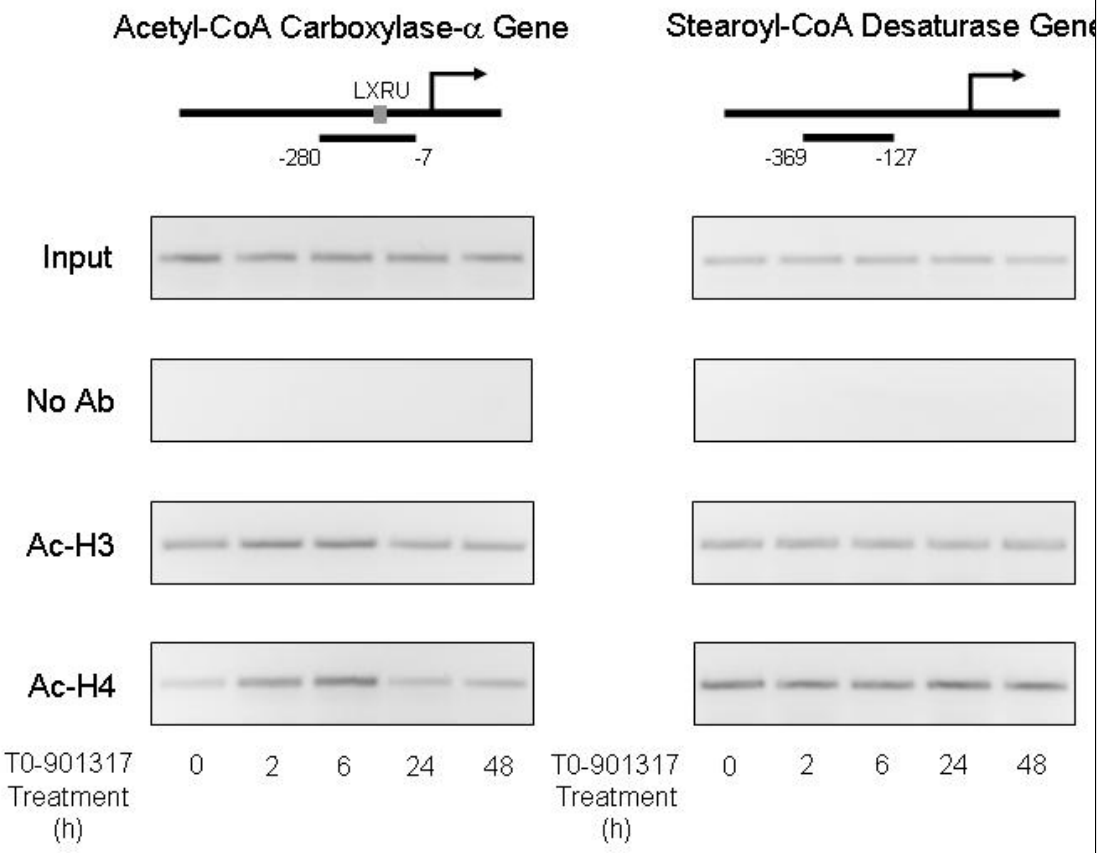

A
Hours of Treatment.....
- T0-901317
0 24 48
+ T0-901317
2 6 12 24 48
LXR•RXR
1
2
TR•RXR
4
Probe: ACCα LXRE/T3RE (-108 to -82 bp)
B
- T0-901317
+ T0-901317
Complex 1
Relative Binding Activity
Hours of Treatment
Complex 2
Relative Binding Activity
Hours of Treatment
Complex 4
Relative Binding Activity
Hours of Treatment

Figure 7


Acetyl-CoA Carboxylase-α Gene
Stearoyl-CoA Desaturase Gene
LXRU
-280
-7
-369
-127
Input
No Ab
Ac-H3
Ac-H4
T0-901317 Treatment (h)
0
2
6
24
48
T0-901317 Treatment (h)
0
2
6
24
48

Chapter 3

Chenodeoxycholic Acid Suppresses the Activation of Acetyl-CoA Carboxylase-α Transcription by Liver X Receptor Agonists

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INTRODUCTION

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 in 1995 as orphan members of the nuclear receptor superfamily (61, 70). Two isoforms exist with different expression patterns. LXRα is expressed at high levels in liver, adipose tissue and macrophages, whereas LXRβ is expressed ubiquitously (64, 70). The majority of the LXR proteins are localized in the nucleus and require heterodimerization with the retinoid X receptor (RXR) in order to bind DNA and regulate transcription (70).

The screening of organic tissue extracts using a cell-based reporter assay led to the breakthrough discovery that oxysterols were the endogenous ligands that bound and activated LXRα and LXRβ (35, 40). The most potent endogenous LXR ligands are 24(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, and 24(S) epoxycholesterol. LXR agonists activate a battery of genes involved in cholesterol efflux (ABCA1, ABCG1, ABCG5, and ABCG8), cholesterol clearance (cholesterol ester transfer protein and apolipoprotein E), and cholesterol catabolism (cholesterol 7α-hydroxylase) (reviewed in (18, 57). Because oxysterols are produced in proportion to cellular cholesterol content, LXRs have been proposed to function as 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 elevated cholesterol levels in the blood and liver when fed a high-cholesterol diet (2, 50).

The effects of LXR agonists on gene transcription are mediated by cis-acting sequences that are comprised of hexameric half-sites arranged as direct repeats with a 4 bp spacer separating the half-sites (70). These sequences, termed LXR response elements (LXREs), bind heterodimers comprised of LXR and RXR. Ligand-bound nuclear receptors activate transcription by recruiting auxiliary transcriptional regulatory proteins referred to as coactivators (71). Examples of coactivators of LXR include steroid receptor coactivator-1 (69) and PGC-1α (48). Coactivators facilitate the ability of LXR

to activate transcription by directly interacting with the basal transcriptional machinery, by modulating interactions between LXR and the basal transcriptional machinery, and by modifying chromatin structure.

We and other investigators have shown that LXR agonists also activate the transcription of genes involved in triglyceride synthesis including ATP-citrate lyase, fatty acid synthase, stearoyl-CoA desaturase, and ACCα (36, 60). In the case of ACCα, we have shown that this effect is mediated by both a direct mechanism involving the activation of LXR/RXR heterodimers on the ACCα gene and by an indirect mechanism involving the increased expression of SREBP-1. SREBP-1 binds a site adjacent to the ACCα LXRE and enhances the ability of LXR/RXR to activate ACCα transcription. LXR agonists increase the expression of SREBP-1 by activating a LXRE on the SREBP-1 gene (56).

HMG-CoA reductase inhibitors (statins) are currently the first-line agents to treat and prevent atherosclerosis in humans. Unfortunately, statins are not effective in reducing circulating cholesterol and LDL levels in a significant fraction of patients with dyslipidemia (11). 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. Accordingly, several laboratories have identified non-steroidal, synthetic compounds that are more effective than endogenous oxysterols in stimulating LXR activity (15, 60). The atheroprotective properties of two of these synthetic LXR agonists, designated T0901317 (*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 T0901317 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 (36, 46, 65). These exciting findings are tempered by the observation that treatment with T0901317 or GW3965 also causes hypertriglyceridemia and the development of a fatty liver (14, 26, 60). These undesired effects of T0901317 and GW3965 are due to an increase in hepatic fatty acid synthesis. One approach to overcome this problem is to

activate another signaling pathway that selectively inhibits the effects of LXR agonists on lipogenic gene transcription without altering their ability to stimulate genes involved in reverse cholesterol transport.

In the present study, we show that, in primary hepatocyte cultures, chenodeoxycholic acid (CDCA) inhibits the stimulatory effects of T0-901317 on the expression of ACCα and other lipogenic enzymes and enhances the stimulatory effects of T0-901317 on expression of ABCA1, a key gene controlling reverse cholesterol transport. We also demonstrate that CDCA decreases ACCα transcription by inhibiting the activity of LXR and SREBP-1 and that extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK) are components of the signaling pathway(s) mediating this response.

EXPERIMENTAL PROCEDURES

Preparation of isolated hepatocytes

Hepatocytes were isolated from livers of 19-day-old chick embryos as previously described (Chapter 2). 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. The triacylglycerol concentration of the culture medium was measured using an enzymatic kit (Sigma).

Isolation of RNA and quantitation of mRNA levels

Medium was removed and RNA was extracted from hepatocytes by the guanidinium thiocyanate/phenol/chloroform method as described (Chapter 2). Total RNA (20 μg) was separated by size in 1% agarose, 0.7 M formaldehyde gels, and then transferred to a Nytran membrane (Schleicher & Schuell) using a Vacuum blotting apparatus (Pharmacia Biotechnology). The RNA was crosslinked to the membrane by UV and baked at 80°C for 15 min. RNA blots were hybridized with ^{32}P-labeled DNA probes labeled by random priming. Hybridization and washes were as described. Membranes were subjected to storage phosphor autoradiography. Hybridization signals

were quantified using ImageQuaNT software (Molecular Dynamics). cDNAs for chicken ACCα, FAS, SCD, SREBP-1, ABCA1 have been described (Chapter 2).

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]CAT have been previously described (Chapter 2). An ACCα promoter construct containing a mutation of the SRE-1 between -79 and -72 bp in the context of p[ACC-108/+274]CAT has been previously described. p[ACC-108/-66]TKCAT, p[ACC-84/-66]TKCAT and pTKCAT constructs containing mutations in the -108 to -66 bp ACCα fragment are described (Chapter 2).

Transient transfection

Chick embryo hepatocytes were transfected as described (Chapter 2). 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 with or without T0-901317 (6 μM). At 66 h of incubation, chick embryo hepatocytes were harvested, and cell extracts were prepared as described in (4). CAT activity (20) and protein (53) were assayed by the indicated methods.

Western blot analysis

For detection of mature and precursor SREBP-1, nuclear extracts were prepared from chick embryo hepatocytes as described (69). The proteins of the nuclear extract were subjected to electrophoresis in 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Millipore) using an electroblotting apparatus (BIORAD). Immunoblot analysis was carried using a mouse monoclonal antibody against SREBP-1 (IgG-2A4) (American Type Culture Collection, Manassas, VA).

Antibody/protein complexes on blots were detected using enhanced chemiluminescence and or fluorescence, ECL-Plus (Amersham Biosciences). Chemiluminescence on the blots was visualized using a FluorChem 8000 imager (Alpha Innotech Corporation) and signals for mature form of SREBP-1 were quantified using FluorChem V200 software. Fluorescence was detected by scanning the membrane on Typhoon 9400 scanner (Amersham Biosciences) and the signals quantified using ImageQuaNT software.

For detection of phospho- and total MAP kinase, western analysis was performed as described (56). Briefly, total cell lysates were prepared in a buffer containing 10 mM Tris, pH 7.4, 1% SDS. Protein concentration was determined using Bradford assay (BIORAD). Proteins were run on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Millipore). Membranes were blocked in 5% nonfat dry milk for 1 h and incubated with primary antibody diluted in 5% bovine serum albumin overnight at 4°C. The primary antibodies against phospho-Akt (Ser473), phospho-p38 MAPK (Thr180/Tyr182), phospho-AMPK (Thr172), phospho-LKB1 (Ser428), phospho-ACC (Ser 79), phospho-PKC (pan, βII Ser660), phospho-S6 kinase (Thr389), phospho-MKK 3/6 (Ser189/207), phospho-MEK 1/2 (Ser 221), phospho-JNK (Thr183/Tyr185), phospho-Raf and total Akt, total p38 MAPK, total AMPK, total ACC, total p38 MAPK, total JNK, total ERK were obtained from Cell Signaling Technology. Anti-rabbit IgG conjugated with horseradish peroxidase (Cell Signaling Technology) was used as the secondary antibody, and the signals were detected by enhanced chemiluminescence, ECL-Plus (Amersham Biosciences). Images were visualized on Typhoon 9400 to detect fluorescence and/or Alpha Innotech to detect chemiluminescence and quantified by densitometry using ImageQuaNT software (Amersham Biosciences). MEK1/2 inhibitor, U0126 was purchased from Cell Signaling Technology. p38 MAPK inhibitors, SB203580 and SB202190 were purchased from Calbiochem.

Real-time RT-PCR

Total RNA from cells were isolated as described. Real-time RT-PCR assays using SYBR-green (BIORAD) were carried out in ninety-six well plates. 100 ng of total RNA was added per well to a total volume of 20 µl. Thermo-cycling conditions were as follows: 15 min at 50°C for reverse transcription; 10 min at 95°C to activate DNA

polymerase and to deactivate reverse transcriptase; 35 cycles of 15 s at 94°C to denature and 1 min at 59°C to anneal and extend DNA template. Reverse transcription and PCR amplification were performed by using iCycler thermal cycler from Bio-Rad. The primers used for genes have been described. Primers of chicken ACCα, SREBP-1 and SCD were used as described (51). Primers for the following genes were designed using PrimerQuest from IDT. The name of the genes and the corresponding Pubmed accession numbers are indicated in parentheses. SHP (AY700583), PGC-1α (AB170013), INSIG-1 (XM_418547), INSIG-2 (XM_422123), HMG-CoA reductase (AB109635), ABCA1 (NM_204145), LXRα (AF492498).

Gel mobility shift analysis

Nuclear extracts were prepared from hepatocytes incubated with or without CDCA in the presence of T0-901317. A double-stranded oligonucleotide containing the ACCα LXRE/T3RE (-108 to -82 bp relative to the transcription initiation site of ACCα promoter 2) was labeled by filling in overhanging 5'-ends using Klenow fragment of *E. coli* DNA polymerase in the presence of [α-^{32}P] dCTP. Binding reactions were carried out as previously described (Chapter 2). DNA and DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gels at 4 ºC in 0.5 X TBE (45 mM Tris, pH 8.3, 45 mM boric acid, 1 mM EDTA). Following 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 (68). Chromatin immunoprecipitations were carried out with antibodies against acetyl-histone H3 (06-599) and acetyl-histone H4 (06-866) (Upstate Biotechnology). Precipitated DNA was analyzed in PCR reactions using Taq DNA polymerase (New England Biolabs) and primers specific for the ACCα and malic enzyme promoters. The cycling parameters were: 1 cycle of 95°C for 4 min, 30 cycles of 95°C for 1 min, 61°C for 1 min, 72°C for 1 min, and 1 cycle of 72°C for 10 min. The forward primer of the ACCα gene was 5'-TCCCCTCCGTCAGCAGCCAATGGG-3'; the reverse

primer was 5'-ATCCCCGGTCCCGCCCTCGGCTCC-3'. The forward primer of the SCD1 gene was 5'-AGCGAACAGCAGATTGCGGCAG-3'; the reverse primer was 5'-AGGAAGGATGCTGAGGAAGAGGA-3'. Amplified products were subjected to electrophoresis in 2% agarose gels and visualized by ethidium bromide staining.

RESULTS

Regulation of LXR signaling by Hexanoate and cAMP

Previous work from our laboratory has shown that cAMP and hexanoate inhibit the activation of ACCα expression caused by insulin and T3. cAMP and hexanoate inhibit ACCα transcription at least in part by repressing the ability of T3 and insulin to increase the levels of mature SREBP-1 resulting in a disruption of the positive interaction between SREBP-1 and nuclear T3 receptor on the ACCα gene (72). We have also shown in Chapter 2 Figure 1, that T0-901317 increases the mRNA abundance of lipogenic genes such as ACCα, FAS and SCD. We wanted to determine whether cAMP and hexanoate inhibited the stimulation of ACCα, FAS, SCD, SREBP-1, LXR and ABCA1 caused by T0-901317. Our experiments showed that cAMP had no effect on T0-901317 induced expression of ACCα and other lipogenic genes (data not shown). In contrast, hexanoate inhibited T0-901317-induced mRNA abundance of lipogenic genes such as ACCα, FAS and SCD in a dose-dependent manner **Figure 3.1**. Hexanoate had no effect on the abundance of these genes in the absence of T0-901317. Hexanoate also inhibited T0-901317-induced ABCA1 mRNA expression in hepatocytes in a dose dependent manner. Thus, hexanoate does not qualify as an agent to selectively inhibit the actions of LXR agonist on lipogenic gene expression. As shown previously in Chapter 2, T0-901317 increases SREBP-1 and LXRα mRNA. Hexanoate had little or no effect on SREBP-1 and LXRα mRNA expression both in the absence and presence of T0-901317.

Chenodeoxycholic acid inhibits T0-901317-induced expression of lipogenic genes in primary cultures of chick embryo hepatocytes

As discussed previously, an increase in bile acids has been shown to be inversely correlated with a decrease in triglycerides, and vice versa (5, 10, 28, 158, 207, 185, 168).

The above observations prompted us to determine whether bile acids could inhibit the stimulation of ACCα and other lipogenic genes caused by T0-901317. Chick hepatocytes were incubated in the presence of insulin and insulin and T0-901317. Chenodeoxycholic acid (CDCA) was added in the absence and presence of T0-901317 in increasing concentrations. Incubation of chicken embryo hepatocytes with CDCA in the absence of T0-901317 had no effect on expression of ACCα, FAS and SCD mRNA abundance **Figure 3.2**. In contrast, CDCA inhibited T0-901317-induced mRNA abundance of lipogenic genes such as ACCα, fatty acid synthase (FAS), stearoyl CoA desaturase (SCD) in a dose dependent manner. Interestingly, CDCA further potentiated T0-901317-induced expression of ABCA1 mRNA, indicating that the effects of bile acids on LXR signaling are gene specific. CDCA has no effect on the mRNA abundance of ABCA1 in the absence of T0-901317.

3-hydroxy-3-methyglutaryl-CoA (HMG-CoA) reductase serves as the rate-limiting enzyme in cholesterol biosynthesis (45). Inhibitors of HMG-CoA reductase, collectively referred to as statins, are extensively used to lower plasma cholesterol both as a preventive measure, and cure for heart disease resulting from increased LDL (21). One of the rare, but major side effects of statins is the occurrence of rhabdomyolysis, that limits the use of these drugs in some patients (58). Therefore, it is important to investigate other agents that inhibit HMG-CoA reductase, or agents that inhibit other genes of the cholesterol synthesis pathway. CDCA has no effect on HMG-CoA reductase mRNA abundance in presence of insulin and causes a slight reduction at 75 μM. T0-901317 increases HMG-CoA reductase mRNA by 3-fold in presence of insulin. CDCA inhibits T0-901317-induced expression of HMG-CoA reductase in a dose dependent manner.

The decrease in ACCα mRNA by CDCA was correlated with a decrease of the ACCα protein by CDCA. Primary chick hepatocytes were incubated in the presence of T0-901317 in the absence and presence of CDCA. Total protein was harvested at the indicated time points and western blot analysis was performed to detect the amount of total ACC. CDCA inhibited total ACC protein as early as 2 h and further inhibited at 6 h,

12 h and 24 h, with maximal inhibition obtained at 24 h **Figure 3.3A**. To determine whether CDCA mediated decrease in ACCα expression was associated with a decrease in triacylglycerol production, the triacylglycerol concentration in the culture medium was measured in hepatocytes treated with T0-901317 in the absence and presence of CDCA. Medium was collected and total triglyceride in the medium was determined as outlined in Materials and methods. CDCA inhibited triacylglycerol accumulation in the medium at 24 hours **Figure 3.3B**. These results support that T0-901317-induced hypertriglyceridemia can be inhibited upon administration of CDCA.

CDCA inhibits T3-induced expression of lipogenic genes

Previous work in our laboratory showed that thyroid hormone stimulates ACCα expression, and that this effect is mediated at least in part, by the binding of the nuclear T3 receptor (TR) to a composite T3RE/LXRE (73). We next asked if bile acids also modulated the activity of the nuclear T3 receptor signaling pathways. Our results showed that bile acids inhibit T3-induced expression of ACCα, FAS, SCD and malic enzyme (ME) in a dose dependent manner **Figure 3.4**. CDCA had no effect on the mRNA abundance of these genes in the absence of T3. T3 causes a small decrease in ABCA1 mRNA. However, as shown in the previous figure, CDCA also increases expression of ABCA1 in a dose dependent manner with a two-fold induction at 75 μM, in the presence of T3. We propose that a common mechanism may be involved in mediating the effects of bile acids on T0-901317 and T3 signaling.

Identification of a bile acid response unit on the ACCα promoter that mediates the effect of CDCA on ACCα transcription

We have previously shown that T0-901317 regulation of ACCα is mediated by a 23 bp region in promoter 2 of the ACCα gene. This region consists of a DR-4 element (-101 to -86 bp) that binds heterodimers comprised of TR•RXR and LXR•RXR and a SRE (-82 to -71 bp) that binds SREBP-1 (Chapter 2). To determine if these cis-acting elements or other sequences in the ACCα gene mediate the effects of CDCA on ACCα transcription, transient transfection experiments were performed using reporter constructs

containing portions of ACCα promoter 2 linked to chloramphenicol acetyl transferase (CAT) gene. In chick embryo hepatocytes transfected with a reporter construct containing -2054 bp of 5'-flanking DNA, CDCA caused a 58% decrease in promoter activity **Figure 3.5A**. 5'-deletion of ACCα sequences to -391, -136, and -108 bp had no effect on CDCA responsiveness. Deletion of ACCα sequences containing the DR-4 element (-108 to -84 bp) abolished the inhibitory effect of CDCA on ACCα transcription. 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 caused a 35% attenuation of CDCA responsiveness on the ACCα promoter. These results indicate that the DR-4 element (LXRE) is required for CDCA regulation of ACCα transcription and that the SRE enhances the ability of the LXRE to inhibit ACCα transcription in the presence of CDCA.

