EFFECTS OF LONG-CHAIN FATTY ACIDS ON LIPID METABOLIZING GENES AND HIGH-DENSITY LIPOPROTEIN CHOLESTEROL PRODUCTION IN CULTURED HUMAN AND RAT HEPATOCYTES

By

ELIZABETH SARAH GREENE

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2006

To my parents, Dave and Hilary Johnson, for instilling in me a love of learning and supporting me through my seemingly never-ending quest for knowledge.

ACKNOWLEDGMENTS

I would like to thank my supervisory committee chair, Dr. Lokenga Badinga, for allowing me the opportunity to be a part of his laboratory. I am grateful for his support and advice in helping me become a better researcher. I would also like to thank my committee members, Dr. Joel Brendemuhl, Dr. Bobbi Langkamp-Henken and Dr. Charles Staples for their guidance and dedication to my education.

I would also like to thank the other members of the laboratory, Carlos Rodriguez-Sallaberry and Cristina Caldari-Torres for many important problem-solving and stress-relieving conversations during morning coffee breaks. Special thanks go to Teri Woodham for being one of the best friends anyone could ever ask for. Additionally, I could not have succeeded without all of the friends I made during the last four years. Finally, I thank my husband, Nic, for his love, patience, strength, and never-ending faith in my abilities. His belief in me made everything possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS KEY

AA	arachidonic acid
ABCA1	adenosine triphosphate-binding cassette transporter-A1
ACC	acetyl-CoA carboxylase
ACO	acyl-CoA oxidase
ACP	acyl carrier protein
AI	adequate intake
Apo	apolipoprotein
BMI	body mass index
CoA	coenzyme A
CHD	coronary heart disease
CLA	conjugated linoleic acid
CM	chylomicron
CPT-I or -II	carnitine-palmitoyl transferase I or -II
CVD	cardiovascular disease
DGAT	diacylglygerol acyltransferase
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
ETF	electron transfer flavoprotein

FA	fatty acid
FABPc	cytosolic fatty acid binding protein
FAS	fatty acid synthase
GLA	gamma-linolenic acid
HDL	high-density lipoprotein
HMG-R	3-hydroxy, 3-methylglutaryl CoA reductase
HODE	hydroxyoctadecadienoic acid
IUPAC	International Union of Pure and Applied Chemistry
LA	linoleic acid
LCAT	lecithin:cholesterol acyltransferase
LNA	linolenic acid
LPL	lipoprotein lipase
MGAT	monoacylglycerol acyltransferase
MTP	microsomal transfer protein
MUFA	monounsaturated fatty acid
NCEP	National Cholesterol Education Program
NEFA	non-esterified fatty acid
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
PUFA	polyunsaturated fatty acid
RXR	retinoid X receptor
SCD	stearyl-CoA desaturase
ST	stearic acid

TAG	triacylglycerol
TPN	total parenteral nutrition
TZD	thiazolidinediones
VLDL	very low-density lipoprotein

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

EFFECTS OF LONG-CHAIN FATTY ACIDS ON LIPID METABOLIZING GENES AND HIGH-DENSITY LIPOPROTEIN CHOLESTEROL PRODUCTION IN CULTURED HUMAN AND RAT HEPATOCYTES

By

Elizabeth Sarah Greene

May 2006

Chair: Lokenga Badinga
Major Department: Animal Sciences

A series of experiments were conducted to examine the short-term effects of long-chain fatty acids (FA) on acyl CoA oxidase (ACO), 3-hydroxy, 3-methylglutaryl CoA reductase (HMG-R) and apolipoprotein A-I (Apo A-I) gene expression, and high-density lipoprotein cholesterol (HDL-C) production in HepG2 (human) and H-4-II-E (rat) hepatocytes. In the three experiments, the FA studied were 1) FA of differing saturation and chain length, 2) conjugated linoleic acid (CLA), and 3) *cis* (c9, c11) and *trans* (t9, t11) isomers of octadecenoic acid.

In HepG2 cells, ACO mRNA was up-regulated by *trans*-10, *cis*-12 CLA and *cis*-vaccenic acid (c11). HMG-R gene expression was increased by stearic acid (ST) and *trans*-10, *cis*-12 CLA. Steady-state levels of Apo A-I mRNA were increased by all FA in the first experiment, *trans*-10, *cis*-12 CLA, and c11. HDL-C was decreased only by *cis*-9, *trans*-11 CLA. In H-4-II-E cells, ACO mRNA was up-regulated by LA, CLA, ST,

oleic acid, and *trans*-vaccenic acid (t11). HMG-R gene expression was increased by ST, CLA isomers, and t11. Apo A-I was increased by ST and EPA, but decreased by CLA and *cis* and *trans* monounsaturated FA. HDL-C was increased by LNA in the first experiment.

Based on these findings, we investigated the possibility that the FA effects are mediated by peroxisome proliferator-activated receptor α (PPARα). In HepG2 cells, activation or inhibition of PPARα had minimal effects on basal or FA-effects on gene expression, consistent with the low-levels of endogenous PPARα in this cell line. In H-4-II-E cells, activation of PPARα increased the abundance of basal ACO mRNA, enhanced the effect of ST on ACO and Apo A-I mRNA, and enhanced the effects of t11 on ACO, HMG-R, and Apo A-I gene expression. Inhibition of PPARα decreased basal expression of ACO and attenuated the effects of ST and t11 on ACO and effects of *trans*-10, *cis*-12 CLA on Apo A-I gene expression. These results indicate that specific FAs may regulate lipid-metabolizing genes in the liver through a PPARα-dependent mechanism. Because of different responses to FA in human and rat cell lines, however, net effects are likely species specific.

CHAPTER 1
INTRODUCTION

Dietary fat is an important nutrient for the function and survival of all organisms. Historically, body lipids have been considered primarily to serve as an energy source, as constituents of cell membranes, and as precursors for molecules involved in signal transduction, such as steroids and prostaglandins. More recently however, fatty acids (FA) have been shown to affect gene expression, leading to changes in cell differentiation, growth, and metabolism (Clarke and Jump, 1994; Jump et al., 1996). Additionally, dietary fat has been implicated in the progression of several chronic diseases, including type II diabetes, cardiovascular disease, and some types of cancer (Sanders, 2003), though the effects may depend on the composition of dietary fat consumed. Therefore, understanding the molecular basis for FA effects on gene regulation is necessary for further elucidation of the role of fats in human health. To address this issue, our studies focused on the effects of three general classes of FA that may play a significant role in health and metabolism: n-3 and n-6 long-chain polyunsaturated fatty acids, conjugated linoleic acids (CLA), and *cis* and *trans* isomers of fatty acids.

Dietary polyunsaturated fatty acids (PUFA) have been reported to lower blood triglycerides, alter the blood lipid profile, decrease intramuscular lipid droplet size, improve insulin sensitivity, and enhance glucose utilization (Jump and Clarke, 1999). Since the seminal observation that PUFAs could inhibit hepatic lipogenesis in mice (Allmann and Gibson, 1965), numerous studies have demonstrated that diets rich in

PUFAs influence metabolic changes by coordinately suppressing lipid synthesis in the liver and enhancing fatty acid oxidation in both liver and skeletal muscle (Jump and Clarke, 1999). The PUFA induction of genes encoding proteins involved in lipid oxidation include 3-hydroxy, 3-methylglutaryl-CoA synthase (Rodriguez et al., 1994), carnitine palmitoyltransferase, fatty acid binding proteins, and peroxisomal acyl-CoA oxidase (ACO; Reddy and Hashimoto, 2001).

Conjugated linoleic acid (CLA) is a collective term for positional and geometric isomers of linoleic acid (LA). Though over 16 individual isomers have been identified (Rickert et al., 1999), only *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA are known to possess biological activity (Pariza et al., 2000). *Cis*-9, *trans*-11 CLA is the predominant CLA produced as an intermediate in the rumen during biohydrogenation of dietary LA and is commonly found in dairy products and ruminant meat. Dietary sources of *trans*-10, *cis*-12 CLA derive predominantly from synthetic partial hydrogenation and are found in margarines, shortenings, and supplements (Gaullier et al., 2002). First identified in grilled beef as a potential anti-carcinogen (Pariza and Hargraves, 1985), numerous health benefits have been attributed to CLA mixtures, including actions as an antiadipogenic (Park et al, 1997), antidiabetogenic (Houseknecht et al., 1998), and antiatherosclerotic (Kritchevsky et al., 2004) agent. More recently, studies involving individual isomers have shown that the two main isoforms can have different effects on metabolism and cell function and may act through different signaling pathways (Wahle et al., 2004). Metabolic responses to *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA may differ, but both isomers have implications for human health. Most studies have been performed in animal models, with species differences observed. In particular, only some of the

findings attributed to animal models pertain to human subjects, and even when comparing studies in humans, results are often inconclusive (Terpstra, 2004).

Trans-fatty acids are geometrical isomers of unsaturated FA that assume a saturated fat-like configuration that differs from that of their *cis* counterparts. The predominant source of *trans* fats in the human diet is hydrogenated oils (such as margarine and partially hydrogenated soybean oil) commonly found in baked goods and deep fat-fried fast foods (Hu et al., 2001). Metabolic studies in several species have shown that *trans*-FA can negatively alter the lipid profile to a greater extent than saturated fats, because *trans*-FA not only increase small, dense LDL cholesterol (Mauger et al., 2003), but also decrease HDL cholesterol in some studies (Judd et al., 1994; de Roos et al., 2003). Additionally, epidemiological evidence associates *trans*-FA intake with increased risk for cardiovascular disease (Ascherio et al., 1999). Few studies, however, have examined the role of individual *trans*-FA in modulating lipid metabolism. As with other FA, it is possible that *cis* and *trans* isomers of octadecenoic acid may also have differential effects on lipid metabolism.

Based on both dietary and *in vitro* studies of lipid metabolism, we hypothesized that various FA of differing degree of saturation and double-bond position will have differing effects on ACO, 3-hydroxy, 3-methylglutaryl CoA reductase (HMG-R), and apolipoprotein A-I (Apo A-I) gene expression, as well as HDL cholesterol production in HepG2 and H-4-II-E hepatoma cells (Table 1-1). Also, because several FA and their derivatives are known ligands for peroxisome proliferator-activated receptors (PPAR; Schoonjans et al., 1996), we hypothesized that these FA may act on lipid-metabolizing genes through activation of PPARα, the predominant receptor subtype in the liver

(Braissant et al., 1996). If this hypothesis is correct, activation of PPARα should mimic the effects of FA, whereas inhibition of PPARα would be expected to block FA effects in HepG2 and H-4-II-E hepatoma cell lines. The overall aim of our studies was to examine the differential roles of fatty acids on lipid metabolizing genes involved in peroxisomal β-oxidation and cholesterol synthesis in human and rat hepatoma cell lines.

Table 1-1. Biological functions of key genes studied

Gene	Function
ACO	**Rate limiting in peroxisomal β-oxidation**
HMG-R	**Rate limiting in cholesterol synthesis; converts HMG-CoA to mevlonate**
Apo A-I	**Necessary for proper packaging of HDL cholesterol**

CHAPTER 2
LITERATURE REVIEW

Structure and Metabolism of Lipids

Structure and Nomenclature of Lipids

Based on physical properties, the term lipid denotes a heterogeneous group of substances that are insoluble in water, but are soluble in non-polar solvents such as chloroform and alcohols (Smith, 2000). This definition covers a wide range of molecules, including FA, phospholipids, sterols, sphingolipids, terpenes, and others (Christie, 2003). Fatty acids consist of a chain of two or more carbon atoms, with a methyl group at one end, and a carboxyl group at the other end of the chain. The main structural features are their chain length, degree of unsaturation (number of double bonds), and presence of substituent groups. Additionally, the presence of double bonds allows for positional and geometric isomerism. Positional isomers occur when double bonds are located at different positions along the carbon chain. The position of unsaturation is numbered in reference to the first of the pair of carbon atoms between which the double bond occurs. Geometric isomerism refers to the configuration of the hydrogen atoms in respect to the double bond. If the hydrogen atoms are on the same side of the molecule opposite the double bond, it is said to be in the *cis* configuration. Alternately, if the hydrogen atoms are on opposite sides, the configuration is *trans*. Most naturally occurring unsaturated FA are in the *cis* configuration, but natural and synthetic *trans* isomers do exist.

The naming scheme for FA must be able to clearly define a lipid structure in a manner that is amenable to scientists and researchers of all fields. Several systems are currently used, though to different degrees. First, there are the trivial names, such as stearic acid and linoleic acid, which were assigned as the individual FA were discovered. Although these may be used for the most-commonly occurring FA, naming and remembering unusual unsaturated, branched, or hydroxyl-FA becomes unwieldy. Because of this difficulty, two different systems have been developed. The older system used Greek letters to identify carbon atoms, beginning at the carboxyl end. Considering the carboxyl carbon as C1, C2 is called the α-carbon, C3 the β-carbon, and so on, ending with the ω-carbon at the methyl end. Though this system is no longer preferred, it is used to name the ω-3 and ω-6 FAs, in which the last double bond in the chain occurs three and six carbons from the ω-carbon, respectively. In much of the newer literature, the ω is often replaced by an *n*, but the meaning remains the same.

Currently, the preferred system for specifying individual FA is the numbering system standardized by the International Union of Pure and Applied Chemistry (IUPAC) (IUPAC-IUB, 1977). For linoleic acid, an 18-carbon FA with two *cis* double bonds in positions 9 and 12 from the carboxyl end, the systematic name is *cis*-9, *cis*-12 octadecadienoic acid. In the shorthand system, FA are identified by two numbers separated by a colon; the first number indicates the number of carbon atoms, the second indicates the number of double bonds in the structure. For example, a saturated fat such as stearic acid would be 18:0, whereas a polyunsaturated fat, such as linoleic acid would be represented by *cis*-9, *cis*-12 18:2.

Biosynthesis of Fatty Acids

Most naturally occurring FA contain an even number of carbon atoms, leading early researchers to speculate that they were formed by the condensation of two-carbon units. This was confirmed using rats fed acetic acid labeled with ^{13}C in the carboxyl group and ^{2}H in the methyl group. When FA were isolated from the rat tissues, the labeled carbons were found in alternate positions along the chain, showing that the complete FA could be derived from acetic acid (Rittenberg and Bloch, 1944). When the details of β-oxidation were elucidated in the 1950s, it led to speculation that FA synthesis could be the simple reversal FA breakdown. However, several discoveries soon showed that the pathways were distinctly different. First, NADPH (not NAD+ as in oxidation) serves as a cofactor. Second, there is a requirement for bicarbonate (Wakil, 1962; Brady and Gurin, 1952).

Fatty acid synthesis can be broken into two basic processes: condensation of two carbon units to form 16 to18-carbon FA and various modifications of these products. In mammals, the majority of carbon for *de novo* FA synthesis comes from pyruvate, the end-product of glycolysis. To be used in FA synthesis, acetyl coenzyme A (CoA; the activated form of acetic acid) must be generated from pyruvate. To accomplish this, the pyruvate is transported from the cytosol into the mitochondria, where the enzyme pyruvate dehydrogenase acts to produce acetyl-CoA. Acetyl-CoA and oxaloacetate combine to form citrate, which can then be transported back out of the mitochondria via a tricarboxylate anion carrier, where the cycle is completed, and acetyl-CoA is produced by the action of ATP:citrate lyase.

The first and rate-limiting reaction in *de novo* FA synthesis is catalyzed by acetyl-CoA carboxylase (ACC). In this enzymatic reaction, acetyl-CoA is carboxylated, leading to the formation of malonyl-CoA (Knowles, 1989). This reaction requires biotin as a cofactor, as shown by inhibition of carboxylation by avidin, a potent inhibitor of biotin (Wakil et al., 1958). Acetyl-CoA carboxylase is activated by phosphorylation and deactivated by dephosphorylation (Shacter et al., 1986). The malonyl-CoA generated by ACC forms the source of nearly all carbons of the FA. Only the first two carbons arise from the "primer molecule," acetyl-CoA. In order for individual malonyl-CoA units to join into the FA chain, they must be attached to the acyl carrier protein (ACP). The ACP is a small molecular mass protein (8.8 kDa) that is very stable over a range of pH and temperature values (D'Agnolo et al., 1975).

The enzymatic steps involved in adding successive malonyl-CoA units to the chain are collectively known as fatty acid synthase (FAS). In animals, FAS is a multifunctional enzyme, with discrete domains catalyzing the condensation, dehydration, and reduction reactions. Animal FAS complexes consist of homodimers with molecular weight of approximately 450-550 kDa (Smith, 1994). Essentially, to elongate the chain, malonyl-ACP attached to one half of the dimer interacts with the growing acyl chain attached to the active site of the condensing enzyme on the other half of the dimer (Joshi et al., 1998). The typical end product of FAS is palmitic acid (16:0). The thioesterase actions of FAS cleave the product from the enzyme. This specificity for a 16-carbon product is likely due to stearic hindrance of the condensing domain by the large FA (Chirala and Wakil, 2004). Although the production of palmitate is most common, different organisms and tissues can produce FA of shorter chain lengths as necessary.

For example, in the rat mammary gland, where large amounts of 8:0 and 10:0 are necessary for the formation of milk triacylglycerols (TAG), a second thioesterase is present, forming medium-chain FA (Smith, 1994).

Although the main product of FAS is palmitate, many tissues contain longer chain FA, particularly as a component of membrane lipids. The formation of long and very long-chain FA is catalyzed by Type III synthases, often termed elongases due to their lengthening of pre-formed and dietary FA. In mammalian tissues, two separate elongation systems are located in the mitochondria and endoplasmic reticulum (ER). In the mitochondria, two carbon units in the form of acetyl-CoA (not malonyl-CoA as in *de novo* synthesis) are added preferentially to monoenoic over saturated substrates (Moon et al., 2001). Mitochondrial elongation is essentially a reversal of β-oxidation, with a requirement for NADPH and NADH (Seubert and Podack, 1973). Formation of the longer chain FA occurs at the ER. In this case, malonyl-CoA serves as the two carbon donor and NADPH is the reducing agent. This system can produce FA with chain lengths in excess of 20 carbons (Suneja et al., 1990).

Once saturated FA have been produced by the organism, they can be used to produce unsaturated FA, mainly by the process of oxidative desaturation. In this mechanism, a double bond is introduced directly into a pre-formed saturated long-chain FA, using O_2 and a reducing compound (NADH) as cofactors (Scheuerbrandt and Bloch, 1962). Mammalian enzymes normally introduce new double bonds between an existing double bond and the carboxyl group, whereas plant enzymes introduce the new bond between an existing double bond and the terminal methyl group. There are three components to the desaturation complex: NADH-cytochrome b_5 reductase, cytochrome

b_5, and the desaturase enzyme (Stritmatter et al., 1974). Most of what is known about desaturases is derived from early studies showing that Δ9 desaturase is the rate-limiting step in the conversion of stearic acid (18:0) to oleic acid (18:1, n-9) (Schroepfer and Bloch, 1965). Because of its actions, it is also referred to as stearyl-CoA desaturase (SCD). Mammals contain desaturases able to introduce double bonds in the Δ5, Δ6, and Δ9 positions. Plants additionally possess the Δ12 and Δ15 desaturases necessary for the formation of n-6 and n-3 FA.

Degradation of Fatty Acids

In the body, FA from dietary or stored TAG are broken down to provide a source of energy. The main forms of FA oxidation are termed alpha (α), beta (β), and omega (ω), depending on the carbon in the chain that is attacked. Of the three types of oxidation, β-oxidation is the most prevalent.

The basic mechanism for β-oxidation was originally proposed by Knoop in 1904 after feeding labeled FA to dogs, and was confirmed by Dakin's isolation of the proposed intermediates in 1912. Fats degraded in this manner liberate two-carbon units in the form of acetyl-CoA through the introduction of a double bond between the β- and γ-carbons, hence the name β-oxidation. Until relatively recently, mitochondria were considered the only cellular site for β-oxidation. Although all the necessary enzymes are present in mitochondria, the microbodies (peroxisomes in mammals and glyoxysomes in plants) can also complete the process (Lazarow and de Duve, 1976). The contribution of microbodies to total β-oxidation depends on the organism and specific tissue considered. For example, in mammals, peroxisomal β-oxidation of very long-chain FAs is

particularly important in the liver and kidneys, with defects leading to devastating diseases (Fournier et al., 1994).

Fatty acyl-CoAs formed within the cytosol cannot enter the mitochondrion directly, providing a major point of control and regulation of FA metabolism (Eaton, 2002). The observation that carnitine stimulates the β-oxidation of long-chain FA gave the first clue to its function in the mitochondrial uptake of FA (Bremer, 1962; Fritz and Yue, 1963). Acyl residues are transferred to carnitine by carnitine-palmitoyl transferase (CPT-I) at the surface of the outer mitochondrial membrane. This allows the FA to transverse the membrane via porin, where they are then transported through the inner mitochondrial membrane by a carnitine:acylcarnitine translocase (Pande, 1975). The translocase causes a one-to-one exchange of carnitine for acylcarnitine, ensuring a constant level of carnitine within the mitochondria (Ramsay and Tubbs, 1975). Once within the mitochondrial matrix, a second carnitine-palmitoyl transferase, CPT-II, acts to transfer the acyl group from carnitine back to CoA, reforming acyl-CoAs, the substrate for β-oxidation (Bieber, 1988).

The reactions of β-oxidation involve four enzymes in repeated sequence, resulting in the cleavage of two carbons at a time from the acyl chain. Acyl-CoA dehydrogenase acts to produce *trans*-2,3-enoyl-CoA. This step is linked to the respiratory chain via electron transfer flavoprotein (ETF) and ETF-ubiquinone oxireductase (Parker and Engel, 2000). The 2-enoyl-CoA hydratase then acts on the product of the first reaction, producing 3-hydroxyacyl-CoA. The next enzyme in the sequence, 3-hydroxyacyl-CoA dehydrogenase, is linked with NAD^+ and produces 3-oxoacyl-CoA. Finally, 3-oxoacyl-CoA thiolase actions produce a saturated acyl-CoA that has been shortened by

two carbons, in the form of acyl-CoA (Eaton et al., 1996). Each of the enzymes is present in several isoforms with varying chain-length specificities, primarily for short, medium, long, and very long-chain acyl-CoA. This allows for improved efficiency of β-oxidation and prevents buildup of intermediates that could lead to inhibition (Bartlett and Eaton, 2004).

Peroxisomal β-oxidation is important in almost all eukaryotic organisms (Kunau et al., 1995). The peroxisomal and mitochondrial enzymes of β-oxidation differ in several ways. Peroxisomes do not have an electron transport system coupled to energy production as can be found in mitochondria. The first and rate limiting step in peroxisomal β-oxidation is catalyzed by acyl-CoA oxidase (ACO), which introduces a *trans*-2 double bond and produces hydrogen peroxide. Next, a trifunctional enzyme produces β-ketoacyl-CoA, which is acted upon by a thiolase, producing acetyl-CoA and a shortened acyl-CoA (Mannaerts et al., 2000). Due to limited substrate specificities for ACO, peroxisomes are incapable of oxidizing long-chain FA completely (Singh et al., 1984). Medium chain products of peroxisomal β-oxidation are transferred to carnitine, allowing them to be transported into the mitochondria for complete oxidation. Defects in peroxisomal β-oxidation can lead to the accumulation of very long-chain FA in various tissues, producing devastating diseases such as Zellweger syndrome and adrenoleukodystrophy (Kunau et al., 1995).

The above enzymatic cycle assumes that the substrate is a straight-chain, saturated FA with an even number of carbons. For FA of odd-chain lengths, β-oxidation yields propionyl-CoA in addition to acetyl-CoA; therefore, the ability of an organism or tissue to oxidize these FA depends on the ability of that organism or tissue to metabolize

propionate. The liver is well-equipped to oxidize propionate and oxidizes odd-chain FA well, whereas the heart cannot oxidize the product, and β-oxidation of odd-chain FA stops with an increase in propionate (Grynberg and Demaison, 1996). Beta-oxidation of unsaturated FA poses several problems. Most naturally occurring unsaturated FA contain *cis* double bonds and the bonds may be in the wrong position along the chain for effective β-oxidation. Unsaturated FA with odd-numbered double bonds, such as the 9-*cis* bond of LA, are shortened to 3-*cis*-enoyl-CoA and then isomerized to 2-*trans*-enoyl CoA that can be further degraded via β-oxidation (Stoffel and Caesar, 1965). Fatty acids with even-numbered double bonds are shortened to 4-*cis*-enoyl-CoA, which are then dehydrogenated to 2-*trans*, 4-*cis*-dienoyl-CoA. One double bond is then removed by NADPH-dependent 2,4-dienoyl-reductase, allowing β-oxidation to continue (Kunau and Dommes, 1978).

The acyl-CoA produced by chain-shortening can have several different fates, depending on the tissue. In ketogenic tissues such as the liver, acetyl-CoA is used to form the ketone bodies, acetoacetate and β-hydroxybutyrate, for export and peripheral oxidation. In most tissues, however, acetyl-CoA enters the Krebs cycle and generates energy in the form of ATP (Hiltunen and Qin, 2000).

Nutritional and Biological Properties of the Polyunsaturated Fatty Acids

Dietary Requirements of the Essential Fatty Acids

Mammalian cells can synthesize saturated and omega-9 (n-9) unsaturated FA *de novo* from acetyl-CoA, but lack the Δ 12 and Δ 15 desaturase enzymes necessary for the formation of double bonds in the omega-6 (n-6) and omega-3 (n-3) positions. Because of this enzyme deficiency, the linoleic acid (LA; 18:2, n-6) and α-linolenic acid (LNA; 18:3, n-3) are considered essential nutrients in the human diet (Innis, 1991). Once ingested,

LA and LNA can be further elongated and desaturated into biologically important long-chain polyunsaturated fatty acids (PUFA) of 20 or more carbons and three to six double bonds.

Determining the essential requirements of a nutrient generally begins with recognition of a deficiency, continues with the study of intakes that can prevent or reverse the deficiency, and finally concludes with the definition of a range of intakes for optimal biological function. In 1929, Burr and Burr discovered that rats fed a fat-free diet developed dermatitis and grew at a slower rate than their fat-fed counterparts. These deficiencies could only be eliminated by adding certain FAs to the diet, which were later determined to be LA and arachidonic acid (AA; 20:4). This knowledge was applied to produce essential FA deficiency in a variety of species, including man. In all species, the deficiency is characterized by skin symptoms, such as dermatosis or eczema, retarded growth, impaired reproduction, and degeneration or impairment of function in many bodily organs, including the heart and kidneys (Sinclair, 1990). These signs are characterized by changes in the FA composition of many tissues, particularly in biological membranes and mitochondria.

Well-documented essential FA deficiency in man is rare, but was first seen in the 1940s and 50s in infants receiving formula containing skim milk and sugar as a substitute for mother's milk. When fed formula containing increasing concentrations of LA, clinical signs of deficiency disappeared when concentrations in the diet were above 0.1% of dietary energy (Hansen et al., 1958). Adult essential FA deficiency was most commonly seen in patients receiving total parenteral nutrition (TPN), in which early formulas were fat-free (Holman, 1981). In some cases, patients responded to the

application to the skin of fats with a high proportion of LA, showing that the FA do not necessarily have to be absorbed through the gastrointestinal tract to be effective. More frequently, LA deficiency may develop as a secondary condition to other disorders such as severe malnutrition and fat malabsorption.

The n-3 FA can, in part, substitute for a deficiency in n-6 FA, but also have their own distinct roles (Benatti et al., 2004). The understanding of n-3 FA essentiality lagged significantly behind that of n-6 FA, partially because of their naturally lower amounts in the body. The first case of n-3 FA deficiency was induced by an n-3 FA-free TPN formula. Symptoms of n-3 FA deficiency in the patient included numbness, tingling, weakness, inability to walk, leg pain, psychological disturbances and blurred vision. The patient's plasma lipid profile showed the concentration of total n-3 FA to be at 34% of the control value. When soybean oil, a source of LNA, was added back to the TPN formula, the signs of deficiency disappeared (Holman et al., 1982).

As essential FA deficiency is usually associated with a disease state, there is little evidence to determine dietary reference intakes for healthy populations. Therefore, based on the data that are available on health effects of LA and LNA, adequate intake (AI) levels have been recommended. The AI is a value based on experimentally derived intake levels or approximate mean nutrient intakes by a group of healthy individuals. Based on current estimates, PUFAs contribute approximately 5-6% of energy in the Western diet (Grundy et al., 1982). For adults, it is recommended that consumption of LA should be 17 g/d for men and 12 g/d for women. For LNA, recommended intakes are 1.6 g/d for men and 1.1 g/d for women (Food and Nutrition Board, 2005). Also, for cardiovascular health benefits, the long-chain desaturation and elongation products,

eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) together should represent 0.3% of dietary energy, with each FA being at least 0.1% of energy (Simopoulos et al., 2000). These values represent, in general, a decrease in n-6 FA consumption and an increase in n-3 FA consumption for the typical individual, altering the current n-6 to n-3 ratio from 10-20:1 to 1-4:1 (Simopoulos, 1999).

Long-Chain Polyunsaturated Fatty Acids of the n-6 Family

Organs and tissues performing storage (adipose tissue), chemical processing (liver), mechanical work (muscle), and excretion (kidney) have membranes in which the n-6 FA predominate, particularly with AA as the major component (Innis, 1991). Arachidonic acid serves an important role as a precursor for biologically important eicosanoids and it and other n-6 FA may play a role as secondary messengers in the process of signal transduction. As previously stated, LA can be desaturated and elongated in mammals to produce biologically important long-chain PUFAs of the n-6 family (Klenk and Mohrhauer, 1960; Mead, 1968). These include γ-linolenic acid (GLA, 18:3) and AA. Normally, only a small proportion of dietary linoleate can be converted to longer-chain PUFA. Most of it is β-oxidized to provide energy (Cunnane and Anderson, 1997). To produce GLA, Δ6 desaturase acts on LA, introducing a double bond at carbon six in the FA chain. This product is then elongated to dihomo-γ-linoleic acid (20:3, n-6), which is converted to AA by Δ5 desaturase. Arachidonic acid can be elongated to form adrenic and ω-6-tetracosatetraenioc acids (22:4 and 24:4), but since there is no evidence of a functional mammalian Δ4 desaturase, ω-6-docosapentaenoic acid (22:5) must be formed via an alternate pathway. A double bond is added to tetracosatetraenioc acid by Δ6

desaturase, forming tetracosapentaenoic acid, which is then oxidized in peroxisomes to form the 22 carbon product (Sprecher et al., 1995; Ferdinandusse et al., 2001).

Long-Chain Polyunsaturated Fatty Acids of the n-3 Family

Nervous tissue, reproductive organs, and the retina have membranes that contain a large percentage of long-chain FA, particularly PUFAs of the n-3 series (Innis, 1991). As with LA and the n-6 PUFAs, dietary LNA can be elongated and desaturated to form long-chain PUFAs of the n-3 family. The key n-3 PUFAs are EPA and DHA. Within the body, the amounts of n-3 PUFAs are lower than that of the n-6 PUFAs. This is due to the small proportion of LNA in the diet, as well as the competition between FA for the Δ5 and Δ6 desaturases (Dang et al., 1989). To produce EPA, LNA is desaturated by Δ6 desaturase to stearidonic acid (18:4), then elongated by 2 carbons, and further desaturated by Δ5 desaturase. As with PUFA of the n-6 series, no Δ4 desaturase is present in mammals, as in lower eukaryotes, to form DHA directly (Qiu et al., 2001). To form DHA, EPA undergoes two cycles of chain elongation to produce ω-3-tetracosapentaenoic acid, which is then desaturated to ω-3-tetracosahexaenoic acid (24:6) (Sprecher et al., 1995). In mammals, EPA and DHA can be derived not only from dietary LNA, but also in the diet directly from sources such as cold-water fish and fish oil.

Digestion and Assimilation of Dietary Fats

In the Western diet fats constitute approximately 40% of energy in the diet. In the human diet, the majority of fat consumed, whether of animal or plant origin, is in the form of TAG. Triacylglycerols are the major biological form of storage lipid, composed of three FA esterified to a glycerol backbone. Long-chain FA, such as oleic acid (18:1) and palmitic acid (16:0) are the major FA present in dietary TAG, although FA can vary

in chain length from C2 to C24 and from saturated FA to unsaturated FA with six or more double bonds. In addition to TAG, smaller amounts of phospholipids, cholesterol, and other sterols are consumed in the diet. An average adult on a Western diet consumes approximately 150 grams of TAG and 4-8 grams of phospholipids daily. Cholesterol intake can vary depending on diet, but average daily intake of total cholesterol is 400-500 mg (Rizek et al., 1974).

Although the majority of TAG digestion occurs in the small intestine, digestion begins in the stomach. Partial hydrolysis of TAG begins with the actions of lingual or gastric lipase, depending on the species studied (Mu and Hoy, 2004). Lingual lipase is secreted by the von Ebner's glands of the tongue and is transported with the food bolus to the stomach (Hamosh and Scow, 1973). Gastric lipase is secreted from the gastric mucosa. Secretion of either of these lipases can be stimulated mechanically (suckling and swallowing), neurally (sympathetic agonists), and by diet (high fat) (Hamosh, 1978). The relative contribution of these lipases to fat hydrolysis is species dependent. For example, rodents have a relatively high activity of lingual lipase and low activity of gastric lipase, whereas, in primates, gastric lipase has high activity (Mu and Hoy, 2004). Both lingual and gastric lipases show a stereo-specific preference for cleaving TAG at the sn-3 position, regardless of the FA present, although short- and medium-chain FA are hydrolyzed at a faster rate than long-chain FA (Jensen et al., 1983). This preferential cleavage gives rise to diglycerides and non-esterified fatty acids (NEFA) as major digestion products. Approximately 10-30% of dietary fat is partially hydrolyzed in the stomach which facilitates further digestion in the small intestine (Hamosh and Scow, 1973). In addition, the churning action of the stomach creates a coarse emulsion

stabilized by phospholipids, and proteolytic digestion in the stomach serves to release fats from food particles where they are generally associated with proteins (Gurr et al., 2002).

The major digestion of dietary TAG results from the actions of pancreatic lipase. Entry of TAG, TAG degradation products, and acidic stomach contents into the duodenum causes gall bladder emptying and secretion of pancreatic lipase and cholecystokinin (Meyer and Jones, 1974). Bile acids serve to emulsify the fats and increase the available surface area for enzymatic action, where pancreatic lipase and colipase act to hydrolyze TAG. Colipase attaches to the ester bond of the TAG, which in turn strongly binds the lipase (Patton, 1981). Pancreatic lipase cleaves the sn-1 and sn-3 bonds specifically, leading to the formation of 2-monoacylglycerols and free FA, with small amounts of 1,2- and 2,3-diacylglycerols as intermediate products (Mattson and Volpenhein, 1964). Although pancreatic lipase attacks primarily at stereospecific locations, the relative rate of hydrolysis depends on the FA present. The lipase has much slower activity when long-chain FA, particularly the n-3 polyunsaturated FA (20:5 and 22:6), are located in the sn-3 position (Ikeda et al., 1995). Additionally, 2-monoacylglycerols can isomerize to 1-monoglycerides to a small extent in aqueous conditions, allowing for the formation of a small percentage of glycerol and free FA (Mattson and Volpenhein, 1962). Phospholipids undergo a similar hydrolysis as TAG, however the specific enzyme, phospholipase A_2, cleaves FA from the sn-2 position of the phosphoglyceride (van Deenen and deHass, 1963). Dietary cholesterol enters the duodenum as both free and esterified cholesterol. Prior to absorption, the esterified cholesterol is hydrolyzed to free cholesterol and NEFA by cholesterol esterase (Hyun et

al., 1969). Cholesterol esterase may also aid in the hydrolysis of TAGs that contain long-chain PUFA (Carlier et al., 1991).

Lipid absorption in humans begins in the distal duodenum and is completed in the jejunum. Non-esterified fatty acids and 2-monoacylglycerols, along with phospholipids, enter into bile micelles, forming mixed micelles. This solubilization allows the non-polar lipids to travel through the unstirred water layer and reach the brush-border membrane of the enterocyte (Dietschy et al., 1971; Wilson et al., 1971). The pH of the unstirred water layer promotes protonation of NEFA, allowing them to more easily leave the micelles and move to the epithelial cell membrane.

Once in close contact with the brush-border, the 2-monoacylglycerols, NEFA, and free cholesterol cross the microvillus membrane. In the past, it had been thought that FA pass into the enterocyte via passive diffusion due to high intraluminal and low cytosolic concentrations of lipids (Keelan et al., 1992). More recently, however, it has been proposed that a specific transport protein facilitates the movement of FA into the cell. Two such proteins that may be involved in intestinal lipid transport are plasma membrane fatty acid binding protein and fatty acid translocase (Frohnert and Bernlohr, 2000). Bile salts and some cholesterol are not absorbed and pass to the ileum, where they are recycled via the portal blood to the liver.

Once within the enterocyte, FAs are re-esterified into TAG and phospholipids in a multi-step process. First, FAs bind to a cytosolic fatty acid binding protein (FABPc), allowing for targeting to the ER (Cartwright et al., 2000). There, acyl-CoA synthetase, a membrane-associated enzyme, activates FAs to their acyl-CoA thioesters via an ATP-dependent mechanism. This activation effectively traps FA within the cell,

maintaining the concentration gradient and increasing the rate of TAG synthesis (DiRusso and Black, 1999). Since the major forms of absorbed lipids in humans and other non-ruminants are 2-monoacylglycerols and NEFAs, resynthesis of approximately 80% of the TAG occurs via the monoacylglycerol pathway (Lehner et al., 1993). In this pathway, the first step is the acylation of 2-monoglygerides with fatty-acyl-CoA to diacylglycerols by monoacylglycerol acyltransferase (MGAT). Monoacylglycerol acyltransferase has a preference for medium-chain saturated and long-chain unsaturated 2-monoacylglycerols (Coleman and Haynes, 1984), but all acyl-CoA studied are incorporated with similar efficiency (Bugaut et al., 1984). The reaction produces predominantly 1,2-diacylglycerols, with only about 10% 2,3-diacylglycerols formed (Lehner and Kuksis, 1996). This stereospecificity allows for the final and rate limiting step in TAG synthesis. Diacylglycerol acyltransferase (DGAT), which will not act on the 2,3-isomer, acetylates diacylglycerol in an acyl-CoA dependent manner (Coleman, 1988). Similarly to MGAT, DGAT shows substrate specificity for di-unsaturated or mixed-diacylglycerols over disaturates.

During fat absorption, the resynthesized TAG are packaged in the enterocyte into lipoproteins, making the lipids stable for transport in the aqueous environment of the blood. The human intestine secretes mainly chylomicrons (CM) and very low-density lipoproteins (VLDL). During fasting, VLDLs are the main lipoproteins secreted by the small intestine, whereas CMs are secreted during fat feeding (Ockner et al., 1969). Chylomicrons are the main route of transport for long-chain dietary FAs. Medium-chain FA (C<12) are absorbed in the non-esterified form, passing directly into the portal blood system. This occurs because short- and medium-chain FA are more likely to occupy

position three of the TAG and are therefore hydrolyzed in the small intestine and not retained as 2-monoacylglycerols (Sethi et al., 1993). Soon after dietary lipids enter the enterocyte, fat droplets can be seen in the ER from the formation of TAG. The rough ER is the site of synthesis of phospholipids and apolipoproteins, which provide a coat to stabilize the lipid droplet. Specifically, apolipoprotein B48 (apo B48) associates with the TAG during its synthesis, forming the immature CM (Cartwright et al., 2000). In the smooth ER, the immature CM accumulates further TAG via the actions of microsomal transfer protein (MTP). The CMs then migrate through the Golgi apparatus, where glycosylation takes place (Leblond and Bennett, 1977) before the fully-formed CM are exported in secretory vesicles. The CM-containing vesicle travels to the basolateral surface of the enterocyte, fuses with the plasma membrane, and is secreted into the extracellular space by exocytosis (Sabesin and Frase, 1977). Very low-density lipoproteins, as mentioned above, are formed in the small intestine when the levels of lipids are too low to form CMs. Very low-density lipoproteins differ from CMs in their density, size, lipid content, and composition, and although both are formed in the same organelles, the two particles are not mixed in individual Golgi vesicles (Mahley et al., 1971).

Lipoproteins secreted from the intestine do not enter the blood stream directly. Instead, they are secreted into minute lymph vessels, known as lacteals, due to their milky appearance when filled with lipid. From there, the CM and VLDL enter the circulation in the subclavian vein via the thoracic duct (Mu and Hoy, 2004). Once in the blood stream, intestinal lipoproteins come into contact with other plasma lipoproteins, where transfer of protein and TAG occurs (Redgrave and Small, 1979). In particular,

CM and VLDL acquire apolipoprotein CII (apo C-II), which is essential for further metabolism. As CM and VLDL pass through capillaries, they come into contact with and bind to lipoprotein lipase (LPL), which is expressed in extrahepatic tissues that use FA, such as adipose tissue, skeletal and cardiac muscle, and the mammary gland (Ginsberg, 1998). Lipoprotein lipase, with apo C-II as a cofactor, hydrolyzes the TAG in the particle, generating NEFA that can diffuse into the tissue for further metabolism or storage (Frayn, 1998). The TAG depleted CM remnant is rapidly removed from plasma and is metabolized by the liver. Once VLDL have interacted with LPL, they also lose surface apolipoproteins C and E, and become low-density lipoprotein (LDL) particles once only apo B remains. The apo B of LDL is recognized by the LDL receptor on the surface of most cells, allowing for LDL uptake and metabolism within peripheral cells. The LDL particles are the major carriers of blood cholesterol in humans, pigs, and guinea pigs; however, in most mammalian species, high-density lipoprotein (HDL) serves this function.

The reverse transport from peripheral cells to the liver is an important physiological process necessary to counteract the deposition of cholesterol in tissues from VLDL and LDL cholesterol. In reverse transport, HDL, primarily synthesized by the liver (Wang and Briggs, 2004), takes cholesterol from peripheral tissues and transports it to the liver for metabolism. In 1968, it was first recognized that reverse cholesterol transport involved the active transport of cholesterol, as cellular free cholesterol was converted to the insoluble ester outside of the cell. The enzyme involved in this process is lecithin:cholesterol acyl-transferase (LCAT), and is a component of HDL that increases the cholesterol esters within this lipoprotein fraction (Glomset, 1968). The rate of LCAT

is affected primarily by the surface properties of individual lecithin molecules (Pownell et al., 1985). Two additional proteins contribute to HDL remodeling, both by working down concentration gradients in an energy-independent manner. Phospholipid transfer protein supplies lecithin to HDL (Tollefson et al., 1988), and a cholesterol ester transfer protein can move cholesterol esters made by LCAT to other lipoproteins, particularly LDL (Tall, 1993). The TAG portion of HDL can be catabolized by the extrcellular hepatic triacylglycerol lipase, and the cholesterol is removed by the liver via several different mechanisms (Nagata et al., 1988; Wang and Briggs, 2004). It is only tissues that actively uptake or synthesize cholesterol that contribute to the reverse cholesterol transport pathway.

Dietary Fats in Relation to Health

Dietary Fats in Relation to Weight Control

According to the World Health Organization (World Health Report, 2002), obesity rates have risen over three-fold since 1980 in most developed and developing countries worldwide. Current estimates count more than one billion adults as overweight and at least 300 million as clinically obese. In the US, approximately 30% of adults are categorized as obese, which is defined as at least 20% heavier than their ideal weight. Obesity is associated with increased early mortality and an increased risk for a variety of diseases, including metabolic, cardiovascular and gastrointestinal diseases. Because of this, the World Health Organization has listed obesity as one of the top ten global health problems in Western cultures (World Health Report, 2002).

Analysis of epidemiological data suggests that dietary fat plays a role in obesity, though the mode of action is not clear (Bray and Popkin, 1998; Astrup et al., 2000). It is evident however, that it is not a simple relationship. Longititudinal measurements of

food intake in both the US and the UK show that fat intake has not increased as a proportion of dietary energy over the last 30 years, unlike the rising trends in obesity (Heini and Weinsier, 1997; Nielsen et al., 2002). Obesity may be due to the types of fat consumed or the interaction of dietary fats or FAs with other dietary compounds.

If high intakes of dietary fat are a factor in the development of obesity, then reducing the fat in the diet would be expected to produce weight loss. Studies examining the relationship between fat in the diet and changes in body weight reported several conclusions. When animals were fed a high fat diet, almost all species develop obesity, as demonstrated in primates, rodents, pigs, dogs, and cats (West and York, 1998). Exceptions include animals with a strong genetic component to obesity, such as C57/BL mice and Osborne-Mendel rats (Bray et al., 2004). Reductions in body weight of subjects consuming a low-fat diet are modest and tend to extend over only a short period of time (Jeffery et al., 1995), and the higher the body mass index (BMI) of the subject, the greater the weight loss (Astrup et al., 2000). There is also a positive relationship between the percent reduction in dietary fat and the decrease in energy intake, suggesting that the major mechanisms for weight loss associated with reduced-fat diets may be primarily through a lower energy intake (Bray et al., 2002). Dietary fat, therefore, may not be an independent cause for obesity. Excessive energy intake, whatever the source, and decreased energy expenditure generally are considered the main causes of obesity (Foreyt and Poston, 2002). Current recommendations still call for a low-fat intake, due to fat's higher caloric density than other nutrients, coupled with less energy expenditure of an increasingly sedentary population (Astrup et al., 2002). When this theory is considered, it is evident that the type or composition of dietary fat may have little effect on obesity.

However, the type of fat consumed can play significant, differential, and more direct roles in other health and disease states.

Dietary Fats and Blood Cholesterol

Dyslipidemia is a condition in which plasma concentrations of LDL cholesterol and TAG are elevated and HDL cholesterol is lower than found in normal, healthy individuals. According to the most recent guidelines set by the US National Cholesterol Education Program (NCEP), total cholesterol should be <200 mg/dL, with LDL cholesterol <100 mg/dL and HDL cholesterol >40 mg/dL (Grundy et al., 2004). Abnormally high LDL cholesterol and low HDL cholesterol outside the recommended values are considered significant risk factors for cardiovascular disease, therefore maintaining optimum blood concentrations is beneficial. Dietary fat has the ability to modify blood cholesterol components in both a positive and negative manner, depending on the types of fat consumed.

As a group, consumption of saturated FAs raises total and LDL cholesterol in blood, but individual saturated fats can have differing effects (Reddy and Katan, 2004). Several feeding studies have demonstrated that individuals consuming diets high in saturated fat had increased concentrations of both HDL and LDL cholesterol (Kromhout et al., 1995). Myristic (14:0) and lauric (12:0) acids have a greater effect on elevating LDL cholesterol than palmitic acid (16:0), but, among these, palmitic acid is greatest in the food supply. Stearic acid, in contrast, decreases plasma and liver cholesterol concentrations, primarily by reducing intestinal cholesterol absorption. The mechanism by which stearic acid reduces cholesterol is thought to be by reducing solubility of cholesterol and altering the population of microflora that can synthesize secondary bile acids (Cowles et al., 2002).

In the human diet, the predominant monounsaturated fatty acid (MUFA) is oleic acid (18:1, n-9). It is found at high levels in olive oil, canola oil, and nuts. The Mediterranean diet, which is not low in fat but is associated with a healthy blood lipid profile, contains a high percentage of fat as oleic acid. When saturated fats in the diet are replaced with oleic acid, total and LDL cholesterol concentrations are lowered (Gardner and Kramer, 1995). This seems to be caused by a passive mechanism; when saturated fats are decreased and MUFA are increased, the fat induced-suppression of LDL receptor activity is less and LDL uptake into cells is increased (Dietschy et al., 1993). Effects of MUFA on HDL cholesterol are less clear. Some studies have indicated that MUFA have no effects on blood HDL concentrations (Delaplanque et al., 1991; Mata et al., 1992). This combined with the LDL-lowering effects suggest that MUFA can shift the LDL:HDL ratio towards a healthier profile. However, upon extensive meta-analysis, the effects of MUFA on HDL cholesterol in blood could not be confirmed (Gardner and Kramer, 1995).

Polyunsaturated fatty acids of the n-6 family, particularly LA, lower total and LDL cholesterol when they are supplied in the diet in place of saturated fats (Kris-Etherton and Yu, 1997). In addition to the passive mechanism described for MUFA, PUFA actively increase receptor-dependent LDL uptake, although this is a small effect (Dietschy et al., 1993. Dietschy, 1998). In some studies in which n-6 PUFA replaced saturated fats, a significant decrease in HDL cholesterol was reported (Shepherd et al., 1978; Jackson et al., 1984), although this is not a consistent effect (Iacono and Dougherty, 1991). When n-3 PUFA were supplemented with the regular diet, LDL cholesterol was raised in some studies (Harris et al., 1988; Fumeron et al., 1991) and HDL cholesterol was either

unchanged or slightly increased (Harris, 1989). These effects tend to be more pronounced in hyperlipidemic subjects. For example, an increase in LDL cholesterol occured in isolated hypertriglyceridemiac subjects when more than 10 g of n-3 FA were supplemented per day (Schmidt and Dyerberg, 1994). When very long-chain n-3 FA, such as EPA and DHA were specifically supplemented, they not only have the ability to significantly lower serum TAG, but also to increase LDL cholesterol more so than supplementing with LNA or a mixture of n-3 FA (Harris, 1997).

Trans-FA are geometrical isomers of unsaturated FA that assume a saturated fat-like configuration. The predominant source in the human diet is from hydrogenated oils, such as margarine and partially hydrogenated soybean oil, commonly found in baked goods and deep fat-fried fast foods (Hu et al., 2001). Metabolic studies have shown that consumption of *trans*-FA has the ability to negatively alter the lipid profile to a greater extent than saturated fats, because they not only increase small, dense LDL cholesterol (Mauger et al., 2003), but also decrease HDL cholesterol (Judd et al., 1994; de Roos et al., 2003). This leads to an increase in the ratio of total to HDL cholesterol that is approximately double that observed with saturated fats (Willett and Ascherio, 1994). Additionally, diets high in trans-FA are associated with raised TAG concentrations (Katan and Zock, 1995), an independent risk marker of cardiovascular disease.

Dietary Fats and Cardiovascular Disease

Most of the FAs in the Western diet are derived from meats, oils, and dairy products, leading to a large intake of saturated and MUFAs, with a relatively small proportion of PUFA consumed. Saturated fat and cholesterol represent two of the most established dietary risk factors for cardiovascular disease (CVD), whereas MUFA and PUFA are likely to provide beneficial effects with increased amounts in the diet. These

effects are partially due to the effects on the blood lipid profile, but the risks associated with intake of certain fats are greater than would be expected from cholesterol effects alone.

As stated above, intake of certain saturated fats can increase LDL and decrease HDL cholesterol, creating an atherogenic lipid profile. A recent analysis of the Nurses' Health Study revealed that intake of short to medium chain FA was not associated with increased coronary heart disease (CHD) risk. In the same analysis, however, intakes of longer chain saturates, particularly stearic acid, were associated with an increased risk of CHD (Hu et al., 1999). Additionally, stearic acid may negatively impact other markers of atherogenesis. Stearic acid can increase lipoprotein(a) concentration (Aro et al., 1997), and may activate Factor VII (Mitropoulos et al., 1994) and impair fibrinolysis (Ferguson et al. 1970). On the positive side however, when compared with consumption of palmitic acid, stearic acid decreases platelet volume, platelet aggregation, and coagulation factor VII activity (Kelly et al., 2001). Due to the many negative health implications of a diet high in saturated fats, there is a consensus to reduce the intake of saturated fats to less than 10% of the total daily energy supply (American Heart Association, 2000).

The Seven Countries Study gave the first epidemiological evidence for a negative correlation between dietary intake of MUFA and mortality from CHD. Mortality was noticeably low in Mediterranean countries, where olive oil is the main source of fat (Keys et al., 1986). In addition to the positive effects on plasma LDL cholesterol levels, diets rich in olive oil can improve endothelial function, as compared to a high saturated fat diet (Fuentes et al., 2001), and attenuate postprandial endothelial dysfunction that follows a fatty meal (Vogel et al., 2000). Intake of MUFA is also protective against LDL

oxidation. Due to their structure, MUFA are more stable and less susceptible to lipid peroxidation. A high intake of MUFA results in a greater incorporation into LDL cholesterol (Mata et al., 1996). Oxidation of LDL cholesterol prevents its recognition by the LDL receptor and subsequent uptake into cells. It is instead taken up by the scavenger receptors of macrophages, leading to the accumulation of cholesterol and the formation of fatty streaks. These processes promote the development of atherosclerosis (Westhuyzen, 1997). Several studies have shown that dietary sources of MUFA other than olive oil are associated with an increased CHD risk (Posner et al., 1991; Esrey et al., 1996). However, these studies did not correct for potential confounding effects, such as the intake of other FAs and antioxidants.

Epidemiological evidence supports a role for dietary LA in reducing the risk of CHD. High adipose LA in healthy men is associated with lower CHD mortality (Riemersma et al., 1986), while low dietary intake of LA predisposes to myocardial infarction (Simpson et al., 1982). A more recent study of Japanese subjects found reduced serum LA in patients with ischemic stroke as compared to healthy controls (Iso et al., 2002). Similarly to LA, LNA intake was inversely associated with mortality from CHD in the Multiple Factor Intervention Trial (Dolecek, 1992). Large prospective studies in both men and women have found that LNA protected against both cardiac deaths and nonfatal myocardial infarction (Ascherio et al., 1996; Hu et al., 1999). The effects of LNA on plasma lipids are not large; therefore the reduction in CHD risk may have more to do with cardiac function, such as arrhythmia, inflammation, and thrombosis.

The low rate of CVD seen in several communities consuming a diet rich in fish (Bang et al., 1980; Kromhout et al., 1985; Hirai et al., 1989; Oomen et al., 2000) has prompted investigations into how fish and its nutritional components may lower the risk of CVD. Fish, particularly fatty fish such as tuna, mackerel, and salmon, are rich in the n-3 FA EPA and DHA (Parkinson et al., 1994). High serum and adipose tissue long-chain n-3 PUFA have been associated with reduced risk of fatal myocardial infarction (Simon et al., 1995; Pedersen et al., 2000; Lemaitre et al., 2003), primary cardiac arrest (Siscovick et al., 1995), and sudden cardiac death (Albert et al., 2002). Upon meta-analysis of 11 randomized controlled trials comparing long-chain n-3 PUFA intake to placebo or control diets, intake of long-chain n-3 PUFA was associated with lower cardiac fatalities in patients with CHD (Bucher et al., 2002). However, these FA did not protect against nonfatal cardiac events or total morbidity (Erkkila et al., 2003; Lemaitre et al., 2003), suggesting that the hypolipidemic effects of DHA and EPA on atherosclerosis are distinct from those effects associated with arrhythmic myocardial dysfunction. In a canine model with dogs made susceptible to fatal ventricular fibrillation and sudden cardiac death, infusion of EPA and DHA reduced cardiac deaths by preventing ventricular fibrillation (Billman et al., 1997; Billman et al., 1999). As the fat infusion was given only one hour prior to inducing ischemia, the effects are not likely by membrane incorporation of n-3 PUFA, but rather by direct action of nonesterified PUFA on the myocytes. In support of this, induction of arrhythmias in cultured neonatal rat myocytes was abolished by the addition of EPA or DHA to the culture medium (Kang and Leaf, 1996), and supplementation of four g/d of EPA and DHA increased heart rate variability in survivors of myocardial infarction, reducing the risk of subsequent

arrhythmic events (Christensen et al., 1996). The anti-arrhythmic actions of EPA and DHA seem to be associated with the ability of these FA to prevent calcium overload in cardiac myocytes during periods of stress (Leaf and Kang, 1997).

As stated previously, *trans*-FA negatively impact the blood lipid profile in several ways. However, the relationship between consumption of *trans*-FA and cardiovascular risk is greater than is predicted based on these lipid changes (Ascherio et al., 1999), suggesting effects on other distinct risk markers for CVD. In humans, it has been shown that *trans*-FA increase lipoprotein (a) levels (Nestel, et al., 1992; Sundram et al., 1997), which are positively associated with increased risk of CHD (Utermann, 1989). Additional studies have examined the effects of *trans*-FA on markers of low-grade chronic inflammation. The Nurses' Health Study showed that a high intake of *trans*-FA was positively associated with concentrations of tumor necrosis factor α receptors 1 and 2 (Mozaffarian et al., 2004). A dietary intervention study in which 8% of dietary energy from carbohydrates, oleic acid, or stearic plus *trans*-FA was replaced with *trans*-FA supported this epidemiological data, and additionally found increases in plasma C-reactive protein, IL-6 and E-selectin with the *trans*-FA diet (Baer et al., 2004).

Conjugated Linoleic Acid

Conjugated linoleic acid (CLA) is the collective term for a group of positional and geometric conjugated dienoic isomers of LA. These FA are considered conjugated because, unlike other FA, the double bonds occur on adjacent carbons, and are not separated by a methylene group. To date, 16 CLA isomers have been identified (Rickert et al., 1999), with double bonds ranging in position from carbons 6 and 8 to carbons 12 and 14. The double bonds can occur in pairs of geometric isomers as *cis*-*cis*, *cis*-*trans*, *trans*-*cis*, and *trans*-*trans*. However, only two isomers (*cis*-9, *trans*-11 CLA and

trans-10, *cis*-12 CLA) are known to possess biological activity (Pariza et al., 2000). Sources of CLA in the human diet are ruminant products and synthetic supplements, though the specific makeup of CLA differs among sources. In milk, cheese, and ruminant meat, which can contain 2-8 mg of CLA/g lipid depending on the source (Lin et al., 1995; Chin et al., 1992), approximately 80% of the CLA is *cis*-9, *trans*-11 CLA and 10% is *trans*-10, *cis*-12 CLA (Fogerty et al., 1988). Based on this, recent studies suggest average intakes of 150-200 mg of CLA per day (Jiang et al., 1999; Ritzenthaler et al., 2001), with intakes as high as 650 mg/day on a diet rich in animal fats (Park et al., 1999a). Conjugated linoleic acid dietary supplements, produced by the chemical isomerization of LA, contain predominantly *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA in equal amounts (Gaullier et al., 2002).

The *cis*-9, *trans*-11 CLA isomer is produced as an intermediate in the rumen during the biohydrogenation of dietary LA. A key anaerobic bacterium in this process is *Butyrivibrio fibrisolvens* (Kepler et al., 1966). The *cis*-12 bond of LA is acted upon by the microbial isomerase, forming *cis*-9, *trans*-11 CLA. In some of the literature, this isomer is also referred to as rumenic acid. This CLA product can leave the rumen and be directly absorbed, or it can be further metabolized by ruminal microbial hydrogenases, forming *trans*-vaccenic acid (*trans*-11, 18:1) before being completely hydrogenated to stearic acid (Kepler et al., 1966). This product may also exit the rumen and be absorbed and transported to peripheral tissues. In the mammary tissue and muscle, a Δ9-desaturase is present that can act on *trans*-vaccenic acid to produce *cis*-9, *trans*-11 CLA (Holman and Mahfouz, 1980; Pollard et al., 1980). This has been shown to occur in several mammalian species, including ruminants (Griinari et al., 2000), mice (Santora et al.,

2000), and humans (Turpeinen et al., 2002). Certain ruminal bacteria also have the capability to convert LA to *trans*-10, *cis*-12 CLA by isomerizing the *cis*-9 bond (Griinari and Bauman, 1999). This can be hydrogenated to form *trans*-10 octadecenoic acid, which may be absorbed and transported to peripheral tissues, but since mammals do not possess a Δ12-desaturase, it would not be converted back to *trans*-10, *cis*-12 CLA.

Numerous beneficial physiological effects have been attributed to CLA. The seminal observation came when CLA isolated from grilled beef inhibited chemically-induced skin neoplasia in mice (Ha et al., 1987). This discovery led to research examining the effects of CLA on cancer (Ha et al., 1990), immune function (Miller et al., 1994), atherosclerosis (Lee et al., 1994), weight gain and food intake (Chin et al., 1994), and body composition (Park et al., 1997). As previously stated, the two biologically active isomers of CLA are *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA. Though derived from the same parent molecule, the two isomers are structurally and functionally distinct. Both isomers contain a *trans* double bond, creating a straighter carbon chain, as opposed to the "kink" created by the *cis* configuration. Many enzymes recognize specific configurations in FA; therefore it is not surprising that differences in bond position and orientation of CLA isomers give them differing biological activities. Numerous studies now indicate that the various physiological and biological effects of CLA may be due to the separate actions of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers (Pariza et al., 2000).

Dietary CLA modulates body composition through decreases in adiposity and increases in lean mass in various animal models. The effects in mice are the most dramatic, with a 50-60% reduction in total adipose mass in animals fed mixed isomers of

CLA over a 4-5 week period, as compared to mice fed the control diet (Park et al., 1997). The effect in mice can be sustained, even after removal of CLA from the diet (Park et al. 2001). Additionally, when the *trans*-10, *cis*-12 isomer was fed to mice, it was more effective in lowering adipose tissue mass than *cis*-9, *trans*-11 CLA (Park et al., 1999b). Similar reductions in adipose mass have been noted in Sprague-Dawley and Zucker lean rats fed CLA, although the effects are not as large as in mice (25-30% reduction) (Sisk et al., 2001). In contrast to lean rats, obese Zucker rats exhibit an adipose-enhancing effect of dietary CLA (Szymczyk et al., 2000). In pigs, CLA-feeding decreased fat deposition and increased lean tissue (Dugan et al., 1997; Thiel-Cooper et al., 2001). In humans, however, the results are not as clear. Several studies have shown no effects of mixed CLA or individual isomers in the diet on changes in body composition in human subjects (Terpstra, 2004), and to date, no studies have shown changes in body weight (Larsen et al., 2003). Conversely, studies feeding mixed CLA and the *trans*-10, *cis*-12 isomer have reported reductions in body fat mass but no changes in body mass index (Blankson et al., 2000; Smedman and Vessby, 2001; Riserus et al., 2004). These changes are much less than those observed in pigs and mice; however, pigs and mice are generally fed at least five times more CLA per kilogam of body weight than humans (House et al., 2005). Comparable doses as used in animal studies would correspond to a daily intake of 130 g in humans (Larsen et al., 2003). Long term supplementation with mixed CLA isomers by healthy overweight individuals also seems to be well tolerated, although reductions of body fat mass may or may not be maintained (Gaullier et al., 2005; Larsen et al., 2006).

Mechanisms by which CLA reduces adiposity may involve pathways that involve energy expenditure. This is shown by increased metabolic rates and reduced nighttime

respiratory quotients in mice fed CLA for six weeks (West and York, 1998). Effects of CLA also have been linked with the induction of adipocyte apoptosis, both *in vivo* (Tsuboyama-Kasaoka et al., 2000) and *in vitro* (Evans et al., 2000), and with decreased uptake of TAG into adipocytes, particularly due to the suppression of lipoprotein lipase activity by *trans*-10, *cis*-12 CLA (Park et al., 2001).

Obesity puts an individual at a greater risk for other diseases, including type II diabetes. It would be expected then, that reduction in fat mass due to CLA intake would help decrease this risk. Evidence, however supports an additional direct effect of CLA on diabetes, which can vary, depending on the species studied. In animal models of diabetes, such as the Zucker diabetic fatty rat, CLA-enriched diets reduce fasting glucose, insulinemia (Houseknecht et al., 1998), tryglyceridemia and blood NEFA concentrations (Belury and Vanden Huvel, 1999) as compared with controls. These beneficial effects may be due, in part, to enhanced muscle uptake of glucose (Ryder et al., 2001). It is important to note that these effects are seen when a mixture of CLA isomers are fed. When fed butter enriched with *cis*-9, *trans*-11 CLA, little or no effect was seen, indicating that the effects on glucose tolerance are likely due to the *trans*-10, *cis*-12 isomer (Ryder et al., 2001). In contrast with diabetic animals, CLA modestly increases fasting serum insulin in nondiabetic pigs (Stangl et al., 1999), mice (Tsuboyama-Kasaoka et al., 2000), and humans (Medina et al., 2000). These negative effects on insulin resistance may result from decreased plasma leptin concentrations (Wang and Jones, 2004) or an increase in TAG concentration in muscle due to feeding *trans*-10, *cis*-12 CLA (Terpstra, 2004).

Similar to other classes of FA, CLA can affect the blood lipid profile and cardiovascular risk factors. In rabbits fed an atherogenic diet, supplementation with CLA lowered serum TAG and LDL cholesterol concentrations, as compared to controls (Lee et al., 1994). These animals fed CLA also showed a decrease in atherosclerotic plaque formation. Another study with rabbits showed a regression of established atherosclerosis, despite an increase in total cholesterol and decrease in HDL cholesterol (Kritchevsky et al., 2000). In a similar model in hamsters fed the *cis*-9, *trans*-11 isomer, there was no effect on plasma lipids (Gavino et al., 2000). Culturing of human platelets with either CLA isomer inhibited induced platelet aggregation (Truitt et al., 1999), but in human subjects supplemented with a mixture of CLA isomers, no difference was observed in platelet aggregation or prothrombin time (Benito et al., 2001). Together, these findings potentially implicate *trans*-10, *cis*-12 CLA to have positive effects on the blood lipid profile and coronary risk factors.

In addition to the effects on disease states, CLA can alter lipid metabolism. When consumed, CLA is incorporated into membrane phospholipids and alters FA homeostasis, particularly in the liver (Belury, 2002). Conjugated linoleic acid that is not broken down via β-oxidation is desaturated and elongated to other conjugated metabolites (Belury and Kempa-Steczko, 1997). The competition of CLA with LA for Δ6-desaturase may result in decreased AA, and can explain the reduced eicosanoid production in several systems (Belury, 2002; Brown and McIntosh, 2003). It has also been found that mice (Degrace et al., 2003) and hamsters (de Deckere et al., 1999) supplemented with CLA, particularly the *trans*-10, *cis*-12 isomer, develop enlarged, fatty livers. This effect has been attributed to an increase in liver TAG, cholesterol, cholesterol esters, and NEFA (Kelley et al.,

2004), though the mechanism is unclear. Supplementation with *trans*-10, *cis*-12 CLA *in vivo* and *in vitro* in various animal and human models leads to an increase in the ratio of saturated to monounsaturated fats (House et al., 2005). This is likely due to a reduction in stearoyl-CoA desaturase, which catalyzes the biosynthesis of MUFA from stearic and palmitic acids (Lee et al., 1998). *Trans*-10, *cis*-12 CLA also inhibits transcription of other genes involved in de novo FA synthesis, desaturation, and TAG synthesis, which may partially explain its effects on changes in lipid metabolism in the liver (Baumgard et al., 2002).

Roles of the Peroxisome Proliferator-Activated Receptors in Lipid Metabolism

Peroxisome proliferator-activated receptors (PPAR) belong to the steroid hormone receptor superfamily that are ligand-activated transcription factors (Wahli and Martinez, 1991), and act by modulating a network of responsive genes. They have been identified in many species, including *Xenopus* (Dreyer et al., 1992), mouse (Issemann and Green, 1990), rat (Gottlicher et al., 1992), and human (Sher et al., 1993). The name PPAR derives from the ability of the first-identified member to induce hepatic peroxisome proliferation in mice, but this phenomenon seems to be rodent-specific, and does not occur in other mammals. The PPARs consist of a family of three isoforms: PPARα, -γ, and –β/δ (Issemann and Green, 1990; Dreyer et al., 1992; Kliewer et al., 1994). Though encoded by separate genes, and different in their tissue distribution and metabolic actions, all three isoforms are structurally similar and can be activated by FA and their metabolic derivatives, making them the first recognized lipid sensors in the body (Schoonjans et al., 1996). Genes whose expression is modified by PPARs are numerous and control glucose homeostasis, cell cycle, inflammation, immune response, and lipid metabolism (Desvergne and Wahli, 1999).

Similar to other nuclear receptors, the PPARs possess structural features composed of functional domains. The DNA-binding domain consists of two zinc fingers that specifically bind peroxisome proliferator response elements (PPRE) in enhancer sites of regulated genes (Wahli and Martinez, 1991). The PPRE are specific DNA sequences formed by the direct repeat of a hexanucleotide sequence (AGGTCA), separated by one or two nucleotides (Torra et al., 2001). Unlike other steroid receptors which function as homodimers, to bind to the PPRE, PPAR must form a heterodimer with the retinoid X receptor (RXR) in the cytoplasm, allowing for transport to the nucleus (Miyata et al., 1994). The ligand binding domain appears to be quite large in comparison with other nuclear receptors (Nolte et al., 1998; Xu et al., 1999), potentially allowing PPARs to interact with a broad range of structurally distinct natural and synthetic ligands.

As PPARs play a critical role in lipid metabolism, the search for natural ligands began with the FAs and eicosanoids. Cell-based transactivation assays and direct binding studies have identified and characterized the endogenous receptor effectors. In general, all isoforms of PPAR are more responsive to n-6 and n-3 PUFA than to saturated or monounsaturated FAs (Krey et al., 1997). However, the affinities for the receptor vary, suggesting a role for site-specific availability and metabolism of particular FA, as well as different affinities for the specific PPAR isoforms (Sampath and Ntambi, 2005). It has been shown that FA such as LA, LNA, and AA can activate PPARα at a concentration of 100 μM (Lehmann et al., 1997). Additionally, EPA is a much more potent activator of PPARα than arachidonic acid in primary hepatocytes (Ren et al., 1997). Since the concentration of NEFA in human blood can be greater than 1 mM, these FA can be considered potent endogenous ligands for PPARα. It is important to note, however, that

the intracellular concentrations of PUFA are not known. Like PPARα, PPARγ has affinity for the PUFAs, as well as metabolic derivatives of PUFAs, such as 9-hydroxyoctadecadienoic acid (HODE) and 13-HODE (Nagy et al., 1998), and CLA (Hontecillas et al., 2002). Peroxisome proliferator-activated receptor δ also interacts with saturated and unsaturated FA, but with a ligand specificity that is intermediate between that of PPARγ and PPARα (Berger and Moller, 2002). Even with the abundance of natural ligands for PPARs, the emphasis in recent years has been on the development of synthetic ligands, due to their greater therapeutic and commercial value. Fibrates, which are ligands for PPARα, and thiazolidinediones (TZD), which are ligands for PPARγ, are two classes of drugs used to treat hypocholesterolemia and type II diabetes.