To determine whether the functional interaction between the ACCα LXRE and SRE required the presence of additional cis-acting sequences, hepatocytes were transfected with constructs containing fragments of the ACCα gene linked to the minimal promoter of the herpes simplex virus thymidine kinase (TK) gene **Figure 3.5B**. The TK promoter alone was unresponsive to CDCA. 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 23% 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 almost completely abolished ACCα promoter activity. These data demonstrate that the ACCα SRE can function alone to enhance CDCA regulation conferred by the ACCα LXRE.

CDCA inhibits ACCα gene transcription by inhibiting the abundance of mature SREBP-1 via a post-translational mechanism in chick embryo hepatocytes

In our previous studies analyzing the regulation of the ACCα gene by T0-901317, we showed that T0-901317 treatment increased the abundance of mature, active form of SREBP-1 in chick embryo hepatocytes and this effect contributed to the activation of ACCα transcription by T0-901317 **Chapter 2, Figure 5**. Further, our studies in **Figure**

3.5 suggest the involvement of the ACCα-SRE in CDCA regulation of the ACCα gene. We wanted to determine the mechanism by which CDCA inhibits ACCα gene transcription, and whether SREBP-1, at least in part, mediates the inhibition. We performed time course experiments to determine the effects of CDCA on the abundance of mature SREBP-1 protein, ACCα mRNA, SREBP-1 precursor and SREBP-1 mRNA in hepatocytes cultured in the presence of T0-901317. Nuclear and membrane extracts and total RNA was isolated at the indicated time points and Northern and Western blot analysis was performed to detect levels of RNA and protein respectively. CDCA inhibited the abundance of the mature, active form of SREBP-1 as early as 2 h with maximal inhibition of about 40% at 12 h **Figure 3.6A**. The decrease in mature SREBP-1 by CDCA is correlated with the fact that treatment with CDCA caused a 20% decrease in the abundance of ACCα mRNA as early as 2 h, with maximal inhibition of about 50% obtained at 6 h **Figure 3.6B**. This observation is consistent with the proposal that alterations in SREBP-1 levels play a role in mediating the regulation of ACCα transcription by CDCA. Interestingly, CDCA increased the abundance of the inactive precursor form of SREBP-1 in the membrane, significantly at 12 h and 24 h by 3- and 5-fold respectively **Figure 3.6C**. CDCA had no effect on SREBP-1 mRNA abundance **Figure 3.6D**. An increase in precursor SREBP-1 suggests a mechanism by which CDCA increases retention of SREBP-1 in the membrane and implicates a posttranslational mechanism that is involved in regulation of ACCα.

Several factors are associated with retention of inactive precursor SREBP-1 in the membrane. For example, Insig-1 and Insig-2 are two proteins that have been shown to play a role in transporting SREBP-1 from the ER to the Golgi (16, 24, 65-67). We measured the mRNA abundance of these two genes upon addition of CDCA in presence of T0-901317. CDCA caused a transient increase in mRNA abundance of Insig-1 about 200% at 2 h, but inhibited the abundance of the mRNA at 12 h and 24 h **Figure 3.6E**. CDCA inhibited mRNA abundance of INSIG-2 mRNA transiently at 2 h **Figure 3.6F**. CDCA had no effect on INSIG-2 mRNA at later time points. It is unclear whether the transient increase of INSIG-1 mRNA at 2 h by CDCA, is sufficient to cause retention of the precursor (inactive) form of SREBP-1 in the membrane. At the same time, CDCA significantly inhibits INSIG-2 mRNA which would cause a decrease of the precursor

accumulation in the membrane. Thus, the opposing effects of CDCA on INSIG-1 and -2 mRNA at 2 hours should have no effect on SREBP-1 processing. Therefore, our data would suggest that other factors independent of the INSIG proteins may be mediating the post-translational modification of SREBP-1 by CDCA.

CDCA modulates the binding of nuclear receptor complexes to the ACCα LXRE/T3RE

In previous work, we showed that the ACCα LXRE/T3RE (-101 to -86 bp) bound four protein complexes in nuclear extracts prepared from chick embryo hepatocytes. 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 (73). In the absence of nuclear receptor agonists, complexes 1 and 2 were the predominant complexes that bound to the ACCα LXRE/T3RE. Further, we have shown that T0-901317 has opposing effects on the binding of LXR•RXR and TR•RXR complexes to the ACCα-LXRE/T3RE **Chapter 2, Figure 6**. These findings have led us to hypothesize that changes in the binding of nuclear receptor complexes to the ACCα-T3RE/LXRE may play a role in mediating the inhibition of ACCα transcription by CDCA. Gel mobility shift assays were performed using nuclear extracts from chick embryo hepatocytes treated with or without CDCA in the presence of T0-901317. As reported previously, a DNA probe containing the ACCα-LXRE/T3RE bound to complex 1, complex 2, and complex 4 in nuclear extracts from hepatocytes incubated in the presence of nuclear receptor agonists.

CDCA had no effect on the binding of Complex 1 to the ACCα-LXRE upto 12 h, but caused a slight decrease in Complex 1 binding at 24 h **Figure 3.7**. CDCA inhibited the binding of Complex 2 to ACCα-LXRE at 6 h and further inhibited the binding at 12 and 24 h. As discussed previously, Complex 4 is composed of TR•RXR heterodimers, that bind to ACCα-LXRE in the presence of thyroid hormone. We have previously shown that T0-901317 increased the binding of Complex 4 to the promoter, in the absence of T3 and presence of T0-901317 **Chapter 2, Figure 6**. CDCA inhibits the binding of Complex 4 to the ACCα-LXRE significantly at 2 hours, and further inhibits the binding over time. The inhibition of Complex 4 by CDCA is more dramatic than CDCA inhibition of Complex 2. The inhibition of TR•RXR heterodimer binding to the

ACCα-LXRE could be due to the fact that CDCA inhibits TRα mRNA abundance over time **Figure 3.8D**.

The latter phenomenon would suppress the inhibitory effects of T0-901317 on ACCα transcription because unliganded TR•RXR functions as a repressor of gene transcription (73). It is possible that CDCA-mediated changes in the binding of nuclear receptor complexes to the ACCα LXRE/T3RE constitutes a mechanism to prevent further inhibition of ACCα transcription by the LXR signaling pathway. The mechanism by which CDCA alters the binding of nuclear receptor complexes to the ACCα LXRE/T3RE is presently unclear but does not involve changes in the expression of LXRα mRNA **Figure 3.8E**.

CDCA modulates expression of several genes that may mediate inhibition of CDCA on ACCα mRNA expression

Short heterodimeric partner (SHP) is an unusual nuclear hormone receptor that lacks a DNA binding domain. SHP functions by dimerizing with other nuclear hormone receptors, such as TR and LXR, and represses their ability to activate transcription. In rodents, bile acids stimulate SHP expression via activation of the LXR signaling pathway. Bile acid induction of SHP expression has been shown to play a role in mediating the inhibitory effects of bile acids on cholesterol 7α hydroxylase transcription, the rate limiting gene for synthesis of bile from cholesterol (19, 40). Human SHP promoter has been shown to be activated by SREBP-1. Overexpression of SREBP-1 activated the human but not mouse SHP promoter (31). Thus SREBP-1 may mediate the species-specific regulation of cholesterol and bile acid homeostasis via modulating SHP gene expression.

To assess the role of SHP in mediating the inhibitory effects of bile acids on T0-901317 induced lipogenic gene expression, we performed time course experiments with CDCA treatment to determine whether CDCA stimulated SHP expression in chick hepatocytes in presence of T0-901317. Surprisingly, CDCA inhibits the SHP mRNA abundance in the presence of T0-901317 as early as 2 h and further inhibited over time **Figure 3.8A**. This result suggests that SHP does not play a role in mediating the inhibitory effects of bile acids on lipogenic gene expression in chick hepatocytes.

PGC-1α has been shown to be a coactivator for LXRα. In transient transfection studies, PGC-1α potentiates the LXR-mediated autoregulation and transactivation of the LXRα promoter via the LXRE on the cholesteryl ester transfer-protein (CETP) gene promoter in a ligand-dependent manner. Further, mutational analyses showed that the LXXLL motif of PGC-1α is essential for coactivation of LXR-mediated transcription (46). We wanted to determine if CDCA inhibits LXR activity by inhibiting PGC-1α mRNA. CDCA inhibits PGC-1α mRNA abundance maximally at 2 h by 50% **Figure 3.8B**.

Recently, PGC-1β has been shown to be a coactivator of SREBP-1 and LXR. This increase in SREBP-1 and LXR activity increases de novo lipogenesis and lipoprotein secretion. PGC-1β coactivation of SREBP and LXR by high fat leads to hypertriglyceridemia and hypercholesterolemia (39). We wanted to determine if CDCA inhibition of ACCα mRNA is mediated by a decrease in PGC-1β levels. Our data shows that there is a transient decrease in PGC-1β mRNA at 2 h by 25% **Figure 3.8C**. However, CDCA has no effect on PGC-1β mRNA at later time points. It is unclear whether this decrease of PGC-1β at 2 hours results in ACCα inhibition.

We have shown that CDCA inhibits the binding of unliganded TR•RXR on the ACCα-LXRE. We wanted to determine whether this decrease in the binding of the complex is due to changes in TR mRNA levels. Our results showed that CDCA inhibits TR mRNA maximally at 2 h **Figure 3.8D**. This may explain the decreased binding of the TR•RXR on the ACCα-LXRE. We have also shown that CDCA inhibits the binding of LXR•RXR heterodimers on the ACCα-LXRE. This decrease in binding could be either due to the inhibition of LXR mRNA abundance or the inhibition of LXR•RXR binding to the ACCα-LXRE. Our results showed that CDCA had little or no effect on the abundance of LXRα RNA in presence of T0-901317 **Figure 3.8E**.

Recent work has shown that bile acids stimulate fibroblast growth factor-19 (FGF-19) expression in human hepatocytes via a FXR-mediated mechanism. FGF-19 is a member of the fibroblast growth factor (FGF) family of secreted signaling molecules. FGF-19's action is initiated by binding to the membrane-bound, tyrosine kinase receptor, FGFR4. Studies with transgenic mice have shown that overexpression of FGF-19 enhances basal energy metabolism and reduces adipose stores. Expression of malic

enzyme (ME) and stearoyl CoA desaturase (SCD) are reduced in FGF-19 transgenic mice. (22, 23, 59, 64, 71).

Based on this information, we hypothesized that bile acids stimulate FGF-19 expression and that increased FGF-19 levels inhibit lipogenic gene expression. Consistent with results reported for human hepatocytes, CDCA caused a transient increase of FGF-19 mRNA abundance in chick hepatocytes in presence of T0-901317 by 750% at 2 h **Figure 3.8F**. CDCA increased expression of FGF-19 mRNA by 450% at 6h and 12 h. FGF-19 mRNA levels were further increased to 600% by CDCA at 24 h.

CDCA increases the phosphorylation and activation of the mitogen activated protein kinase (MAP Kinase) pathways

Bile acids have been shown to increase phosphorylation and activation of MAP kinase pathways in several cell types (44). Recently, two reports showed an involvement of ERK and p38 MAP kinase pathways as inhibitors of lipogenic gene expression (7, 56). We hypothesized that CDCA inhibits ACCα mRNA by activating MAP kinase pathways. Primary chick hepatocytes were treated with T0-901317 in the absence and presence of CDCA, for the time points indicated in the figure. Total protein was isolated and western blot analysis was performed to determine the levels of phosphorylated, active ERK, JNK and p38 MAPK **Figure 3.9**. CDCA increased the phosphorylation of ERK, JNK and p38 MAP kinases at 5 min and this increase in phosphorylation was sustained upto 24 h. CDCA also increased the phosphorylation and activation of MEK1/2, a MAPK kinase (MAPKK), responsible for phosphorylation and activation of ERK. This increase in phosphorylation and activation of MEK1/2 is correlated with the activity of ERK. CDCA also increases phosphorylation and activation of MKK3/6, another MAPKK, that is responsible for phosphorylation and activation of p38 MAPK. Again, this activation of MKK3/6 is correlated with the activity of p38 MAPK. We also measured the phosphorylation of Raf and found that CDCA has no effect on phosphorylation of the protein. This shows that the effects of CDCA on phosphorylation of MAP kinases are selective.

ERK and p38 MAPK mediate the inhibitory effects of CDCA on ACCα mRNA abundance

In **Figure 3.9** we have shown that CDCA increases the phosphorylation and activation of ERK, JNK and p38 MAPK. To determine whether one or all of these MAPKs mediate CDCA inhibition of ACCα mRNA, we used inhibitors of MAPK activity, such as U0126 for ERK, SP600125 for JNK and SB203580 for p38 MAPK. Primary chick hepatocytes were treated with T0-901317 in the absence and presence of CDCA. Medium was changed after 12 hours with the addition of insulin and T0-901317 in all plates and CDCA in the indicated plates. 12 hours later, cells were treated with either the inhibitors or DMSO as control. 6 hours after addition of inhibitors and DMSO, total RNA was isolated and ACCα mRNA was measured using real time RT-PCR. In vehicle treated cells, CDCA inhibited the ACCα mRNA expression by about 60% **Figure 3.10**. However, in presence of the ERK inhibitor, U0126, the inhibition of ACCα mRNA by CDCA was significantly decreased to about 27% **Figure 3.10A**. As U0126 inhibits MEK1/2 activity, it inhibits phosphorylation and activation of ERK. Western blot analyses with proteins isolated from identically treated plates showed that U0126 completely inhibits the phosphorylation and activation of ERK **Figure 3.10C**.

In presence of the p38 MAPK inhibitor SB203580, CDCA inhibition of ACCα mRNA was almost completely abolished **Figure 3.10B**. We also observed similar effects with another p38 MAPK inhibitor, SB202190 (data not shown). Our studies with the JNK inhibitor SP600125 remain inconclusive. At the least, our data would suggest that JNK mediates the activation of ACCα mRNA abundance by T0-901317. In presence of T0-901317, SP600125 completely abolishes the increase in ACCα mRNA abundance by T0-901317. More about the role of JNK in ACCα expression will be discussed in Chapter 4, **Figure 4.10**.

CDCA increases FGF-19 mRNA abundance via both ERK and p38 MAPK and inhibits PGC-1α mRNA via p38 MAPK, but not ERK

Our data upto this point shows that CDCA increases FGF-19 mRNA abundance and increases the phosphorylation and activation of ERK and p38 MAPK. To determine whether the increase in FGF-19 is mediated by the MAP kinases, we measured mRNA

abundance of FGF-19 in the absence and presence of CDCA in presence of vehicle, U0126 and SB203580. In vehicle treated cells, CDCA increases FGF-19 by about 640% **Figure 3.11**. This data is consistent with our previous results showing that CDCA increases FGF-19 over time. In presence of the ERK inhibitor U0126, the increase of FGF-19 mRNA by CDCA was completely abolished **Figure 3.11A**. Similarly, in presence of p38 MAPK inhibitor SB203580, the increase in FGF-19 mRNA by CDCA was completely abolished **Figure 3.12B**. These results show that CDCA increases FGF-19 mRNA via ERK and p38 MAPK.

Next, we wanted to determine whether the inhibition of PGC-1α mRNA by CDCA is mediated by ERK and/or p38 MAPK. Chick hepatocytes were plated in the absence and presence of CDCA in the presence of vehicle, U0126 and SB203580. Total RNA was isolated and real time RT-PCR was performed to measure relative mRNA abundance. In vehicle treated cells, CDCA inhibited PGC-1α mRNA by 60%, in agreement with our previous data **Figure 3.12**. U0126 had no effect on CDCA inhibition of PGC-1α mRNA **Figure 3.12A**. However, SB203580 completely abolished CDCA inhibition of PGC-1α expression **Figure 3.12B**. These results show that CDCA inhibition of PGC-1α is mediated by p38 MAPK, but not ERK.

Hepatocytes infected with adenovirus vectors expressing PGC-1α does not reverse CDCA inhibition of ACCα mRNA

As discussed previously, PGC-1α is a coactivator of LXR. Our data show that CDCA inhibits PGC-1α mRNA in chick hepatocytes, and that p38 MAPK but not ERK mediates this effect. To establish a role of PGC-1α in mediating inhibition of ACCα mRNA by CDCA we infected hepatocytes with adenovirus containing PGC-1α in frame with GFP. Cells were also infected with adenovirus containing GFP and no DNA (null), and no virus as controls. Hepatocytes were treated with equal amounts (2.2×10^{12}) of null, GFP and PGC-1α virus particles. The fluorescence of cells treated with GFP and PGC-1α was determined to ensure infectivity. After 24 hours, 90-100% of cells treated with both GFP and PGC-1α expressed green fluorescence (data not shown). CDCA inhibits ACCα mRNA in uninfected cells and cells infected with adenovirus containing null, GFP and PGC-1α **Figure 3.13**. Cells treated with adenovirus containing PGC-1α

had a 10-fold increase in PGC-1α protein levels over control cells (data not shown). One interpretation of these findings maybe that PGC-1α does not mediate CDCA inhibition of ACCα mRNA in chick hepatocytes. However, as we cannot measure endogenous PGC-1α protein for lack of a suitable antibody, our results are inconclusive. Adenovirus was prepared by Callee M. Walsh in Dr. Salati's lab.

Inhibition of p38 MAPK activity, but not ERK activity attenuates the effects of CDCA on mature SREBP-1

Previous reports have shown that phosphorylation of Thr426 and Ser430 of SREBP-1a by Gsk3β facilitates binding of the ubiquitin ligase, SCFFbw7, which targets SREBP for 26S proteasomal degradation. Inhibition of Gsk3β activity by LiCl or insulin increases the accumulation of SREBP (55), (32). Another report showed that n-3 polyunsaturated fatty acids (PUFAs) decrease the mature, active form of SREBP-1 in primary rat hepatocytes through 26S proteasome and Erk-dependent pathways. Specific inhibitors of these pathways attenuate the inhibitory effect of PUFA on mature SREBP-1 levels (7). The mechanisms by which Erk phosphorylation, and 26S proteasomal mediate degradation of mature SREBP-1 are yet to be identified.

Based on these reports, we wanted to determine whether CDCA inhibition of mature SREBP is mediated by ERK and/or p38 MAPK. We plated hepatocytes in the absence and presence of CDCA in presence of either vehicle, U0126 or SB203580. As shown previously, CDCA inhibits mature SREBP by 60% in vehicle treated cells **Figure 3.14**. In presence of U0126, CDCA inhibition of mature SREBP was not affected as compared to vehicle treated controls. However, inhibition of p38 MAPK activity by SB203580 significantly attenuated CDCA inhibition of mature SREBP-1 by about 35%. We propose that CDCA mediates inhibition of ACCα mRNA by increasing p38 MAPK pathway, which in turn, phosphorylates SREBP-1 and targets it for degradation.

Effects of CDCA on ACCα mRNA abundance, ERK and p38 MAPK activity are selective

CDCA increases low density lipoprotein receptor (LDLr) gene expression via ERK activation-mediated stabilization of LDLr mRNA via activating SHP (44). These

authors reported that ursodeoxycholic acid (UDCA) had no effect on LDLr and SHP mRNA. These results suggested that the structural difference between CDCA and UDCA, specifically the 7β-hydroxy epimer of CDCA, is critical for the activation of ERK and binding to FXR (41, 48). The lack of inhibitory effect of UDCA on LDLr can be accounted for the fact that UDCA does not activate ERK (8). Octyl β-D-glucopyranoside, a detergent also had no effect on ERK activity (43). These results suggest that bile acids activate MAP kinases in a specific manner, not by their detergent effects, but by specific side chain residues and three-dimensional structure.

We wanted to determine whether other bile acids besides CDCA, demonstrated similar effects on ACCα mRNA, ERK and p38 MAPK. We tested cholic acid (CA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), taurocholic acid (TCA), hyodeoxycholic acid (HDCA), and taurodeoxycholic acid (TDCA) for their ability to modulate ACCα mRNA abundance, ERK and p38 MAPK activity. Chick hepatocytes were plated in presence of T0-901317 in absence and presence of the indicated bile acids. 6 hours after incubation with bile acids, total RNA and protein was isolated. Real time RT-PCR analysis showed that CDCA inhibits ACCα mRNA about 50%, and all other bile acids tested had no effect **Figure 15**. Western blot analysis showed that only CDCA robustly activates ERK and p38 MAPK. There may be a slight effect of CA and DCA on p38 MAPK activity. These results showed that the effects of CDCA are highly selective.

CDCA is the most potent modulator of gene expression

As discussed in Chapter 1, relative hydrophobicity is a major determinant of bile acid action. CDCA is one of the most hydrophobic bile acids. We wanted to determine whether effects of CDCA on gene expression are specific and also wanted to determine the effects of other bile acids on gene expression. We performed Northern blot and real time RT-PCR analysis to determine mRNA abundance of FAS, SCD, FGF-19, ABCA1, PGC-1α and PGC-1β **Figure 3.16**. We have shown that CDCA inhibits FAS, SCD and PGC-1α, increases FGF-19 and ABCA1, and has no effect on PGC-1β mRNA. Both CA and DCA inhibit FAS and SCD mRNA abundance by about 50%. CDCA inhibits both these genes by about 80%. Other bile acids had no effect on FAS and SCD mRNA

abundance. CDCA causes an 8-fold increase in FGF-19 mRNA. CA and DCA cause a two-fold increase in FGF-19 mRNA. Other bile acids had no effect on FGF-19 mRNA.