PPARα

The first PPAR discovered (Issemann and Green, 1990), PPARα is expressed predominantly in the liver, kidney, heart, brown fat, and skeletal muscle (Braissant et al., 1996; Auboeuf et al., 1997), as well as in monocytic (Chinetti et al., 1998), vascular endothelial (Inuoe et al., 1998), and vascular smooth muscle cells (Staels et al., 1998). It plays an important role in lipid metabolism via regulation of the expression of genes involved in cellular free FA uptake, β-oxidation, and cellular cholesterol trafficking (Li et al., 2002). It has been reported that PPARα is greatly induced during fasting or starvation in which a switch from carbohydrates and fats to mostly fats as an energy source is required. During fasting, FA released from the adipose tissue are taken up by the liver, where they are re-esterified to TAG or broken down via β-oxidation to ketones. Peroxisome proliferator-activated receptor α induces expression of fatty acid translocase (Motojima et al., 1998) and fatty acid transport protein (Martin et al., 1997), genes involved in transport of FA into the cell, as well as CPT-I (Brady et al., 1999), which

catalyzes the rate limiting step for transport of FA into the mitochondria for oxidation. Activation of PPARα also directly upregulates genes involved in peroxisomal β-oxidation, including acetyl-CoA synthase (Schoonjans et al., 1995) and ACO (Tugwood et al., 1992). The importance of PPARα in this response has been demonstrated by studies involving PPARα-null mice, which are unable to induce the change in energy source, resulting in hypoglycemia, hyperlipidemia, hypoketonemia and fatty liver (Kersten et al., 1999). In rodents, activation of PPARα induces peroxisome proliferation, hepatomegaly, and hepatocarcinogenesis (Issemann and Green, 1990). Fortunately, these effects are not present in humans, possibly due to the 10-fold greater concentrations of PPARα in rodent as compared to human liver (Palmer et al., 1998) or to differences in the PPREs of responsive genes, such as ACO (Lambe et al., 1999).

Fibrates have been a commonly prescribed drug to treat dyslipidemia in humans for over 30 years, but the direct role of PPARα in the lipid-lowering actions of fibrates has only recently been established. In humans, fibrate administration lowers plasma TAG and increases plasma concentrations of HDL and its major constituents, apolipoproteins A-I (apo A-I) and A-II (apo A-II) (Malmendier and Delcroix, 1985; Mellies et al., 1987). Peroxisome proliferator-activated receptor α activation affects several key genes in HDL metabolism, including apo A-I, apo A-II, ABCA1, LPL, and scavenger receptor class B type I (Fruchart, 2001). Peroxisome proliferator-activated receptor α also has been shown to down-regulate apo C-III (Hertz et al., 1995; Staels et al., 1995), a protein that inhibits TAG hydrolysis by LPL, further contributing to the lipid-lowering effects of fibrates.

Surprisingly, the role of PPARα in cardiovascular disease appears to be negative. In a mouse model, over-expression of PPARα in the heart increases FA oxidation and decreases glucose use, similar to that seen in the diabetic heart. Upon fibrate administration, these mice develop greater cardiomyopathy than the wild-type controls (Finck et al., 2002). Peroxisome proliferator-activated receptor α null mice do not show this effect (Finck et al., 2003). This knowledge, when combined with research indicating that PPARα and apolipoprotein E double knockout mice are resistant to insulin-resistance and atherosclerotic lesions induced by a high-fat diet (Tordjman et al., 2001), suggests that PPARα senses FAs and induces their use, thereby playing a potential causative role in cardiovascular disease. Unlike humans, in rodent models, fibrate administration decreases apo A-I and apo A-II expression, suggesting differential regulation in the different species (Berthou et al., 1995). Overall reduction in TAG and increase in HDL cholesterol in humans, even with potential for negative cardiovascular events, would still result in less fat accumulation in the vessel walls, and would be beneficial to heart health.

PPARβ/δ

. Peroxisome proliferator-activated receptor β/δ (hereafter referred to as PPARδ) has been slighted in its importance in the body because of its ubiquitous expression and unavailability of selective ligands, despite the fact that it is the predominant isoform in skeletal muscle – one of the most insulin responsive and metabolically demanding tissues of the body. The importance of PPARδ in FA metabolism was first realized from studies using knockout animals. Most PPARδ null mice die during early embryogenesis, and the numbers that do survive show a marked decrease in fat mass (Peters et al., 2000). In exercised or fasted PPARα null mice, the liver, but not the muscle glycogen levels

deplete as compared to wild-type litter mates, indicating that a factor other than PPARα may be in control of energy homeostasis (Muoio et al., 2002).

Recently, synthetic, highly selective PPARδ-agonists have been developed, and its role in FA catabolism and energy homeostasis has been further elucidated (Peters et al., 2000; Barak et al., 2002). Activation of PPARδ increases FA oxidation in human and rodent myocytes, showing the redundancy of PPARs α and δ in FA homeostasis (Muoio et al., 2002). In genetically obese ob/ob mice, PPARδ activation not only enhances β-oxidation in skeletal muscle, but protects against diet-induced obesity, improves glucose tolerance, and improves insulin sensitivity, showing its potential as a target in treating and preventing obesity and type II diabetes (Tanaka et al., 2003).

Similarly to PPARα, PPARδ activation up-regulates adenosine triphosphate-binding cassette transporter-A1 (ABCA1) gene expression and increases cholesterol efflux from cells and increases HDL cholesterol in mice (Leibowitz et al., 2000) and non-human primates (Oliver et al., 2001). The PPARδ selective agonist GW501516, in particular, has shown therapeutic potential for the treatment of dyslipidemia, by dramatically improving the serum lipid profile of insulin-resistant rhesus monkeys. This occurs through decreases in concentrations of blood TAG and insulin and increases in HDL to a greater extent than is achieved with fibrates in fasting individuals. Activation of PPARδ has the added normal-lipidemic effect of lowering the blood concentrations of small dense LDL cholesterol (Oliver et al., 2001).

Recent research suggests a role for PPARδ in the heart as well. In cultured human cardiomyocytes, PPARδ is highly expressed, and its activation leads to an increase in FA oxidation (Cheng et al., 2004), leading to a potential increase in energy to an energy

demanding organ, and implicating PPARδ as a modulator of cardiac energy homeostasis. Regarding foam cell formation, research with different PPARδ activators have given different results. In one study, activation increased cholesterol efflux through the ABCAI pathway (Oliver et al., 2001), whereas another study demonstrated enhanced lipid accumulation (Vosper et al., 2001). Though these discrepancies may be due to different experimental models and structurally different agonists, further research can help elucidate the role of PPARδ.

PPARγ

Because it is primarily found in adipose tissue, PPARγ is a prime suspect in the regulation of lipid metabolism. In support of this, many studies have shown the importance of PPARγ in the formation and functioning of adult fat cells (Rosen et al., 2000). As obesity is a primary risk factor for incidence of the metabolic syndrome, it is highly likely that PPARγ plays a role in the associated diseases and their treatment. Thiazolidinediones are pharmacologic activators of PPARγ, which significantly improve insulin sensitivity in humans with type II diabetes (Sood et al., 2000). The mechanism of action, however, still remains unclear, especially considering the fact that muscle is the major insulin responsive tissue, and PPARγ is present at very low levels in muscle and liver and high in adipose. Resolving this apparent paradox had proved difficult. Most research has stemmed from clinical trials and rodent models of obesity and diabetes. Unlike the readily available PPARα null mice, PPARγ knockout mice die early in gestation, preventing valuable loss-of-function studies (Barak et al., 1999). By the use of microarrays for gene expression profiling, several key metabolic genes were identified, all primarily in the adipocyte. Changes induced by TZD administration to Zucker

diabetic fatty rats include modulation of genes involved in glucose uptake, lipid uptake and storage, and energy expenditure (Way et al., 2001). The small increases in glucose disposal by the adipose, coupled with greater sequestration of fat into adipose, thereby relieving some of the metabolic burden of muscle and liver and allowing for greater glucose use by these tissues, is a potential explanation for the profound activity of the TZD class of drugs.

In addition to the action of PPARγ ligands on adipose, there is mounting evidence that these compounds can exert some effects on other tissues. aP2/DTA mice, whose white and brown adipose tissue has been eliminated by fat-specific expression of diphtheria toxin A chain, develop hyperglycemia, hyperinsulinemia, and hyperlipidemia indicative of insulin-resistant diabetes (Ross et al., 1993). Thiazolidinedione administration to these animals improves the serum lipid profile, but results are conflicting on the effects on glucose tolerance, with one study showing decreases in insulin (Burant et al., 1997) and another showing no change (Chao et al., 2000). Using tissue-specific PPARγ knock-out mice, the question of whether TZDs directly or indirectly affect insulin resistance has been researched. Targeted deletion of PPARγ in adipose results in adipose hypertrophy, elevated plasma NEFA and TAG, increased hepatic gluconeogenesis and insulin resistance, without changes in insulin sensitivity of muscle (He et al., 2003). These observations indicate that changes in adipose function via PPARγ result in changes in hepatic function with minimal effects in muscle.

In addition to the effects on adipose tissue and insulin resistance, activation of PPARγ seems to play a role in atherosclerosis. Again, the positive effects of TZD treatment on decreased risk of atherosclerosis may be secondary to the improvement in

lipid profile, but PPARγ activation may also have a direct effect on the formation and progression of atherosclerotic lesions. Peroxisome proliferator-activated receptor γ activation inhibits leukocyte-endothelial cell interaction, a critical inflammatory response in the formation of atherosclerotic plaques (Jackson et al., 1999). Activation by TZDs also inhibits the expression of vascular cell adhesion molecule (Pasceri et al., 2000) and E-selectin (Nawa et al., 2000), which would reduce the "homing" of monocyte and macrophage cells to atherosclerotic plaques.

CHAPTER 3
EFFECTS OF N-3 AND N-6 FATTY ACIDS ON LIPID METABOLIZING GENES AND HIGH-DENSITY LIPOPROTEIN CHOLESTEROL PRODUCTION IN CULTURED HUMAN AND RAT HEPATOCYTES

Introduction

Dietary polyunsaturated fatty acids (PUFA) have been shown to lower blood triglycerides, alter the blood lipid profile, decrease intramuscular lipid droplet size, improve insulin sensitivity, and enhance glucose utilization (Jump and Clarke, 1999). Since the observation that PUFAs could inhibit hepatic lipogenesis in mice (Allmann and Gibson, 1965), numerous studies have demonstrated that diets rich in PUFAs influence metabolic changes by coordinately suppressing lipid synthesis in the liver and enhancing fatty acid oxidation in both liver and skeletal muscle (Jump and Clarke, 1999). The PUFA induction of genes encoding proteins involved in lipid oxidation include 3-hydroxy, 3-methylglutaryl CoA synthase (Rodriguez et al., 1994), carnitine palmitoyltransferase, fatty acid binding proteins and peroxisomal acyl-CoA oxidase (ACO; Reddy and Hashimoto, 2001). With the discovery of a new member of the steroid hormone receptor superfamily, the peroxisome proliferator-activated receptor (PPAR; Issemann and Green, 1990) and the discovery that certain fatty acids (FA) and their derivatives can specifically bind PPARs (Gottlicher et al., 1992), the possibility arose that PUFAs mediate metabolic effects via alteration of PPAR activity. In the liver, the predominant isoform is PPARα; therefore this isoform has become the primary focus of studies involving the liver.

The objective of this study was to examine the short term effects of FAs of differing levels of saturation and bond position on lipid metabolizing gene expression and high-density lipoprotein (HDL) cholesterol production in HepG2 and H-4-II-E cells. Based on both dietary and *in vitro* studies of lipid metabolism, we hypothesized that FAs of differing saturation and double bond position may have differing effects on ACO, 3-hydroxy, 3-methylglutaryl CoA reductase (HMG-R), and apolipoprotein A-I (Apo A-I) gene expression. Also, because several fatty acids and their derivatives are known ligands for PPARs, we hypothesized that fatty acids may act on lipid metabolizing genes through activation of PPARα in the liver.

Materials and Methods

Materials

Polystyrene tissue culture dishes (100 x 20 mm) were purchased from Corning (Corning Glass Works, Corning, NY). The antibiotic/antimycotic (ABAM), sodium pyruvate, fatty acid-free bovine serum albumin (BSA), stearic acid (ST), WY 14,643, and MK886 were from Sigma Chemical Co. (St. Louis, MO). Minimum Essential Medium (MEM), phenol red-free MEM, Hanks Balanced Salt Solution (HBSS) and TriZol reagent were from GIBCO BRL (Carlsbad, CA). The fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA). Linoleic, linolenic, and eicosapentaenoic acids were from Cayman Chemicals (Ann Arbor, MI). BioTrans nylon membrane and [α-^{32}P] deoxycytidine triphosphate (SA 3000 Ci/nmol) were from MP Biolomedicals (Atlanta, GA). The Enzyme Color Solution, Reacting Solution, and HDL Calibrator were from Wako Diagnostics (Richmond, VA).

Cell Culture and Treatment

HepG2 (ATCC # HB-8065; Manassas, VA) and H-4-II-E (ATCC # CRL-1548; Manassas, VA) cells were suspended in 10 mL of growth medium (MEM), containing 2.2 g/L sodium bicarbonate, 1.0 mM sodium pyruvate, 1% (v/v) ABAM and 10% FBS. Cells were cultured at 37°C in a humidified atmosphere containing 95% O_2 and 5% CO_2. Cultures were replenished with fresh medium every 2 d until cells were approximately 90% confluent. Cells were washed twice with HBSS, and cultured in fresh serum-free medium containing appropriate treatments for an additional 24 h.

Stock solutions of fatty acids were stored at -20°C. At preparation of treatments, fatty acids were mixed with serum-free culture medium containing 33 mg/mL of fatty acid-free BSA to a concentration of 1 mM. This mixture was incubated for 2 h at 37°C to allow complexation of the fatty acids with BSA and then further diluted in culture medium to a final treatment concentration of 100 μM of fatty acids.

To investigate the effects of supplemental PUFAs on hepatic gene expression and cholesterol synthesis, HepG2 and H-4-II-E cells were treated with stearic (ST), linoleic (LA), linolenic (LNA) or eicosapentoenoic (EPA) acid (100 μM). Sub-confluent cells were incubated with serum-free medium alone (Control) or with appropriate treatments (listed above) complexed with BSA, for a period of 24 h. Cells were rinsed twice with 10 mL of HBSS. The remaining cell monolayer was then lysed in 3 mL of TriZol reagent, and stored at -80°C for subsequent mRNA analysis. The same fatty acid treatments were repeated, using phenol red-free MEM. After incubation, conditioned media were collected and stored at -20^{o}C until lipid extraction and HDL cholesterol analysis.

To determine whether fatty acid effects on gene expression involves PPARα activation, confluent HepG2 and H-4-II-E cells were treated with ST (100 μM), the

PPARα agonist WY 14,643 (10 μM), or a combination of fatty acid and WY 14,643. Additional sets of culture dishes were incubated with ST alone, the PPARα inhibitor MK886 (10 μM; Kehrer et al., 2001), or a combination of ST and MK886. After a 24 h incubation, cells were washed twice with 10 mL of HBSS, lysed with TriZol, and stored at -80^{o}C until mRNA analysis.

RNA Isolation and Analysis

Total cellular RNA was isolated from cells using TriZol reagent according to the manufacturer's instructions. Ten micrograms of total RNA was fractioned in a 1.0% agarose formaldehyde gel following previously described protocols (Ing et al., 1996) using the MOPS buffer (Fisher Scientific, Pittsburgh, PA) and transferred to a Biotrans nylon membrane by downward capillary transfer in 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) using the TurboBlotting system (Schleicher and Schuel, Keene, NH). Nylon membranes were cross-linked by exposure to a UV light source for 90 sec and baked at 80°C for 1 h. Membranes were incubated for 2 h at 50°C in ultrasensitive hybridization buffer (ULTRAhyb; Ambion, Austin, TX) followed by an overnight incubation at 50°C in the same ULTRAhyb solution containing the ^{32}P-labeled acyl-CoA oxidase (ACO), 3-hydroxy, 3-methylglutaryl CoA reductase (HMG-R) and apolipoprotein A-I (Apo A-I) cDNA probes. Probes were generated by RT-PCR for ACO (forward 5'-CCGGAGCTGCTTACACACAT-3'; reverse 5'-GGTCATACGTGGC TGTGGTT-3'), HMG-R (forward 5'-TCCTTGGTGATGGGAGCTTGTTGTG-3'; reverse 5'-TGCGAACCCTTCAGATGTTTCGAGC-3'), human Apo A-I (forward 5'-AAGACAGCGGCAGAGACTAT-3'; reverse 5'-ATCTCCTCCTGCCACTTCTT-3'), and rat Apo A-I (forward 5'-AAGGACAGCGGCAGAGACTA-3'; reverse 5'-CCACAACCTTTAGATGCCTT-3'). The sizes and sequences of these cDNA probes

were verified by DNA sequencing prior to their use in Northern blot analysis. Filters were sequentially washed in 2X SSC (1X= 0.15 M sodium chloride, 0.015 M sodium citrate)-0.1% SDS and in 0.1x SSC-0.1% SDS twice each at 50°C and then exposed to X-ray film to detect radiolabeled bands. Equal loading of total RNA for each experimental sample was verified by comparison to 18S rRNA ethidium bromide staining.

Lipid Extraction

Total lipids were extracted from conditioned media as described by Bligh and Dyer (1959), with modifications. For each sample, 2 mL of conditioned media was aliquotted into a 20 mL glass screw-top vial. Fourteen mL of chloroform:methanol (2:1, v/v) was then added and the vials were vortexed for 5 minutes. The vials were then centrifuged at 1700 rpm for 5 minutes. The bottom lipid-containing chloroform layer was transferred to a clean, dry, pre-weighed vial, placed in a 37^{o}C water bath, and dried under nitrogen gas. Dry samples were placed in a 50^{o}C oven for 10 minutes and placed in a desiccator to cool to room temperature. Samples were weighed, and lipid weight was determined by difference. The sample was resuspended in chloroform and stored at -20^{o}C until HDL cholesterol analysis.

HDL Cholesterol Assay

Lipid extracts from conditioned media were analyzed using a commercially available L-Type HDL-C kit, following the manufacturer's directions. Briefly, using a 96-well plate, 3 µL of sample was pipetted into each well. Two hundred seventy µL of Enzyme Color Solution (R1) was added, and the plate was incubated for 5 minutes at 37^{o}C. Ninety µL of Reacting Solution (R2) was then added, and the plate was incubated another 5 minutes at 37^{o}C. The absorbance at 600 nm was measured using the

SpectraMax 340 PC microplate reader (Molecular Devices, Sunnyvale, CA), and the concentration of the samples was calculated by plotting against a standard curve.

Statistical Analysis

All hybridization signals as measured by densitometry were evaluated by least squares analysis of variance (ANOVA) using the General Linear Model (GLM) procedure of the SAS software package (SAS Institute Inc, Cary, NC). In each experiment, treatments were run in duplicate, and the whole experiment was also duplicated, giving n=4 plates per treatment. The general model for mRNA analysis included experiment, treatment, and experiment x treatment interaction. In mRNA analyses, densitometric values for target genes were expressed as ratios of target gene densitometric values over the corresponding 18S rRNA densitometric values. For HDL cholesterol concentration, the sources of variation included experiment, treatment, experiment x treatment interaction, and plate (experiment x treatment). The plate, nested within experiment and treatment, was considered a random variable, and therefore the plate variance was used as an error term to test the effects of experiment, treatment, and experiment x treatment interaction. Treatment means were further compared using preplanned orthogonal contrasts. These contrasts were control vs. fat treatment (ST, LA, LNA, EPA), saturated fat (ST) vs. PUFA (LA, LNA, EPA), n-6 (LA) vs. n-3 (LNA, EPA); and LNA vs. EPA. For all responses, the two cell lines were analyzed separately.

Results

Effects of Fatty Acids on HepG2 Cells

Steady-state levels of ACO mRNA were not affected by any FA treatment in HepG2 cells ($P = 0.3$; Figure 3-1). Concentrations of HMG-R mRNA transcript were greater (+24%, $P = 0.006$) in HepG2 cells treated with ST than in PUFA-treated cells

(Figure 3-2). Concentrations of Apo A-I mRNA transcript were greater (+15%, P = 0.05) in HepG2 cells treated with FA than in control cells (Figure 3-3). There were no differences in HDL cholesterol concentration among any of the treatments (P = 0.9; Figure 3-4).

Effects of Fatty Acids on H-4-II-E Cells

In the H-4-II-E cells, ACO mRNA expression was greater (+26%, P = 0.004) in ST-treated cells as compared to PUFA-treated cells (Figure 3-5). Concentrations of HMG-R mRNA were greater in ST-treated as compared to PUFA-treated cells (+27%; P = 0.002), in n-3 (EPA and LNA)-treated as compared to n-6 (LA)-treated cells (+30%; P = 0.004), and in EPA-treated as compared to LNA-treated cells (+49%; P < 0.001), with the EPA treatment showing the greatest induction of HMG-R mRNA transcript (Figure 3-6). Similarly, steady-state levels of Apo A-I mRNA were increased in ST-treated cells as compared to PUFA-treated cells (+39%; P < 0.001) and in EPA-treated cells as compared to LNA-treated cells (+31%; P = 0.008; Figure 3-7). As compared to n-6 FA, n-3 FA increased (+79%; P = 0.0002) HDL cholesterol concentration by H-4-II-E cells, with the effect predominantly deriving from the large increase (+84%; P < 0.0001) in production with LNA as compared to EPA (Figure 3-8).

Role of PPARα in Stearic Acid-Induced Effects on Gene Expression

Co-incubation of HepG2 cells with ST and 10 μM WY 14,643, a specific PPARα agonist, decreased (-9%; P = 0.04) ACO mRNA expression as compared to ST alone. There was no detectible effect on ACO mRNA with the use of the agonist alone (P = 0.8; Figure 3-9). WY 14,643 decreased both basal (-32%; P = 0.0002) and ST-induced (-10%; P = 0.02) expression of HMG-R mRNA (Figure 3-10). Use of the PPARαagonist

alone (P = 0.5) or in combination with ST (P = 0.4) had no effects on Apo A-I mRNA (Figure 3-11).

In HepG2 cells, incubation with 10 μM MK886, a specific PPARαinhibitor increased (P < 0.05) basal production of all three gene transcripts (Figures 3-12, 3-13, and 3-14). Co-incubation with MK886 had no effects on ST-induced expression of any of the genes.

Co-incubation of H-4-II-E cells with WY 14,643 increased (+22%; P = 0.04) basal levels and enhanced (+38%; P = 0.0003) the effect of ST on ACO gene expression (Figure 3-15). Both basal (-45%; P = 0.01) and ST-induced (-32%; P = 0.03) HMG-R mRNA expression were decreased with the use of the PPARα agonist (Figure 3-16). The abundance of ST-induced Apo A-I mRNA transcript was enhanced (+29%; P = 0.001) by the use of WY 14,643 (Figure 3-17). Basal levels of Apo A-I mRNA were unaffected (P = 0.2).

In H-4-II-E cells, incubation with MK886 attenuated (-28%; P = 0.001) the effects of ST on ACO mRNA expression (Figure 3-18). The PPARα inhibitor increased (+29%; P = 0.003) the basal concentration of HMG-R mRNA transcript, but had no effects (P = 0.8) on ST-induced gene expression (Figure 3-19). The concentration of both basal (-96%; P = 0.01) and ST-induced (-39%; P = 0.03) Apo A-I mRNA transcript was reduced by the use of MK886 (Figure 3-20).

Discussion

Dietary fat has been implicated as a major factor in many areas of health and disease. However, it has been suggested by numerous studies that all fats may not have the same effects. In this study, both human and rat hepatoma cells were used as models, as it also has been suggested that species differences exist in fat metabolism (Bergen and

Mersmann, 2005). In HepG2 (human) liver cells, ACO mRNA expression was unaffected by any FA treatment. In contrast, in the H-4-II-E (rat) liver cells, ACO mRNA expression was induced by ST only. Other studies, however, have shown up-regulation of ACO mRNA in rat liver by dietary PUFAs as well as by saturated fats (Berthou et al., 1995). In HepG2 cells, it has been shown that PUFAs of differing saturation and length can regulate ACO mRNA in a dose-dependent and differential manner (Rise and Galli, 1999). In a human retinoblastoma cell line, low concentrations of supplemental n-3 PUFA increased ACO mRNA, whereas high concentrations of the FA decreased it (Langelier et al., 2003). Consistent with our findings in rat cells, pigs fed a tallow-based diet high in saturated fat had an increased concentration of ACO mRNA as compared to fish-oil fed animals (Ding et al., 2003).

3-hydroxy, 3-methylglutaryl CoA reductase is the rate limiting enzyme in cholesterol synthesis, and its inhibition is the target of the statin class of drugs, used in the treatment of hyperlipidemias. In this study, we showed that in HepG2 cells, HMG-R mRNA was up-regulated by ST as compared to the PUFAs, whereas in the H-4-II-E cells, it was up-regulated by both ST and EPA. Consistent with our findings in rodent cells, in C3H mice fed diets differing in fat composition, HMG-R mRNA was increased to a greater extent in mice fed the PUFA diet than in those fed the saturated fat diet (Cheema and Agellon, 1999). In Reuber H35 rat hepatoma cells, incubation with either saturated fats or PUFAs increased HMG-R enzyme activity (Garcia-Pelayo et al., 2003). Enzyme activity of HMG-R also has been shown to be increased in mice fed a diet high in PUFAs (Kuan and Dupont, 1989).

Apolipoprotein A-I is the predominant lipoprotein associated with HDL cholesterol and is essential for its normal metabolism. Deletion of the Apo A-I gene in humans results in very low plasma concentrations of HDL cholesterol and premature coronary artery disease (Schaefer et al., 1982). Dietary fat has the ability to modulate plasma lipids, and may act, in part, by effects on apolipoproteins. In this study, we showed that, in HepG2 cells, Apo A-I mRNA was up-regulated by all FA. However, no effects were seen in HDL cholesterol concentration in the culture media. This is supported in a study by Dashti and coworkers (2002) in which HDL concentration was not different between LA- and saturated fat-treated HepG2 cells. In Golden-Syrian hamsters, an effective model for human diet and blood lipid interactions, canola and soybean oils increased Apo A-I mRNA as compared to a butter diet, though HDL concentrations were lowered in the diets containing unsaturated as compared to saturated fats (Dorfman et al., 2005). In the H-4-II-E cells, ST increased Apo A-I mRNA concentration as compared to the PUFA-treated cells. In contrast to current findings, Sprague-Dawley rats fed diets high in saturated fat or PUFAs showed no differences in Apo A-I amounts (Hatahet et al., 2003). However, the saturated fat diet contained primarily palmitic acid, not stearic acid, as in this study.

As fatty acids and their derivatives have been identified as potential ligands for peroxisome proliferator-activated receptors (PPAR), we investigated the possibility that fatty acid effects in the two cell lines may be mediated by PPARα. Incubation of HepG2 cells with WY 14,643, a PPARα agonist, had no effects on basal expression of ACO or Apo A-I mRNA. In the H-4-II-E cells, however, incubation with the agonist not only enhanced ST-induced ACO and Apo A-I mRNA expression but also increased basal

expression of ACO mRNA. Not unexpectedly, use of the PPARα inhibitor, MK886, was able to cause the opposite effect, blocking the effects of ST on ACO and Apo A-I mRNA expression in H-4-II-E cells. Although ACO is an established PPARα responsive gene (Tugwood, et al., 1992), species differences do exist. It is questionable whether the PPAR response element of human ACO is active (Woodyatt et al., 1999). Dietary studies have shown that rodents are responsive to the effects of PPARα activation, but non-rodent species, such as primates and guinea pigs, are resistant or unresponsive to some of the negative effects (Bentley et al., 1993; Cattley et al., 1998). In a comprehensive analysis of gene expression in human and rat hepatoma cells by microarray analysis, only rat ACO mRNA was responsive to WY 14,643 (Vanden Heuvel et al., 2003). Other genes that may be differentially regulated in human and rat liver include cytosolic aspartate aminotransferase (Tomkiewicz et al., 2004), peroxisomal 3-oxoacyl-CoA thiolase (Lawrence et al., 2001), and catalase (Ammerschlaeger et al., 2004). Additionally, different PPARα agonists may regulate lipid metabolism in a compound-dependent manner. A recent study by Duez and coworkers (2005) showed that, in mice, fenofibrate and gemfibrozil, both stimulate ACO mRNA expression, but only fenofibrate greatly induces Apo A-I gene expression. Interestingly, although effects of PPARα activation or inhibition on ACO and Apo A-I mRNA were different between the human and rat cell lines, effects on HMG-R mRNA were similar. In both cell lines, activation of PPARα by WY 14,643 caused a decrease in basal and ST-induced HMG-R mRNA expression. Inhibition of PPARα by MK886 increased HMG-R mRNA expression to a level similar to that induced by ST treatment alone, suggesting that ST

effects are mediated by PPARα. The findings of this study, in combination with other reports, strongly suggest a species-specific role for PPARα in gene regulation.

Summary

In HepG2 cells, ST up-regulated HMG-R gene expression as compared to PUFAs. As compared to control, in this cell line, all FA in this experiment up-regulated Apo A-I gene expression. When PPARα was selectively activated, the effect of ST on ACO gene expression was decreased, whereas both basal and ST-induced HMG-R gene expression were decreased. Incubation with the PPARα inhibitor was able to decrease the basal production of all three genes, but had no effects on ST-induced gene expression.

In H-4-II-E cells, ST up-regulated ACO, HMG-R, and Apo A-I gene expression as compared to the PUFAs. Selective activation of PPARα increased basal levels of ACO and further enhanced the effect of ST on ACO and Apo A-I mRNA. Conversely, selective activation of PPARα decreased basal levels of HMG-R and blocked the effect of ST on HMG-R mRNA. Incubation with the PPARα inhibitor was able to decrease the effects of ST-induced ACO and Apo A-I mRNA, as well as decrease the basal concentration of Apo A-I mRNA and increase the basal concentration of HMG-R mRNA. Together, these results indicate that FAs likely regulate lipid metabolizing genes in the liver through a PPARα-dependent mechanism. However, due to different responses in the human and rat hepatoma cell lines, the net effects are likely species specific.

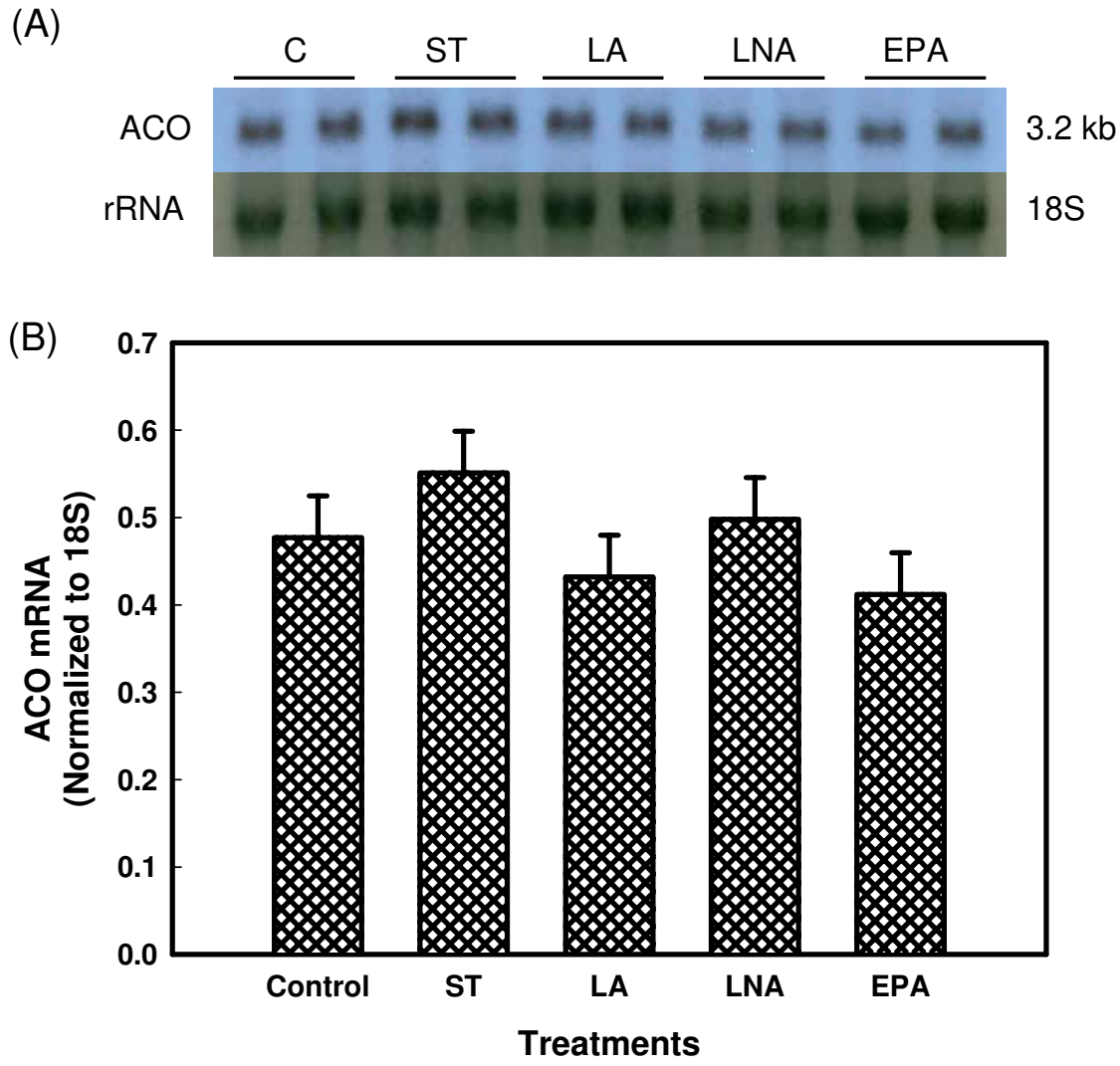

Figure 3-1. Effect of long-chain FA on ACO mRNA expression in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and FA-treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (ST + LA + LNA + EPA), P = 0.9; Contrast 2: ST vs. (LA + LNA + EPA), P = 0.09; Contrast 3: LA vs. (LNA + EPA), P = 0.7; Contrast 4: LNA vs. EPA, P = 0.2.