CDCA causes a two-fold increase in ABCA1 mRNA. TDCA causes a significant (1.5-fold) increase in ABCA1 mRNA, although the extent of increase is not as great as by CDCA. All other bile acids had no effect on ABCA1 mRNA. CDCA causes a 60% decrease in PGC-1α mRNA, but other bile acids had no effect on PGC-1α mRNA. CDCA had no effect on PGC-1β mRNA. Both DCA and UDCA significantly inhibited PGC-1β by 30% and 25% respectively. Other bile acids have no effect on PGC-1β mRNA. The effect of CDCA on all these genes is consistent with results shown in this Chapter.

CDCA inhibits the recruitment of acetylated histones H3 and H4 on the ACCα promoter

The decrease in ACCα transcription by CDCA may be mediated in whole or in part by alterations in histone acetylation on the ACCα promoter. Previous studies have shown that the activation of LXR•RXR heterodimers by LXR ligands/agonists recruits coactivator complexes containing histone acetyltransferase (HAT) activity (1, 61). We have shown that T0-901317 causes a transient increase in histone acetylation on the ACCα promoter **Chapter2, Figure 7**. Increased histone acetylation causes a chromatin decondensation that enhances the accessibility of the basal transcriptional machinery and other transcription factors to the target promoter. On the other hand, decreased histone acetylation causes chromatin condensation that inhibits the recruitment of basal transcription machinery and cofactors on the promoter.

To investigate the role of histone acetylation in mediating the inhibition of ACCα transcription by CDCA, chromatin immunoprecipitation (ChIP) were performed in chick embryo hepatocytes incubated in the absence or presence of CDCA. Hepatocytes were treated with 1% formaldehyde to cross-link DNA to associated proteins. Protein-DNA complexes were immunoprecipitated with an antibody against acetylated histone H3 or acetylated histone H4. Immunoprecipitated DNA was analyzed by PCR using primers that flanked the ACCα LXRE/T3RE. In hepatocytes incubated in the absence of CDCA, acetylation of histone H3 and histone H4 was detected at the ACCα LXRE **Figure 3.17**.

Addition of CDCA to the culture medium caused a significant decrease in the acetylation of histone H3 and H4. We also performed ChIP analysis in an uncharacterized region of the SCD1 promoter using a primer set that amplified SCD sequences between -369 and -193 bp. In contrast to the data for ACCα, CDCA had no effect on histone acetylation on SCD.

DISCUSSION

Data so far demonstrated that the previously identified ACCα-LXRE confers CDCA regulation on ACCα promoter 2. We also demonstrated that SREBP-1 is an accessory factor that enhances the ability of LXRE to inhibit ACCα transcription by CDCA **Figure 3.5**. Our results showed that CDCA decreases the concentration of mature, active form of SREBP-1 in chick embryo hepatocytes. As shown previously, CDCA inhibits mature SREBP-1 by inhibiting post-translational modification of SREBP-1 **Figure 3.6**.

We have shown that p38 MAPK but not ERK mediates inhibitory effects of CDCA on ACCα expression by modulating mature SREBP-1 levels. It is possible that activation of p38 MAPK by CDCA results in phosphorylation of SREBP-1 at Thr426 and/or Ser430 as these residues are conserved in chicken. Dr. Johan Ericsson has kindly provided us with a phospho-Thr426 antibody. Data from western blot experiments performed with this antibody are inconclusive. We speculate that p38 MAPK phosphorylates SREBP-1 at Thr426 and/or Ser430 and targets the protein for degradation.

Although we have provided evidence that shows LXRE mediates ACCα inhibition by CDCA, data from in vitro binding assay showed that CDCA does not directly modulate binding activity of LXR•RXR heterodimers on the ACCα-LXRE **Figure 3.7**. We have shown that CDCA enhances the recruitment of unliganded TR•RXR on the LXRE. This would result in an increase in ACCα expression as previous reports have shown that unliganded TR•RXR is an inhibitor of ACCα expression. Therefore it is possible that the increase in binding activity of TR•RXR acts to prevent

inhibition of ACCα mRNA below basal levels to allow the cells to have a steady supply of fatty acids that are an integral part of membranes.

Our data shows that CDCA mediates its inhibitory effects on ACCα mRNA expression by activation of p38 MAPK. Using SB203580, a p38 MAPK inhibitor, the inhibition of ACCα mRNA by CDCA was completely abolished. It is interesting to note that SB203580 alone causes a 60% increase in ACCα mRNA in the absence of CDCA **Figure 3.10**. In the absence of CDCA, P-p38 MAPK levels are readily detected, as shown in **Figure 3.9**. Taken together, these results would suggest that basal levels of P-p38 MAPK have an inhibitory effect on ACCα mRNA expression. Upon CDCA addition, p38 MAPK is further activated, and this results in ACCα mRNA inhibition as shown in **Figure 3.10B**. The role of basal P-p38 MAPK as an inhibitor of ACCα mRNA is also consistent with the fact that SB203580 alone increases mature SREBP-1 levels **Figure 3.14**, Lane 5. We have previously shown that an increase in mature SREBP-1 is correlated with an activation of ACCα mRNA expression **Chapter 2, Figure 5**. As discussed in Chapter 1, oxysterols (oxidized derivatives of cholesterol) are endogenous ligands/agonists of LXR. An increase in oxysterols would lead to an increase in lipogenic enzyme expression that would lead to the formation of triglycerides. Thus, p38 MAPK prevents the over-activation of the lipogenic pathway, thereby preventing the accumulation of plasma triglycerides.

One interesting finding in our work is the sustained activity of the MAPKs **Figure 3.9**. The sustained activation of ERK and p38 MAPK may be required for the sustained inhibition of ACCα mRNA. Early activation of ERK and p38 MAPK trigger cellular processes that inhibit ACCα initially. Once ACCα mRNA levels are inhibited, other processes may be involved to ensure that ACCα mRNA levels are still reduced. Our studies on CDCA inhibition show the involvement of multiple pathways that mediate CDCA inhibition of ACCα such as SREBP-1, histone acetylation and possibly FGF-19. Activated MAPKs at later time points act to ensure low abundance of ACCα mRNA in presence of T0-901317.

The mechanisms responsible for the sustained activity of MAPKs are unclear. One possibility is that CDCA inhibits the activity of the specific phosphatases that inactivate MAPKs. For example, it has been reported that MAPK phosphatase-3 (MKP-

3), which is a highly selective phosphatase that inactivates ERK, inhibits insulin-mediated repression of the PEPCK promoter (11). However, this study also showed that the effect of MKP-3 is not due to dephosphorylation and inactivation of ERK. It is possible that in hepatocytes the activity of other MKPs is inhibited by CDCA.

We have also determined that CDCA increases the phosphorylation and activation of JNK. Our data with ERK and p38 MAPK inhibitors showed that both these MAPKs mediate inhibitory effects of CDCA on ACCα mRNA. In contrast, studies with the JNK inhibitor SP600125 showed that JNK mediates activation of ACCα mRNA by T0-901317 (Chapter 4). The mechanism for such selective activity by the MAPKs is unclear.

The role of bile acids in recent times have emerged from that of lipid solubilizers to signaling molecules that modulate several important cellular processes. Recently the identification of a cell surface receptor TGR5, a member of the G-protein coupled receptor (GPCR) family has further established an endocrine role for bile acids. In fact reports have shown that bile acid treatment increases cAMP levels, that is a classic measure of GPCR activity (30). Another report has shown that bile acids promote energy expenditure by increasing cAMP. GPCRs have been shown to activate MAPKs (42). It is therefore tempting to speculate that bile acids bind to a cell surface receptor on hepatocytes that belong to the GPCR family. The activated GPCR increases cAMP levels that would in turn activate MAPKs, which in turn would lead to an inhibition of lipogenic genes. This theory although attractive, is probably not the mechanism for ACCα inhibition by CDCA in chick hepatocytes. First, cAMP does not inhibit T0-901317-induced ACCα mRNA expression in chick hepatocytes (Data not shown). Second, cAMP does not activate any MAPK pathways in chick hepatocytes (Data not shown). Taken together, these reports suggest that in hepatocytes, bile acids inhibit lipogenic enzyme expression by a mechanism not involving cAMP.

Finally, this report shows that CDCA inhibits T0-901317-induced expression of lipogenic genes by increasing MAPKs such as p38 MAPK. CDCA mediates inhibition of ACCα by modulating mature SREBP-1. Identification of the signaling pathways mediating CDCA inhibition of lipogenic genes may lead to identification of compounds that would combat atherosclerosis.

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FIGURE LEGENDS

Figure 3.1: Hexanoate inhibits the stimulatory effect of T0-901317 on acetyl CoA carboxylase α (ACCα), fatty acid synthase (FAS), stearoyl CoA desaturase (SCD) and ATP-binding cassette protein A1 (ABCA1) mRNA, but not sterol regulatory element binding protein-1 (SREBP-1) and liver X receptor α (LXRα) mRNA expression, in a dose dependent manner. Chicken embryo hepatocytes were plated in the absence of hormones overnight. Medium was changed and the cells were treated with indicated amounts of hexanoate, T0-901317 (6 μM), and insulin (50 nM) for 24 hours. Total RNA was isolated and Northern blot analysis was performed to determine the mRNA levels of the genes. Data shown are representative of two experiments for SREBP-1 and LXRα, and three experiments for ACCα and ABCA1.

Figure 3.2: Chenodeoxycholic acid (CDCA) inhibits the T0-901317 induced expression of ACCα, FAS, SCD and HMG-CoA reductase mRNA, but increases the T0-901317 induced expression of ABCA1 mRNA in a dose dependent manner. Chicken embryo hepatocytes were plated in the absence of hormones overnight. Medium was changed and the cells were treated with the indicated amounts of CDCA, insulin (50 nM) and T0-901317 (6 μM) for 24 hours. Total RNA was isolated and Northern blot analysis was performed to determine mRNA levels. HMG-CoA reductase mRNA was detected using real time RT-PCR as described in Methods. Data represents mean ± SE of at least four experiments. The values for mRNA abundance for the treatments have been calculated with respect to the insulin treatment (Lane 1), the value of which was set to 1.

Figure 3.3: CDCA inhibits total ACC protein and triglyceride secretion in medium. A) Chick hepatocytes were plated in the absence of hormones overnight. Medium was changed and insulin and T0-901317 were added to all plates. 24 hours later, medium was changed again with the addition of insulin and T0-901317 to all plates and CDCA (75 μM) was added in the indicated plates. Total protein was isolated from plates at the

indicated time points and western blot analysis was performed to determine total ACC. B) For triglyceride measurements, cells were plated in the absence of hormones overnight. Medium was changed with the addition of insulin (50 nM) and T0-901317 (6 μM) to all plates in the absence and presence of CDCA (75 μM). Medium from the plates were collected 24 hours later, and triglyceride assay was performed according to manufacturer's protocol (Sigma). Data shown are mean ± SE of three experiments.

Figure 3.4: Chenodeoxycholic acid (CDCA) inhibits the T3-induced expression of ACCα, FAS, SCD and ME mRNA, but increases the expression of ABCA1 mRNA in a dose dependent manner. Chicken embryo hepatocytes were plated in the absence of hormones overnight. Medium was changed and the cells were treated with the indicated amounts of CDCA, insulin (50 nM) and T3 (1.5 μM) for 24 hours. Total RNA was isolated and Northern blot analysis was performed to determine mRNA levels. Data represents mean ± standard deviation of two experiments. The values for mRNA abundance for the treatments have been calculated with respect to the insulin treatment (Lane 1), the value of which was set to 1.

Figure 3.5: A) Effects of deletions of the 5'-flanking region of ACCα promoter 2 on transcriptional activity in the absence and presence of CDCA. CEHs were transiently transfected with p[ACC-2054/+274] chloramphenicol acetyltransferase (CAT) or equimolar amounts of other plasmids as described under Experimental Procedures. After transfection, cells were treated with T0-901317 and insulin in the absence or presence of CDCA for 48 h. Protein extracts prepared as described in Materials and methods, and CAT assays performed. Left: The constructs used in these experiments. 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 was +274 bp. The location of the LXR response element (LXRE) is between -101 to -86 bp and SRE-1 is between -80 to -71 bp is indicated by the vertical lines. Right: CAT activity of cells transfected with p[ACC-108/+274]SRE(mut2)CAT and treated with T0-901317 and

insulin was set at 1, and the other activities were adjusted proportionately. The percent inhibition by CDCA is the CAT activity of cells treated with T0-901317, insulin, and CDCA expressed as a percentage of that in cells treated with T0-901317 and insulin and subtracted from 100. The results are the means ± SEM of at least four experiments. Significant differences between means within the column ($P < 0.05$) are as follows: a indicates that p[ACC-108/+274]SRE(mut 2)CAT is different from all other constructs. b indicates that p[ACC-82/+274]CAT is significantly different from all other constructs.

B) Fragments of the ACCα gene containing the LXRE and/or SRE-1 were linked to the minimal thymidine kinase (TK) promoter in TKCAT. CEHs were transiently transfected with these constructs and treated with T0-901317 and insulin in the absence or presence of CDCA as described in Part A (above) and under Experimental Procedures. Left: Constructs used in these experiments. Numbers indicate the 5' and 3' boundaries of ACCα DNA relative to the transcription initiation site of promoter 2. Right: CAT activity in CEH transfected with p[ACC-TKCAT and treated with T0-901317 and insulin was set at 1, and the other activities were adjusted proportionately. The percent inhibition by CDCA on ACCα promoter activity was calculated as described in the legend to Part A. The results are the means ± SEM of at least five experiments. Significant differences between means within the column ($p < 0.05$) are as follows: a, versus any other construct.

Figure 3.6: CDCA inhibits ACCα mRNA by inhibiting mature SREBP-1 via a post-translational mechanism. CEHs were plated in the absence of hormones overnight. Medium was changed with the addition of insulin (50 nM) and T0-901317 (6μM) in all plates. 24 hours later, medium was changed again, with the addition of CDCA (75 uM) in the indicated plates. Total RNA, nuclear and membrane protein extracts were prepared at the indicated time points. ACCα, SREBP-1, INSIG-1 and INSIG-2 mRNA abundance was measured using Northern blot analysis. Mature and precursor SREBP-1 was determined using Western blot analysis. The signals from blots were quantified using ImageQuaNT software. The value of insulin and T0-901317 treated sample at time 0,

was set at 1. Data represents mean ± SE for at least three experiments. * indicates significant values at $p<0.05$.

Figure 3.7: Effect of CDCA on the binding of hepatic nuclear proteins to the ACCα LXRE. A) Eighteen hours after being placed in culture, CEHs were incubated in Waymouth's medium containing insulin (50 nM), and T0-901317 (6 μM), with or without CDCA 75 μM for the indicated times. Cells were harvested and 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 (-108 to -82 bp). Specific protein-DNA complexes are indicated by arrows. Previous studies have shown that complexes 1 and 2 contain liver X receptor (LXR)•retinoid X receptor (RXR) heterodimers, whereas complex 4 contains nuclear T3 receptor (TR)•RXR heterodimers. These data are representative of five experiments employing independent preparations of nuclear extract. B) Signal from the complexes was quantified using ImageQuaNT software. The value for the samples treated with insulin and T0-901317 at 0 h was set to 1. Data represents mean ± SE of four experiments.

Figure 3.8: CDCA modulates the mRNA abundance of short heterodimeric partner (SHP), PPAR-gamma coactivator -1α (PGC-1α), PPAR-gamma coactivator -1β (PGC-1β), thyroid receptor (TR), liver X receptor (LXR) and fibroblast growth factor-19 (FGF-19). CEHs were plated in the absence of hormones overnight. Medium was changed with the addition of insulin and T0-901317 in all plates. 24 hours later, medium was changed again, with the addition of insulin (50 nM), T0-901317 (6 μM) and CDCA (75 μM) as indicated. Total RNA was harvested from the plates at the indicated time points and relative mRNA abundance was measured using realtime RT-PCR. The value for insulin and T0-901317 treated sample at time 0 was set at 1. Data represents mean ± SE of at least three experiments.

Figure 3.9: CDCA phosphorylates and activates mitogen-activated protein kinase (MAPK) pathways. CEHs were plated in the absence of hormones overnight. Medium was changed with the addition of insulin (50 nM) and T0-901317 (6 μM) in all plates. 24 hours later, medium as changed again, with the addition of insulin and T0-901317 in all plates and CDCA (75 μM) in the indicated plates. Total protein was harvested from the plates at the indicated time points. Western blot analysis was performed to determine MAPK levels using antibodies as described in Methods. Phosphorylation of Raf at all the corresponding time points serves as a negative control. Data are representative of at least three experiments.

Figure 3.10: CDCA mediates inhibitory effects on ACCα mRNA abundance via ERK and p38 MAPK pathways. CEHs were plated in the absence of hormones overnight. Medium was changed with the addition of insulin and T0-901317 in all plates. 12 hours later, medium was changed again with the addition of insulin and T0-901317. 12 hours later, ERK inhibitor U0126 (20 μM) A), and p38 MAPK inhibitor SB203580 (20 μM) B), was added to the indicated plates. Control cells were treated with equal volume of DMSO. DMSO by itself has no effect on ACCα mRNA levels (data not shown). 1 hour after the addition of inhibitors, CDCA was added to the indicated plates. Total RNA was isolated 6 hours after the addition of CDCA and relative mRNA was determined using real time RT-PCR. The column indicates percent inhibition by CDCA for vehicle treated, U0126 treated A), and SB203580 treated B), plates. The percent inhibition by CDCA relative mRNA abundance of cells treated with T0-901317, insulin, and CDCA expressed as a percentage of that in cells treated with T0-901317 and insulin and subtracted from 100. * represents that the data is significant at $p<0.05$. Data represents mean ± SE of five experiments.

Figure 3.11: CDCA mediates stimulatory effects on ACCα mRNA abundance via ERK and p38 MAPK pathways. CEHs were plated in the absence of hormones overnight. Medium was changed with the addition of insulin (50 nM) and T0-901317 (6 μM) in all

plates. 12 hours later, medium was changed again with the addition of insulin and T0-901317. 12 hours later, ERK inhibitor U0126 (20 μM) A), and p38 MAPK inhibitor SB203580 (20 μM) B), was added to the indicated plates. Control cells were treated with equal volume of DMSO. DMSO by itself has no effect on FGF-19 mRNA levels (data not shown). 1 hour after the addition of inhibitors, CDCA was added to the indicated plates. Total RNA was isolated 6 hours after the addition of CDCA and relative mRNA was determined using real time RT-PCR. The column indicates percent activation by CDCA for vehicle treated, U0126 treated A), and SB203580 treated B), plates. The percent activation by CDCA relative mRNA abundance of cells treated with T0-901317, insulin, and CDCA expressed as a percentage of that in cells treated with T0-901317 and insulin. * represents that the data is significant at $p<0.05$. Data represents mean ± SE of four experiments.

Figure 3.12: CDCA mediates inhibitory effects on PGC-1α mRNA abundance via p38 MAPK, but not ERK pathway. CEHs were plated in the absence of hormones overnight. Medium was changed with the addition of insulin and T0-901317 in all plates. 12 hours later, medium was changed again with the addition of insulin and T0-901317. 12 hours later, ERK inhibitor U0126 (20 μM) A), and p38 MAPK inhibitor SB203580 (20 μM) B), was added to the indicated plates. Control cells were treated with equal volume of DMSO. DMSO by itself has no effect on PGC-1α mRNA levels (data not shown). 1 hour after the addition of inhibitors, CDCA was added to the indicated plates. Total RNA was isolated 6 hours after the addition of CDCA and relative mRNA was determined using real time RT-PCR. The column indicates percent inhibition by CDCA for vehicle treated, U0126 treated A), and SB203580 treated B), plates. The percent inhibition by CDCA relative mRNA abundance of cells treated with T0-901317, insulin, and CDCA expressed as a percentage of that in cells treated with T0-901317 and insulin and subtracted from 100. * represents data is significant at $p<0.05$. Data represents mean ± SE of five experiments.

Figure 3.13: Chick hepatocytes infected with adenovirus overexpressing PGC-1α does not reverse the inhibitory effects of CDCA on ACCα mRNA expression. CEH were plated in the absence of hormones. Four hours later, medium was changed with the addition of insulin (50 nM) and T0-901317 (6 μM) and infected with adenovirus containing the indicated proteins. Cells were treated with approximately 2.2 x 10^{12} virus particles of null, GFP and PGC-1α per plate. Hepatocytes that were uninfected by virus and treated in parallel served as controls. 24 hours after addition of the adenovirus, medium was changed again with addition of insulin and T0-901317 in all plates and CDCA in the indicated plates. 24 hours later, total RNA was harvested from the cells and real time RT-PCR was performed to determine relative ACCα mRNA levels. Data is representative of three experiments.

Figure 3.14: CDCA inhibits mature SREBP-1 via p38 MAPK, but not ERK. CEHs were plated in the absence of hormones overnight. Medium was changed with the addition of insulin and T0-901317 in all plates. 12 hours later, medium was changed again with the addition of insulin and T0-901317 in all plates. 12 hours later, DMSO (vehicle), U0126 and SB203580 were added in the indicated plates. One hour after the addition of inhibitors, CDCA was added to the indicated plates. 6 hours later, nuclear extracts were prepared and western blot analysis was performed to determine mature SREBP-1 levels. The signals for SREBP-1 were quantified by ImageQuaNT. The values for CDCA, insulin and T0-901317 treated cells were expressed as a percent of cells treated with T0-901317 and insulin. * represents significance at $p<0.05$. Data represents mean ± SE of five experiments.

Figure 3.15: CEHs were plated in the absence of hormones. Eighteen hours later, medium was changed with the addition of insulin and T0-901317 to all plates. 12 hours later, medium was changed again, with the addition of insulin and T0-901317 in all plates. 12 hours later, cells were treated with CDCA, cholic acid (CA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), taurocholic acid (TCA), hyodeoxycholic acid

(HDCA) and taurodeoxycholic acid (TDCA). The concentration of all bile acids in the medium was 75 μM. 6 hours after addition of bile acids, total RNA and total protein was harvested from cells. ACCα mRNA abundance was determined using real time RT-PCR analysis. ERK and p38 MAPK were determined by western blot analysis using antibodies as described. Data are representative of three experiments.

Figure 3.16: CEHs were plated in the absence of hormones. Eighteen hours later, medium was changed with the addition of insulin and T0-901317 to all plates. 12 hours later, medium was changed again, with the addition of insulin and T0-901317 in all plates. 12 hours later, cells were treated with CDCA, cholic acid (CA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), taurocholic acid (TCA), hyodeoxycholic acid (HDCA) and taurodeoxycholic acid (TDCA). The concentration of all bile acids in the medium was 75 μM. 6 hours after addition of bile acids, total RNA and total protein was harvested from cells. FAS, SCD, FGF-19 and ABCA1 mRNA was determined by Northern blot analysis, and PGC-1α and -1β mRNA was determined by real time RT-PCR analysis. The value of the sample treated with insulin and T0-901317 was set to 1. Data represents mean ± STDEV for FAS, SCD, FGF-19 and ABCA1 mRNA and mean ± SE of three experiments for PGC-1α and -1β.

Figure 3.17: CDCA inhibits recruitment of acetylated histones on the ACCα promoter. Chick hepatocytes were plated in presence of insulin (50 nM) and T0-901317 (6 μM) n the absence and presence of CDCA (75 μM). The association of acetylated histones H3 and H4 with ACCα and SCD gene was measured by chromatin immunoprecipitation assay as described in Methods. Immunoprecipitates were analyzed by PCR using primers described in Methods and indicated in the figure. Chromatin samples that were processed identically in the absence of primary antibody served as controls. Data are representative of three independent experiments.

FIGURES

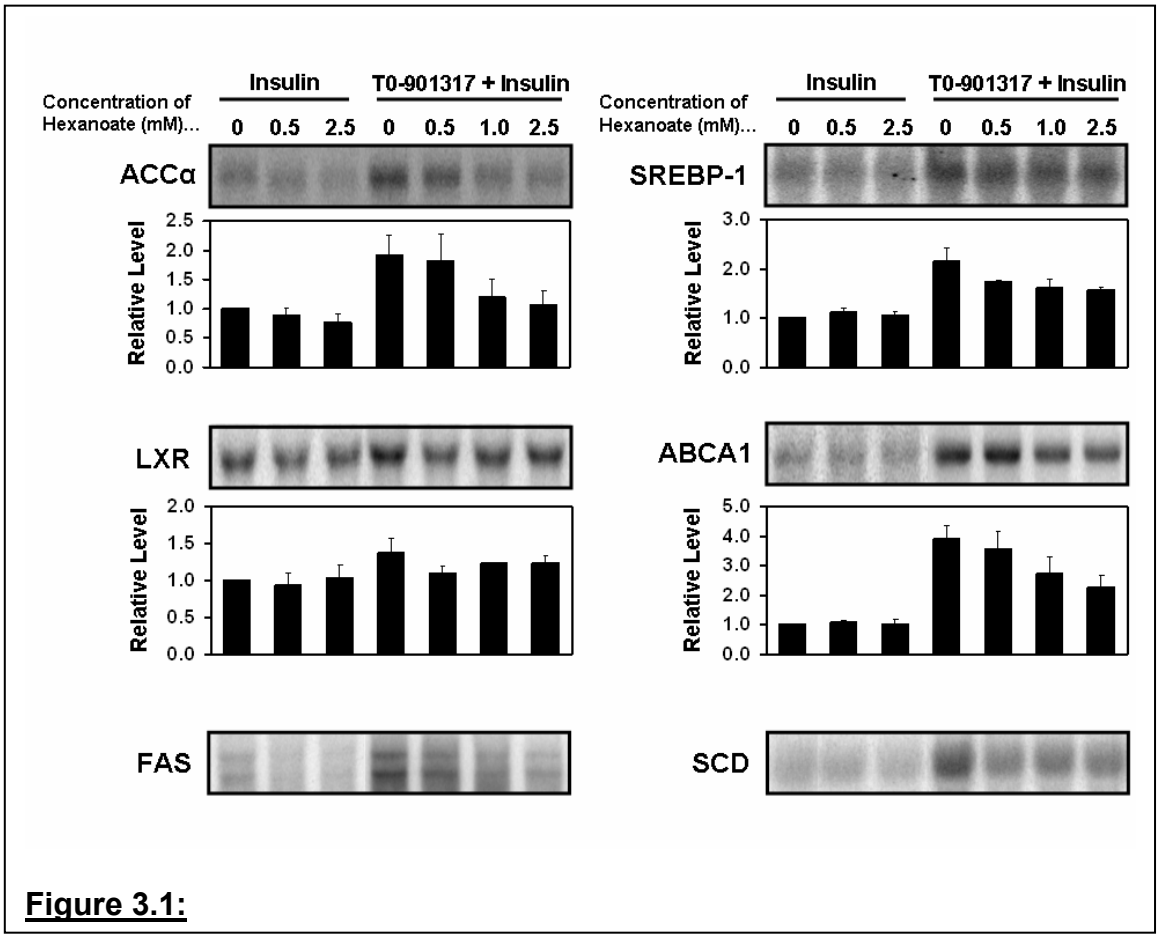


Figure 3.1:

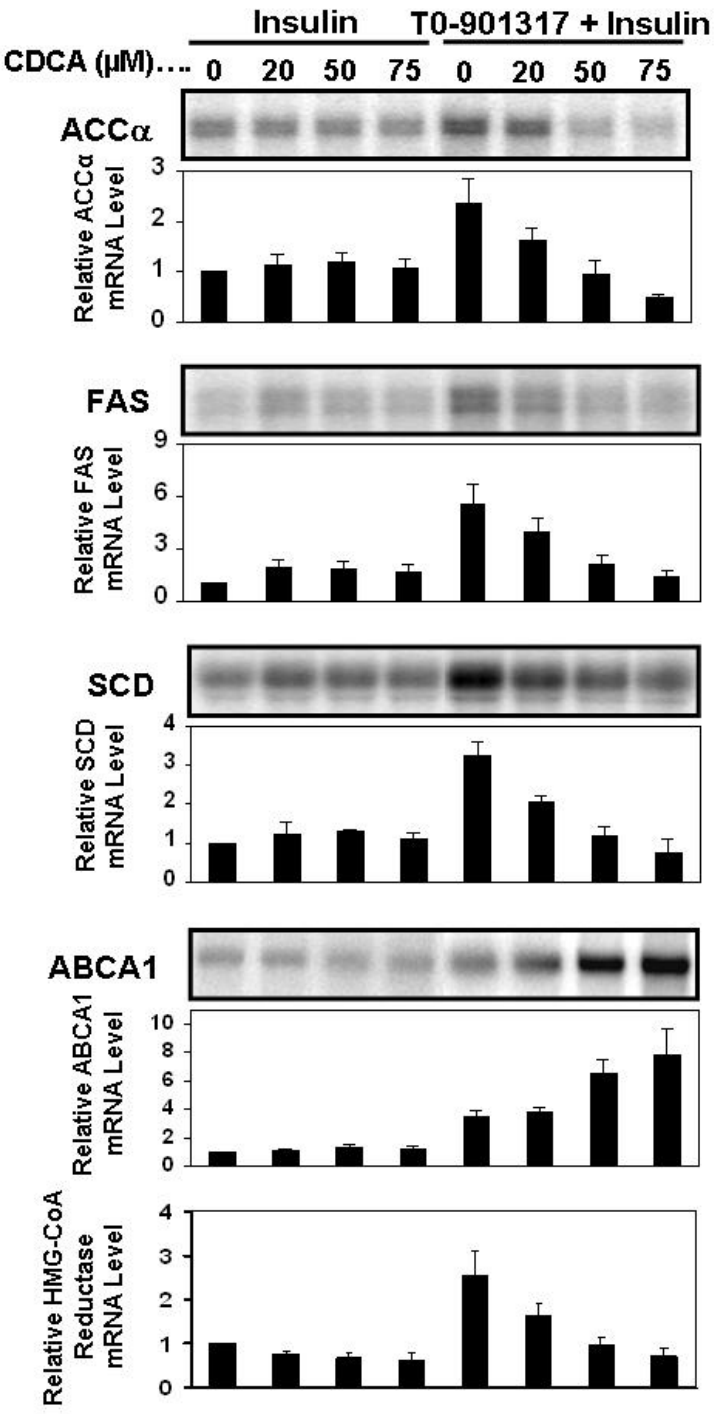

Insulin
T0-901317 + Insulin
CDCA (μM)…. 0 20 50 75 0 20 50 75
ACCα
Relative ACCα mRNA Level
3
2
1
0
FAS
Relative FAS mRNA Level
9
6
3
0
SCD
Relative SCD mRNA Level
4
3
2
1
0
ABCA1
Relative ABCA1 mRNA Level
10
8
6
4
2
0
Relative HMG-CoA Reductase mRNA Level
4
3
2
1
0

Figure 3.2

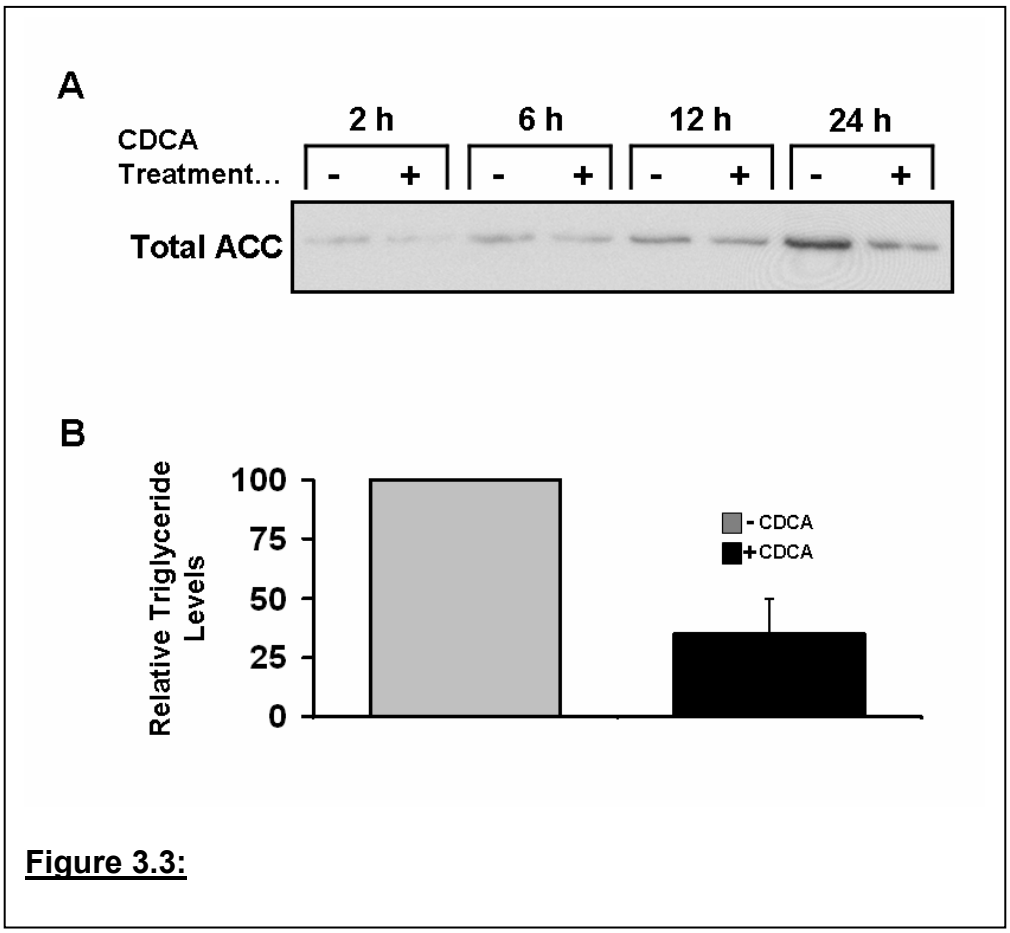

A
2 h
6 h
12 h
24 h
CDCA
Treatment…
- + - + - + - +
Total ACC
B
Relative Triglyceride Levels
100
75
50
25
0
- CDCA
+CDCA

Figure 3.3:

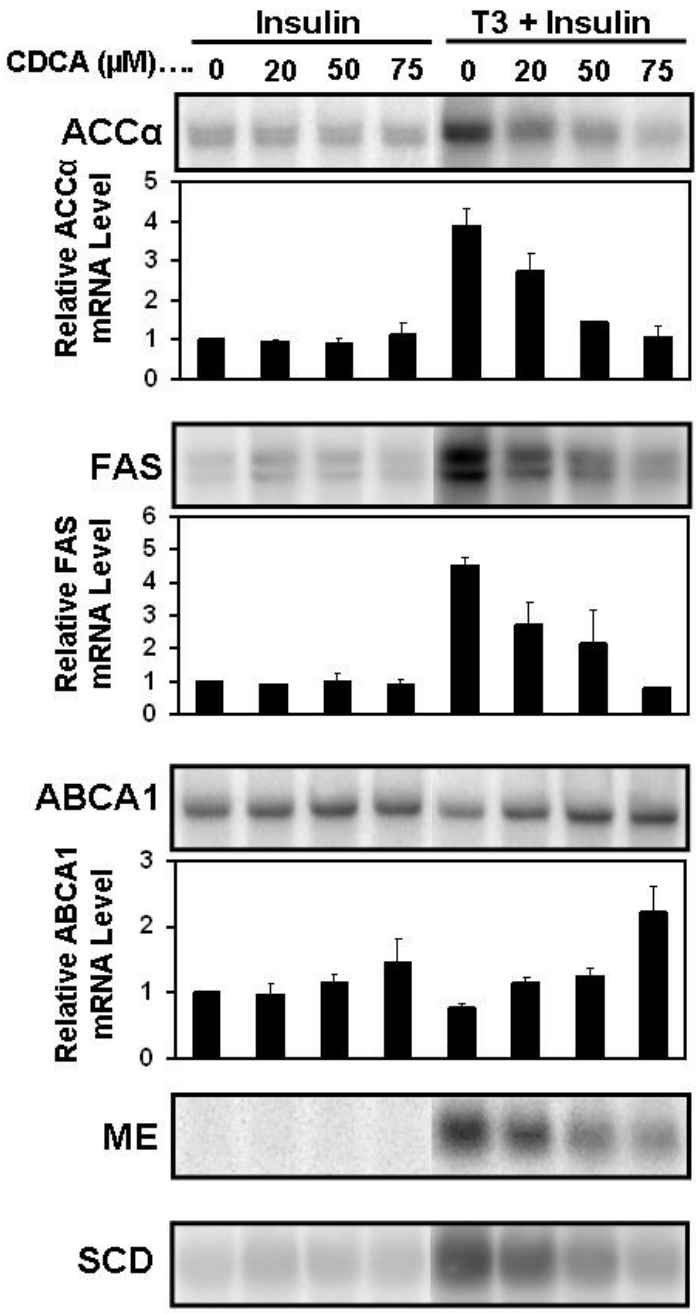

Insulin
T3 + Insulin
CDCA (μM)…. 0 20 50 75 0 20 50 75
ACCα
Relative ACCα mRNA Level
0 1 2 3 4 5
FAS
Relative FAS mRNA Level
0 1 2 3 4 5 6
ABCA1
Relative ABCA1 mRNA Level
0 1 2 3
ME
SCD

Figure 3.4

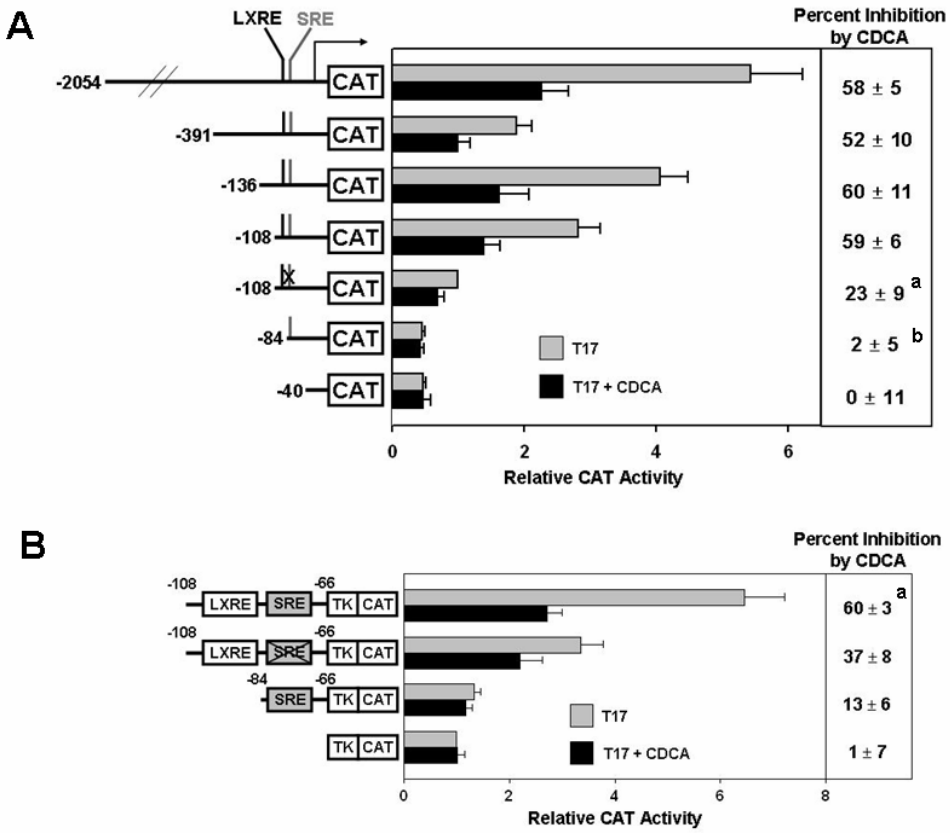

A
LXRE
SRE
Percent Inhibition by CDCA
-2054
CAT
58 ± 5
-391
CAT
52 ± 10
-136
CAT
60 ± 11
-108
CAT
59 ± 6
-108
CAT
23 ± 9 a
-84
CAT
2 ± 5 b
-40
CAT
0 ± 11
T17
T17 + CDCA
0
2
4
6
Relative CAT Activity
B
Percent Inhibition by CDCA
-108
-66
LXRE
SRE
TK
CAT
60 ± 3 a
-108
-66
LXRE
SRE
TK
CAT
37 ± 8
-84
-66
SRE
TK
CAT
13 ± 6
TK
CAT
1 ± 7
T17
T17 + CDCA
0
2
4
6
8
Relative CAT Activity

Figure 3.5:

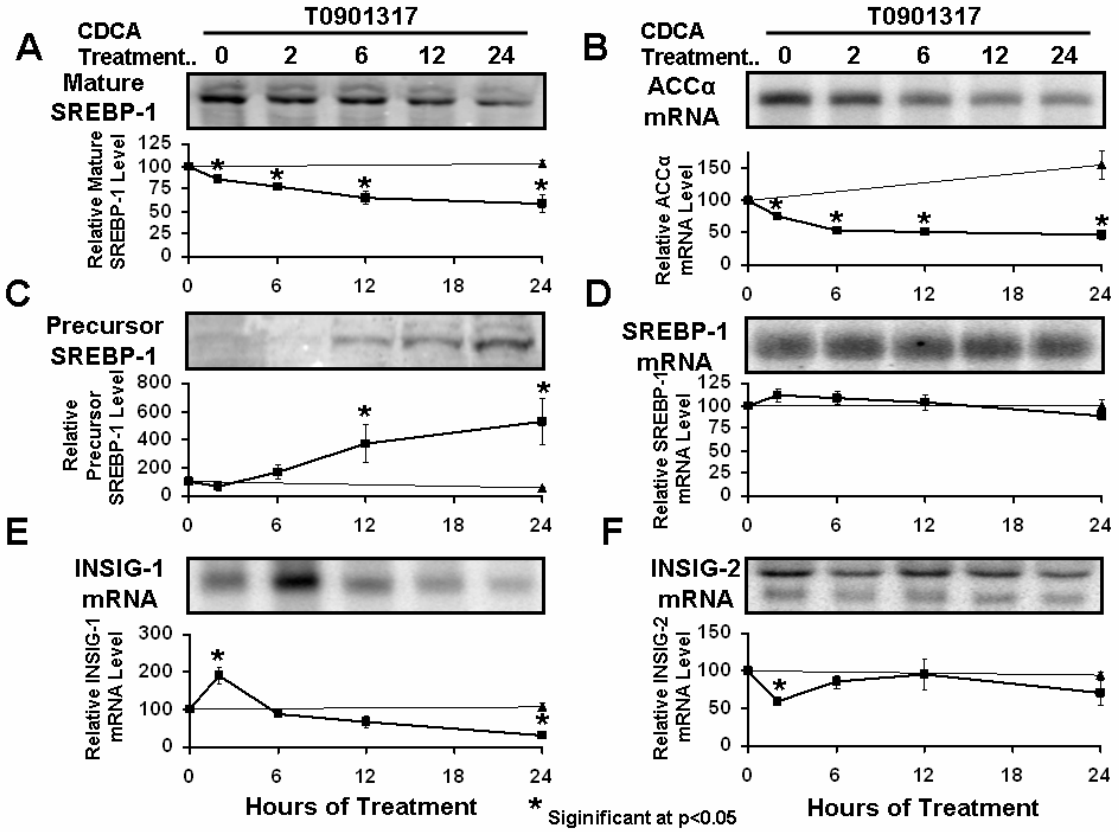

A
CDCA Treatment..
T0901317
0 2 6 12 24
Mature SREBP-1
Relative Mature SREBP-1 Level
B
ACCα mRNA
Relative ACCα mRNA Level
C
Precursor SREBP-1
Relative Precursor SREBP-1 Level
D
SREBP-1 mRNA
Relative SREBP-1 mRNA Level
E
INSIG-1 mRNA
Relative INSIG-1 mRNA Level
F
INSIG-2 mRNA
Relative INSIG-2 mRNA Level
Hours of Treatment
* Siginificant at p<0.05

Figure 3.6:

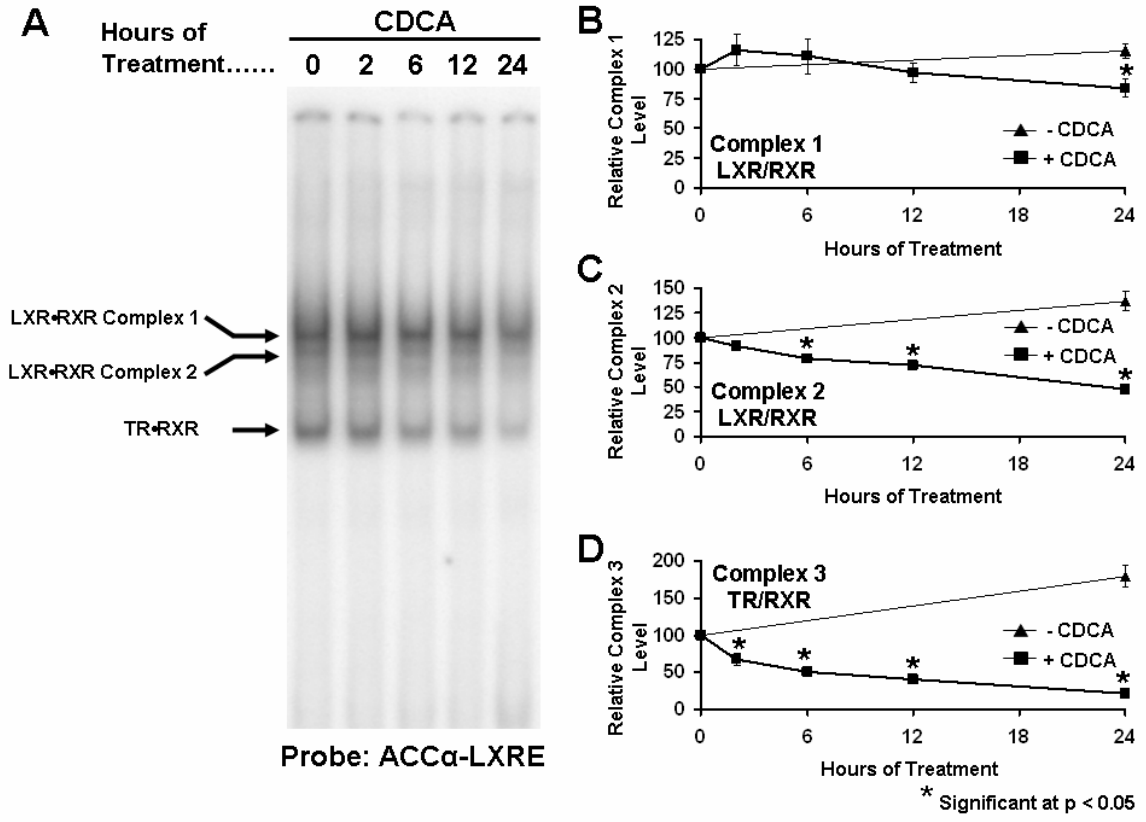

A
Hours of Treatment.....
CDCA
0 2 6 12 24
LXR•RXR Complex 1
LXR•RXR Complex 2
TR•RXR
Probe: ACCα-LXRE
B
Relative Complex 1 Level
Complex 1 LXR/RXR
- CDCA
+ CDCA
Hours of Treatment
C
Relative Complex 2 Level
Complex 2 LXR/RXR
- CDCA
+ CDCA
Hours of Treatment
D
Relative Complex 3 Level
Complex 3 TR/RXR
- CDCA
+ CDCA
Hours of Treatment
* Significant at p < 0.05

Figure 3.7:

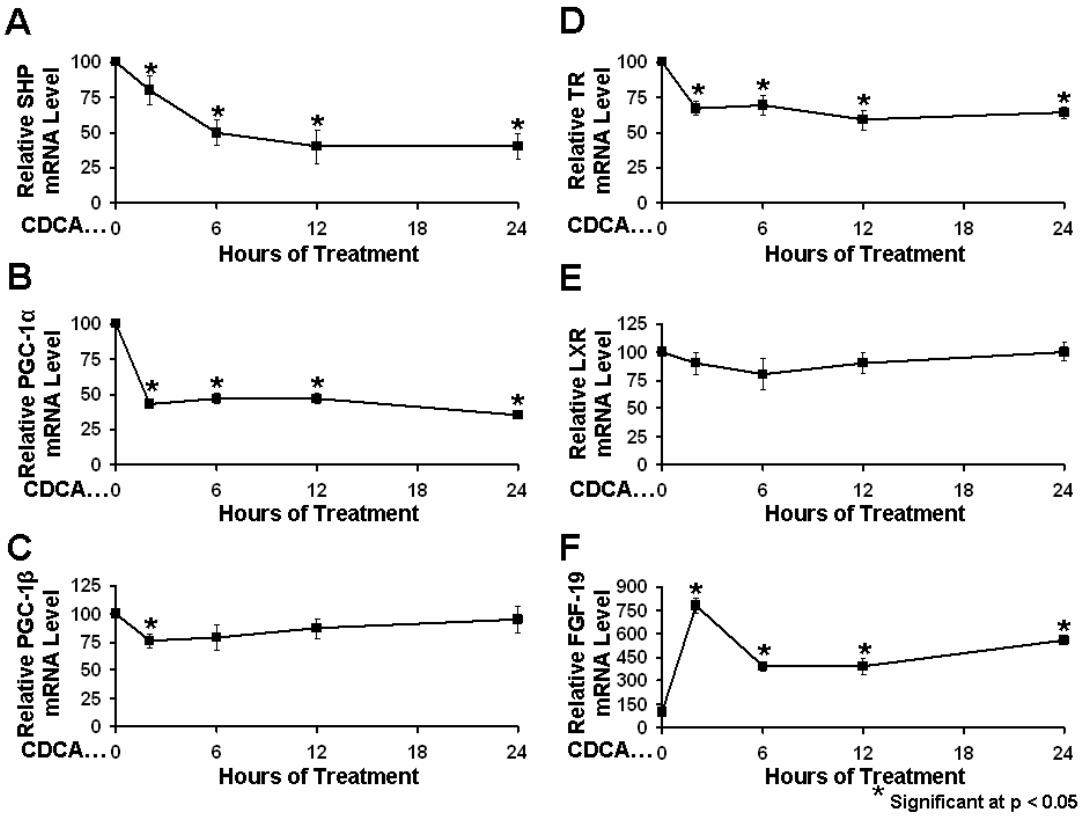

A
Relative SHP mRNA Level
100
75
50
25
0
CDCA…
0
6
12
18
24
Hours of Treatment
D
Relative TR mRNA Level
B
Relative PGC-1α mRNA Level
E
Relative LXR mRNA Level
125
C
Relative PGC-1β mRNA Level
F
Relative FGF-19 mRNA Level
900
750
600
450
300
150
* Significant at p < 0.05

Figure 3.8:

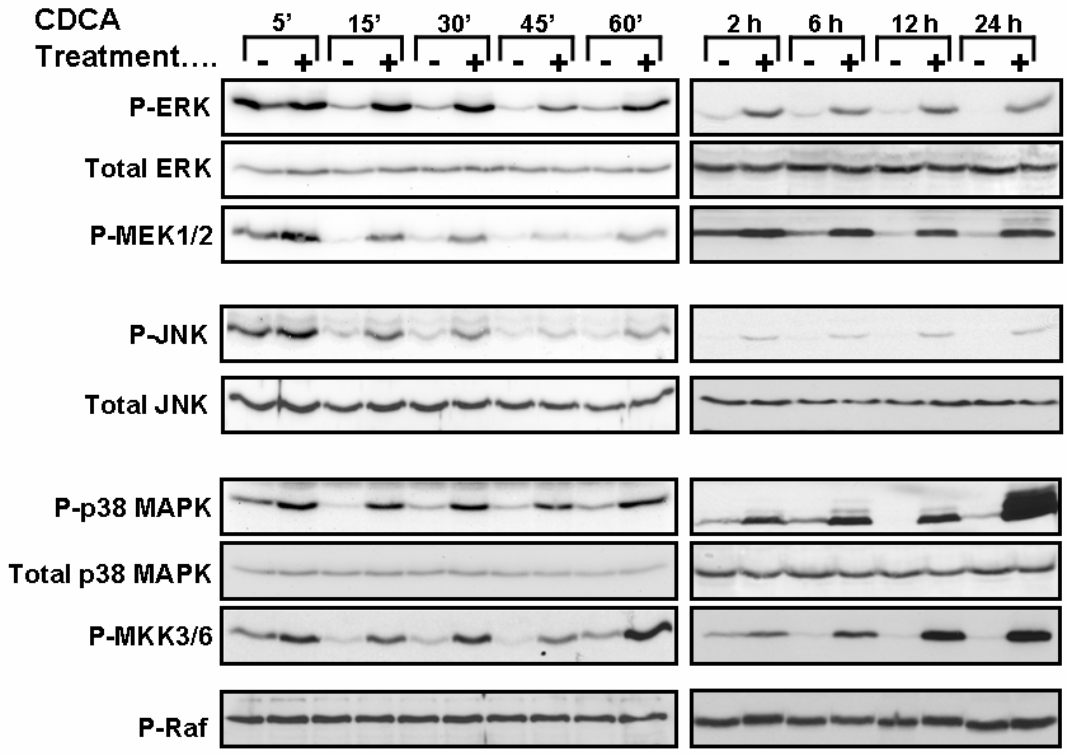
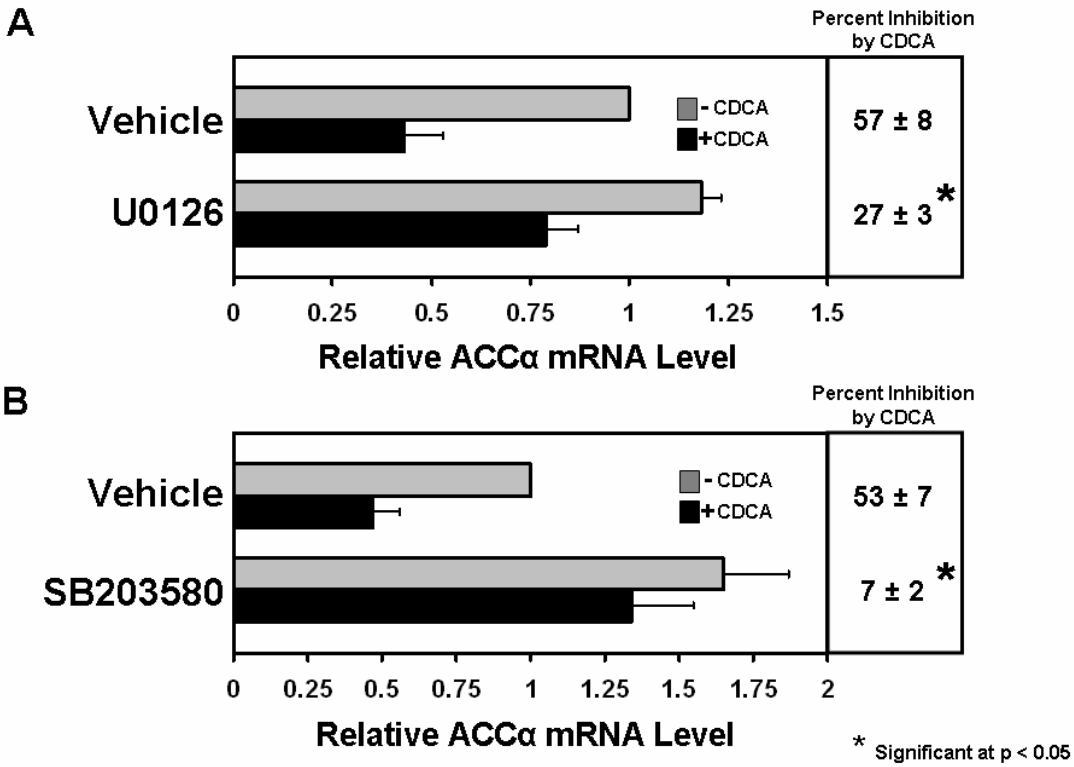

CDCA
Treatment....
5'
15'
30'
45'
60'
2 h
6 h
12 h
24 h
- + - + - + - + - + - + - + - + - +
P-ERK
Total ERK
P-MEK1/2
P-JNK
Total JNK
P-p38 MAPK
Total p38 MAPK
P-MKK3/6
P-Raf

Figure 3.9:


A
Percent Inhibition by CDCA
Vehicle
U0126
- CDCA
+CDCA
57 ± 8
27 ± 3 *
0
0.25
0.5
0.75
1
1.25
1.5
Relative ACCα mRNA Level
B
Percent Inhibition by CDCA
Vehicle
SB203580
- CDCA
+CDCA
53 ± 7
7 ± 2 *
0
0.25
0.5
0.75
1
1.25
1.5
1.75
2
Relative ACCα mRNA Level
* Significant at p < 0.05

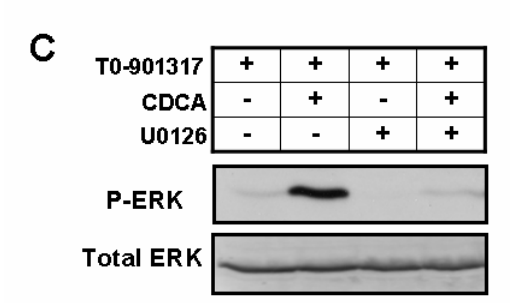

C
T0-901317 + + + +
CDCA - + - +
U0126 - - + +
P-ERK
Total ERK

Figure 3.10

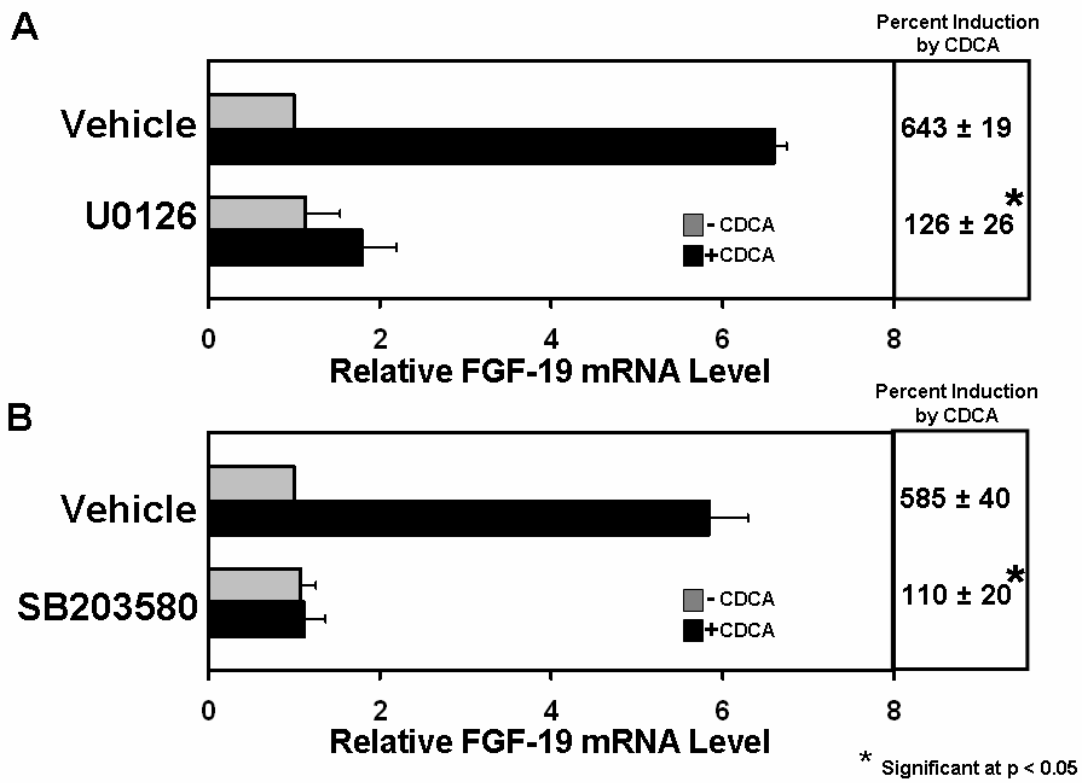

A
Percent Induction by CDCA
Vehicle
U0126
643 ± 19
126 ± 26 *
- CDCA
+CDCA
0
2
4
6
8
Relative FGF-19 mRNA Level
B
Percent Induction by CDCA
Vehicle
SB203580
585 ± 40
110 ± 20 *
- CDCA
+CDCA
0
2
4
6
8
Relative FGF-19 mRNA Level
* Significant at p < 0.05

Figure 3.11:

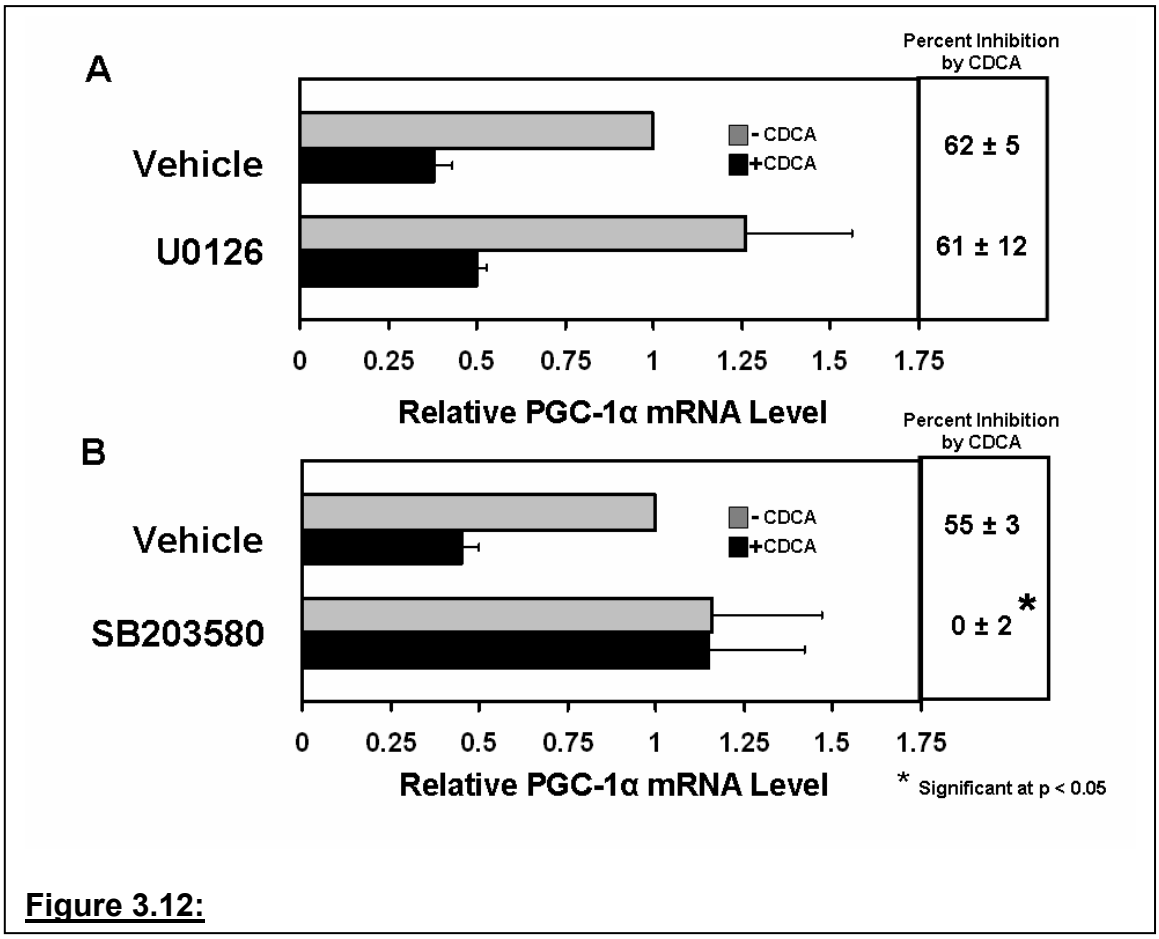

A
Percent Inhibition by CDCA
Vehicle
U0126
- CDCA
+CDCA
62 ± 5
61 ± 12
0
0.25
0.5
0.75
1
1.25
1.5
1.75
Relative PGC-1α mRNA Level
B
Percent Inhibition by CDCA
Vehicle
SB203580
- CDCA
+CDCA
55 ± 3
0 ± 2 *
0
0.25
0.5
0.75
1
1.25
1.5
1.75
Relative PGC-1α mRNA Level
* Significant at p < 0.05