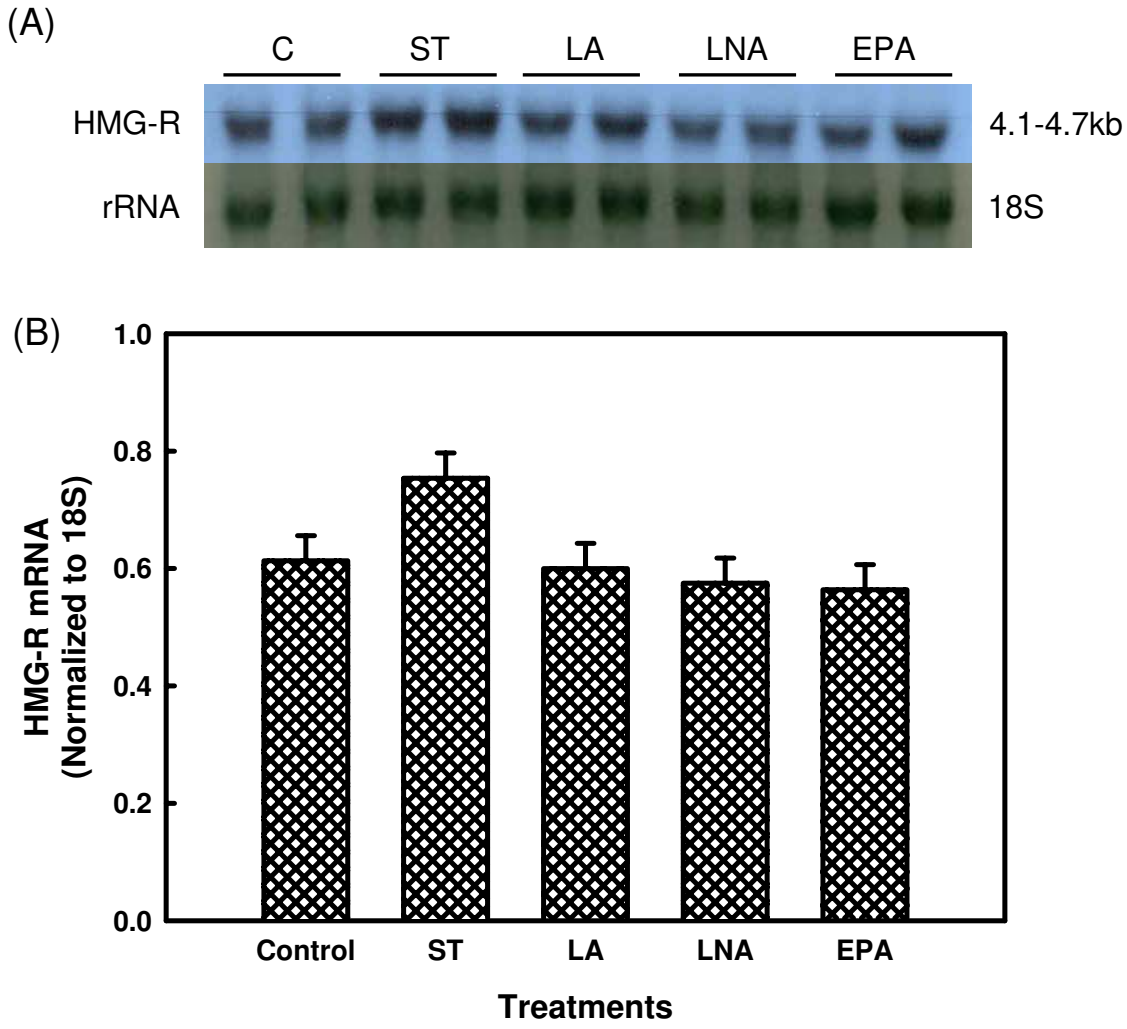

Figure 3-2. Effect of long-chain FA on HMG-R mRNA expression in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and FA-treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (ST + LA + LNA + EPA), P = 0.8; Contrast 2: ST vs. (LA + LNA + EPA), P = 0.006; Contrast 3: LA vs. (LNA + EPA), P = 0.6; Contrast 4: LNA vs. EPA, P = 0.9.

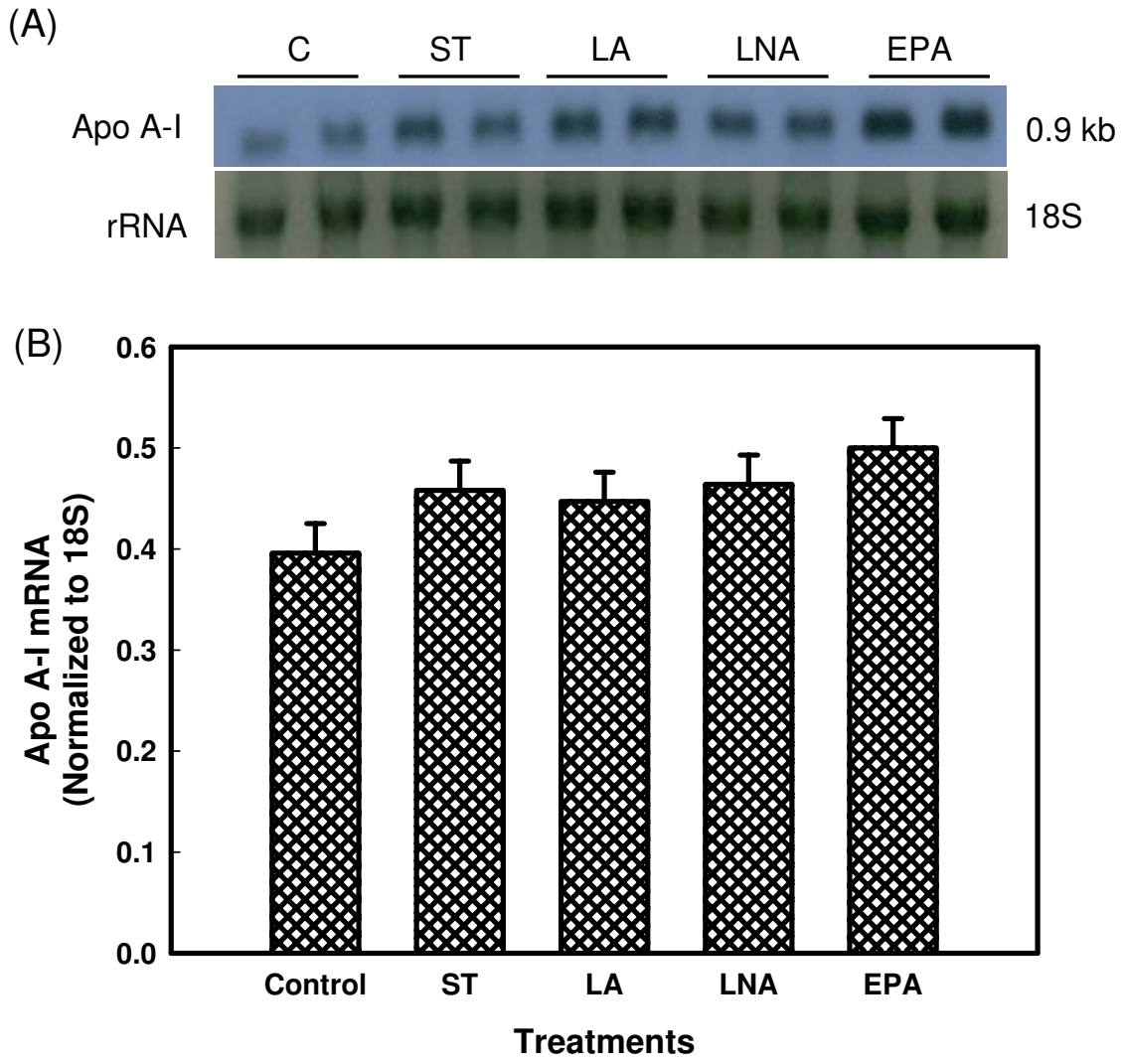

Figure 3-3. Effects of long-chain FA on Apo A-I mRNA expression in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and FA-treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (ST + LA + LNA + EPA), P = 0.05; Contrast 2: ST vs. (LA + LNA + EPA), P = 0.7; Contrast 3: LA vs. (LNA + EPA), P = 0.3; Contrast 4: LNA vs. EPA, P = 0.4.

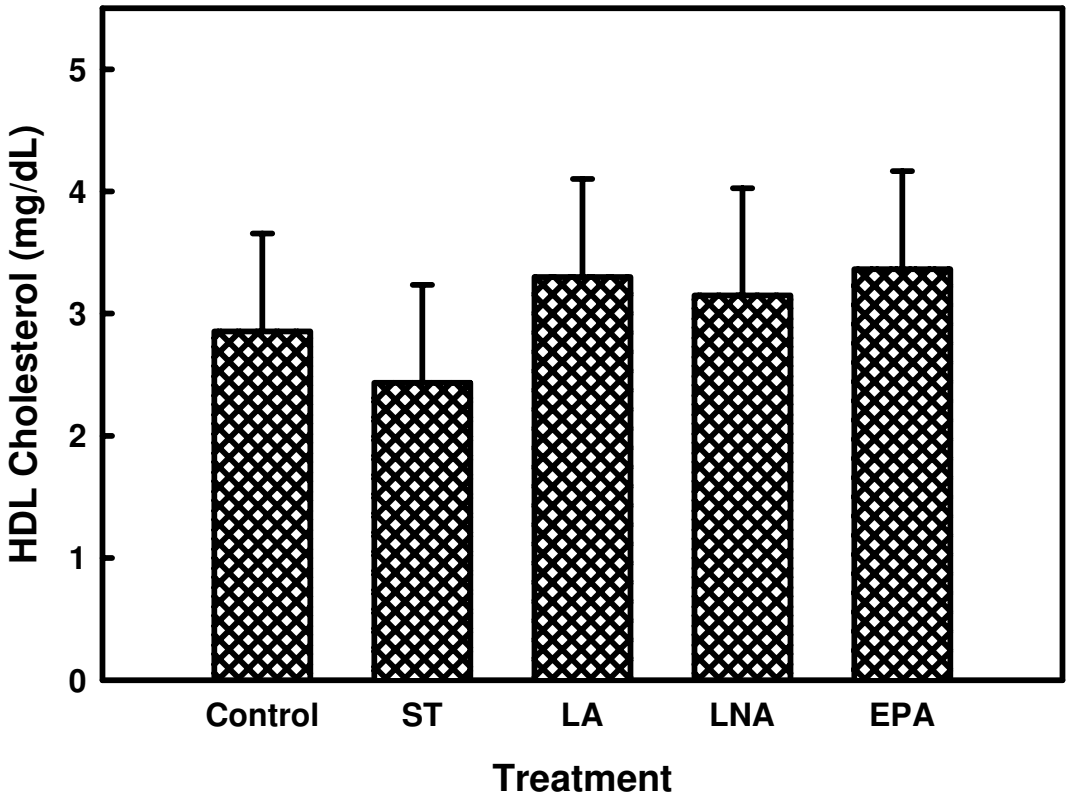

Figure 3-4. Effects of long-chain FA on HDL cholesterol production in HepG2 cells. Data represents least square means ± SEM calculated over two experiments. To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (ST + LA + LNA + EPA), P = 0.8; Contrast 2: ST vs. (LA + LNA + EPA), P = 0.4; Contrast 3: LA vs. (LNA + EPA), P = 0.9; Contrast 4: LNA vs. EPA, P = 0.9.

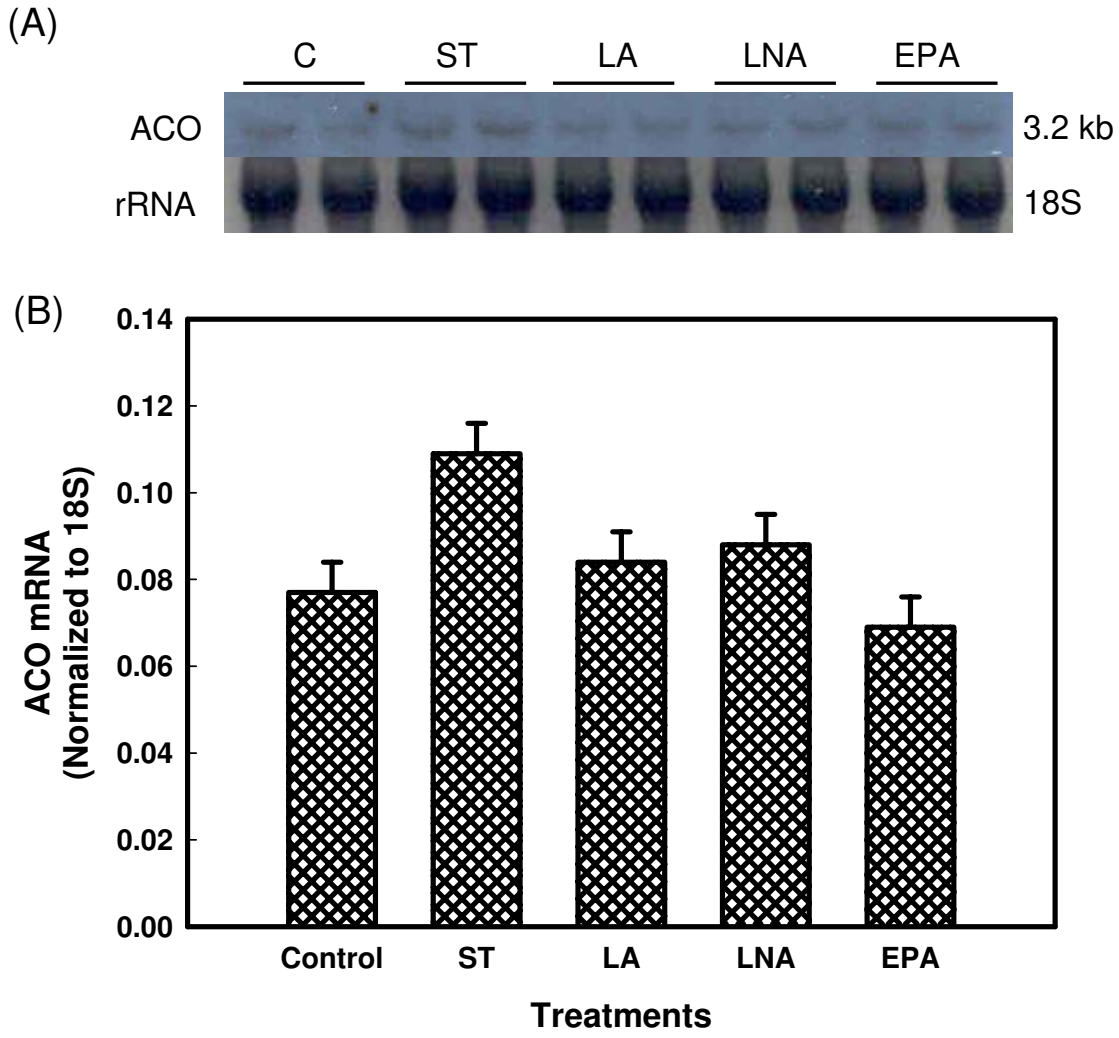

Figure 3-5. Effects of long-chain FA on ACO mRNA expression in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and FA-treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (ST + LA + LNA + EPA), P = 0.2; Contrast 2: ST vs. (LA + LNA + EPA), P = 0.004; Contrast 3: LA vs. (LNA + EPA), P = 0.6; Contrast 4: LNA vs. EPA, P = 0.07.

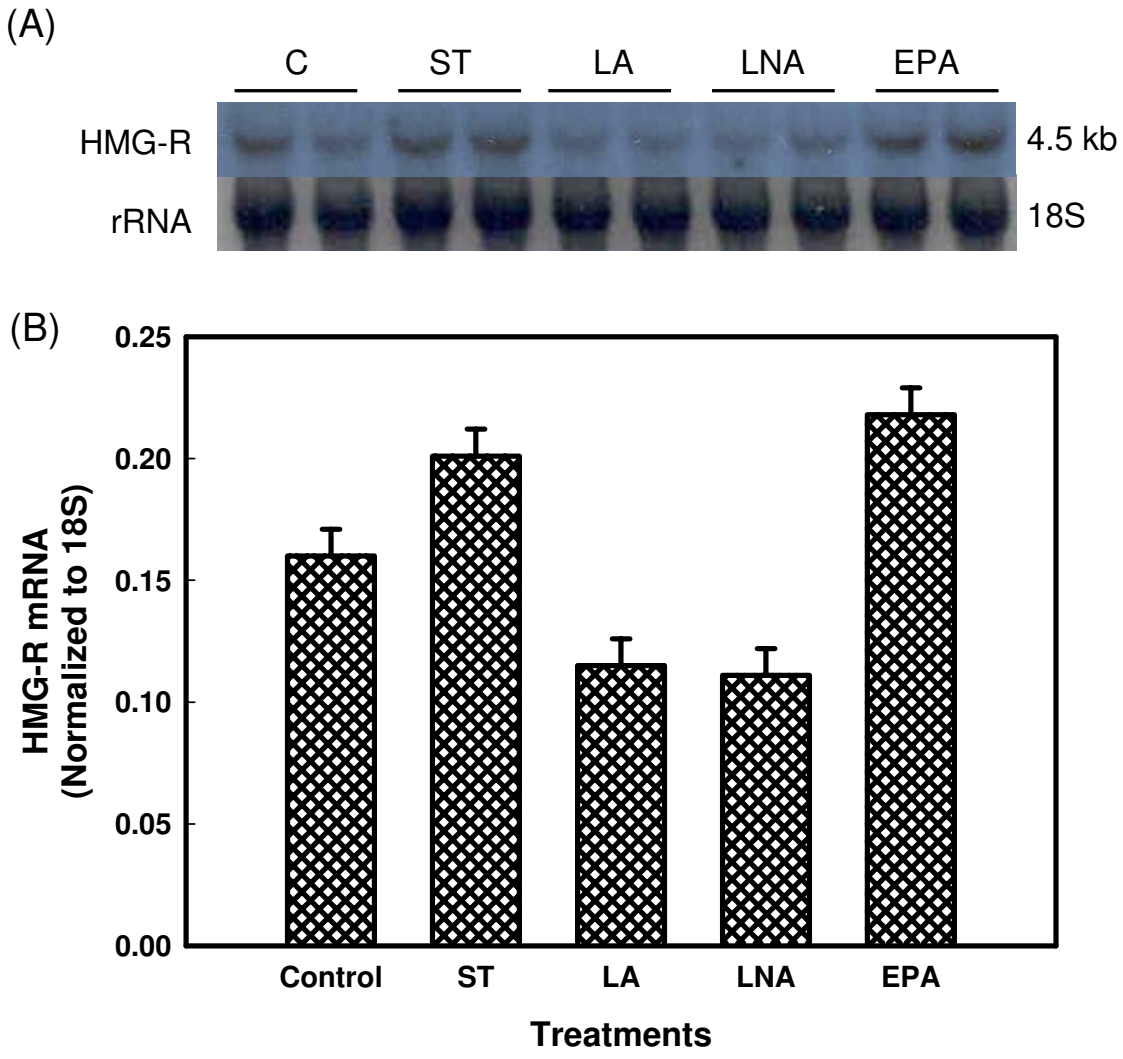

Figure 3-6. Effects of long-chain FA on HMG-R mRNA expression in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and FA-treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (ST + LA + LNA + EPA), P = 0.9; Contrast 2: ST vs. (LA + LNA + EPA), P = 0.002; Contrast 3: LA vs. (LNA + EPA), P = 0.004; Contrast 4: LNA vs. EPA, P < 0.001.

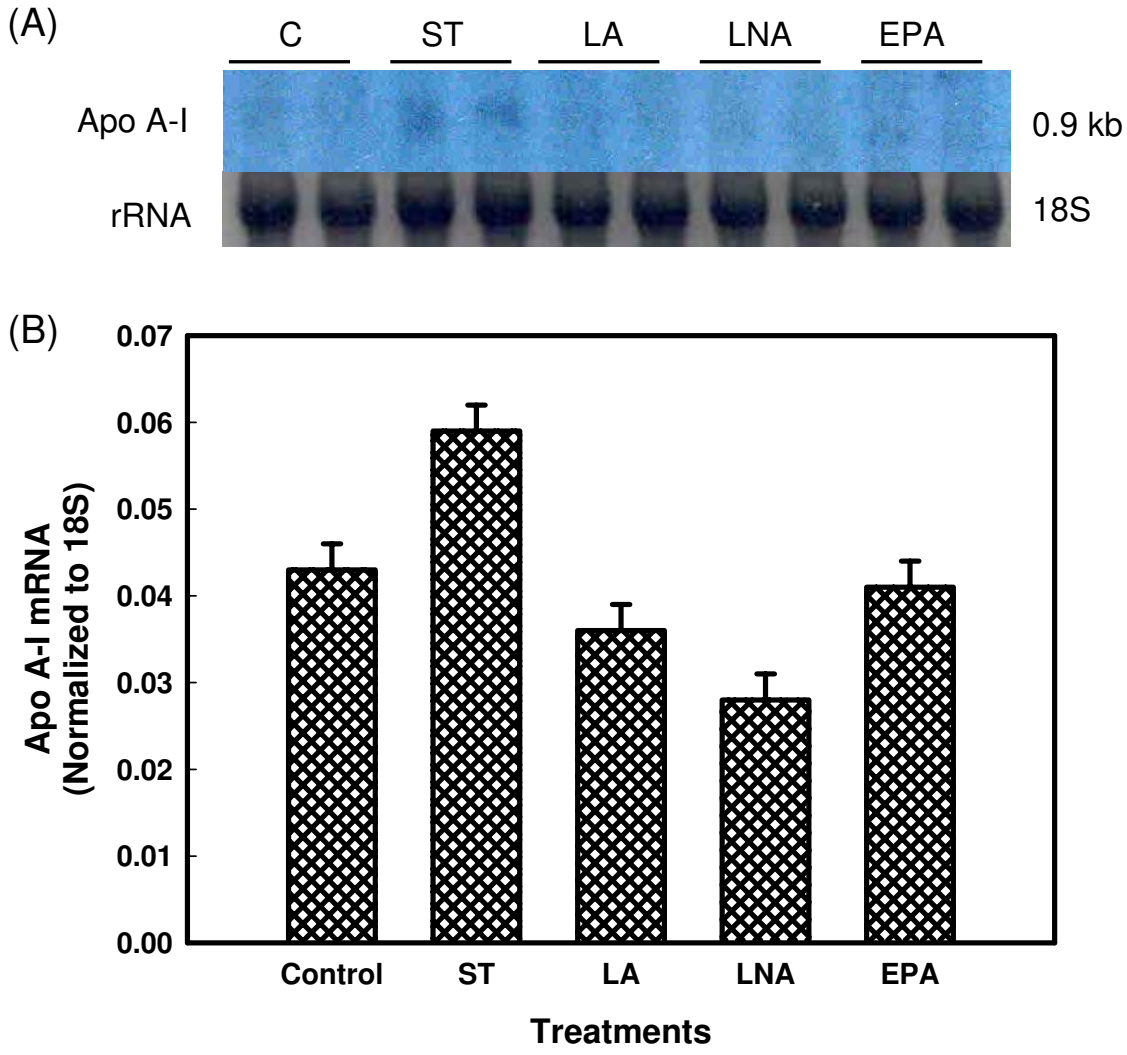

Figure 3-7. Effects of long-chain FA on Apo A-I mRNA expression in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and FA-treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (ST + LA + LNA + EPA), $P = 0.6$; Contrast 2: ST vs. (LA + LNA + EPA), $P < 0.001$; Contrast 3: LA vs. (LNA + EPA), $P = 0.6$; Contrast 4: LNA vs. EPA, $P = 0.008$.

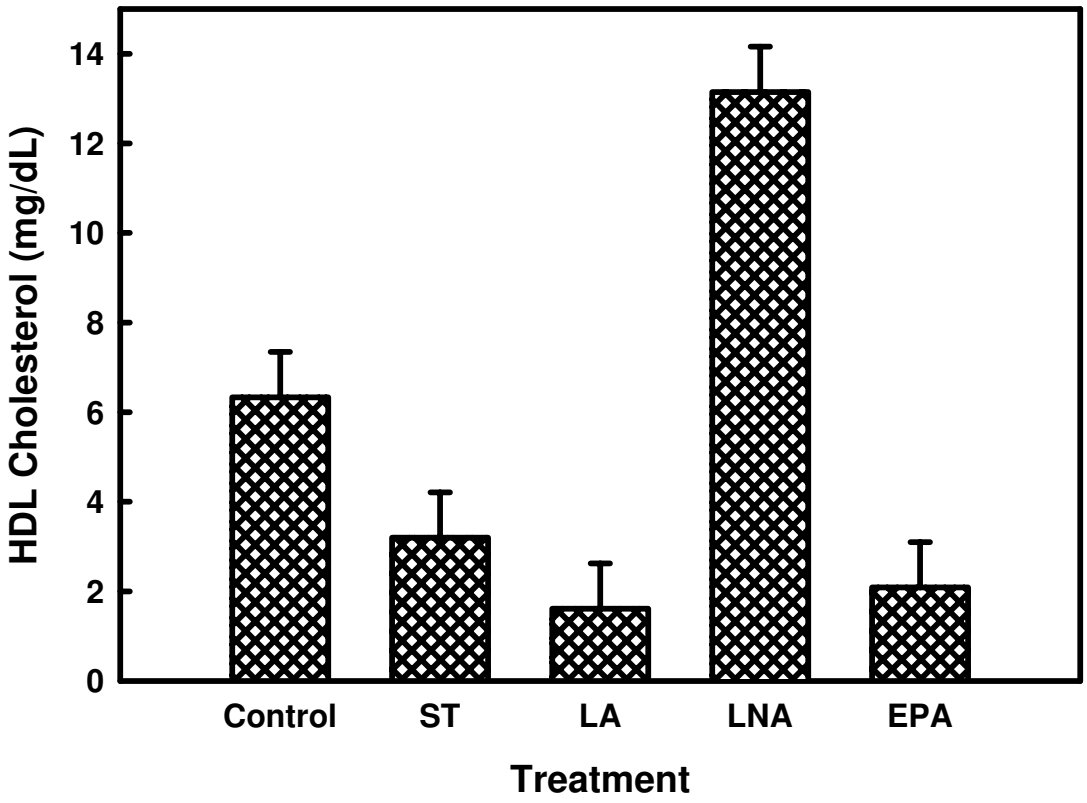

Figure 3-8. Effects of long-chain FA on HDL cholesterol production in H-4-II-E cells. Data represents least square means ± SEM calculated over two experiments. To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (ST + LA + LNA + EPA), P = 0.3; Contrast 2: ST vs. (LA + LNA + EPA), P = 0.06; Contrast 3: LA vs. (LNA + EPA), P = 0.0002; Contrast 4: LNA vs. EPA, P < 0.0001.

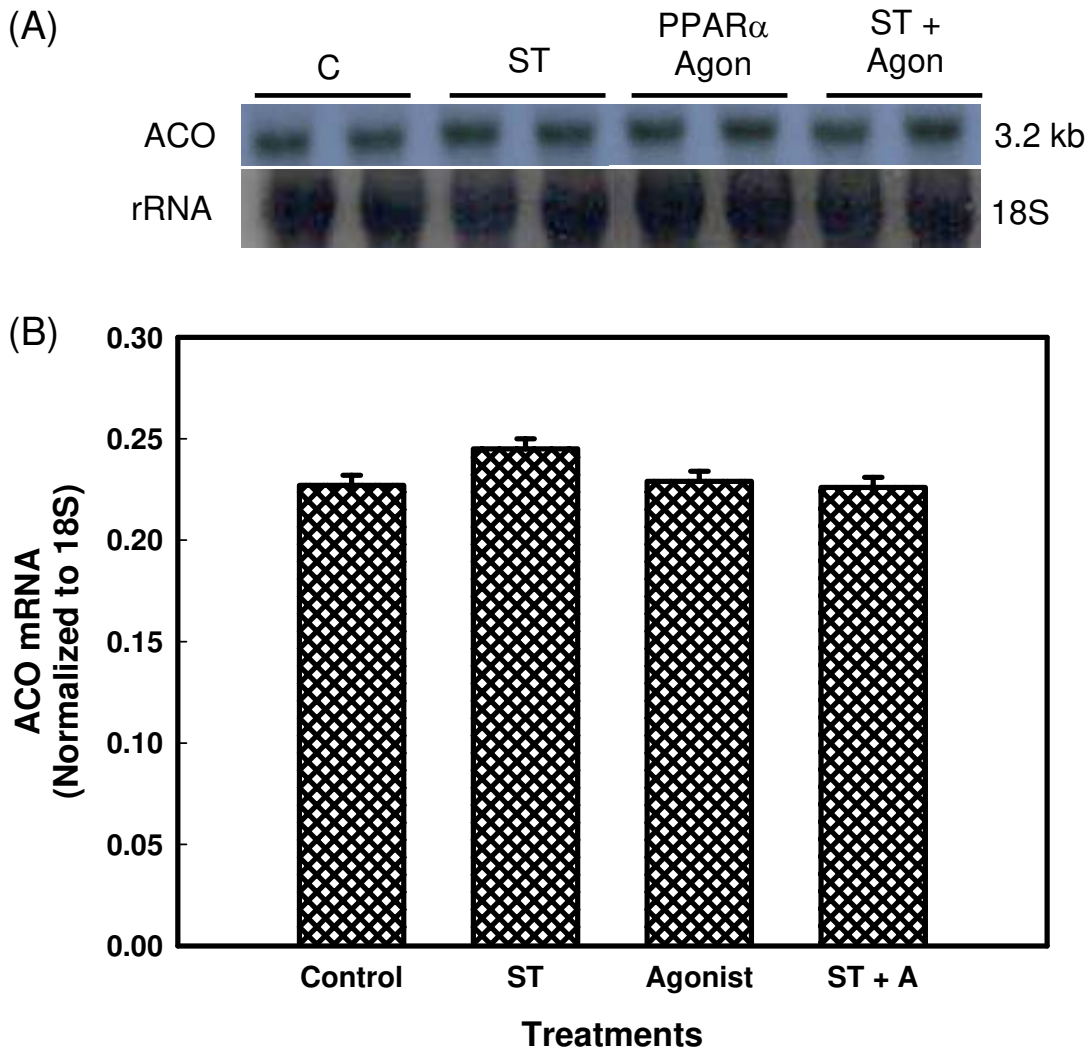

Figure 3-9. Effect of WY 14,643 on ACO mRNA response to ST in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (ST + ST+A), P = 0.2; Contrast 2: ST vs. ST+A, P = 0.04; Contrast 3: Control vs. Agonist, P = 0.8.

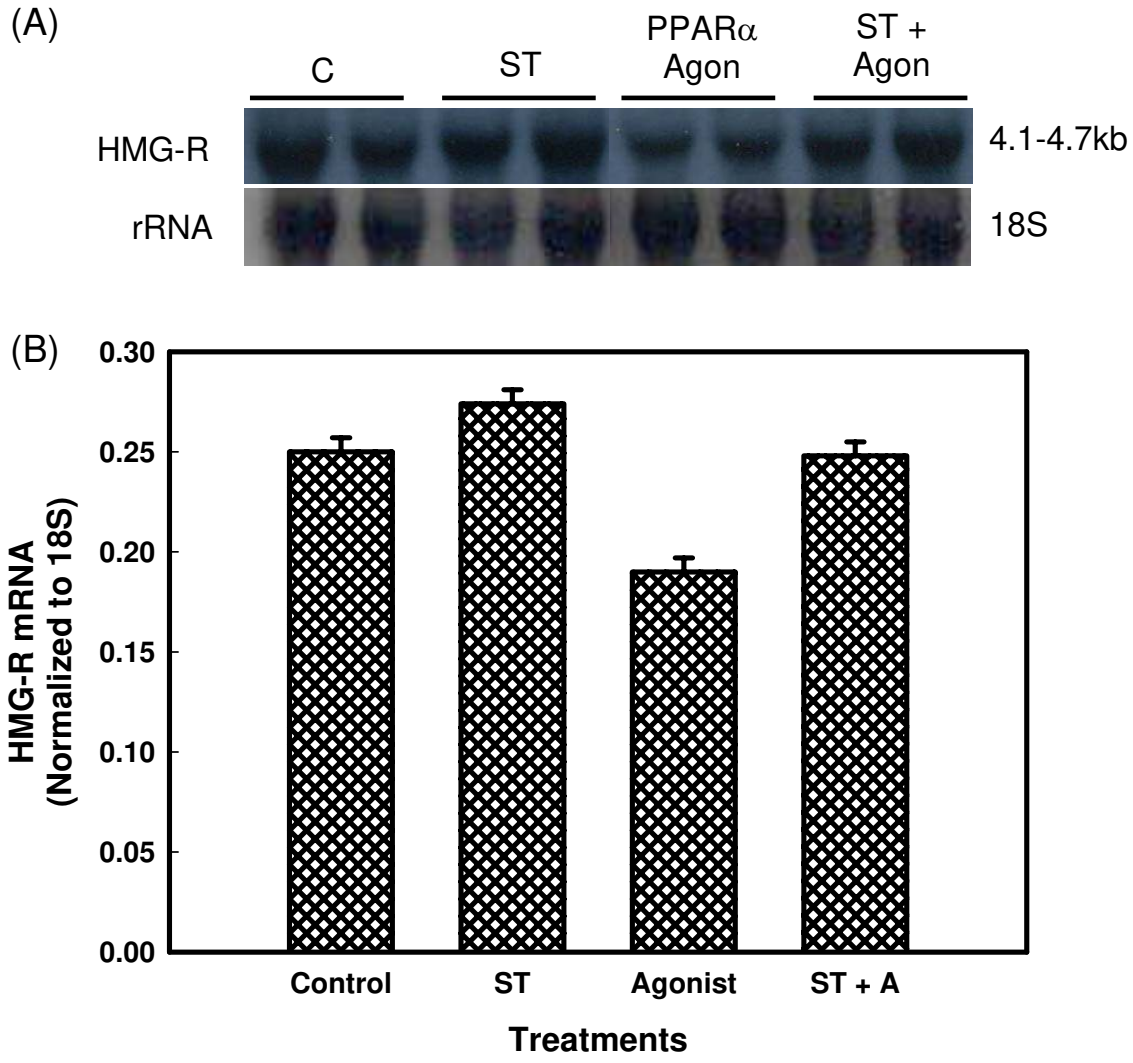

Figure 3-10. Effect of WY 14,643 on HMG-R mRNA response to ST in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (ST + ST+A), P = 0.003; Contrast 2: ST vs. ST+A, P = 0.02; Contrast 3: Control vs. Agonist, P = 0.002.