Figure 3.12:

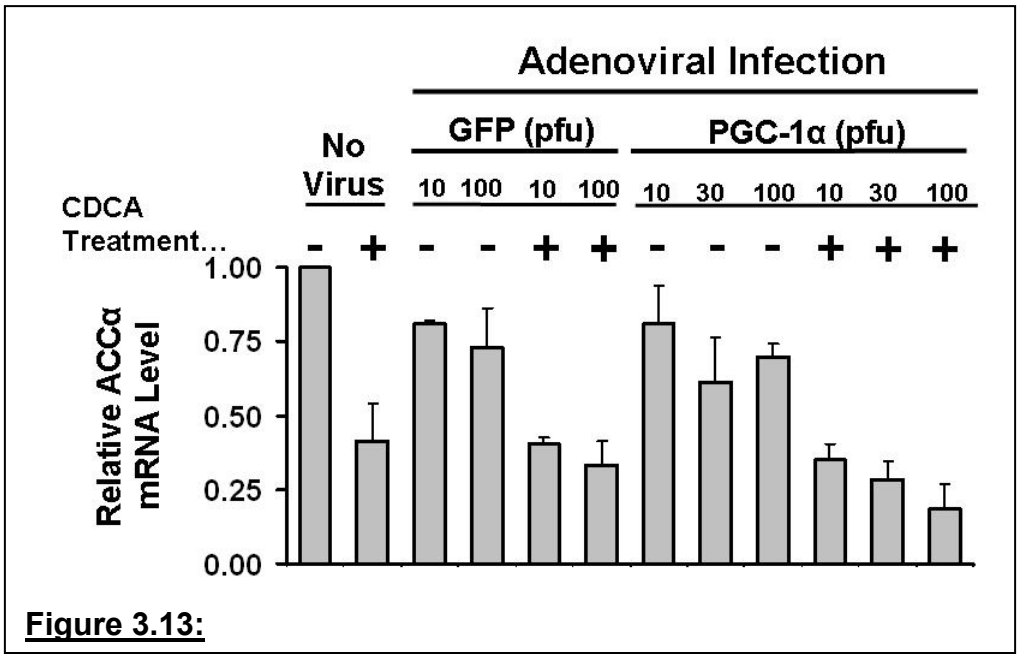

Adenoviral Infection
GFP (pfu)
PGC-1α (pfu)
No Virus
10 100 10 100
10 30 100 10 30 100
CDCA Treatment…
- + - - + + - - - + + +
Relative ACCα mRNA Level
1.00
0.75
0.50
0.25
0.00

Figure 3.13:

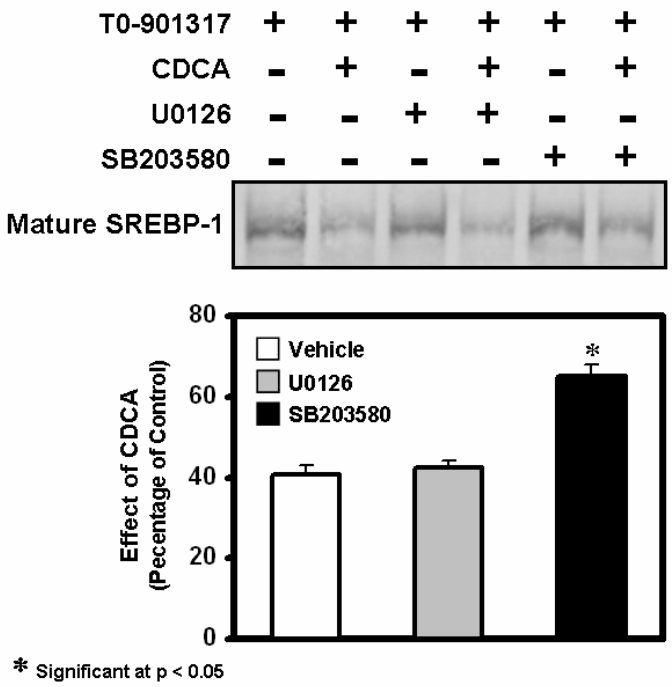

T0-901317 + + + + + +
CDCA - + - + - +
U0126 - - + + - -
SB203580 - - - - + +
Mature SREBP-1
Effect of CDCA (Pecentage of Control)
80
60
40
20
0
Vehicle
U0126
SB203580
*
* Significant at p < 0.05

Figure 3.14:

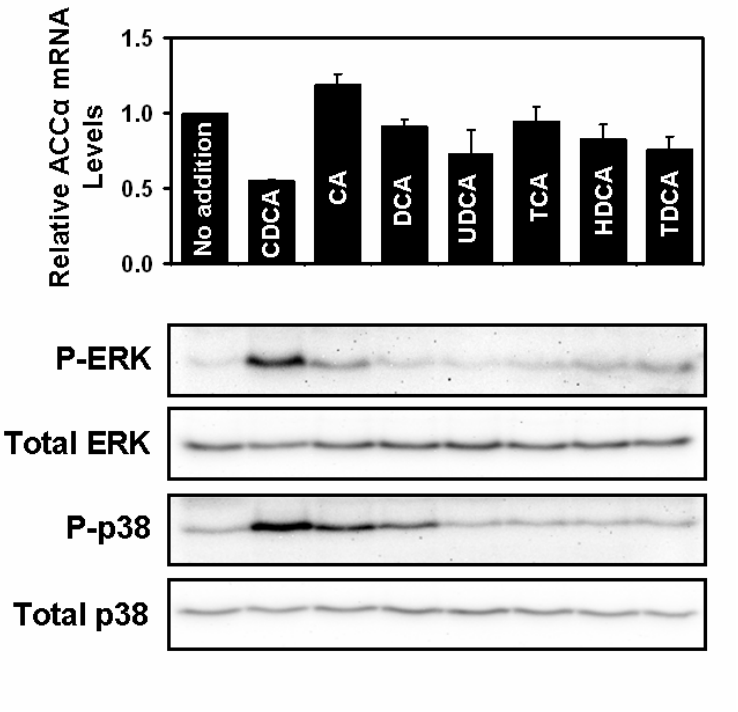

Relative ACCα mRNA Levels
1.5
1.0
0.5
0.0
No addition
CDCA
CA
DCA
UDCA
TCA
HDCA
TDCA
P-ERK
Total ERK
P-p38
Total p38

Figure 3.15:

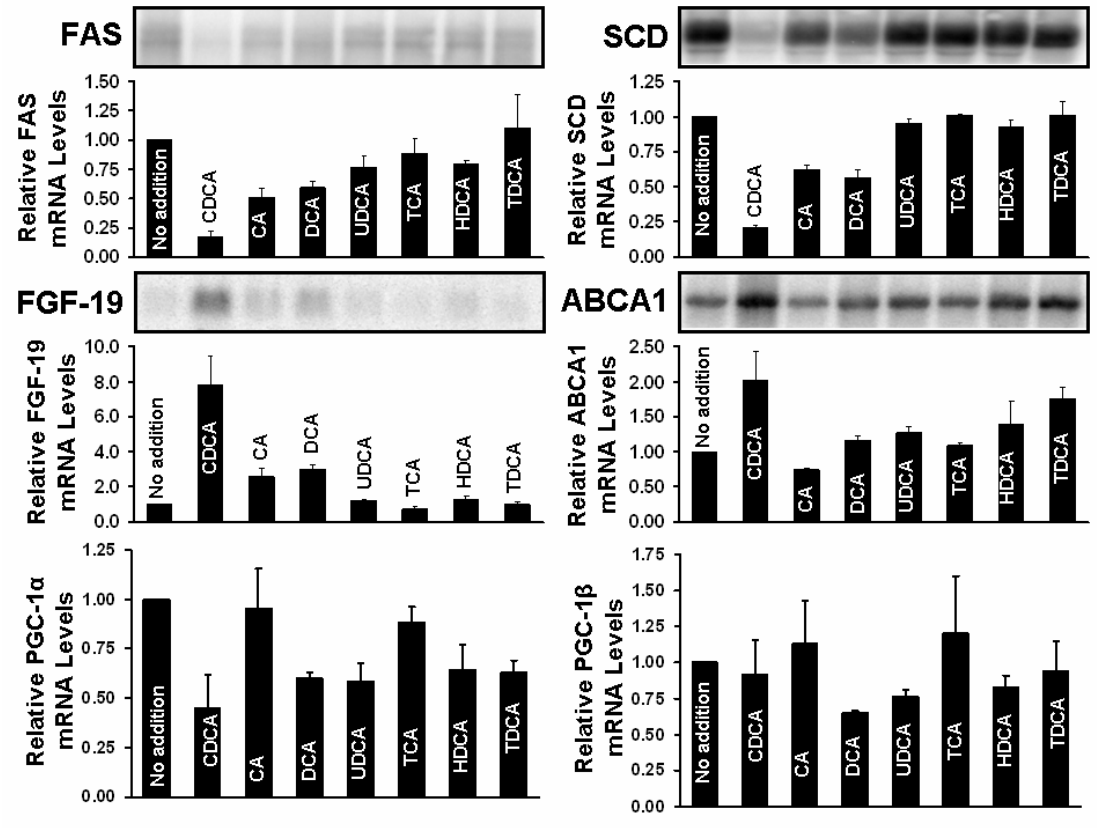

FAS
SCD
Relative FAS mRNA Levels
Relative SCD mRNA Levels
FGF-19
ABCA1
Relative FGF-19 mRNA Levels
Relative ABCA1 mRNA Levels
Relative PGC-1α mRNA Levels
Relative PGC-1β mRNA Levels
No addition
CDCA
CA
DCA
UDCA
TCA
HDCA
TDCA

Figure 3.16:

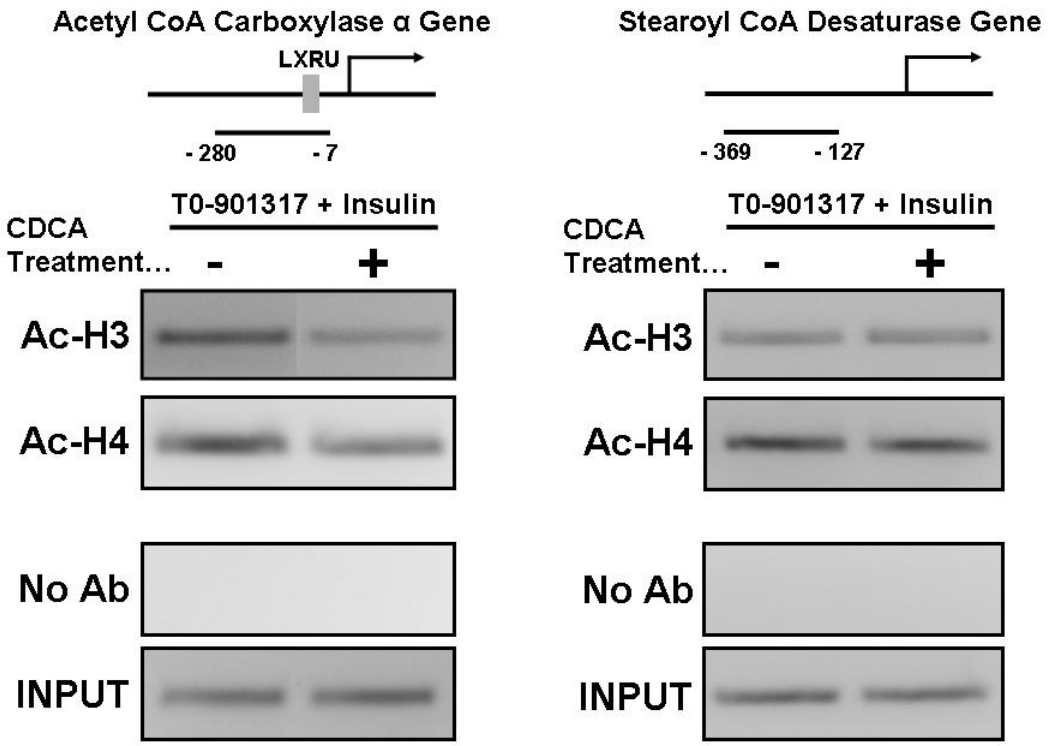

Acetyl CoA Carboxylase α Gene
LXRU
- 280
- 7
T0-901317 + Insulin
CDCA Treatment…
-
+
Ac-H3
Ac-H4
No Ab
INPUT
Stearoyl CoA Desaturase Gene
- 369
- 127
T0-901317 + Insulin
CDCA Treatment…
-
+
Ac-H3
Ac-H4
No Ab
INPUT

Figure 3.17:

Chapter 4
Appendix

RESULTS

CDCA activates PKC, but synthetic activators of PKC do not mimic the effects of CDCA in chick embryo hepatocytes

Previous reports have shown that bile acids induce the expression of inflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin (IL-1β). These cytokines are recognized by high affinity receptors on hepatic parenchymal cells, that activate protein kinase-C (PKC) (13). Activation of PKC has been shown to increase Mg^{2+} accumulation in hepatocytes via ERK and p38 MAPK (18). We wanted to determine whether bile acids activate PKC in chick hepatocytes, and whether activation of PKC results in ACCα mRNA inhibition via ERK and/or p38 MAPK. Chick hepatocytes were plated and treated with CDCA and inhibitors as described in Chapter 3. 6 hours after addition of CDCA, total protein was harvested from cells. Western blot analysis showed that CDCA increases phoshorylation and activation of phospho-PKC at 6 h **Figure 4.1**. ERK inhibitor U0126, and p38 MAPK inhibitor SB203580 did not reverse the effects of CDCA on PKC activation. These results suggest that PKC activation by CDCA is not mediated by ERK and p38 MAPK and that both these MAPKs are probably downstream of PKC.

Next, we wanted to determine whether phorbol esters that are very well documented activators of PKC (11, 12, 20) were able to mimic the inhibitory effects of CDCA on ACCα mRNA expression and activation of ERK and/or p38 MAPK. Chick hepatocytes were treated as described above. 12-O-tetradecanoyl-phorbol-13-acetate (TPA) was added to plates as indicated and total RNA and protein was isolated at the indicated time points. ACCα mRNA was detected using real time RT-PCR. TPA had no effect on ACCα mRNA both at 6 h and 12 h. Moreover, addition of the p38 MAPK inhibitor SB203580, also had no effect on ACCα mRNA in the presence of TPA. To determine whether TPA activated PKC and ERK and/or p38 MAPK, we performed western blot analysis to determine the levels of phospho-PKC, phospho-ERK and phospho-p38 MAPK. Our results showed that TPA had no effect on the phosphorylation

of PKC, ERK and p38 MAPK (Data not shown). One explanation for this observation is that TPA is not active in chick hepatocytes.

To further determine the role of PKC in mediating CDCA inhibition of ACCα, we incubated chick hepatocytes in presence of insulin and T0-901317, insulin and T0-901317 and CDCA, and insulin and T0-901317 and CDCA plus the PKC inhibitor GF-109203X. The PKC inhibitor did not attenuate or abolish the effects of CDCA on ACCα mRNA (Data not shown). It is possible that GF-109203X is not active in chick hepatocytes or that PKC does not mediate CDCA inhibition of ACCα. At present our results are inconclusive.

CDCA increases phosphorylation of AMP-activated protein kinase in presence of insulin and T0-901317

We have shown in Chapter 3 that CDCA inhibits ACCα mRNA levels by inhibiting mature SREBP-1. We wanted to determine whether CDCA activates other agents that inhibit SREBP-1 expression. Reports in the literature has shown that phosphorylated active AMP-activated protein kinase (P-AMPK) inhibits SREBP-1 (22). Further, studies have also shown that activators of AMPK such as 5-aminoimidazole-4-carboxamide 1-beta-ribofuranoside (AICAR), and the alkaloid drug berberine, inhibits triglyceride and cholesterol synthesis (1, 6, 16). LKB1 is upstream kinase of AMPK (8, 15, 19). CDCA increased phosphorylation of AMPK at Thr172 at 6 h, 12 h and 24 h **Figure 4.2**. The activation of AMPK at 6 h was preceded by an increase in phosphorylation and activation of the AMPK upstream kinase, LKB1. CDCA activates LKB1 at 2 h and the activation is sustained through 24 h. CDCA also activated phosphorylation of ACC at Ser79. Phosphorylation of ACC at Ser 79 is a direct measure of AMPK activity (1, 3, 6). As we showed previously in Chapter 3, CDCA inhibited total ACC levels. Phospho ACC/total ACC ratio was greater than 1.5 from 6 h. CDCA had no effect on total AMPK levels.

AICAR has no effect on ACCα mRNA abundance in presence of insulin and T0-901317

Since CDCA increases AMPK activity, we wanted to determine whether known activators of AMPK such as AICAR, can mimic inhibitory effects of CDCA on ACCα mRNA. We incubated chick hepatocytes in the presence of insulin, insulin and T0-901317 for 40 hours. AICAR was added to the plates after 40 hours. 6 hours after addition of AICAR, total RNA was isolated and real time RT-PCR was performed to determine ACCα mRNA. Surprisingly, AICAR had no effect on ACCα mRNA abundance in presence of insulin and T0-901317 and insulin **Figure 4.3**.

Next, we wanted to determine whether AICAR phosphorylated AMPK, ACC protein and p38 MAPK. Cells were plated and treated with AICAR as described above. Total protein was harvested from the cells 6 hours after addition of AICAR. Western blot analysis showed that AICAR had no effect on phosphorylation of AMPK, ACC and p38 MAPK. The lack of effect of AICAR on AMPK is surprising as AICAR is a very well documented activator of AMPK. These results suggest that increase in AMPK activity by CDCA does not mediate inhibition of ACCα mRNA.

AICAR inhibits T3-induced ACCα mRNA expression by activating AMPK, p38 MAPK and inhibiting mature SREBP-1

Our results showed that CDCA inhibits both T0-901317 and T3 induced expression of lipogenic genes in chick hepatocytes. We also showed that CDCA activated P-AMPK in presence of T0-901317. However, AICAR that is an activator of AMPK, had no effect on ACCα expression or AMPK activation in presence of insulin and insulin and T0-901317. We therefore wanted to determine whether AICAR inhibits ACCα mRNA in presence of T3 by activating AMPK. Chick hepatocytes were plated and treated as described. 40 hours after stimulation with T3, AICAR was added in the indicated concentrations. 6 hours after AICAR addition, total protein and RNA was isolated. Western blot analysis was performed to determine P-AMPK, P-ACC and P-p38 MAPK, and real time RT-PCR was performed to analyze ACCα mRNA levels.

Consistent with our earlier findings, AICAR had no effect on P-AMPK, P-ACC, P-p38 MAPK and ACCα mRNA in presence of insulin **Figure 4.4**. Surprisingly, AICAR phosphorylated and activated AMPK and p38 MAPK, and phosphorylated ACC. AICAR also inhibited mature, active SREBP-1 protein. The increase in p38 MAPK activity and

decrease of mature SREBP-1 by AICAR is correlated with an inhibition of ACCα mRNA inhibition by AICAR in presence of T3 in a dose dependent manner. These data provide evidence that AMPK mediates the inhibitory effect of CDCA on T3-induced ACCα mRNA expression, but not T0-901317-induced ACCα mRNA expression. The implications of AMPK activation and specificity of effects are unclear.

It has already been established that feeding a high carbohydrate diet causes an increase in insulin and T3 that mediate an increase in lipogenic gene transcription in liver (4, 5, 10). Increase in T3 concentration in liver activates the transcription of the genes for ACCα (9), fatty acid synthase (17) and malic enzyme (14). Feeding carbohydrate also increases the levels of nuclear T3 receptors (TRs) in liver that may result in the stimulation of lipogenic gene transcription by dietary carbohydrate (2). Thus inhibition of lipogenic gene expression in presence of insulin and T3 by bile acids may act to inhibit lipogenic gene transcription in a feedback mechanism.