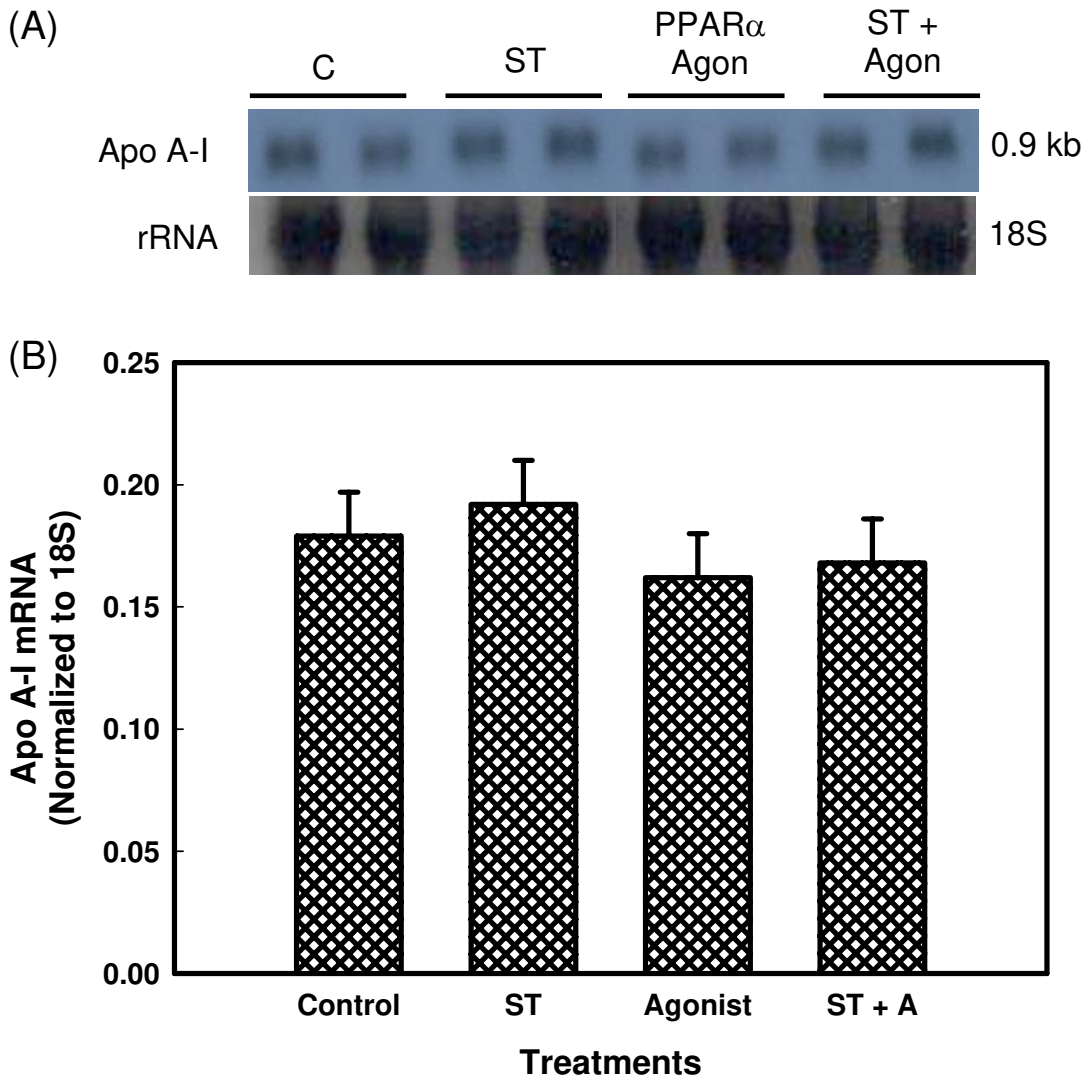

Figure 3-11. Effect of WY 14,643 on Apo A-I mRNA response to ST in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (ST + ST+A), P = 0.6; Contrast 2: ST vs. ST+A, P = 0.4; Contrast 3: Control vs. Agonist, P = 0.5.

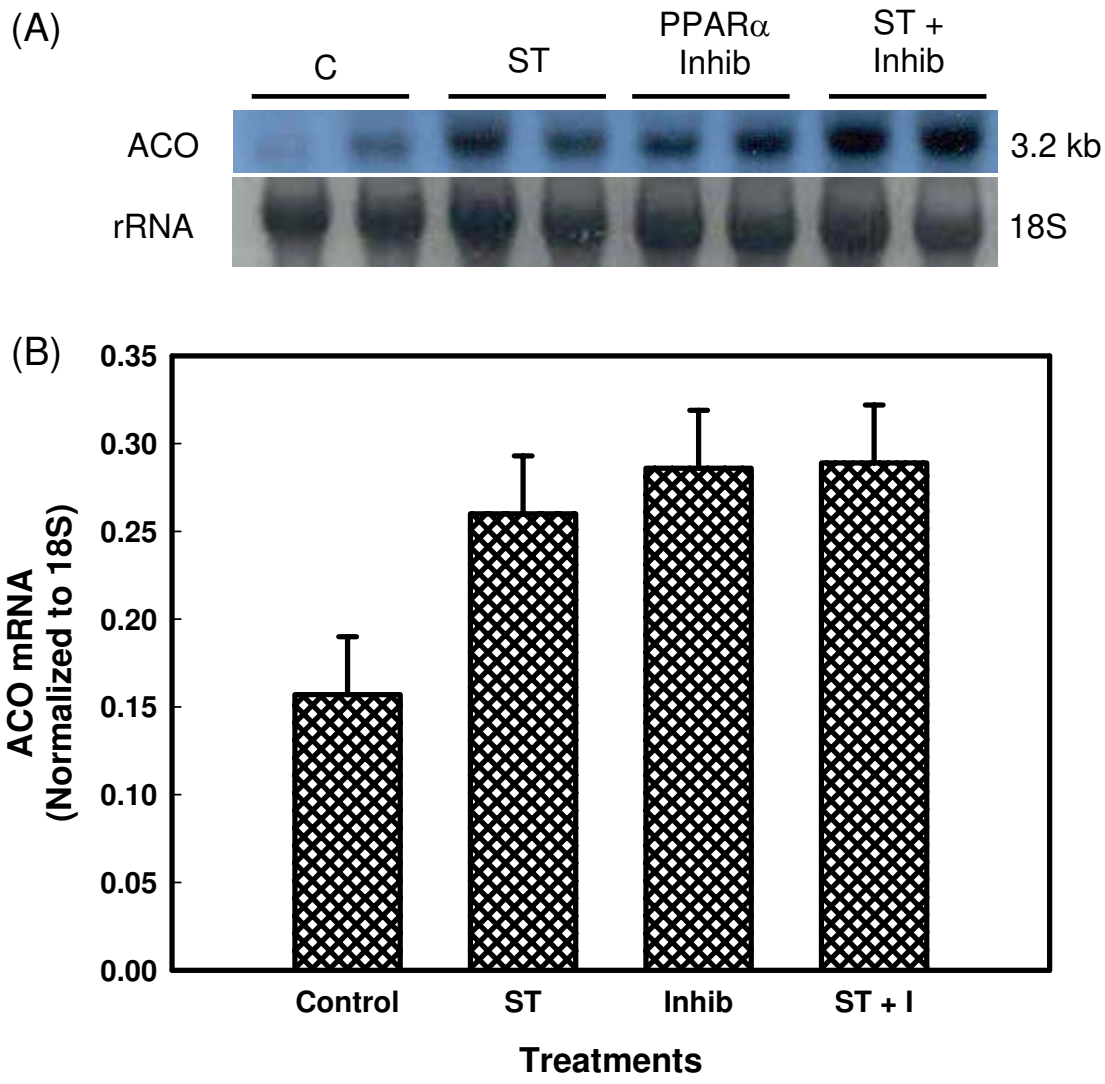

Figure 3-12. Effect of MK886 on ACO mRNA response to ST in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and FA treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Inhib) vs. (ST + ST+I), P = 0.15; Contrast 2: ST vs. ST+I, P = 0.6; Contrast 3: Control vs. Inhib, P = 0.03.

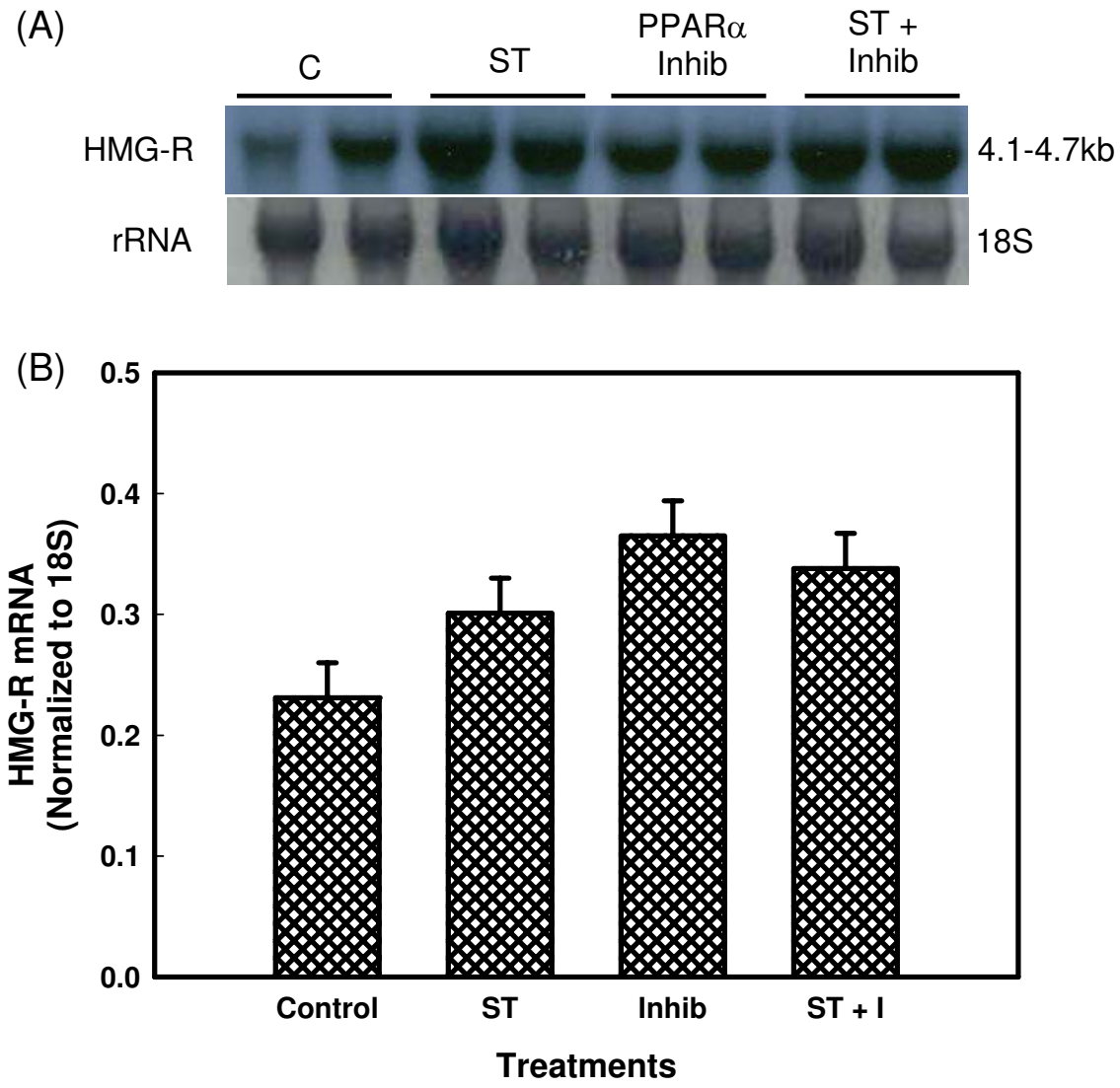

Figure 3-13. Effect of MK886 on HMG-R mRNA response to ST in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Inhib) vs. (ST + ST+I), P = 0.5; Contrast 2: ST vs. ST+I, P = 0.4; Contrast 3: Control vs. Inhib, P = 0.01.

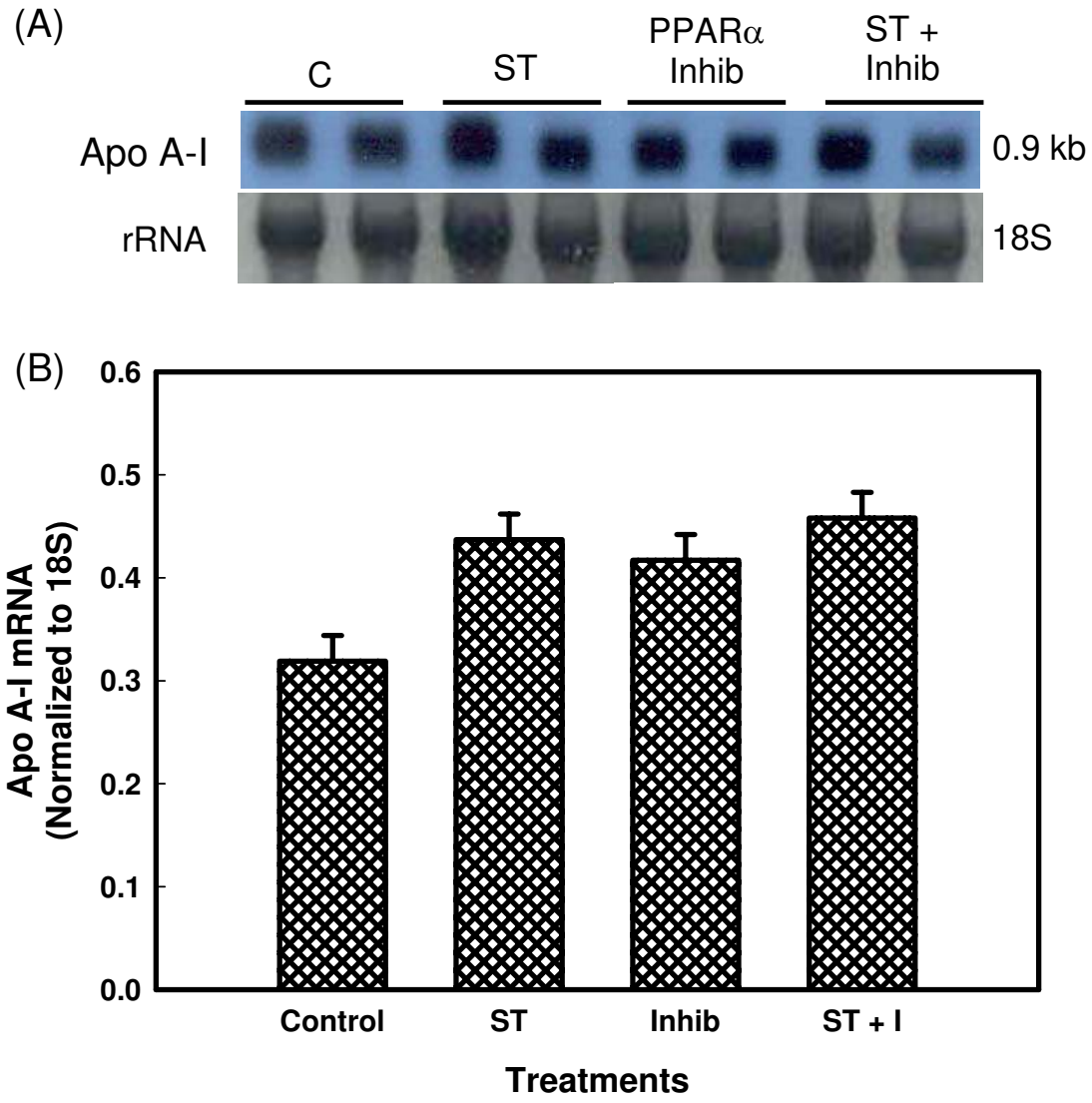

Figure 3-14. Effect of MK886 on Apo A-I mRNA response to ST in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Inhib) vs. (ST + ST+I), P = 0.01; Contrast 2: ST vs. ST+I, P = 0.6; Contrast 3: Control vs. Inhib, P = 0.02.

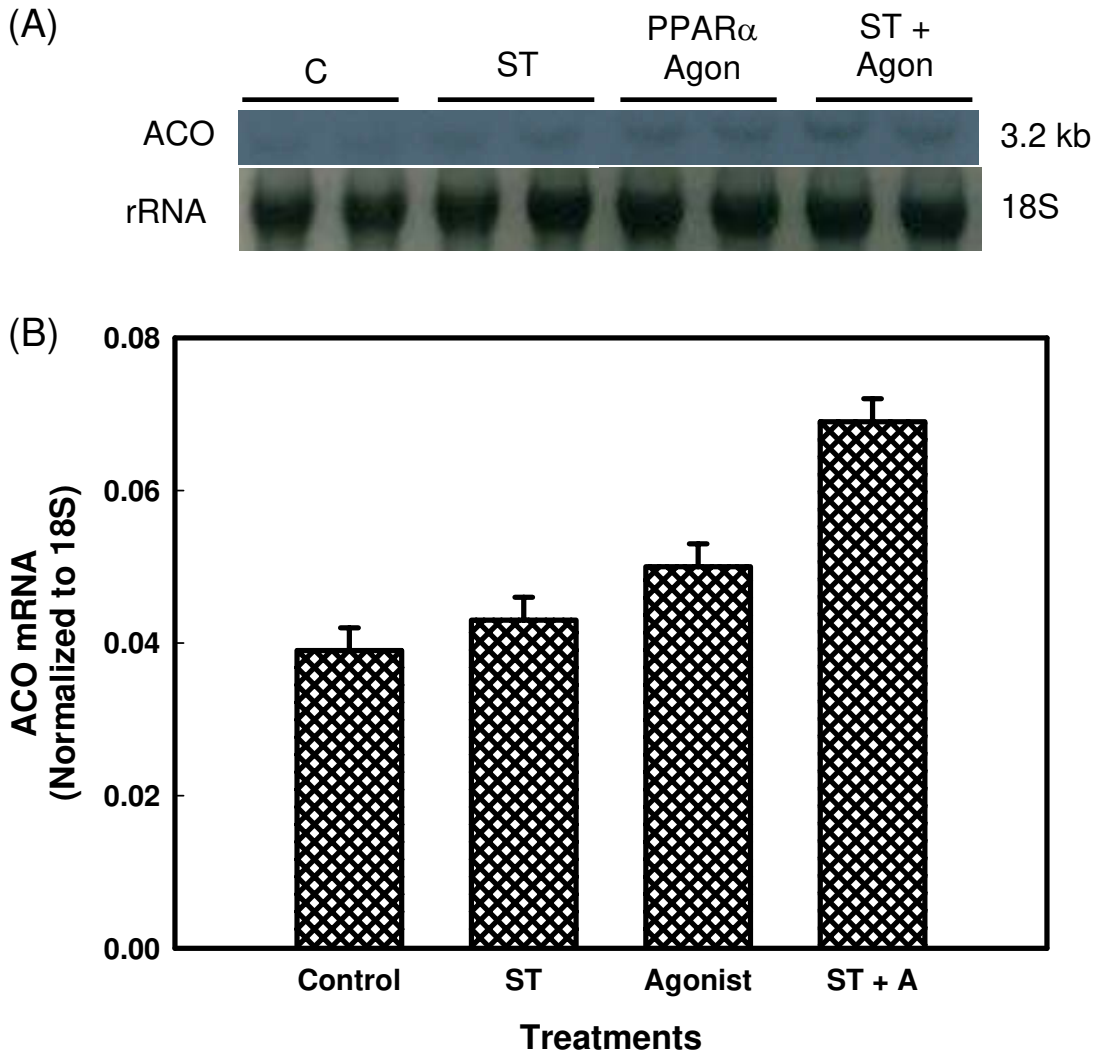

Figure 3-15. Effect of WY14,643 on ACO mRNA response to ST in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (ST + ST+A), P = 0.007; Contrast 2: ST vs. ST+A, P = 0003; Contrast 3: Control vs. Agonist, P = 0.04.

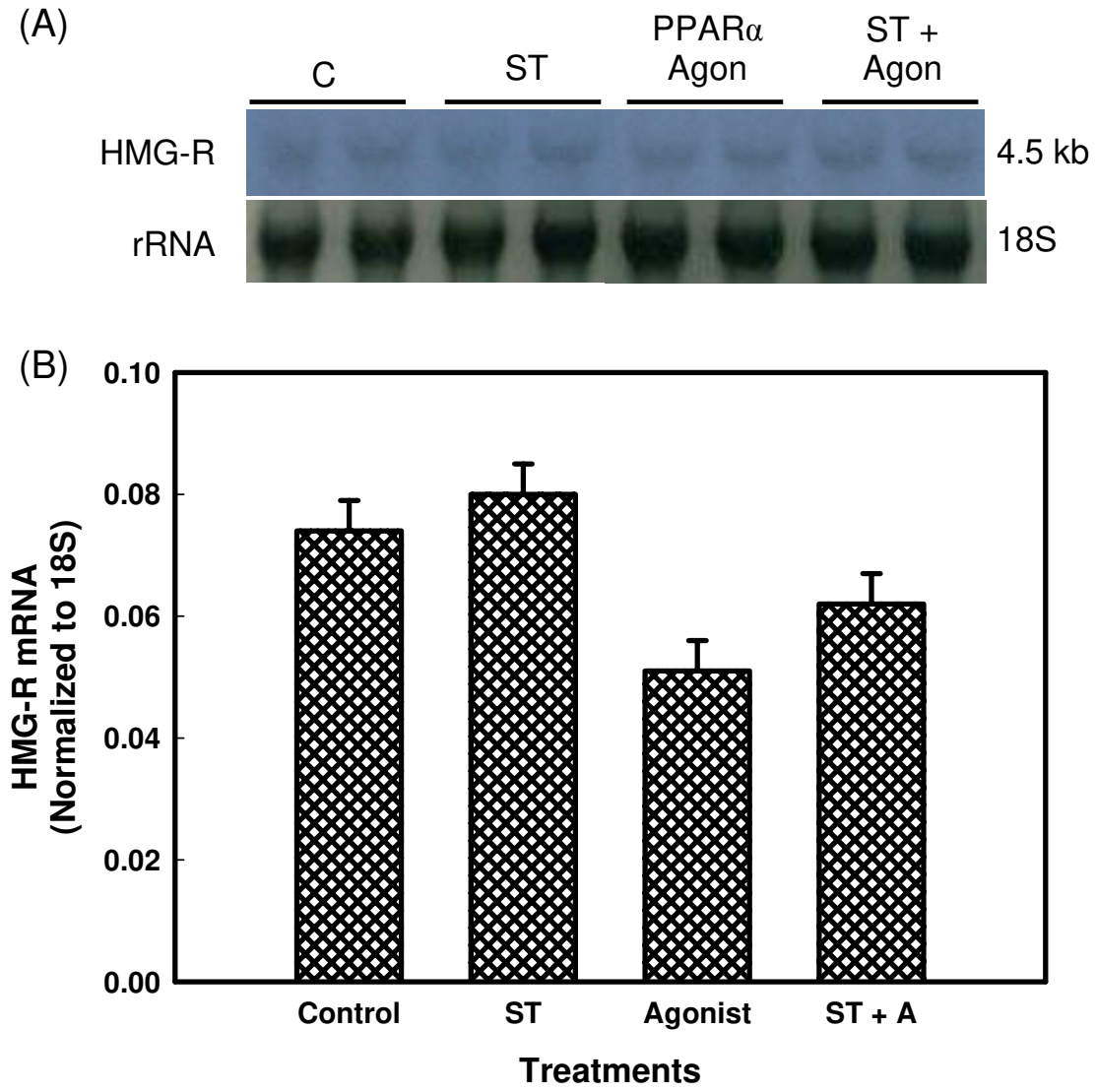

Figure 3-16. Effect of WY 14,643 on HMG-R mRNA response to ST in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (ST + ST+A), P = 0.12; Contrast 2: ST vs. ST+A, P = 0.03; Contrast 3: Control vs. Agonist, P = 0.01.

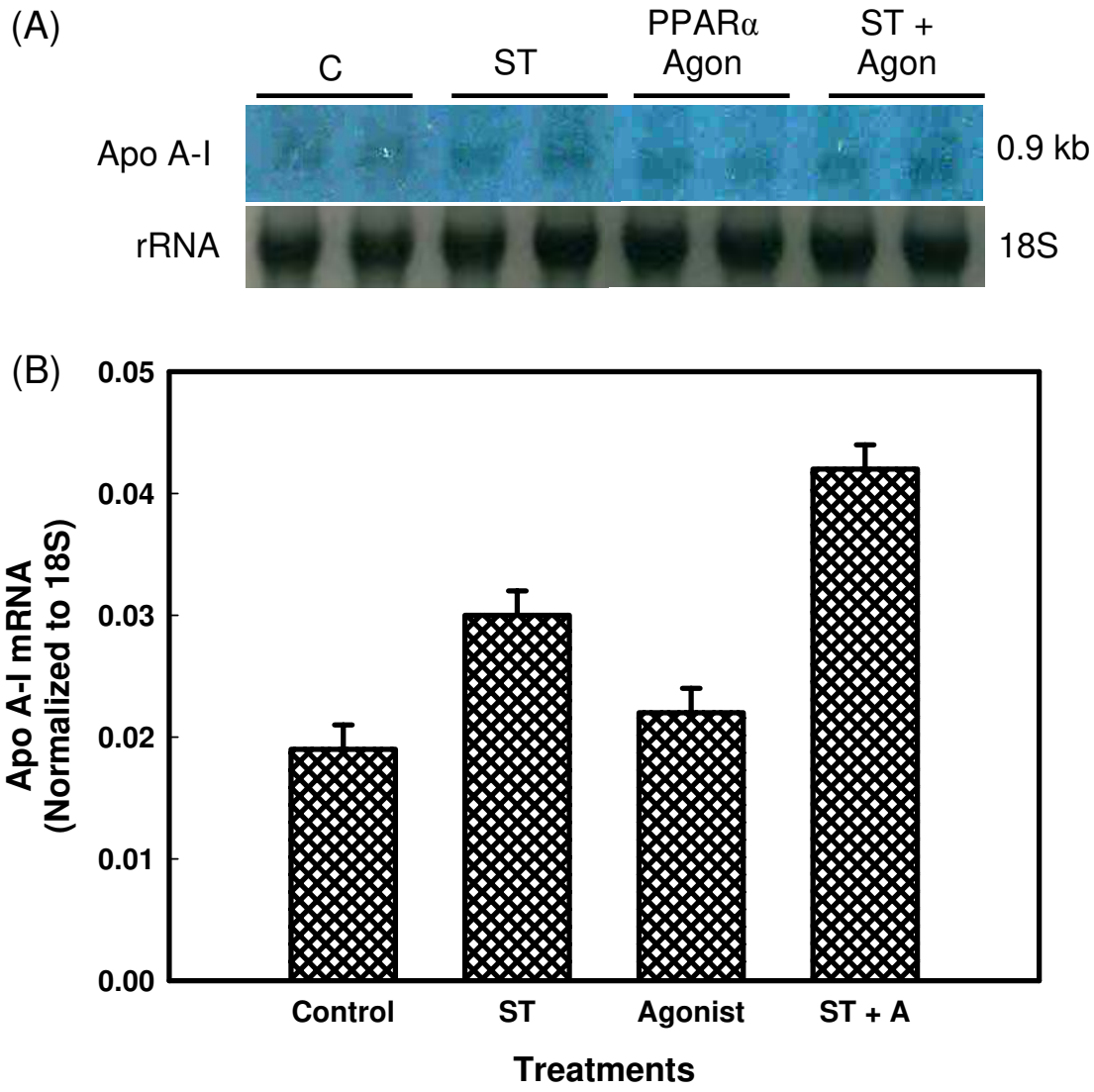

Figure 3-17. Effect of WY 14,643 on Apo A-I mRNA response to ST in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control Agonist) vs. (ST + ST+A), $P < 0.0001$; Contrast 2: ST vs. ST+A, $P = 0.001$; Contrast 3: Control vs. Agonist, $P = 0.2$.

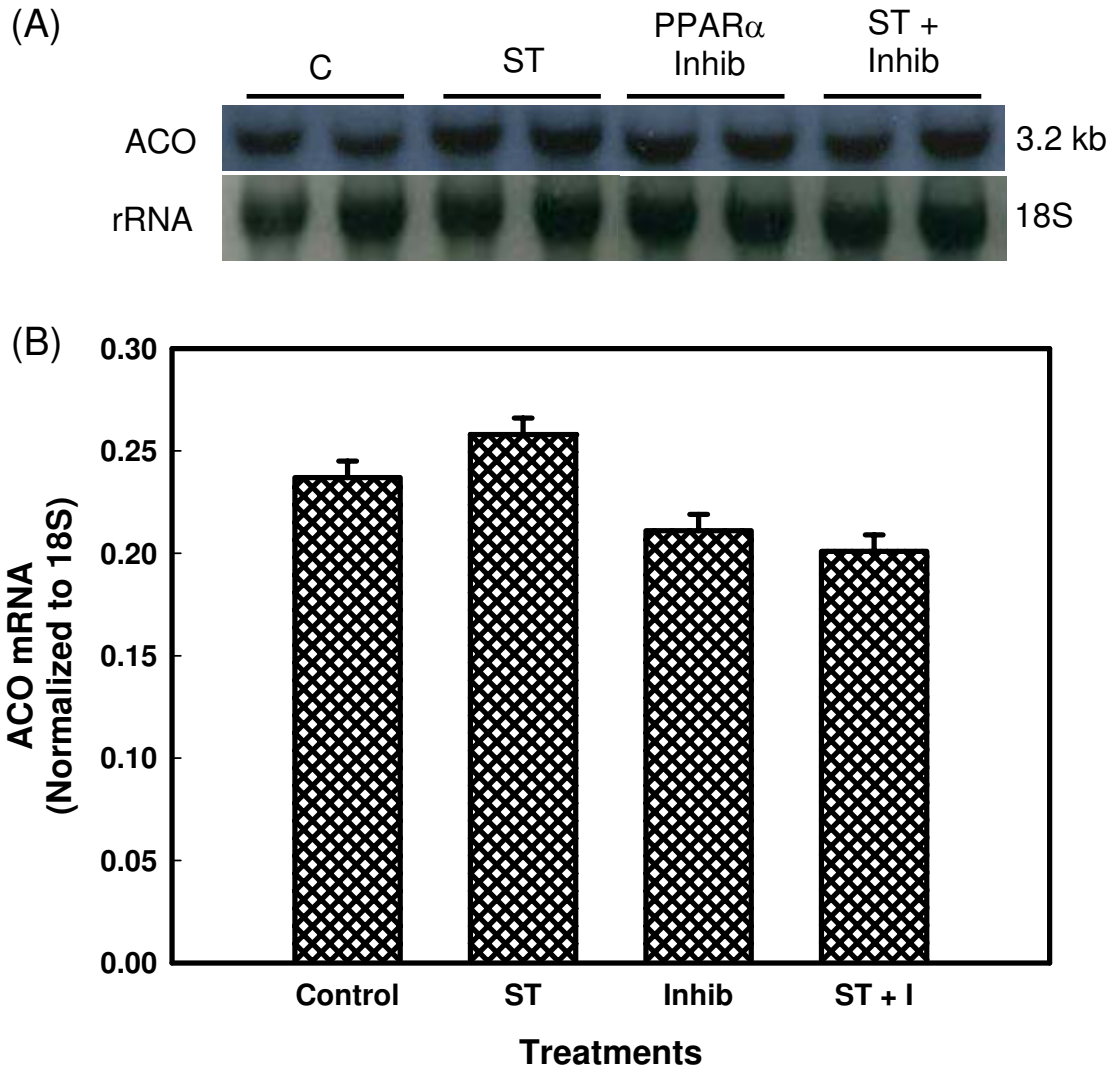

Figure 3-18. Effect of MK886 on ACO mRNA response to ST in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Inhib) vs. (ST + ST+I), P = 0.5; Contrast 2: ST vs. ST+I, P = 0.001; Contrast 3: Control vs. Inhib, P = 0.06.

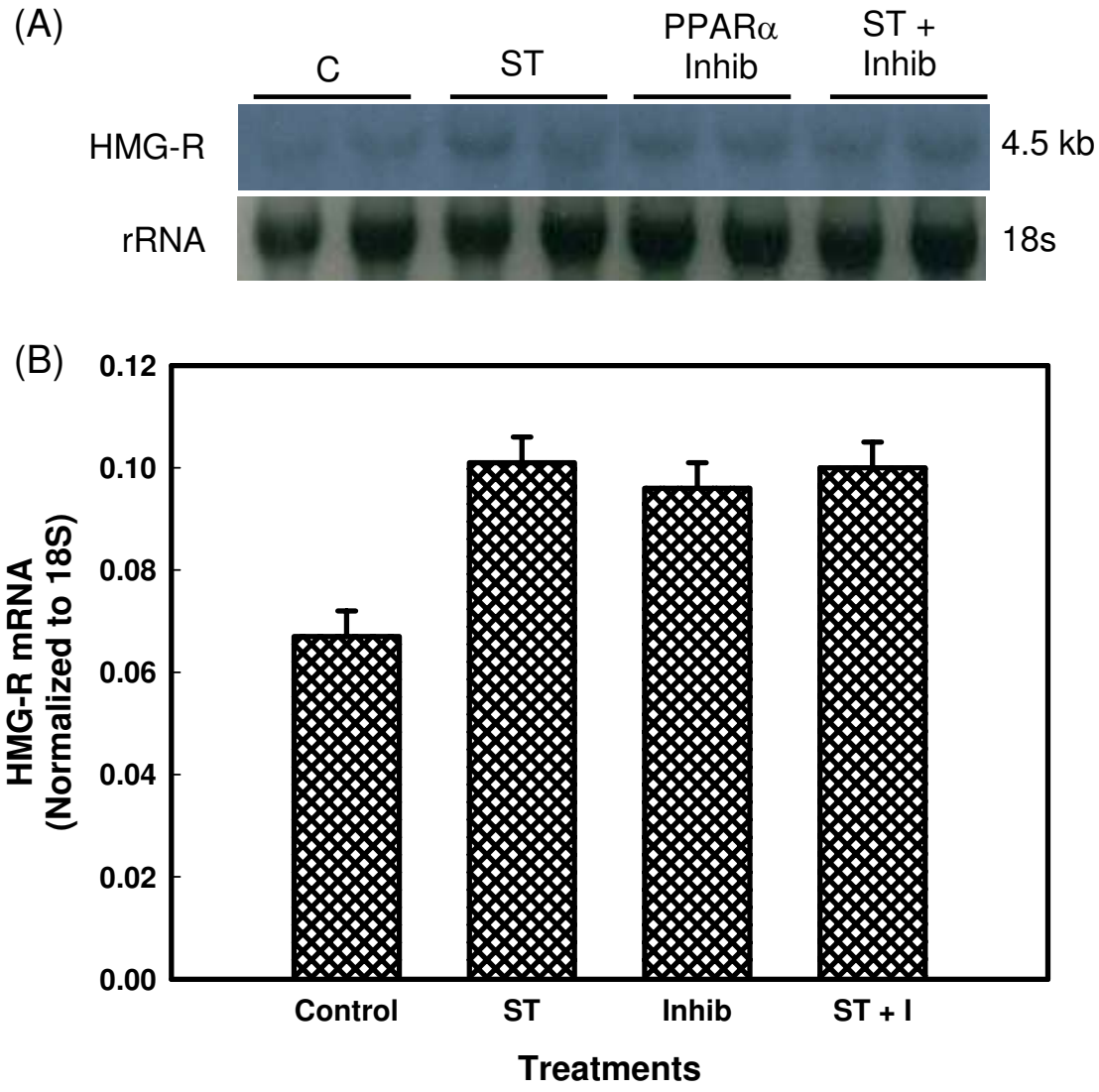

Figure 3-19. Effect of MK886 on HMG-R mRNA response to ST in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Inhib) vs. (ST + ST+I), P = 0.004; Contrast 2: ST vs. ST+I, P = 0.8; Contrast 3: Control vs. Inhib, P = 0.003.