CDCA inhibits T3-induced ACCα mRNA via a cis acting element on the promoter

We have previously shown that T0-901317 and CDCA regulation on ACCα is mediated by a 23 bp region (-101 to -71 bp) in promoter 2 of the ACCα gene. This region consists of a DR-4 element (-101 to -86 bp) that binds heterodimers comprised of TR•RXR and LXR•RXR and a SRE (-82 to -71 bp) that binds SREBP-1. To determine whether the functional interaction between the ACCα T3RE and SRE required the presence of additional cis-acting sequences, hepatocytes were transfected with constructs containing fragments of the ACCα gene linked to the minimal promoter of the herpes simplex virus thymidine kinase (TK) gene. The TK promoter alone was unresponsive to CDCA **Figure 4.5**. When a DNA fragment containing both the ACCα T3RE and ACCα SRE (-108 to -66 bp) was linked to the TK promoter, treatment with CDCA caused a 50% decrease in promoter activity. Mutation of the ACCα SRE in the context of the ACCα -108 to -66 bp fragment had no change in CDCA responsiveness. Similarly, when the ACCα T3RE was linked to the TK promoter, there was no change in promoter activity by CDCA. When a DNA fragment containing the ACCα SRE alone (-84 to -66 bp) was appended to the TK promoter, CDCA treatment almost completely abolished ACCα promoter activity.

CDCA modulates the binding of nuclear receptor complexes to the ACCα LXRE/T3RE in presence of insulin and T3

In previous work, we showed that the ACCα LXRE/T3RE (-101 to -86 bp) bound four protein complexes in nuclear extracts prepared from chick embryo hepatocytes (). 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 the absence of nuclear receptor agonists, complexes 1 and 2 were the predominant complexes that bound to the ACCα LXRE/T3RE. T3 treatment caused an increase in the binding of complexes 3 and 4. These findings have led us to propose that changes in the binding of nuclear receptor complexes to the ACCα-LXRE/T3RE play a role in mediating the activation of ACCα transcription by T3. In this experiment, we wanted to determine whether treatment with AICAR inhibited the binding of nuclear receptor heterodimers on the ACCα-LXRE/T3RE. Gel mobility shift assays were performed using nuclear extracts from chick embryo hepatocytes treated insulin alone, insulin and T0-901317 and insulin and T3, in the absence and presence of AICAR. As reported previously, a DNA probe containing the ACCα LXRE/T3RE bound to complex 1, complex 2, and complex 4 in nuclear extracts from hepatocytes incubated in the absence of nuclear receptor agonists.

There was no change in binding activity of complex 1 in presence of insulin, and insulin and T0-901317, in the absence and presence of AICAR **Figure 4.6**. T3 causes a small increase in complex 1 binding activity in presence of T3 and AICAR caused a small reduction in binding of complex 1. However, the decrease in complex 1 binding activity by AICAR in presence of T3 is not significant. There was no change in binding activity of complex 2 in presence of insulin and insulin and T0-901317 in the absence and presence of AICAR. Treatment of T3 did not change binding activity of complex 2 in the absence of AICAR. However, AICAR caused a slight reduction in complex 2 binding activity in presence of insulin and T3.

T0-901317 caused an increase in binding activity of complex 4. This result is consistent with that shown in Chapter 2. AICAR had no effect on complex 4 binding activity in presence of insulin and insulin and T0-901317. T3 caused a 4-fold increase in

complex 4 binding activity. This result is consistent previous findings from our lab that showed complex 4 mediates the increase in enhancer activity of ACCα-LXRE/T3RE in presence of T3 complex 4 mediate the increase in enhancer activity of ACCα-LXRE/T3RE caused by the addition of T3 (21). AICAR caused a 75% inhibition of complex 4 binding activity. This result shows that AICAR inhibits ACCα mRNA abundance by inhibiting TR•RXR heterodimers recruitment to the ACCα-LXRE/T3RE in presence of insulin and T3.

CDCA increases FGF-19 mRNA in presence of insulin and T3

Our data shows that bile acids inhibit T0-901317-induced ACCα mRNA and that FGF-19 mRNA may mediate this process. We have also shown that bile acids inhibit T3-induced ACCα mRNA expression. We therefore wanted to determine whether FGF-19 may mediate this effect in presence of T3. Consistent with results reported for human hepatocytes, and in chick hepatocytes in presence of insulin and T0-901317, CDCA increased FGF-19 mRNA abundance in chick hepatocytes in a dose dependent manner in absence and presence of T3 **Figure 4.7**. This result would further suggest that CDCA inhibits T0-901317- and T3-induced ACCα mRNA expression by increasing FGF-19.

Recombinant human FGF-19 has no effect on gene expression in chick hepatocytes

We investigated whether recombinant human FGF-19 modulated lipogenic gene expression in chick hepatocytes. Addition of FGF-19 at concentrations that were within or exceeded the physiological range had no effect on mRNA abundance of ACCα, ME, FAS, SCD, ATP-CL, SREBP-1 and ABCA1 in the absence or presence of T0-901317 or T3 **Figure 4.8**. One interpretation of this finding is that FGF-19 is not a mechanism mediating the effects of bile acids on lipogenic gene expression in chick hepatocytes. However, it is possible that human recombinant FGF-19 (61% sequence homology with chicken FGF-19) is not active in the chicken system or that recombinant FGF-19 derived from bacteria has low biological activity due to improper folding. This experiment was performed in collaboration with Sushant Bhatnagar in our lab.

It has been reported that FGF-19 has a unique loop that binds heparin. This loop accounts for the affinity of FGF-19 for the receptor FGFR4. Studies have shown that

heparin also facilitates functioning of FGF-19 (7). We treated chick hepatocytes with insulin and T0-901317 and heparin in the absence and presence of recombinant human FGF-19. FGF-19 had no effect on ACCα mRNA abundance even in the presence of heparin.

Hepatocytes infected with adenovirus vectors expressing chicken FGF-19 inhibits ACCα mRNA expression

As discussed previously, recombinant human FGF-19 had no effect on ACCα mRNA abundance. To establish a role of FGF-19 in mediating inhibition of ACCα mRNA by CDCA we infected hepatocytes with adenovirus containing chicken FGF-19. Cells were also infected with adenovirus containing no DNA (null) and no virus as controls. CDCA inhibits ACCα mRNA in uninfected cells and cells infected with adenovirus containing null virus **Figure 4.9**. Cells infected with chicken FGF-19 virus inhibited ACCα mRNA in a dose dependent manner. Cells treated with adenovirus containing FGF-19 had a 10-25-fold increase in FGF-19 mRNA levels over control cells. Western blot analysis using an antibody against chicken FGF-19 (provided by Dr. Ladher) to detect overexpression of FGF-19 protein was inconclusive. Adenovirus was prepared by Callee M. Walsh in Dr. Salati's lab.

Effect of the specific JNK inhibitor SP600125 on CDCA mediated inhibition of lipogenic genes

Previous work has established that bile acids activate JNK and that this pathway is involved in the bile acid-mediated inhibition of cholesterol 7α hydroxylase transcription in human hepatocytes. We have confirmed this finding in chick hepatocytes. To investigate the role of JNK in mediating the reduction of lipogenic gene expression caused by bile acids, we used a specific inhibitor of JNK, SP600125 to measure lipogenic gene expression.

Consistent with our previous experiments, CDCA inhibits both T3 and T0-901317-induced ACCα, FAS and SCD expression, and increases FGF-19 expression (Lanes 3 and 7) **Figure 4.10**. SP600125 inhibits expression of ACCα and FAS in

presence of both T3 and T0-901317 compared to T3 and T0-901317 alone (Lanes 2 & 6 vs. Lanes 1 & 5). In presence of T3, SP600125 has no effect on expression of SCD, but in presence of T0-901317, SP600125 inhibits expression of SCD (Lanes 2 & 6). In presence T0-901317, SP600125 increases expression of FGF-19 (Lane 6), but SP600125 does not increase expression of FGFG-19 in presence of T3.

In presence of CDCA, SP600125 has an additional inhibitory effect on expression of ACCα, FAS and SCD than CDCA alone, and an additional inductive effect on expression of FGF-19 than CDCA alone, both in the presence of T3 or T0-901317 (Lanes 5 & 11). SP600125 decreases expression of lipogenic genes by almost half and increases FGF-19 expression two fold, with respect to CDCA alone, in presence of T0-901317. Results from this experiment are inconsistent with bile inhibiting lipogenic gene expression via activation of JNK. Results from this experiment suggest that JNK mediates activation of lipogenic genes by T0-901317 and T3. These results would also suggest that JNK inhibits activation of FGF-19, at least in presence of T0-901317. However, this experiment provides further data of an inverse correlation between lipogenic gene expression and FGF-19 expression in chicken embryo hepatocytes.

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FIGURE LEGENDS

Figure 4.1: CDCA activates PKC and phorbol esters have no effect on ACCα mRNA abundance. Chick hepatocytes were plated in presence of insulin (50 nM) and T0-901317 (6 μM). 12 hours later medium was changed with the addition of insulin and T0-901317. 24 hours later, medium was changed again, with the indicated treatments. Total protein and RNA was harvested and western blot and real time RT-PCR was performed.

Figure 4.2: CDCA activates AMPK signaling pathway. CEH were plated in absence of hormones overnight. Medium was changed with addition of insulin (50 nM) and T0-901317 (6 μM) in all plates. 24 hours later medium was changed again with addition of insulin and T0-901317 in all plates and CDCA in the indicated plates. Total protein was isolated from cells at the indicated time points and western blot was performed using antibodies as described in Chapter 3.

Figure 4.3: AICAR has no effect on ACCα mRNA abundance, AMPK and p38 MAPK in presence of insulin and T0-901317. Cells were plated in absence of hormones. 16 hours later medium was changed with addition of insulin (50 nM) and T0-901317 (6 μM) in all plates. 24 hours later, medium was changed again with addition of insulin and T0-901317. 18 hours later, AICAR was added in the indicated concentration. 6 hours after AICAR addition, total RNA and protein was harvested and A) real time RT-PCR analysis was performed to determine ACCα mRNA abundance and B) western blot analysis was performed to determine P-AMPK, P-ACC and P-p38.

Figure 4.4: AICAR activates AMPK signaling pathway, inhibits mature SREBP-1 and inhibits ACCα mRNA abundance in presence of insulin and T3. CEH were plated and in absence of hormones overnight. Medium was changed with addition of insulin (50 nM) and T3 (1.5 μM) in all plates. 24 hours later, medium was changed again with the same

treatments. 18 hours later, AICAR was added to the indicated plates and concentration as indicated. 6 hours later, total RNA, total protein and nuclear protein was harvested. Data represents mean ± SE of three experiments. * represents significance at $p<0.05$.

Figure 4.5: Fragments of the ACCα gene containing the T3RE and/or SRE-1 were linked to the minimal thymidine kinase (TK) promoter in TKCAT. CEHs were transiently transfected with these constructs and treated with T3 (1.5 μM) and insulin (50 nM) in the absence or presence of CDCA as described in Part A (above) and under Experimental Procedures. Left: Constructs used in these experiments. Numbers indicate the 5' and 3' boundaries of ACCα DNA relative to the transcription initiation site of promoter 2. Right: CAT activity in CEH transfected with p[ACC-TKCAT and treated with T3 and insulin was set at 1, and the other activities were adjusted proportionately. The results are the means ± SEM of three experiments.

Figure 4.6: Effect of CDCA on the binding of hepatic nuclear proteins to the ACCα T3RE in presence of insulin and T3. A) Eighteen hours after being placed in culture, CEHs were incubated in Waymouth's medium containing insulin (50 nM), and T3 (1.5 μM), with or without CDCA 75 μM for the indicated times. Cells were harvested and 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 T3RE (-108 to -82 bp). Specific protein-DNA complexes are indicated by arrows. Previous studies have shown that complexes 1 and 2 contain liver X receptor (LXR)•retinoid X receptor (RXR) heterodimers, whereas complex 4 contains nuclear T3 receptor (TR)•RXR heterodimers. These data are representative of three experiments employing independent preparations of nuclear extract. B) Signal from the complexes was quantified using ImageQuaNT software. The value for the samples treated with insulin and T3 at 0 h was set to 1. Data represents mean ± SE of three experiments.

Figure 4.7: CDCA increases expression of fibroblast growth factor 19 (FGF-19) in the absence and presence of T3 in a dose dependent manner. Northern Blot analysis was performed to determine mRNA levels. Chicken embryo hepatocytes were treated with the indicated amounts of CDCA, insulin (50 nM) and T3 (1.5 μM). Data shows mean ± standard deviation of two experiments. The values for mRNA abundance for the treatments have been calculated with respect to insulin treatment, the value of which was set to 1.

Figure 4.8: Recombinant human FGF-19 has no effect on gene expression. CEH were plated in absence of hormones. 16 hours later, medium was changed with addition of insulin (50 nM), T0-901317 (6 μM) and T3 (1.5 μM). FGF-19 was added to the indicated plates at indicated concentrations. 24 hours later total RNA was isolated. A) Northern blot analysis was performed to measure relative mRNA abundance of the genes. This part of the experiment was performed in collaboration with Sushant Bhatnagar. B) Real time RT-PCR was performed to measure relative mRNA levels of ACCα.

Figure 4.9: Hepatocytes infected with adenovirus expressing chicken FGF-19 inhibit ACCα mRNA expression. CEH were plated in the absence of hormones. Four hours later, medium was changed with the addition of insulin (50 nM) and T0-901317 (6 μM) in all plates and infected with adenovirus containing the indicated proteins. The amount of adenovirus is used in plaque forming units (pfu). Hepatocytes that were uninfected by virus and treated in parallel served as controls. 24 hours after addition of the adenovirus, medium was changed again with addition of insulin and T0-901317 in all plates and CDCA (75 μM) in the indicated plates. 24 hours later, total RNA was harvested from the cells and real time RT-PCR was performed to determine relative ACCα and FGF-19 mRNA levels. Data is representative of three experiments. * Significant at $p < 0.05$.

Figure 4.10: Effects of the JNK inhibitor SP600125 on gene expression. CEH were plated in the absence of hormones overnight. Medium was changed with addition of insulin (50 nM) in all plates and T3 (1.5 μM) and T0-901317 (6 μM) in the indicated plates. SP600125 and CDCA were added to the plates indicated. 24 hours later total RNA was isolated and Northern Blot analysis was performed to determine relative mRNA levels.

FIGURES

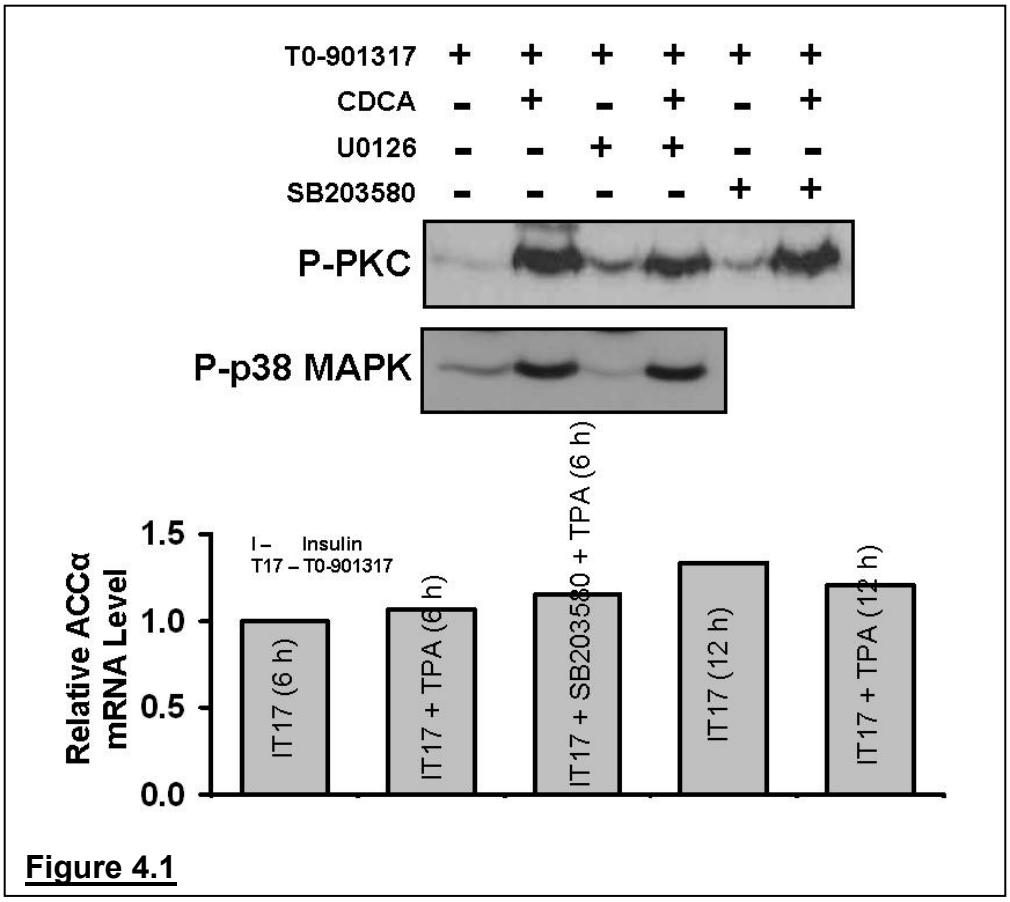

Figure 4.1

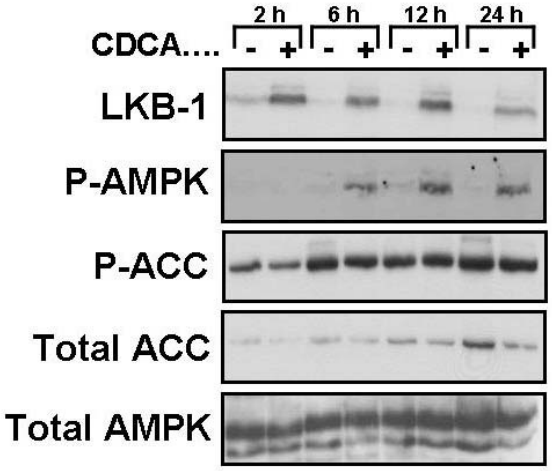

Figure 4.2

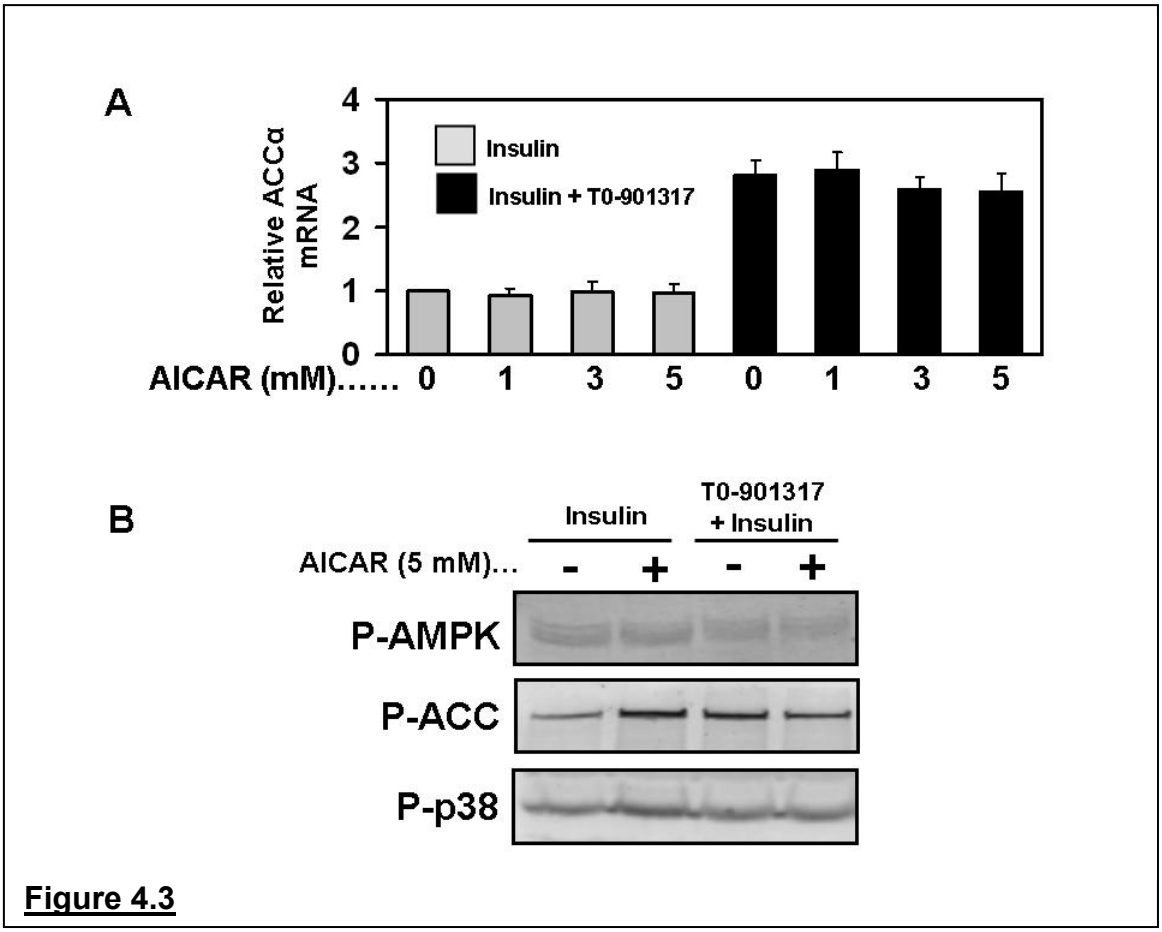
A
Relative ACCα mRNA
4
3
2
1
0
Insulin
Insulin + T0-901317
AICAR (mM)..... 0 1 3 5 0 1 3 5
B
Insulin
T0-901317 + Insulin
AICAR (5 mM)... - + - +
P-AMPK
P-ACC
P-p38