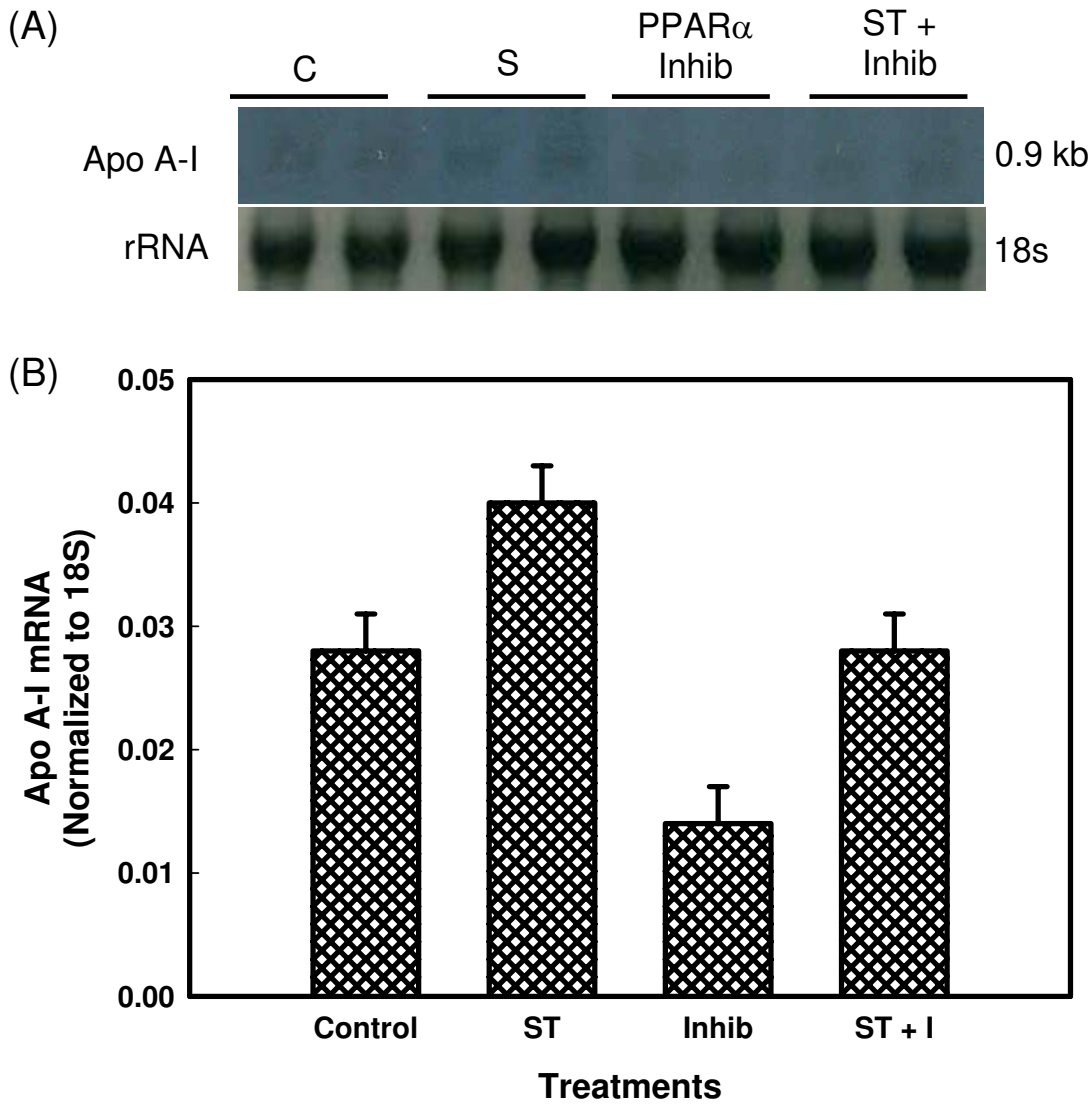

Figure 3-20. Effect of MK886 on Apo A-I mRNA response to ST in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Inhib) vs. (ST + ST+I), P = 0.003; Contrast 2: ST vs. ST+I, P = 0.03; Contrast 3: Control vs. Inhib, P = 0.01.

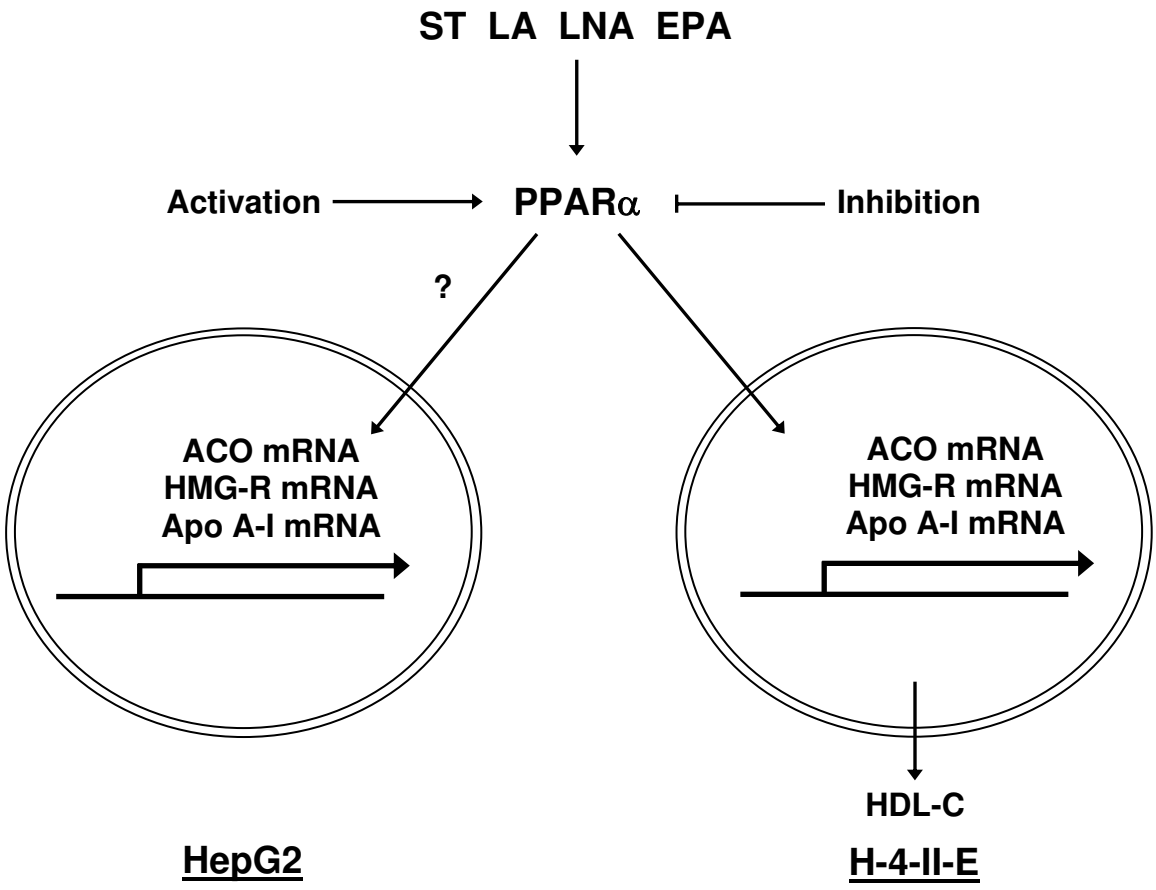

Figure 3-21. Regulation of lipid metabolizing genes and HDL cholesterol production by long-chain fatty acids. In HepG2 cells, HMG-R mRNA was up-regulated by ST as compared to the PUFAs. All fatty acids up-regulated Apo A-I mRNA as compared to control. Activation of PPARα attenuated the effects of ST on ACO and HMG-R gene expression. In H-4-II-E cells, ST up-regulated ACO, HMG-R, and Apo A-I gene expression as compared to the PUFAs. Activation of PPARα increased basal expression of ACO and enhanced ST effects on ACO and Apo A-I mRNA. Both basal and ST-induced HMG-R mRNA levels were decreased by PPARα activation. Inhibition of PPARα decreased basal expression of Apo A-I and attenuated ST-induced expression of ACO and Apo A-I mRNA. Basal concentrations of HMG-R mRNA were increased by PPARα inhibition. As compared to n-6 PUFA, n-3 PUFA increased HDL cholesterol production, with the effect predominantly deriving from the increase due to LNA.

CHAPTER 4
EFFECTS OF ISOMERS OF CONJUGATED LINOLEIC ACID ON LIPID METABOLIZING GENES AND HIGH-DENSITY LIPOPROTEIN CHOLESTEROL PRODUCTION IN CULTURED HUMAN AND RAT HEPATOCYTES

Introduction

Conjugated linoleic acid (CLA) is a collective term for positional and geometric isomers of linoleic acid (LA). Though over 16 individual isomers have been identified (Rickert et al., 1999), only *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA are known to possess biological activity (Pariza et al., 2000). *Cis*-9, *trans*-11 CLA is the predominant CLA produced as an intermediate in the rumen during the biohydrogenation of dietary LA and is commonly found in dairy products and ruminant meat. Dietary sources of *trans*-10, *cis*-12 CLA derive predominantly from synthetic partial biohydrogenation and is found in margarines, shortenings, and supplements (Gaullier et al., 2002). First identified in grilled beef as a potential anti-carcinogen (Pariza and Hargraves, 1985), numerous health benefits have been attributed to CLA mixtures, including actions as an antiadipogenic (Park et al, 1997), antidiabetogenic (Houseknecht et al., 1998), and antiatherosclerotic (Kritchevsky et al., 2004) agent. More recently, studies involving individual isomers have shown that the two main isoforms can have different effects on metabolism and cell function and may act through different signaling pathways (Wahle et al., 2004). The metabolic responses to *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA may differ, but both isomers have implications for human health. Most studies have been performed in animal models, with species differences observed. In particular, only some

of the findings attributed to animal models pertain to human subjects, and even when comparing studies in humans, results are often inconclusive (Terpstra, 2004).

The objective of this study was to examine the short term effects of the two biologically active isomers of CLA on lipid metabolizing gene expression and high-density lipoprotein (HDL) cholesterol production in HepG2 (human) and H-4-II-E (rat) hepatoma cell lines. Based on both dietary and *in vitro* studies of lipid metabolism, we hypothesized that the different isomers of CLA may have differing effects on acyl-CoA oxidase (ACO), 3-hydroxy, 3-methylglutaryl CoA reductase (HMG-R), and apolipoprotein A-I (Apo A-I) gene expression. Also, because several fatty acids and their derivatives are known ligands for peroxisome proliferator-activated receptors (PPAR), we hypothesized that CLA isomers may act on lipid-metabolizing genes through activation of PPARα in the liver.

Materials and Methods

Materials

Polystyrene tissue culture dishes (100 x 20 mm) were purchased from Corning (Corning Glass Works, Corning, NY). The antibiotic/antimycotic (ABAM), sodium pyruvate, fatty acid-free bovine serum albumin (BSA), WY 14,643, and MK886 were from Sigma Chemical Co. (St. Louis, MO). Minimum Essential Medium (MEM), phenol red-free MEM, Hanks Balanced Salt Solution (HBSS) and TriZol reagent were from GIBCO BRL (Carlsbad, CA). The fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA). Linoleic acid, *cis*-9, *trans*-11 CLA, and *trans*-10, *cis*-12 CLA were from Cayman Chemicals (Ann Arbor, MI). BioTrans nylon membrane and [α-^{32}P]deoxycytidine triphosphate (SA 3000 Ci/nmol) were from MP Biolomedicals

(Atlanta, GA). The Enzyme Color Solution, Reacting Solution, and HDL Calibrator were from Wako Diagnostics (Richmond, VA).

Cell Culture and Treatment

HepG2 (ATCC # HB-8065; Manassas, VA) and H-4-II-E (ATCC # CRL-1548; Manassas, VA) cells were cultured and fatty acids were complexed as described in chapter 3. To investigate the effects of supplemental CLA on hepatic gene expression and cholesterol synthesis, HepG2 and H-4-II-E cells were treated with LA, *cis*-9, *trans*-11 CLA, or *trans*-10, *cis*-12 CLA (100 μM). Sub-confluent cells were incubated with serum-free medium alone (Control) or with appropriate treatments (listed above) complexed with BSA, for a period of 24 h. Cells were then rinsed twice with 10 mL HBSS. The remaining cell monolayer was then lysed in 3 mL TriZol reagent, and stored at -80°C for subsequent mRNA analysis. The same fatty acid (FA) treatments were repeated, using phenol red-free MEM. After incubation, conditioned media were collected and stored at -20^{o}C until lipid extraction and HDL cholesterol analysis.

To investigate whether CLA effects on gene expression involves PPARα activation, confluent HepG2 and H-4-II-E cells were treated with *trans*-10, *cis*-12 CLA isomer (100 μM), the PPARα agonist WY 14,643 (10 μM), or a combination of *trans*-10, *cis*-12 CLA and WY 14,643. Additional sets of culture dishes were incubated with *trans*-10, *cis*-12 CLA alone, the PPARα antagonist MK886 (10 μM; Kehrer et al., 2001), or a combination of *trans*-10, *cis*-12 CLA and MK886. After 24 h of incubation, cells were washed twice with 10 mL HBSS, lysed with TriZol, and stored at -80^{o}C until mRNA analysis.

RNA Isolation and Analysis

Total cellular RNA was isolated from cells using TriZol reagent according to the manufacturer's instructions. Ten micrograms of total RNA was fractioned in a 1.0% agarose formaldehyde gel following previously described protocols (Ing et al., 1996) using the MOPS buffer (Fisher Scientific, Pittsburgh, PA) and transferred to a Biotrans nylon membrane by downward capillary transfer in 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) using the TurboBlotting system (Schleicher and Schuel, Keene, NH). Nylon membranes were cross-linked by exposure to a UV light source for 90 sec and baked at 80°C for 1 h. Membranes were incubated for 2 h at 50°C in ultrasensitive hybridization buffer (ULTRAhyb; Ambion, Austin, TX) followed by an overnight incubation at 50°C in the same ULTRAhyb solution containing the ^{32}P-labeled ACO, HMG-R, and Apo A-I cDNA probes. Probes were generated by RT-PCR for ACO (forward 5'-CCGGAGCTGCTTACACACAT-3'; reverse 5'-GGTCATACGTGGCTGT GGTT-3'), HMG-R (forward 5'-TCCTTGGTGATGGGAGCTTGTTGTG-3'; reverse 5'-TGCGAACCCTTCAGATGTTTCGAGC-3'), human Apo A-I (forward 5'-AAGACA GCGGCAGAGACTAT-3'; reverse 5'-ATCTCCTCCTGCCACTTCTT-3'), and rat Apo A-I (forward 5'-AAGGACAGCGGCAGAGACTA-3'; reverse 5'-CCACAACCTTTAG ATGCCTT-3'). The sizes and sequences of these cDNA probes were verified by DNA sequencing prior to their use in Northern blot analysis. Filters were sequentially washed in 2X SSC (1X= 0.15 M sodium chloride, 0.015 M sodium citrate)-0.1% SDS and in 0.1x SSC-0.1% SDS two times each at 50°C and then exposed to X-ray film to detect radiolabeled bands. Equal loading of total RNA for each experimental sample was verified by comparison to 18S rRNA ethidium bromide staining.

Lipid Extraction

Total lipids were extracted from conditioned media as described by Bligh and Dyer (1959), with modifications. For each sample, 2 mL of conditioned media was aliquotted into a 20 mL glass screw-top vial. Fourteen mL of chloroform:methanol (2:1, v/v) was then added and the vials were vortexed for 5 minutes. The vials were then centrifuged at 1700 rpm for 5 min. The bottom lipid-containing chloroform layer was transferred to a clean, dry, pre-weighed vial, placed in a 37^{o}C water bath, and dried under nitrogen gas. Dry samples were placed in a 50^{o}C oven for 10 minutes and placed in a desiccator to cool to room temperature. Samples were weighed, and lipid weight was determined by difference. The sample was resuspended in chloroform and stored at -20^{o}C until HDL cholesterol analysis.

HDL Cholesterol Assay

Lipid extracts from conditioned media were analyzed using a commercially available L-Type HDL-C kit, following the manufacturer's directions. Briefly, using a 96-well plate, 3 µL of sample was pipetted into each well. Two hundred seventy µL of Enzyme Color Solution (R1) was added, and the plate was incubated for 5 minutes at 37^{o}C. Ninety µL of Reacting Solution (R2) was then added, and the plate was incubated another 5 minutes at 37^{o}C. The absorbance at 600 nm was measured using the SpectraMax 340 PC microplate reader (Molecular Devices, Sunnyvale, CA), and the concentration of the samples was calculated by plotting against a standard curve.

Statistical Analysis

All hybridization signals as measured by densitometry were evaluated by least squares analysis of variance (ANOVA) using the General Linear Model (GLM) procedure of the SAS software package (SAS Institute Inc, Cary, NC). In each

experiment, treatments were run in duplicate, and the whole experiment was also duplicated, giving n=4 plates per treatment. The general model for mRNA analysis included experiment, treatment, and experiment x treatment interaction. In mRNA analyses, densitometric values for target genes were expressed as ratios of target gene densitometric values over the corresponding 18S rRNA densitometric values. For HDL cholesterol concentration, the sources of variation included experiment, treatment, experiment x treatment interaction, and plate (experiment x treatment). The plate, nested within experiment and treatment, was considered a random variable, and therefore the plate variance was used as an error term to test the effects of experiment, treatment, and experiment x treatment interaction. Treatment means were further compared using preplanned orthogonal contrasts. These contrasts were control vs. fat treatment (LA, *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA), LA vs. CLA (*cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA), and *cis*-9, *trans*-11 CLA vs. *trans*-10, *cis*-12 CLA. For all responses, the two cell lines were analyzed separately.

Results

Effects of Conjugated Linoleic Acid on HepG2 Cells

Concentrations of ACO mRNA transcript were greater (+17%; P = 0.03) in HepG2 cells treated with CLA as compared to LA. In addition, ACO mRNA concentration was increased (+22%; P = 0.009) in HepG2 cells treated with *trans*-10, *cis*-12 CLA as compared to the *cis*-9, *trans*-11 isomer (Figure 4-1). Steady-state levels of HMG-R mRNA were increased (+38%; P = 0.0003) in cells treated with CLA as compared to those treated with LA. Concentration of HMG-R mRNA was also increased (+22%; P = 0.009) in cells treated with *trans*-10, *cis*-12 as compared to *cis*-9, *trans*-11 CLA (Figure 4-2). On average, the CLA isomers increased (+21%; P = 0.03) Apo A-I mRNA

transcript as compared to LA. Similar to the other genes studied, *trans*-10, *cis*-12 CLA increased (+22%; P = 0.03) Apo A-I gene transcript as compared to *cis*-9, *trans*-11 CLA (Figure 4-3). As compared to LA, incubation with CLA decreased (-18%; P = 0.05) HDL cholesterol production in HepG2 cells. This effect was predominantly derived from *cis*-9, *trans*-11 CLA, which decreased (-29%; P = 0.02) HDL cholesterol concentration in the media as compared to *trans*-10, *cis*-12 CLA (Figure 4-4).

Effects of Conjugated Linoleic Acid on H-4-II-E Cells

In H-4-II-E cells, ACO mRNA transcript was increased (+23%; P = 0.0005) by all FA as compared to control, though among the FA studied, there were no differences (Figure 4-5). As compared to LA, CLA increased (+38%; P = 0.001) HMG-R gene expression; however, there were no differences between the two isomers of CLA (P = 0.2; Figure 4-6). On average, treating H-4-II-E cells with FA decreased (-30%; P = 0.01) Apo A-I mRNA concentration, but there were no differences among the FA (Figure 4-7). There was no effect (P = 0.6) of any of the FA on HDL cholesterol production (Figure 4-8).

Role of PPARα in *trans*-10, *cis*-12 CLA-Induced Effects on Gene Expression

Incubation of HepG2 cells with 10 μm WY 14,643, a specific PPARα agonist, had no effect on either basal (P = 0.99) or *trans*-10, *cis*-12 CLA-induced (P = 0.3) ACO mRNA expression (Figure 4-9). Basal levels of HMG-R mRNA were increased (+23%; P = 0.02) by use of the PPARα agonist, but there was no effect (P = 0.4) on *trans*-10, *cis*-12 CLA-induced gene expression (Figure 4-10). WY 14,643 increased (+38%; P < 0.0001) Apo A-I mRNA basal concentrations to levels similar to that induced by *trans*-10, *cis*-12 CLA, but had no additive effect (P = 0.9; Figure 4-11).

In HepG2 cells, incubation with 10 μm MK886, a specific PPARα inhibitor, had no effect on either basal or *trans*-10, *cis*-12 CLA-induced expression of any of the genes studied (ACO, Figure 4-12; HMG-R, Figure 4-13, Apo A-I, Figure 4-14).

Incubation of H-4-II-E cells with WY 14,643 increased (+24%; P = 0.02) basal expression and attenuated (-27%; P = 0.02) the effects of *trans*-10, *cis*-12 CLA on ACO gene expression (Figure 4-15). There were no effects of the agonist on basal (P = 0.9) or *trans*-10, *cis*-12 CLA-induced (P = 0.2) expression of HMG-R or Apo A-I mRNA concentrations (HMG-R, Figure 4-16; Apo A-I, Figure 4-17).

In H-4-II-E cells, incubation with MK886 had no effects (P = 0.3) on ACO gene expression (Figure 4-18). The PPARα inhibitor decreased (-25%; P = 0.02) basal expression of HMG-R mRNA, but had no effects (P = 0.1) on *trans*-10, *cis*-12 CLA-induced gene expression (Figure 4-19). Basal levels were unaffected (P = 0.9), but co-incubation with MK886 attenuated (-35%; P = 0.002) *trans*-10, *cis*-12 CLA-induced Apo A-I mRNA concentration (Figure 4-20).

Discussion

Numerous beneficial physiological effects have been attributed to CLA, though these effects may be both isomer and species specific. One of the potential mechanisms by which CLA modulates health and disease states is through changes in lipid metabolism. To address these facts, in this study, the two biologically active isomers of CLA were studied in both human and rat hepatoma cell lines. In HepG2 (human) cells, ACO mRNA expression was up-regulated by CLA as compared to LA treated cells, with an increase additionally seen with incubation of *trans*-10, *cis*-12 CLA as compared with *cis*-9, *trans*-11 CLA. In contrast, all FA increased ACO mRNA as compared to control in H-4-II-E cells, but there were no differences among the FA studied. Several animal

and cell models have also shown similar effects. Feeding mice a mix of (Peters et al., 2001) or individual CLA isomers (Warren et al., 2003; Degrace et al., 2004) increases expression of ACO mRNA as compared to control mice. In two studies, the increases seen in ACO mRNA levels in CLA-fed mice also coincided with increases in enzyme activity (Takahashi et al., 2003; Ide, 2005). In FaO cells, a rat hepatoma cell line derived from H4IIEC3 cells (Bayly et al., 1993), ACO gene expression was increased with 200 μM *cis*-9, *trans*-11 CLA. This effect was not seen, however, with lower concentrations of CLA (Moya-Camarena et al., 1999). In a recent study using a hamster model, ACO activity was increased by *trans*-10, *cis*-12 CLA as compared to control or *cis*-9, *trans*-11 CLA (Macarulla et al., 2005). Together with our findings, these studies suggest a potential role for CLA isomers in increasing liver peroxisomal β-oxidation.

Fatty acids have the ability to modulate serum cholesterol levels, though the exact site and mode of regulation may vary from one model to another. One potential gene involved is HMG-R, the rate limiting enzyme in cholesterol synthesis. In HepG2 cells, CLA isomers increased HMG-R mRNA transcript as compared to the parent molecule, LA. Between the CLA isomers, *trans*-10, *cis*-12 CLA up-regulated HMG-R mRNA concentrations as compared with the *cis*-9, *trans*-11 isomer. This differed in the H-4-II-E cells, where, on average, CLA increased gene expression as compared to LA, but no differences were seen between the two CLA isomers. Although the effects of saturated and polyunsaturated fats on HMG-R gene expression and enzyme activity have been examined, few studies have explored the role of CLA. In a recent study, HMG-R activity was decreased in rats fed diacylglycerol-enriched structured lipids containing CLA as compared to those fed lipids without CLA or corn oil (Kim et al., 2006). Though this

differs from our findings, gene expression was not measured, and as with other target genes, it would not be surprising if species and model-specific differences exist relative to transcriptional and/or posttranscriptional regulation of the HMG-R enzyme.

Another factor involved in normal lipoprotein profiles and metabolism is Apo A-I, the predominant apolipoprotein associated with HDL cholesterol. Dietary fat has the ability to modulate plasma lipids, and may act, in part, by effects on apolipoproteins. In general, CLA-induced changes in the blood lipid profile observed in various models are conflicting. In our study, Apo A-I gene transcript was increased by the CLA isomers as compared to LA, and by *trans*-10, *cis*-12 CLA as compared to *cis*-9, *trans*-11 CLA in HepG2 cells. High-density lipoprotein (HDL) cholesterol production in HepG2 cells was decreased by the CLA isomers as compared to LA, with the effect predominantly derived from the decrease due to *cis*-9, *trans*-11 CLA. In H-4-II-E cells, steady-state levels of Apo A-I mRNA was decreased by all FA treatments; however, there were no differences among the FA studied. These decreases in gene expression did not result in increased HDL cholesterol production, as levels were not different among any treatments. The different responses seen in the two cell lines is reflected by conflicting responses in other species studied. Mice supplemented with *cis*-9, *trans*-11 or *trans*-10, *cis*-12 CLA showed no differences in Apo A-I mRNA concentrations in control or CLA-fed animals (Warren et al., 2003). In contrast, in apo-E deficient mice, dietary *trans*-10, *cis*-12 CLA decreased plasma Apo A-I levels as compared to *cis*-9, *trans*-11 CLA (Arbones-Mainar et al., 2006). Similar to the decrease in HDL cholesterol production in HepG2 cells, rabbits fed a mixture of CLA isomers showed an increase in total serum cholesterol and a decrease in HDL cholesterol (Kritchevsky et al., 2000). Additionally, in the Syrian Golden

hamster, diets containing *trans*-10, *cis*-12 CLA increased HDL cholesterol as compared with LA or *cis*-9, *trans*-11 CLA diets (Mitchell et al., 2005). However, some studies in rat models have shown no effect of dietary CLA on serum HDL cholesterol (Kloss et al., 2005). In a human dietary study, both Apo A-I gene transcript and HDL cholesterol were decreased by CLA-enriched butter as compared with pre-supplement levels (Desroches et al., 2005).

Fatty acids and their derivatives have been identified as potential ligands for PPAR. As several CLA isomers have been identified as high-affinity ligands and activators of PPARα (Moya-Camarena et al., 1999), we investigated the possibility that CLA effects in the two cell lines may be mediated by PPARα. Incubation of HepG2 cells with WY 14,643, a specific PPARα agonist, showed no effects on *trans*-10, *cis*-12 CLA-induced gene expression, although it did increase basal expression of HMG-R and Apo A-I gene transcripts. Inhibition of PPARα had no effects on any of the genes in HepG2 cells. In H-4-II-E cells, however, the PPAR response differed. Activation of PPARα had no effects on HMG-R or Apo A-I mRNA concentrations, but basal concentrations of ACO were increased. In contrast, MK886 decreased basal levels of HMG-R mRNA expression and attenuated the effect of *trans*-10, *cis*-12 CLA-induced Apo A-I concentration. Although ACO is an established PPARα responsive gene (Tugwood, et al., 1992), species differences do exist. It is questionable whether the PPAR response element of human ACO is active (Woodyatt et al., 1999). Dietary studies have shown that rodents are responsive to the effects of PPARα activation, but non-rodent species, such as primates and guinea pigs, are resistant or unresponsive to some of the negative effects (Bentley et al., 1993; Cattley et al., 1998). In a

comprehensive analysis of gene expression in human and rat hepatoma cells by microarray analysis, only rat ACO mRNA was responsive to WY 14,643 (Vanden Heuvel et al., 2003). Other genes that may be differentially regulated in human and rat liver include cytosolic aspartate aminotransferase (Tomkiewicz et al., 2004), peroxisomal 3-oxoacyl-CoA thiolase (Lawrence et al., 2001), and catalase (Ammerschlaeger et al., 2004). Consistent with our findings in the rat cell line, Apo A-I gene expression was not different from controls in mice fed fenofibrate, a potent PPARα activator (Warren et al., 2003). However, different PPARα agonists may regulate lipid metabolism in a compound-dependent manner. A recent study by Duez and coworkers (2005) showed that, in mice, fenofibrate and gemfibrozil, both stimulated ACO mRNA expression, but only fenofibrate greatly induced Apo A-I gene expression. The lack of effect of PPARα activation or inhibition on *trans*-10, *cis*-12 CLA-induced gene expression in our study may be due to possible interactions between CLA and PPARδ, which may serve as a PPARα-independent mediator in response to CLA supplementation, as shown in PPARα-null mice (Peters et al., 2001). Warren and coworkers also supported this idea, with reports that PPARα expression in mice decreased with *trans*-10, *cis*-12 CLA, while ACO mRNA expression increased (Warren et al., 2003). Findings from our studies and others suggest that it is probable that the effects of CLA are not solely dependent upon PPARα.

Summary

In HepG2 cells, ACO, HMG-R, and Apo A-I steady-state mRNA levels were up-regulated by both CLA isomers as compared to LA, and the greatest gene induction was seen with the *trans*-10, *cis*-12 CLA isomer. Selective activation or inhibition of PPARα had no effect on *trans*-10, *cis*-12 CLA-induced gene expression. However,

incubation of HepG2 cells with the PPARα agonist increased basal production of HMG-R and Apo A-I mRNA concentration. Consistent with the low level of endogenous expression of PPARs in HepG2 cells (Hsu et al., 2001), both the PPARα activator and PPARα inhibitor had marginal effects on basal and *trans*-10, *cis*-12 CLA-stimulated lipid metabolizing gene expression in the human hepatoma cell line.

In H-4-II-E cells, all of the FA studied increased expression of ACO mRNA and decreased expression of Apo A-I mRNA. On average, CLA isomers increased HMG-R gene expression as compared with LA, although there was no difference between the two isomers. Selective activation of PPARα increased basal expression and attenuated *trans*-10, *cis*-12 CLA-induced expression of ACO mRNA concentration. Activation of PPARα had no effect on HMG-R or Apo A-I gene transcripts. Inhibition of PPARα decreased basal expression of HMG-R gene transcript and attenuated *trans*-10, *cis*-12 CLA effects on Apo A-I mRNA. Taken together, these results indicate that *trans*-10, *cis*-12 CLA likely regulates lipid metabolizing genes in the liver through a PPARα-dependent mechanism. However, due to different responses in the human and rat hepatoma cell lines, the net effects are likely species specific.

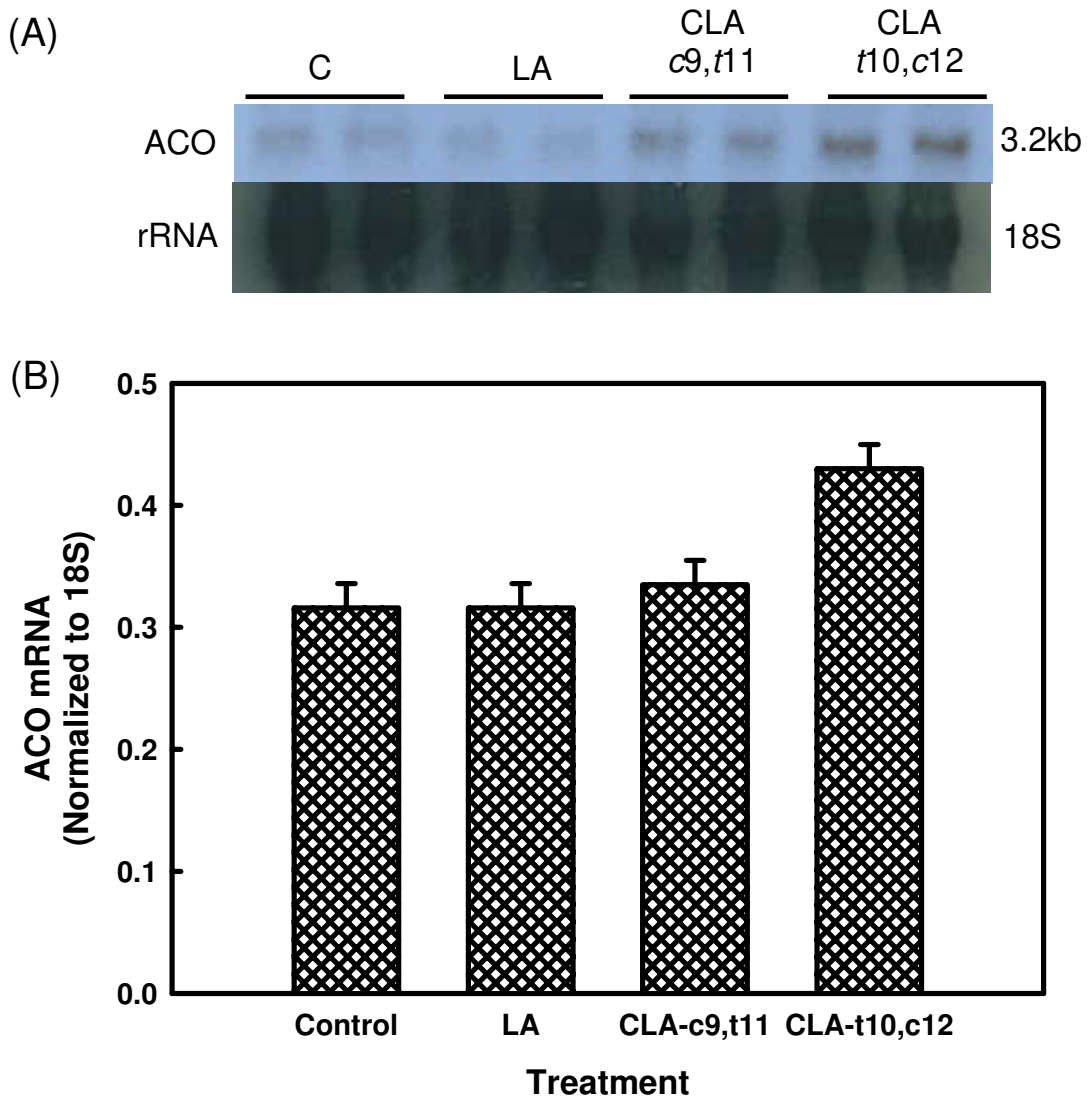

Figure 4-1. Effect of CLA on ACO mRNA expression in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and FA-treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (LA + *cis*-9, *trans*-11 CLA + *trans*-10, *cis*-12 CLA), P = 0.09; Contrast 2: LA vs. (*cis*-9, *trans*-11 CLA + *trans*-10, *cis*-12 CLA), P = 0.03; Contrast 3: *cis*-9, *trans*-11 CLA vs. *trans*-10, *cis*-12 CLA, P = 0.009.

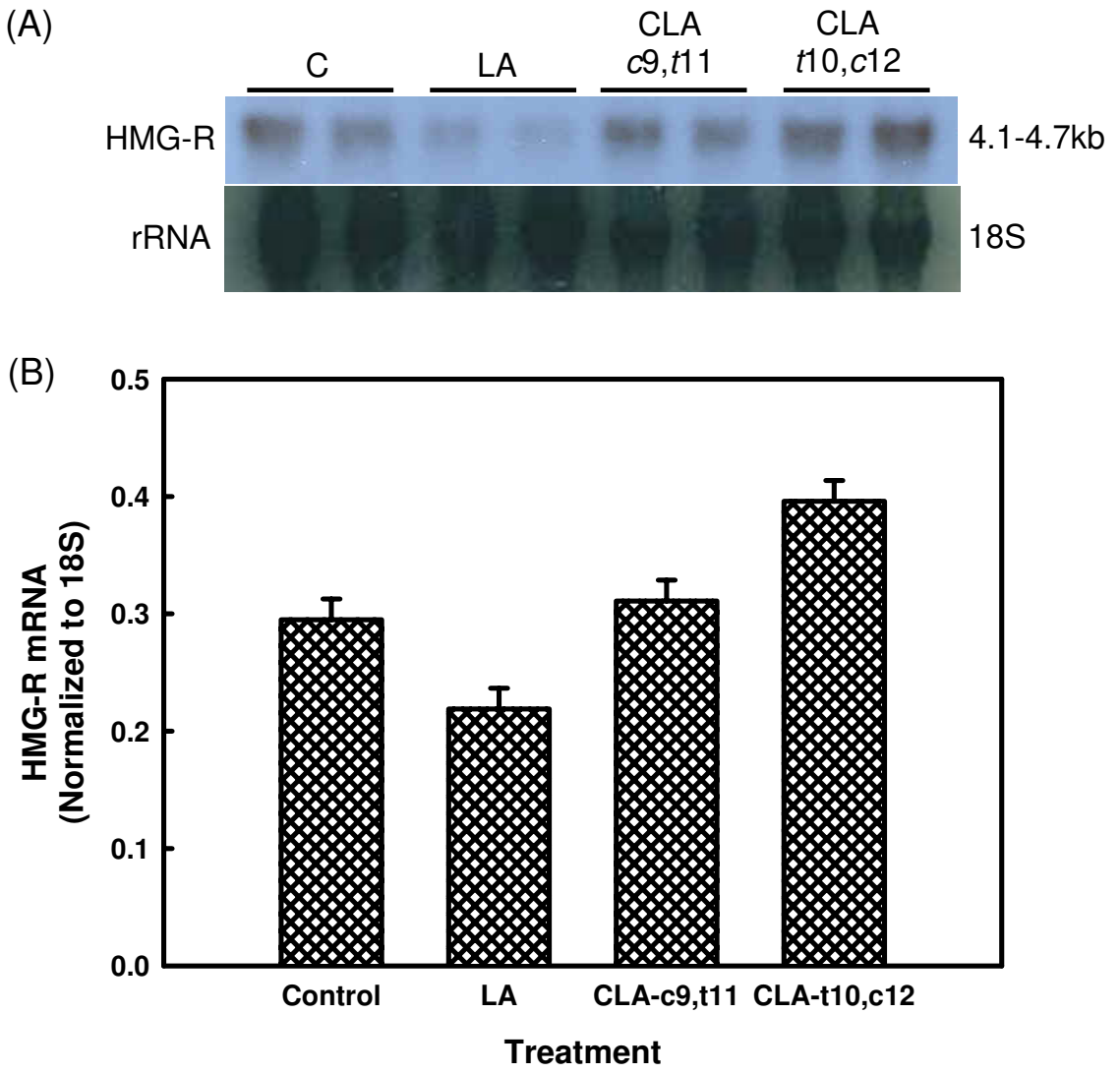

Figure 4-2. Effect of CLA on HMG-R mRNA expression in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and FA-treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (LA + *cis*-9, *trans*-11 CLA + *trans*-10, *cis*-12 CLA), P = 0.5; Contrast 2: LA vs. *cis*-9, *trans*-11 CLA + *trans*-10, *cis*-12 CLA), P = 0.0003; Contrast 3: *cis*-9, *trans*-11 CLA vs. *trans*-10, *cis*-12 CLA, P = 0.009.

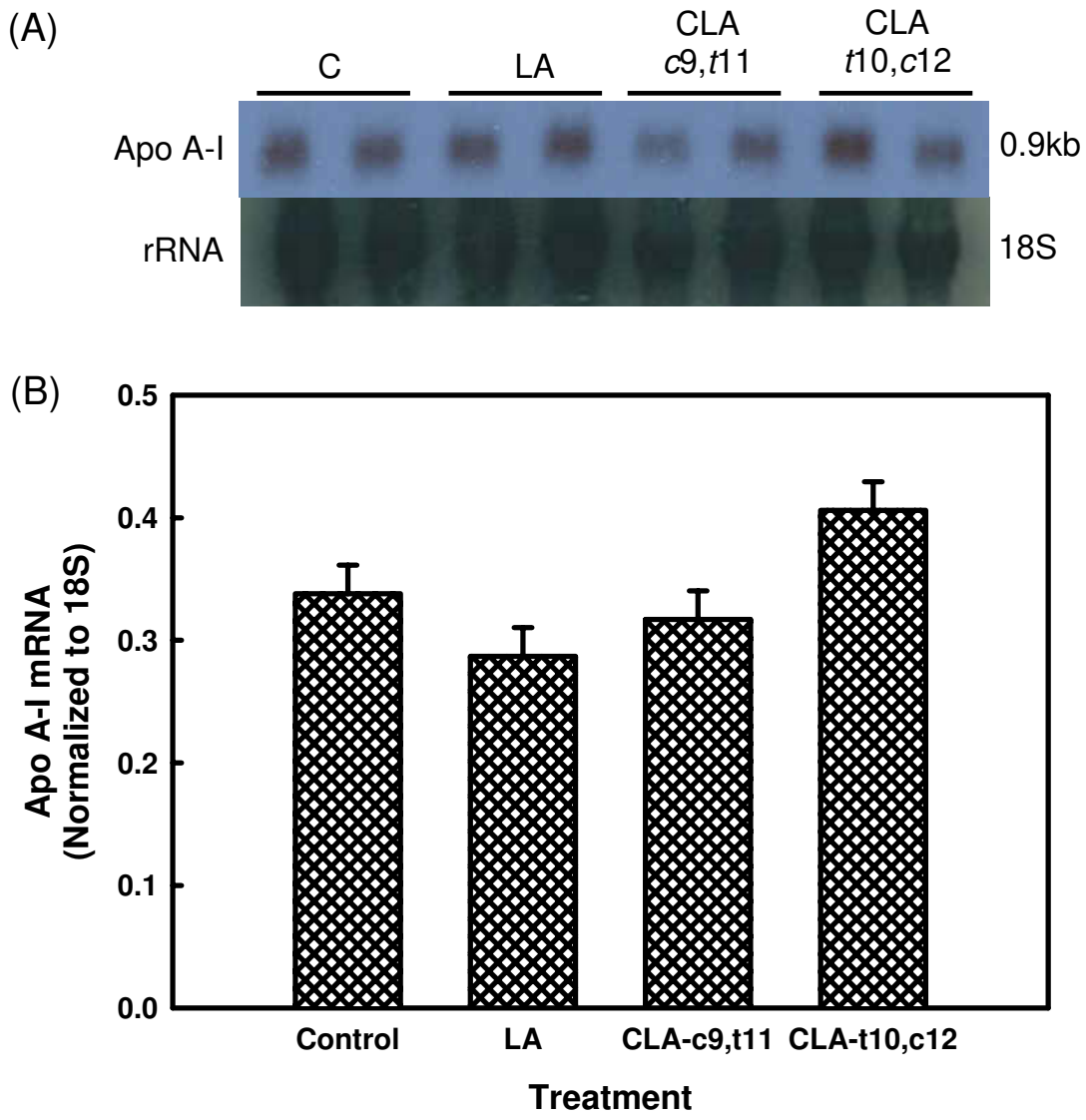

Figure 4-3. Effect of CLA on Apo A-I mRNA expression in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and FA-treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (LA + *cis*-9, *trans*-11 CLA + *trans*-10, *cis*-12 CLA), P = 0.9; Contrast 2: LA vs. *cis*-9, *trans*-11 CLA + *trans*-10, *cis*-12 CLA), P = 0.03; Contrast 3: *cis*-9, *trans*-11 CLA vs. *trans*-10, *cis*-12 CLA, P = 0.03.

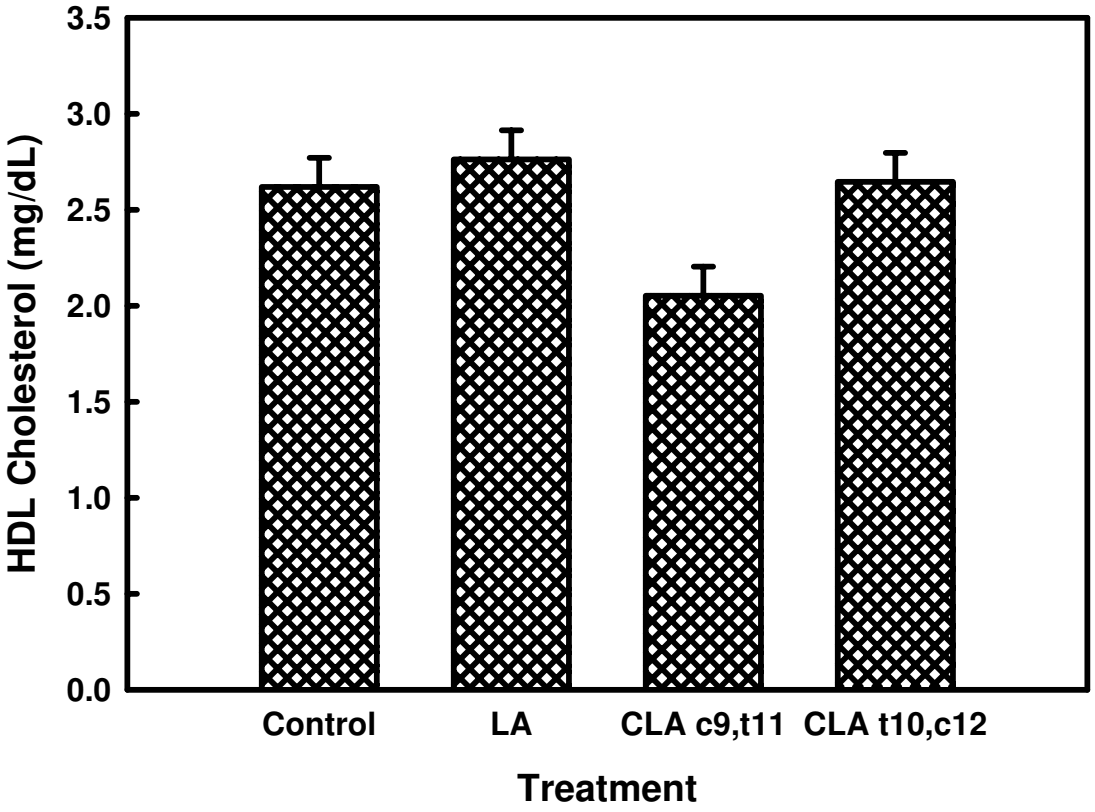

Figure 4-4. Effect of CLA on HDL cholesterol production by HepG2 cells. Data represents least square means ± SEM calculated over two experiments. To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (LA + *cis*-9, *trans*-10 CLA + *trans*-10, *cis*-12 CLA), P = 0.5; Contrast 2: LA vs. (*cis*-9, *trans*-10 CLA + *trans*-10, *cis*-12 CLA), P = 0.05; Contrast 3: *cis*-9, *trans*-10 CLA vs. *trans*-10, *cis*-12 CLA, P = 0.02.

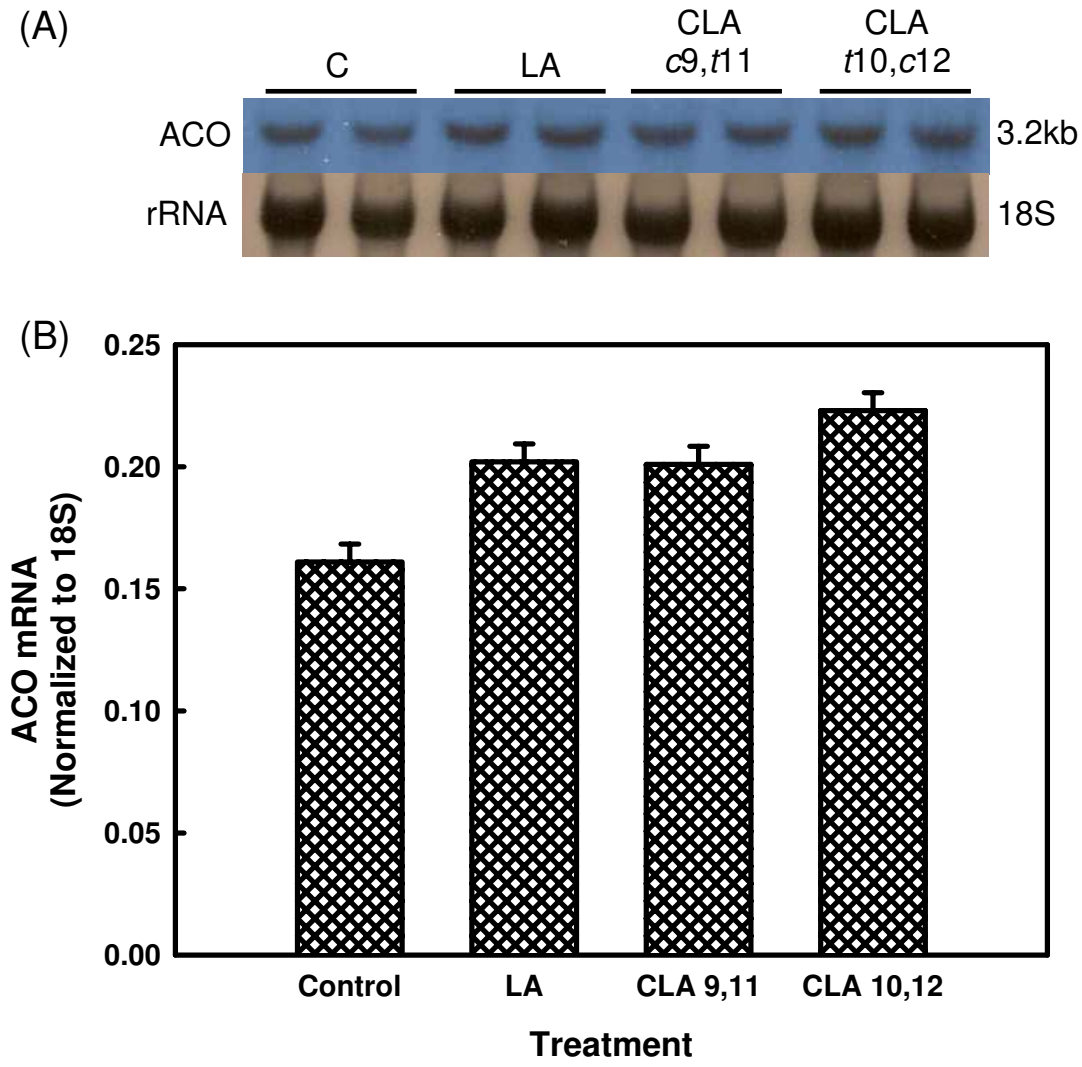

Figure 4-5. Effect of CLA acid on ACO mRNA expression in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and FA-treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (LA + *cis*-9, *trans*-11 CLA + *trans*-10, *cis*-12 CLA), P = 0.0005; Contrast 2: LA vs. (*cis*-9, *trans*-11 CLA + *trans*-10, *cis*-12 CLA), P = 0.3; Contrast 3: *cis*-9, *trans*-11 CLA vs. *trans*-10, *cis*-12 CLA, P = 0.07.

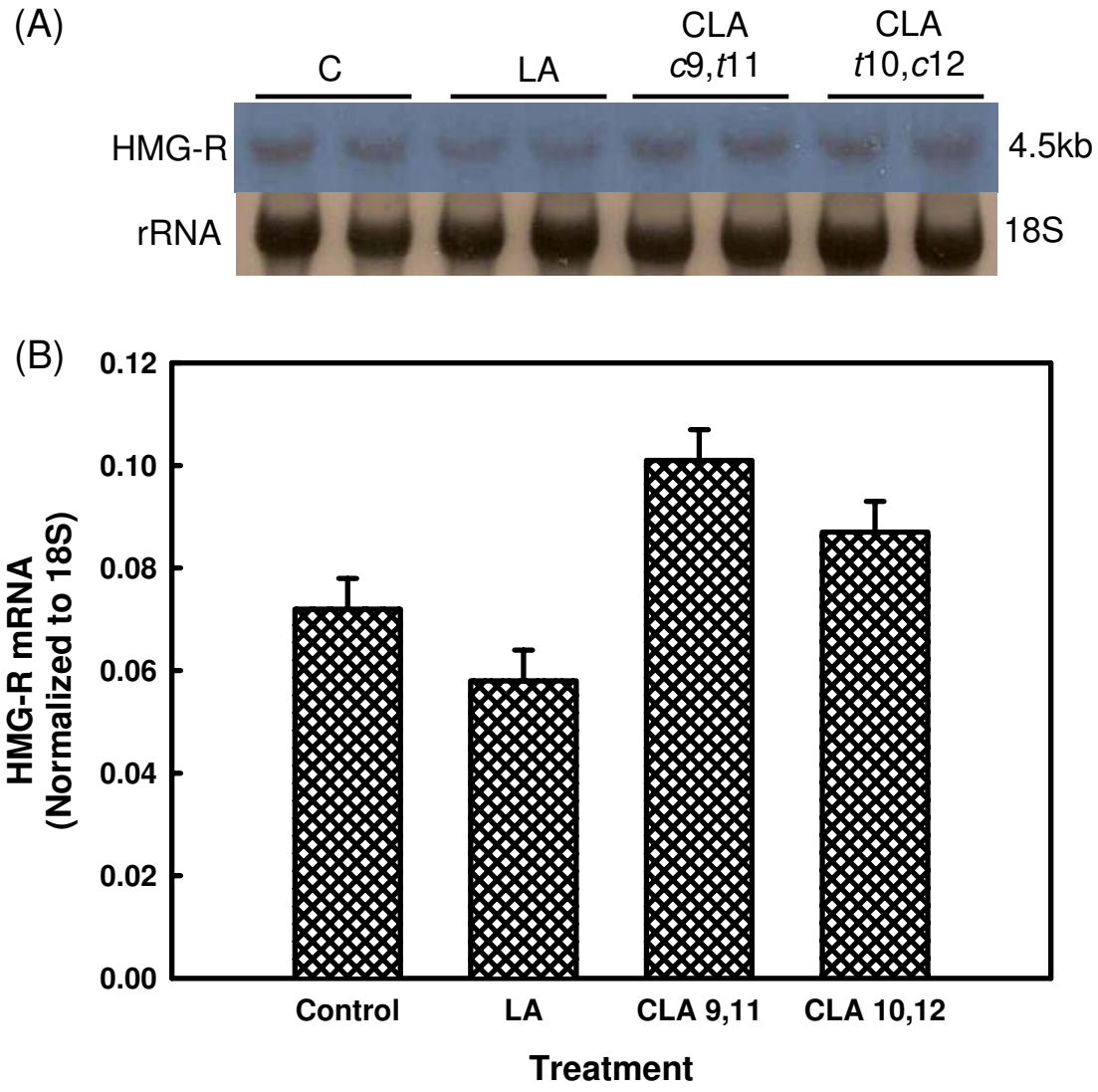

Figure 4-6. Effect of CLA on HMG-R mRNA expression in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and FA-treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (LA + *cis*-9, *trans*-11 CLA + *trans*-10, *cis*-12 CLA), P = 0.2; Contrast 2: LA vs. (*cis*-9, *trans*-11 CLA + *trans*-10, *cis*-12 CLA), P = 0.001; Contrast 3: *cis*-9, *trans*-11 CLA vs. *trans*-10, *cis*-12 CLA, P = 0.2.

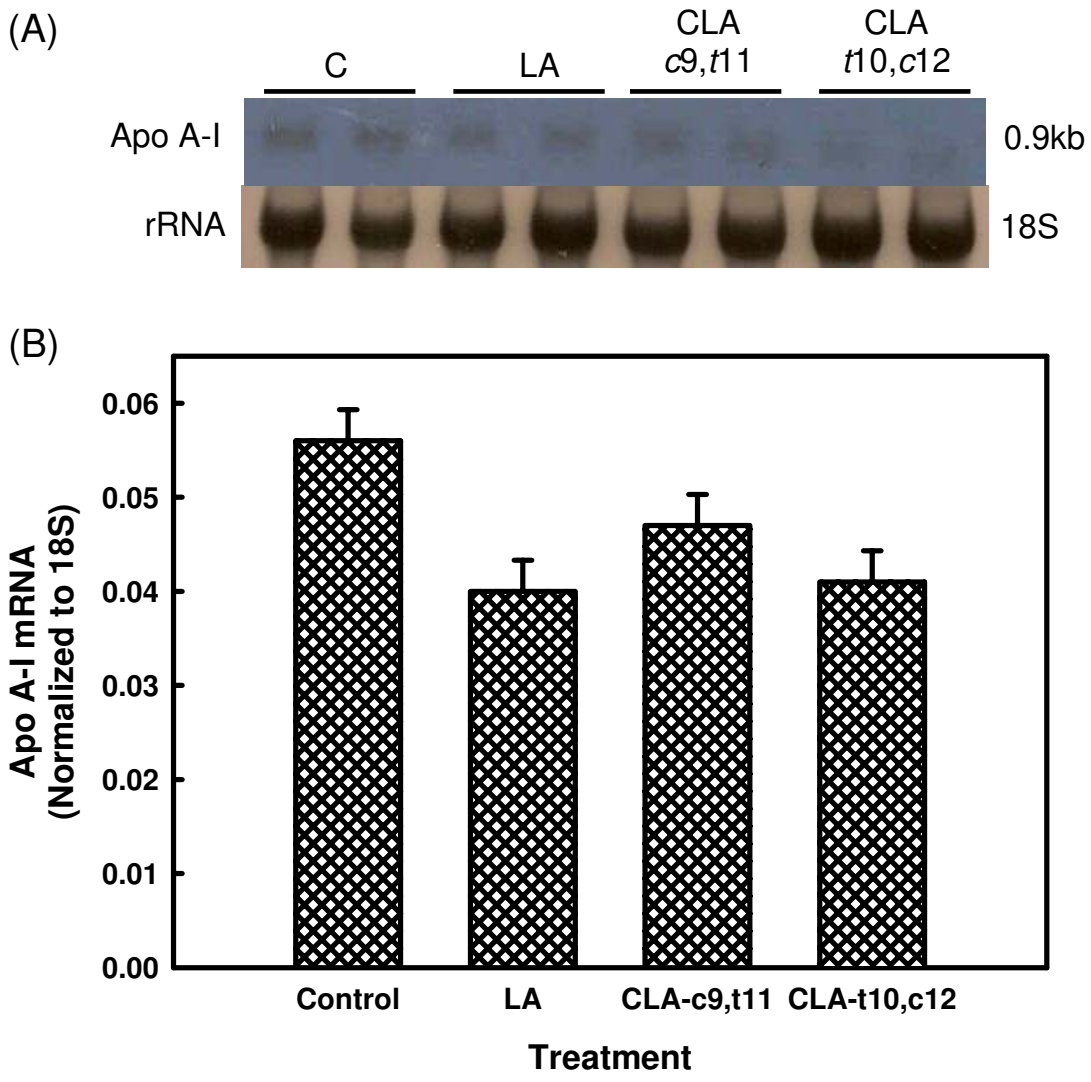

Figure 4-7. Effect of CLA on Apo A-I mRNA expression in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and FA-treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (LA + *cis*-9, *trans*-11 CLA + *trans*-10, *cis*-12 CLA), P = 0.01; Contrast 2: LA vs. (*cis*-9, *trans*-11 CLA + *trans*-10, *cis*-12 CLA), P = 0.3; Contrast 3: *cis*-9, *trans*-11 CLA vs. *trans*-10, *cis*-12 CLA, P = 0.3.

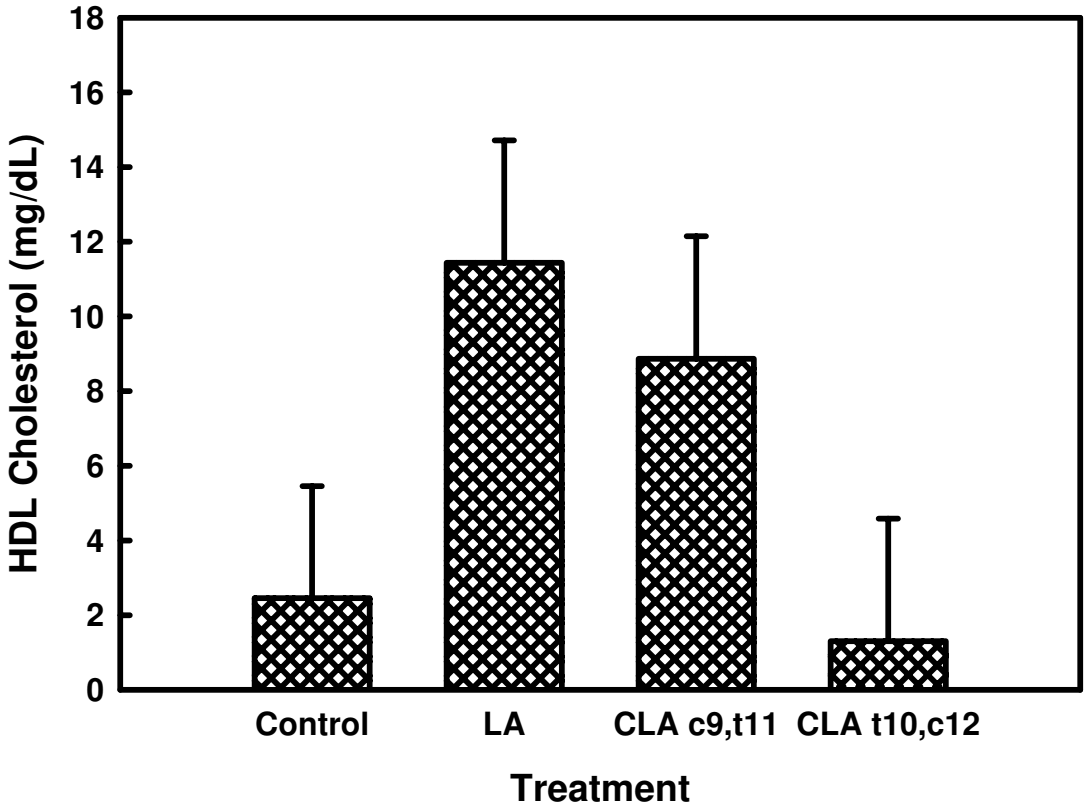

Figure 4-8. Effect of CLA on HDL cholesterol production by H-4-II-E cells. Data represents least square means ± SEM calculated over two experiments. To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (LA + *cis*-9, *trans*-10 CLA + *trans*-10, *cis*-12 CLA), P = 0.2; Contrast 2: LA vs. (*cis*-9, *trans*-10 CLA + *trans*-10, *cis*-12 CLA), P = 0.1; Contrast 3: *cis*-9, *trans*-10 CLA vs. *trans*-10, *cis*-12 CLA, P = 0.1.

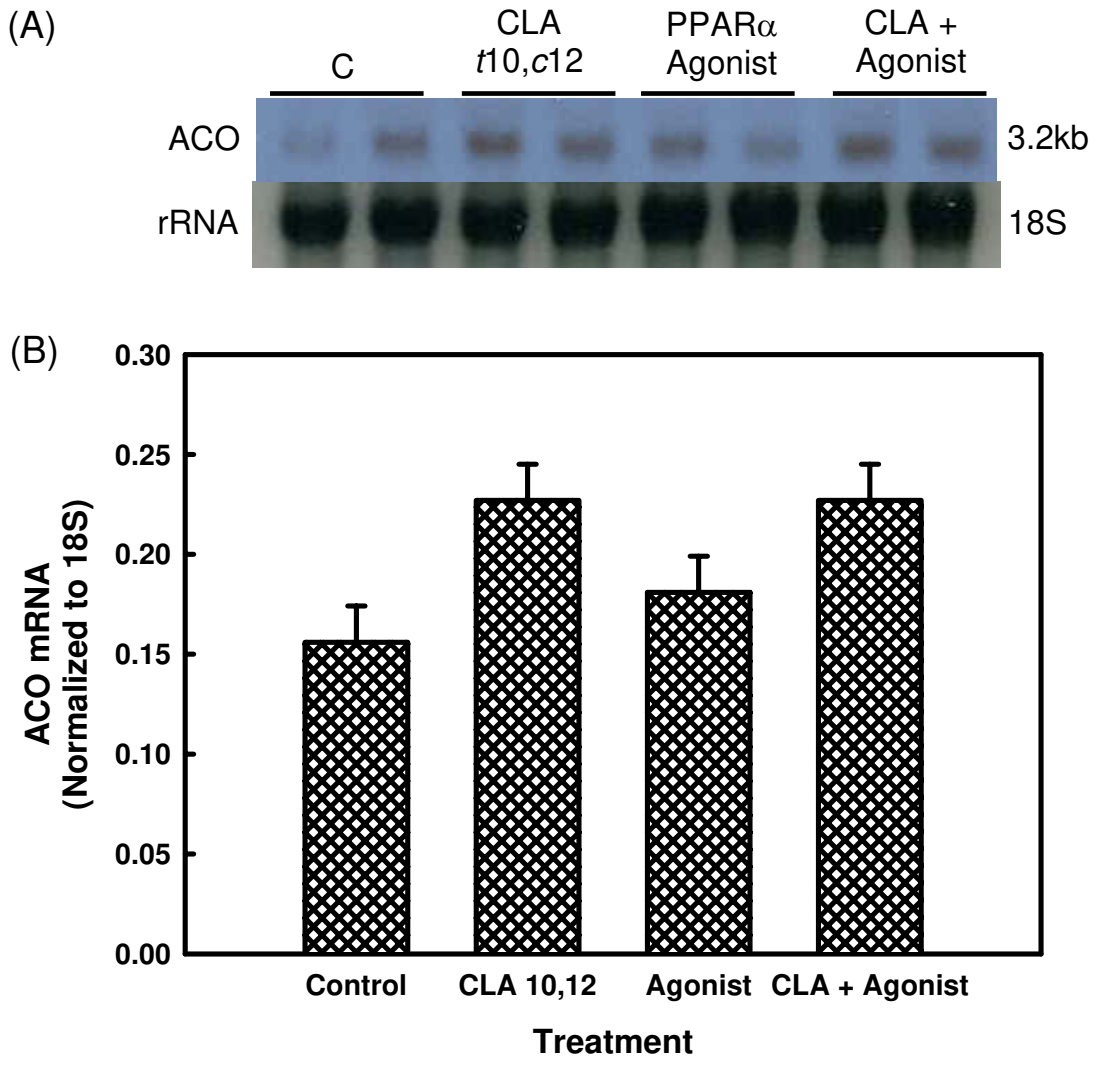

Figure 4-9. Effect of WY 14,643 on ACO mRNA response to *trans*-10, *cis*-12 CLA in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (CLA + CLA+A), P = 0.01; Contrast 2: CLA vs. CLA+A, P = 0.99; Contrast 3: Control vs. Agonist, P = 0.3.