Figure 4.3

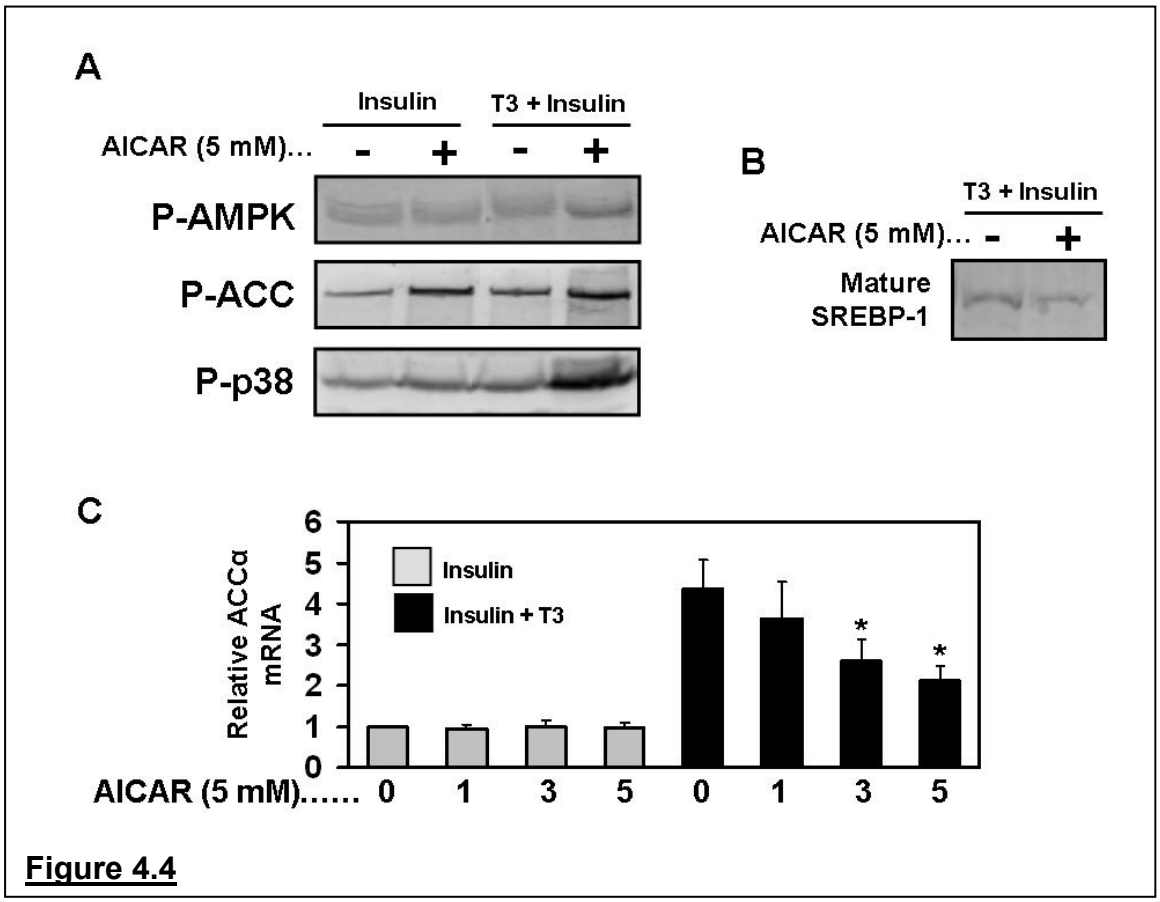
A
Insulin
T3 + Insulin
AICAR (5 mM)…
-
+
-
+
P-AMPK
P-ACC
P-p38
B
T3 + Insulin
AICAR (5 mM)…
-
+
Mature
SREBP-1
C
Relative ACCα mRNA
Insulin
Insulin + T3
0
1
2
3
4
5
6
*
*
AICAR (5 mM)……
0
1
3
5
0
1
3
5

Figure 4.4

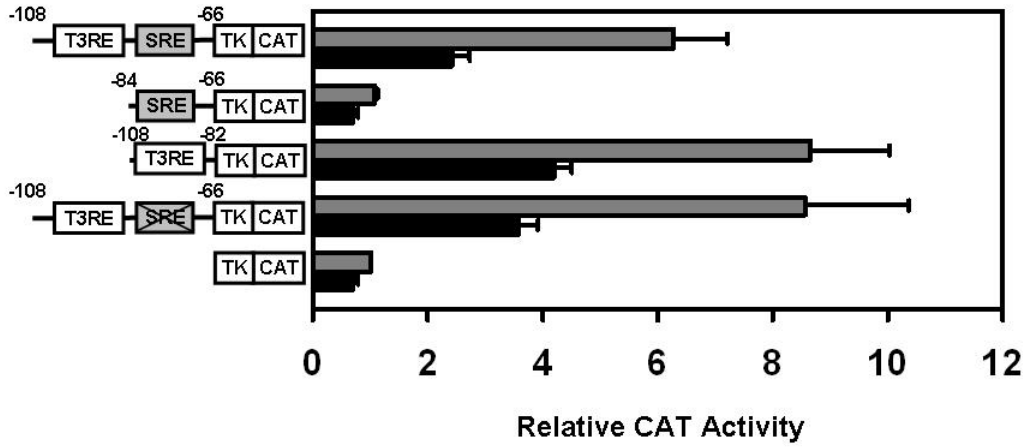
-108
-66
T3RE
SRE
TK
CAT
-84
-66
SRE
TK
CAT
-108
-82
T3RE
TK
CAT
-108
-66
T3RE
SRE
TK
CAT
TK
CAT
0
2
4
6
8
10
12
Relative CAT Activity

Figure 4.5

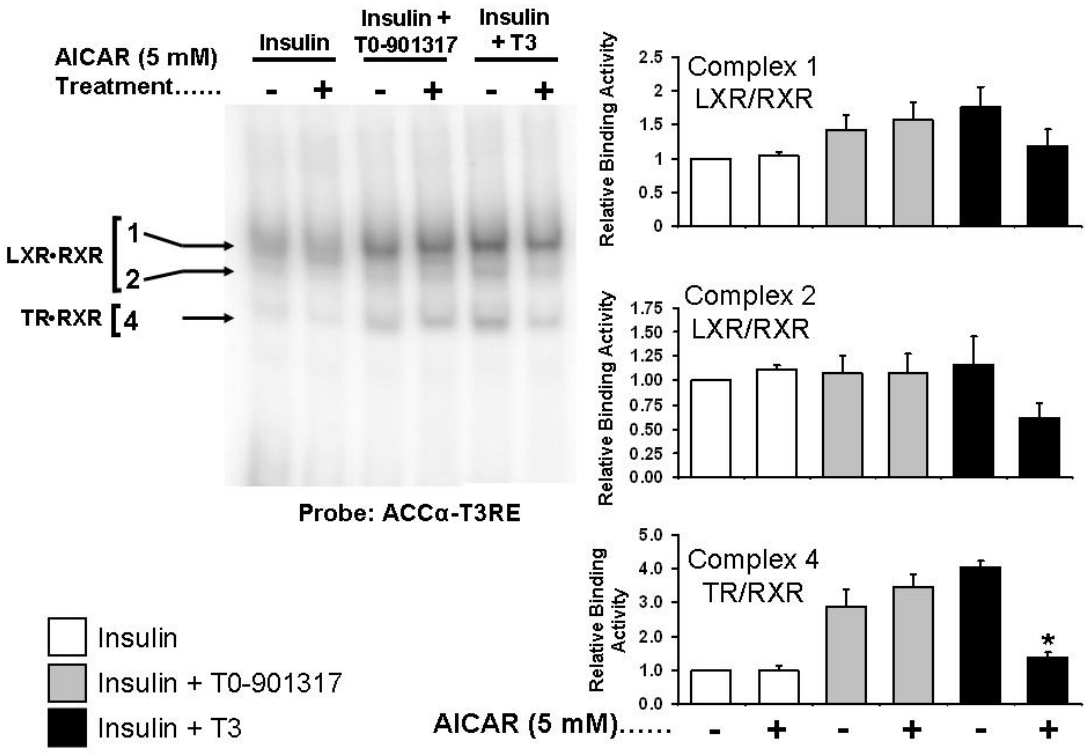
AICAR (5 mM)
Treatment.....
Insulin
Insulin + T0-901317
Insulin + T3
- + - + - +
LXR•RXR
1
2
TR•RXR
4
Probe: ACCα-T3RE
Insulin
Insulin + T0-901317
Insulin + T3
Relative Binding Activity
Complex 1
LXR/RXR
Complex 2
LXR/RXR
Complex 4
TR/RXR
*
AICAR (5 mM).....
- + - + - +

Figure 4.6

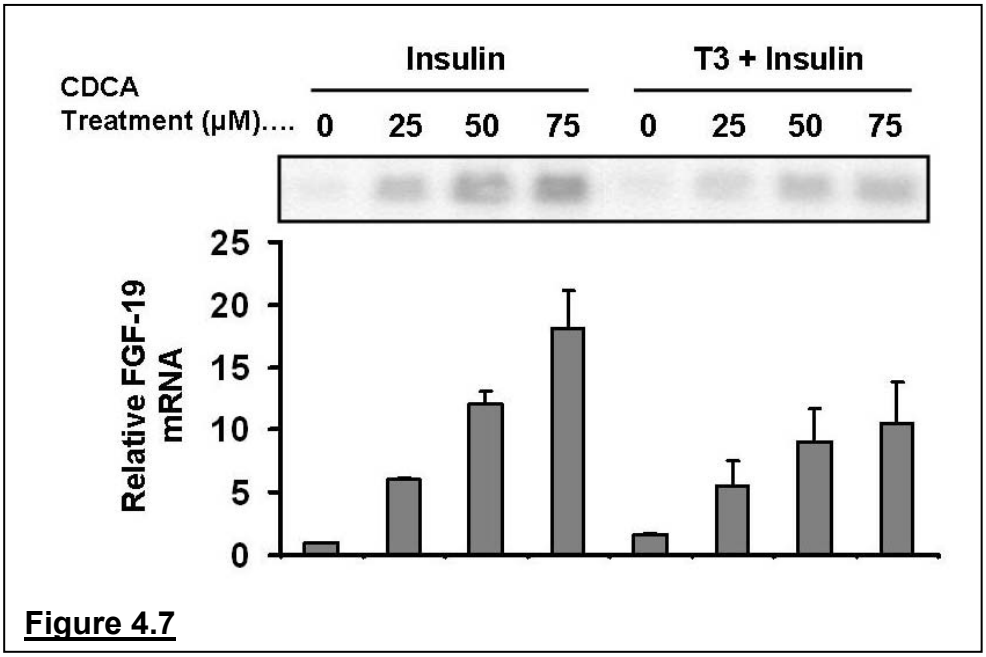
Insulin
T3 + Insulin
CDCA
Treatment (μM)....
0
25
50
75
0
25
50
75
Relative FGF-19 mRNA
25
20
15
10
5
0

Figure 4.7

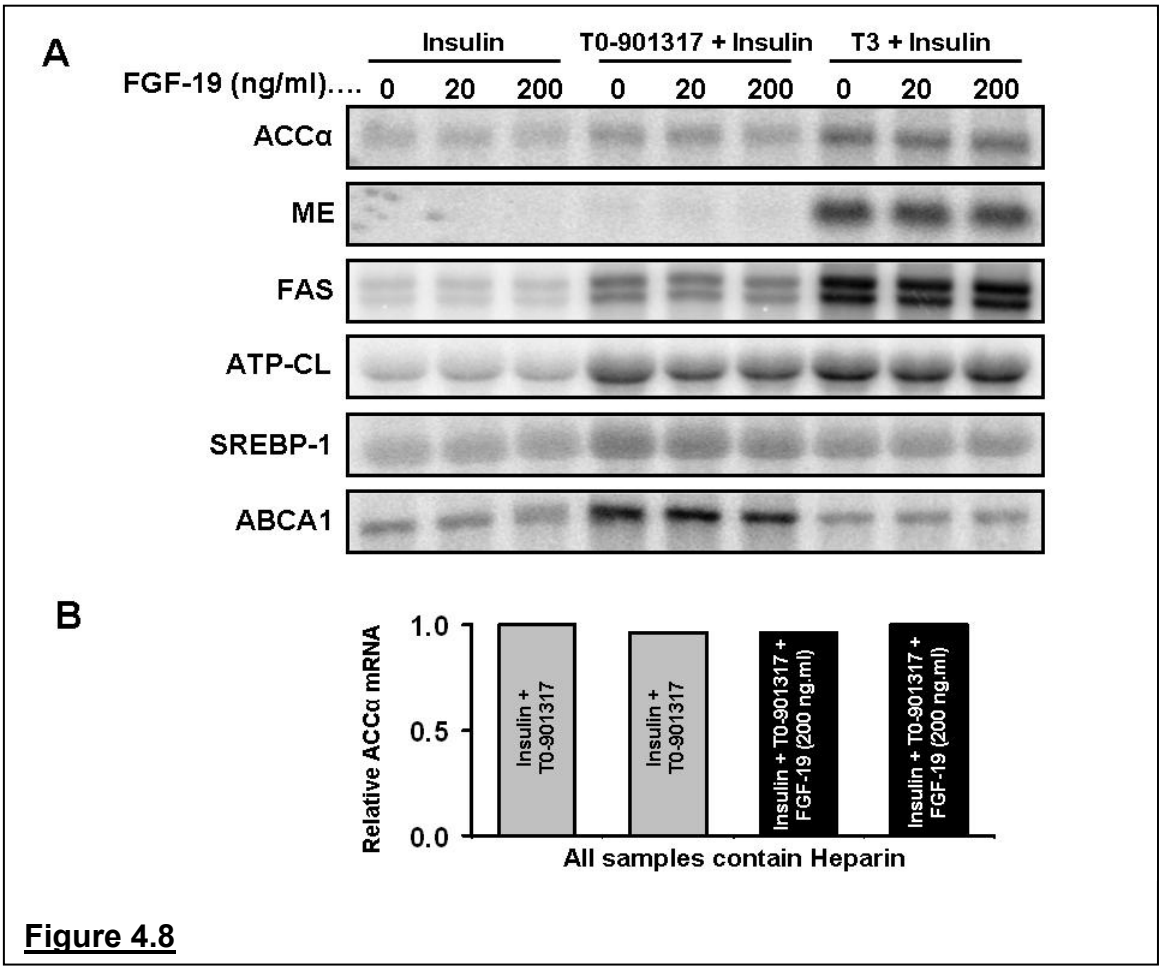
A
Insulin
T0-901317 + Insulin
T3 + Insulin
FGF-19 (ng/ml)....
0
20
200
0
20
200
0
20
200
ACCα
ME
FAS
ATP-CL
SREBP-1
ABCA1
B
Relative ACCα mRNA
1.0
0.5
0.0
Insulin + T0-901317
Insulin + T0-901317
Insulin + T0-901317 + FGF-19 (200 ng.ml)
Insulin + T0-901317 + FGF-19 (200 ng.ml)
All samples contain Heparin

Figure 4.8

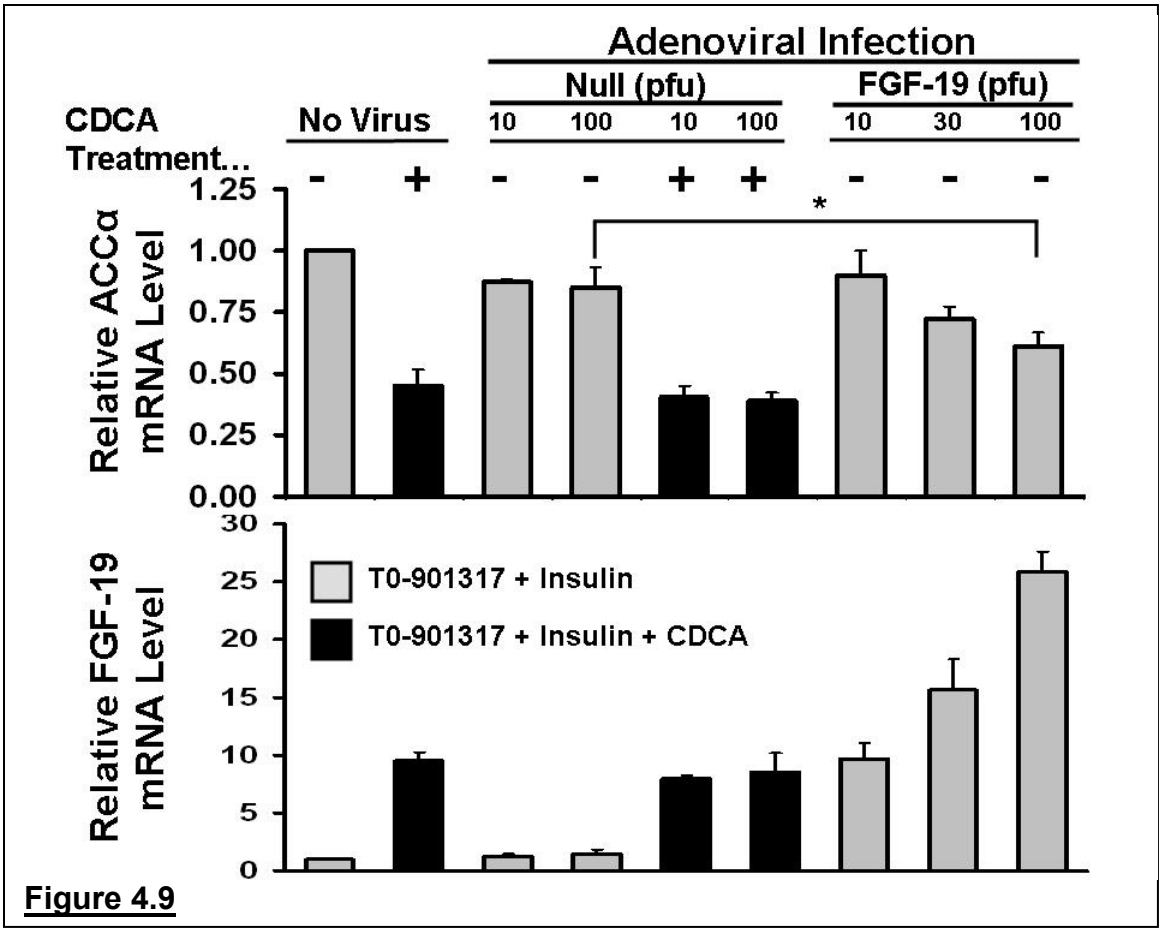
Adenoviral Infection
Null (pfu)
FGF-19 (pfu)
CDCA Treatment…
No Virus
10
100
10
100
10
30
100
- + - - + + - - -
*
Relative ACCα mRNA Level
1.25
1.00
0.75
0.50
0.25
0.00
Relative FGF-19 mRNA Level
30
25
20
15
10
5
0
T0-901317 + Insulin
T0-901317 + Insulin + CDCA

Figure 4.9

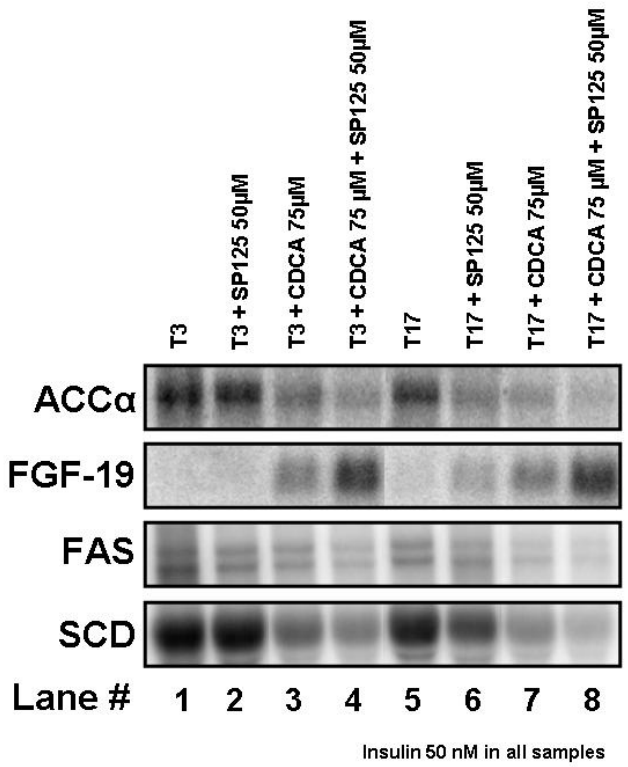

Figure 4.10