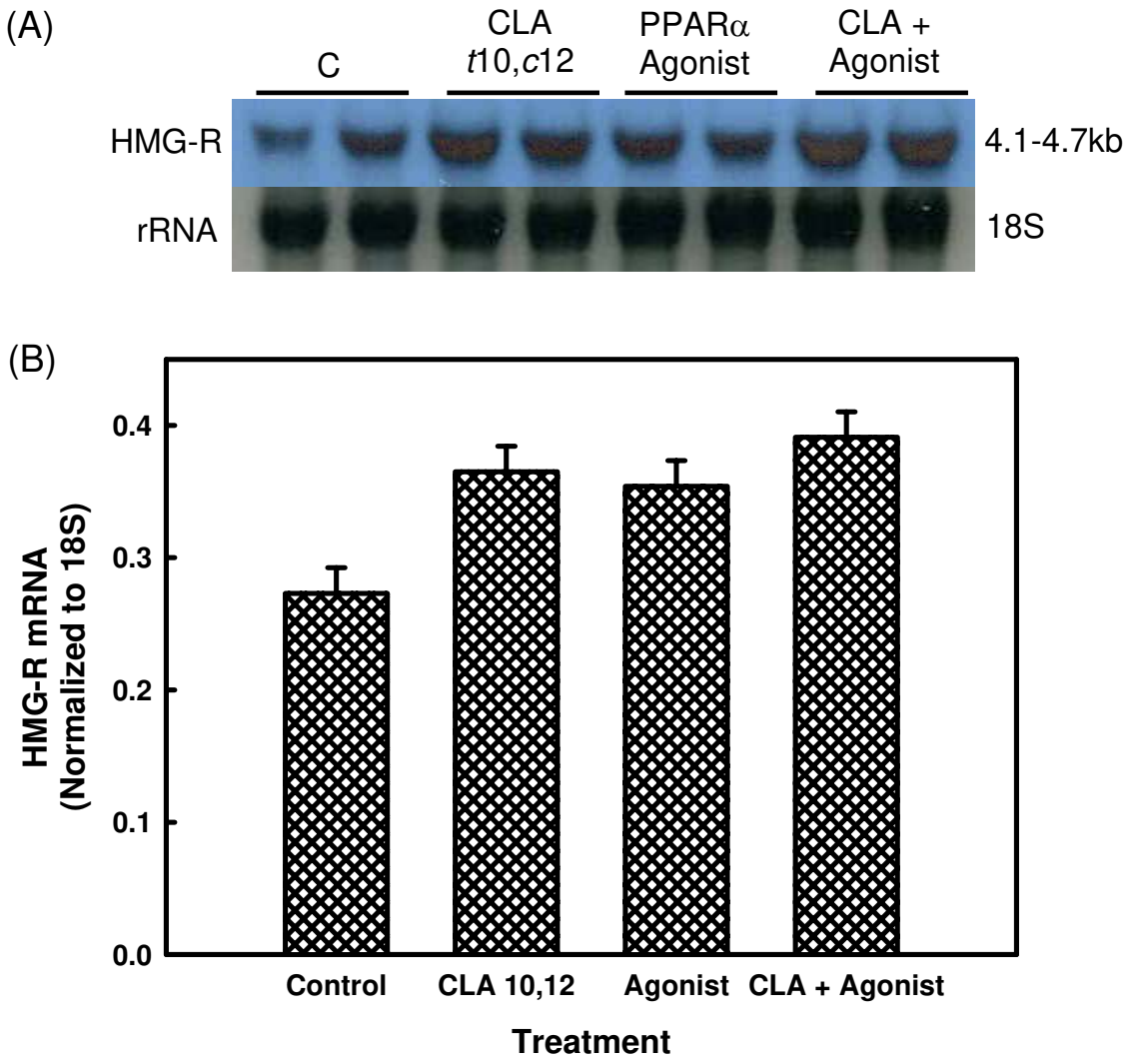

Figure 4-10. Effect of WY 14,643 on HMG-R mRNA response to *trans*-10, *cis*-12 CLA in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (CLA + CLA+A), P = 0.01; Contrast 2: CLA vs. CLA+A, P = 0.4; Contrast 3: Control vs. Agonist, P = 0.02.

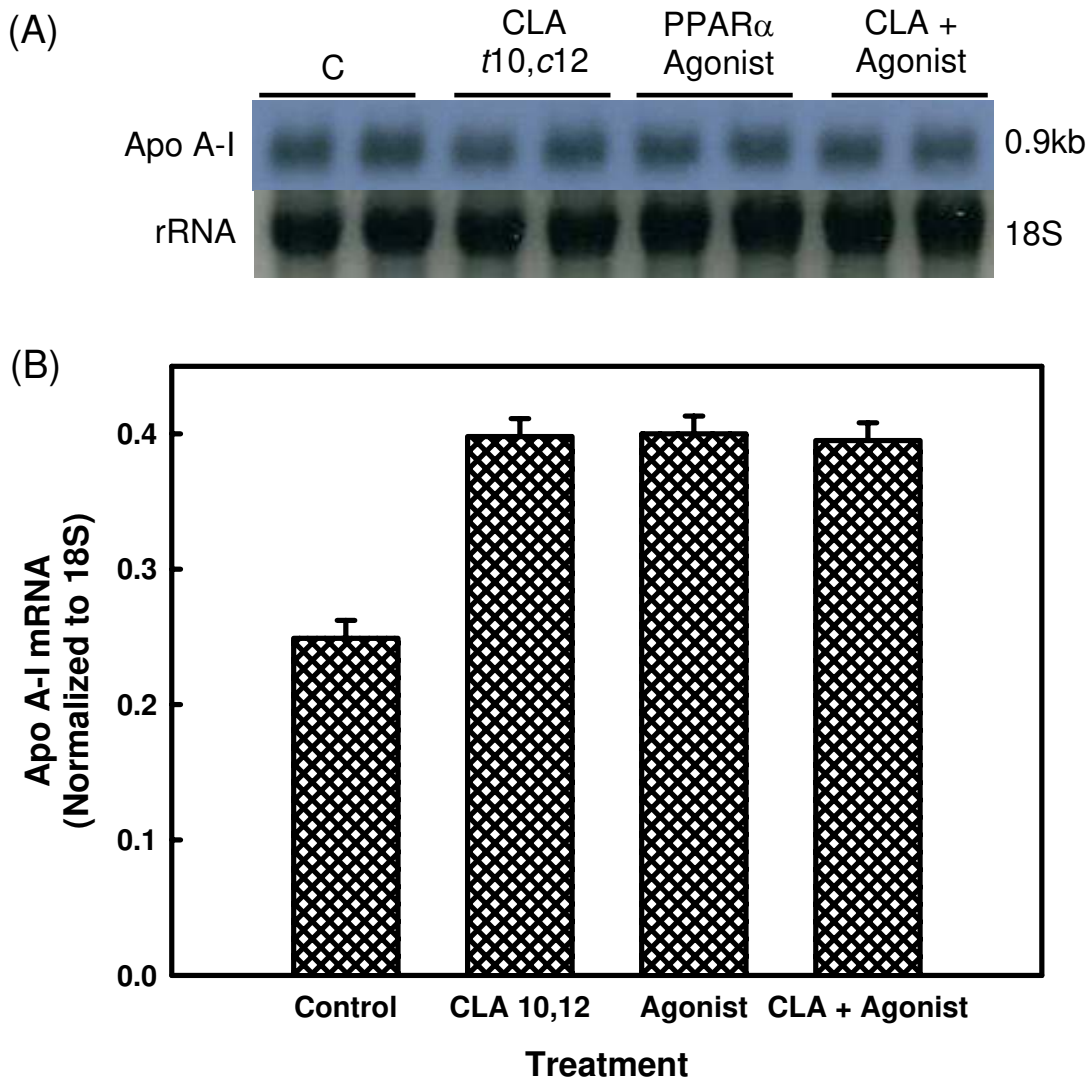

Figure 4-11. Effect of WY 14,643 on Apo A-I mRNA response to *trans*-10, *cis*-12 CLA in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (CLA + CLA+A), P = 0.0006; Contrast 2: CLA vs. CLA+A, P = 0.9; Contrast 3: Control vs. Agonist, P < 0.0001.

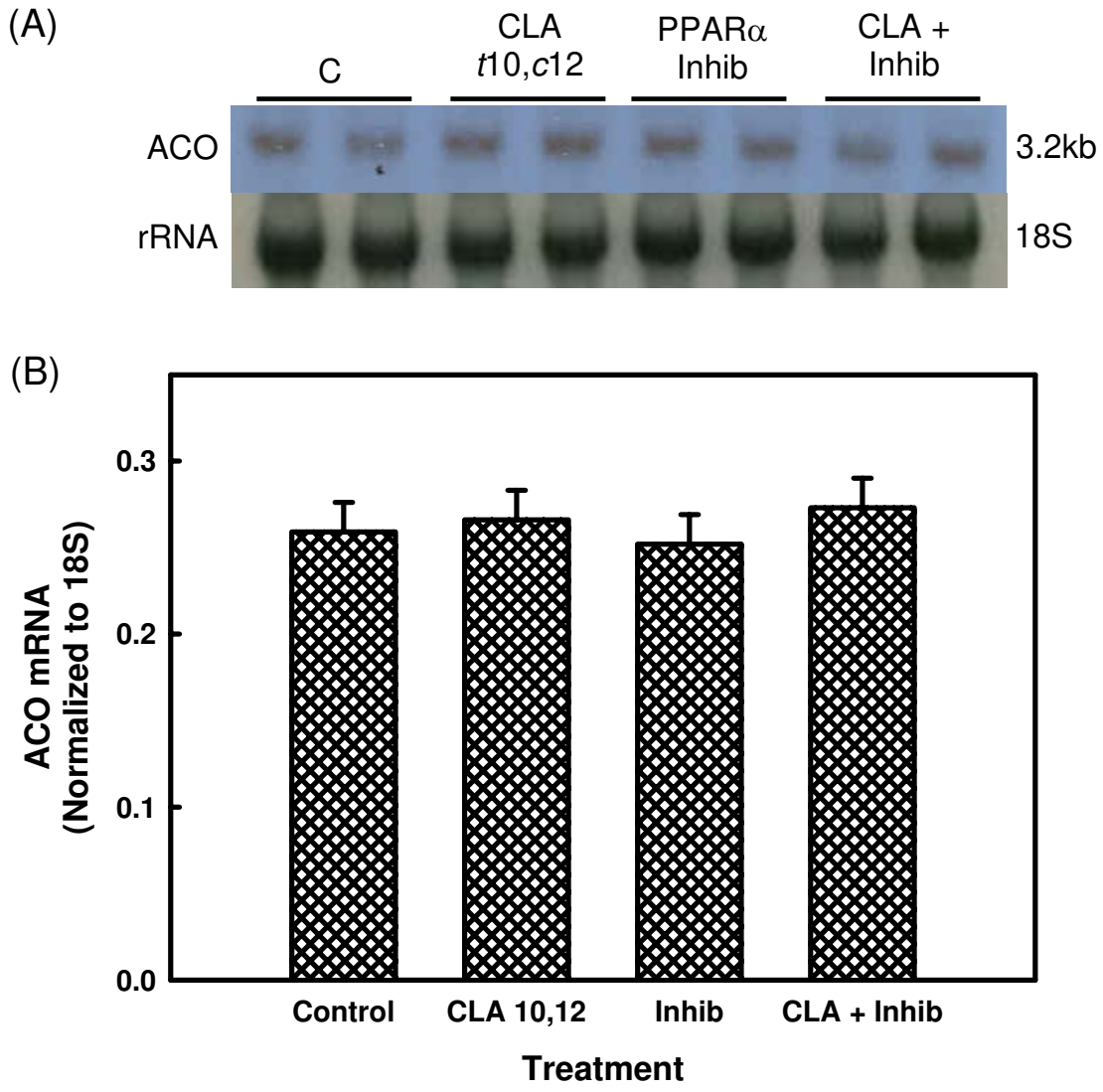

Figure 4-12. Effect of MK886 on ACO mRNA response to *trans*-10, *cis*-12 CLA in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Inhib) vs. (CLA + CLA+I), P = 0.5; Contrast 2: CLA vs. CLA+I, P = 0.8; Contrast 3: Control vs. Agonist, P = 0.8.

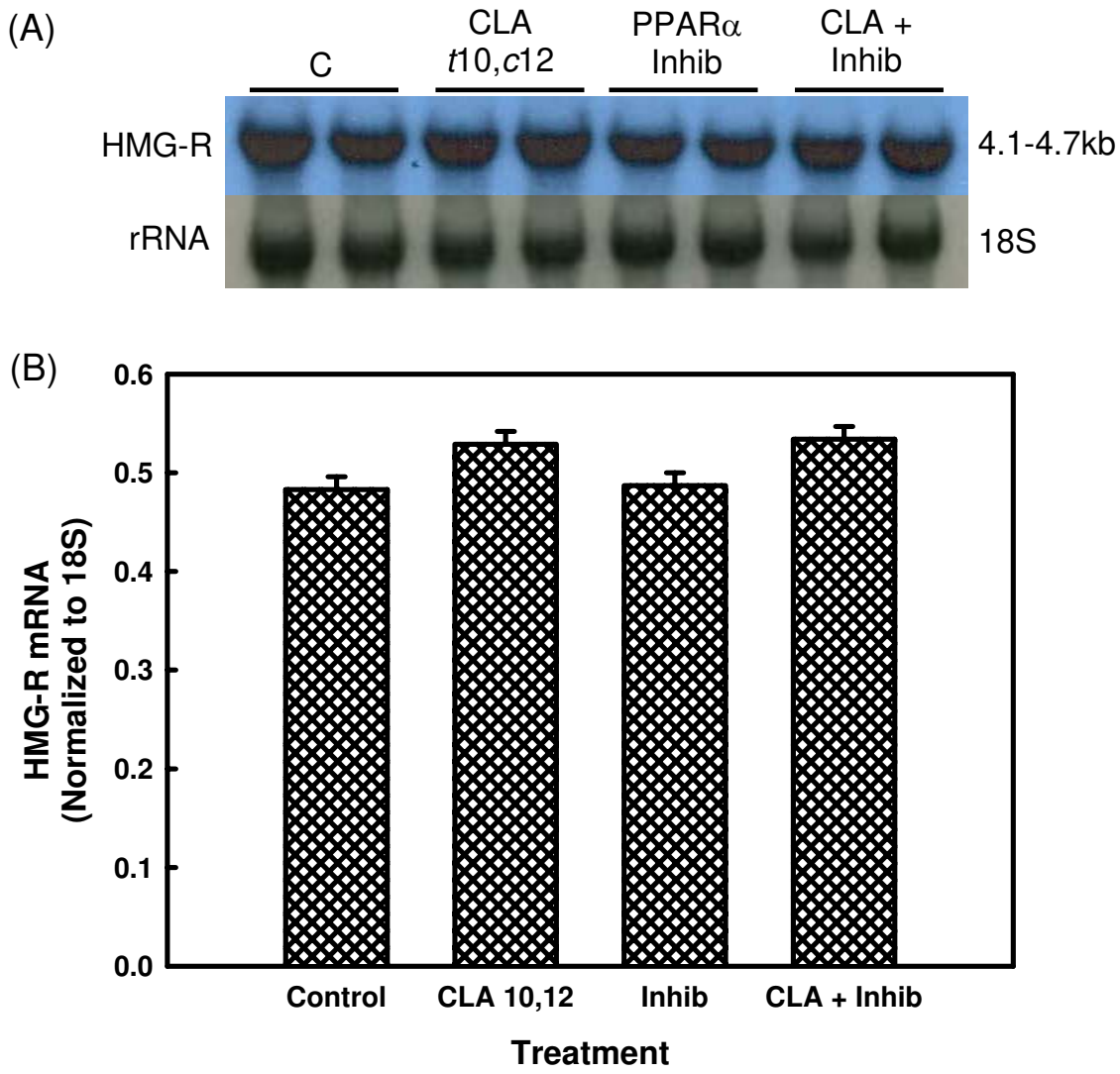

Figure 4-13. Effect of MK886 on HMG-R mRNA response to *trans*-10, *cis*-12 CLA in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Inhib) vs. (CLA + CLA+I), P = 0.007; Contrast 2: CLA vs. CLA+I, P = 0.8; Contrast 3: Control vs. Agonist, P = 0.8.

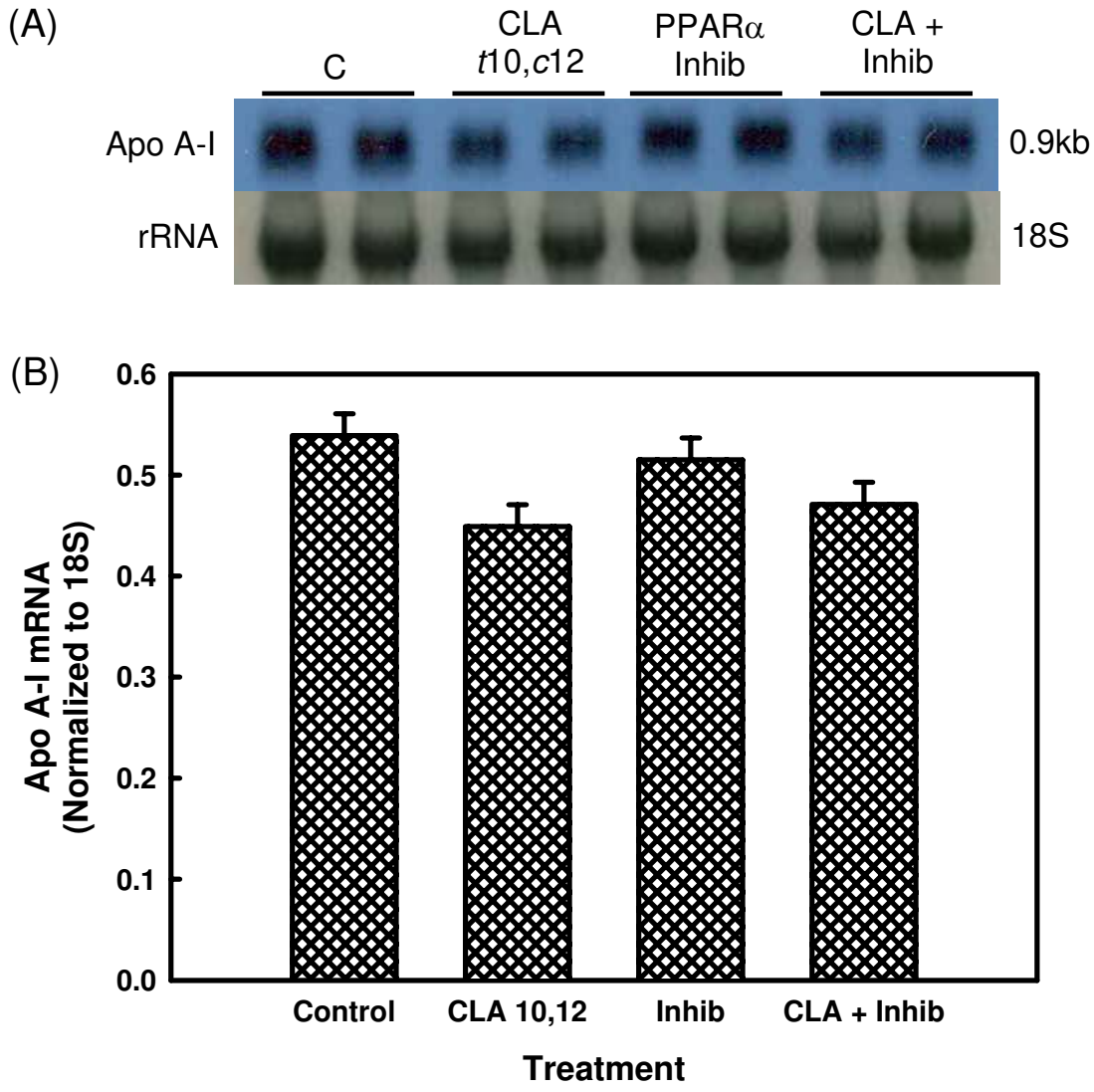

Figure 4-14. Effect of MK886 on Apo A-I mRNA response to *trans*-10, *cis*-12 CLA in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Inhib) vs. (CLA + CLA+I), P = 0.02; Contrast 2: CLA vs. CLA+I, P = 0.5; Contrast 3: Control vs. Agonist, P = 0.5.

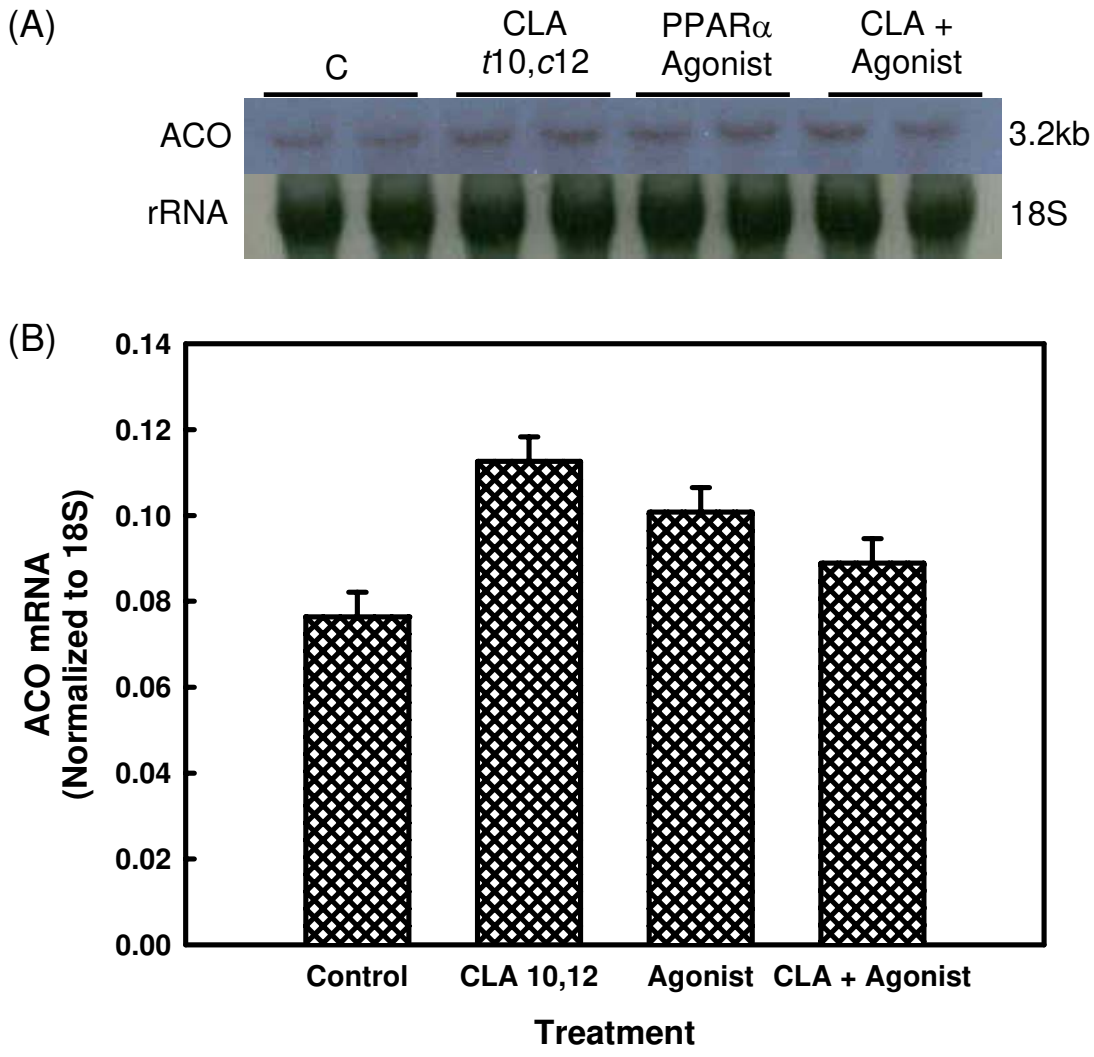

Figure 4-15 Effect of WY 14,643 on ACO mRNA response to *trans*-10, *cis*-12 CLA in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (CLA + CLA+A), P = 0.07; Contrast 2: CLA vs. CLA+A, P = 0.02; Contrast 3: Control vs. Agonist, P = 0.02.

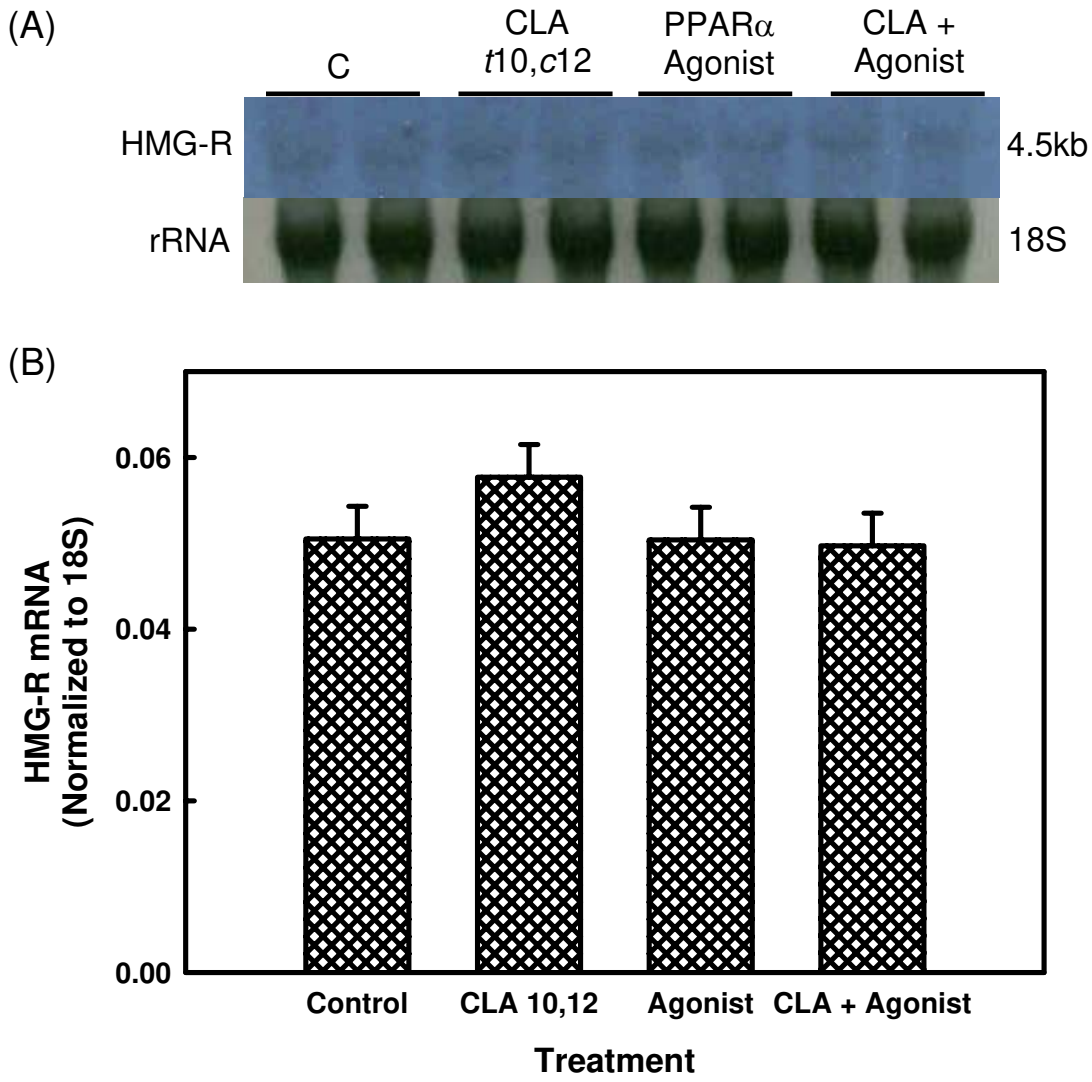

Figure 4-16. Effect of WY 14,643 on HMG-R mRNA response to *trans*-10, *cis*-12 CLA in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (CLA + CLA+A), P = 0.4; Contrast 2: CLA vs. CLA+A, P = 0.2; Contrast 3: Control vs. Agonist, P = 0.9.

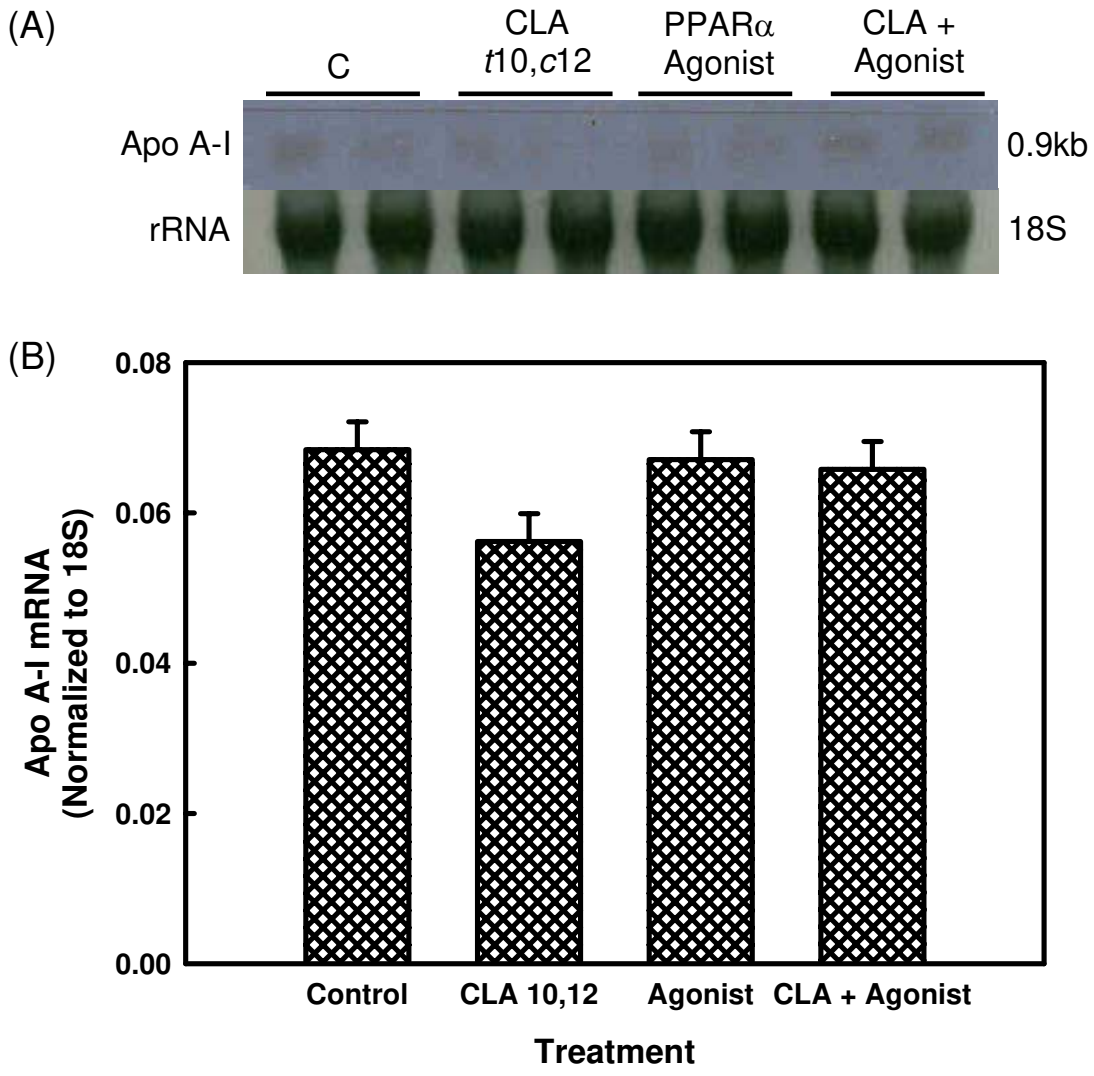

Figure 4-17. Effect of WY 14,643 on Apo A-I mRNA response to *trans*-10, *cis*-12 CLA in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (CLA + CLA+A), P = 0.1; Contrast 2: CLA vs. CLA+A, P = 0.1; Contrast 3: Control vs. Agonist, P = 0.8.

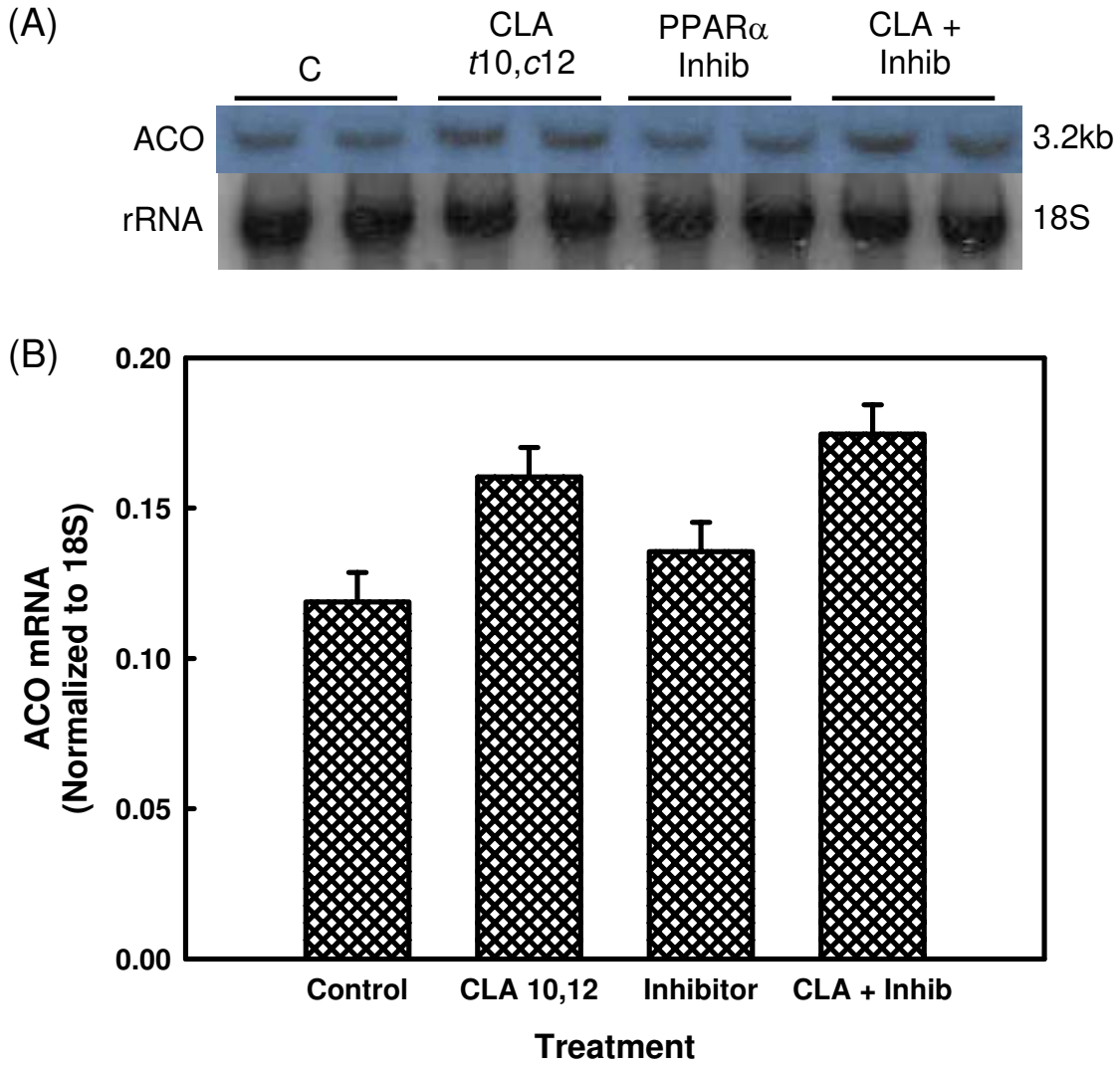

Figure 4-18. Effect of MK886 on ACO mRNA response to *trans*-10, *cis*-12 CLA in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Inhib) vs. (CLA + CLA+I), P = 0.003; Contrast 2: CLA vs. CLA+I, P = 0.3; Contrast 3: Control vs. Inhib, P = 0.3.

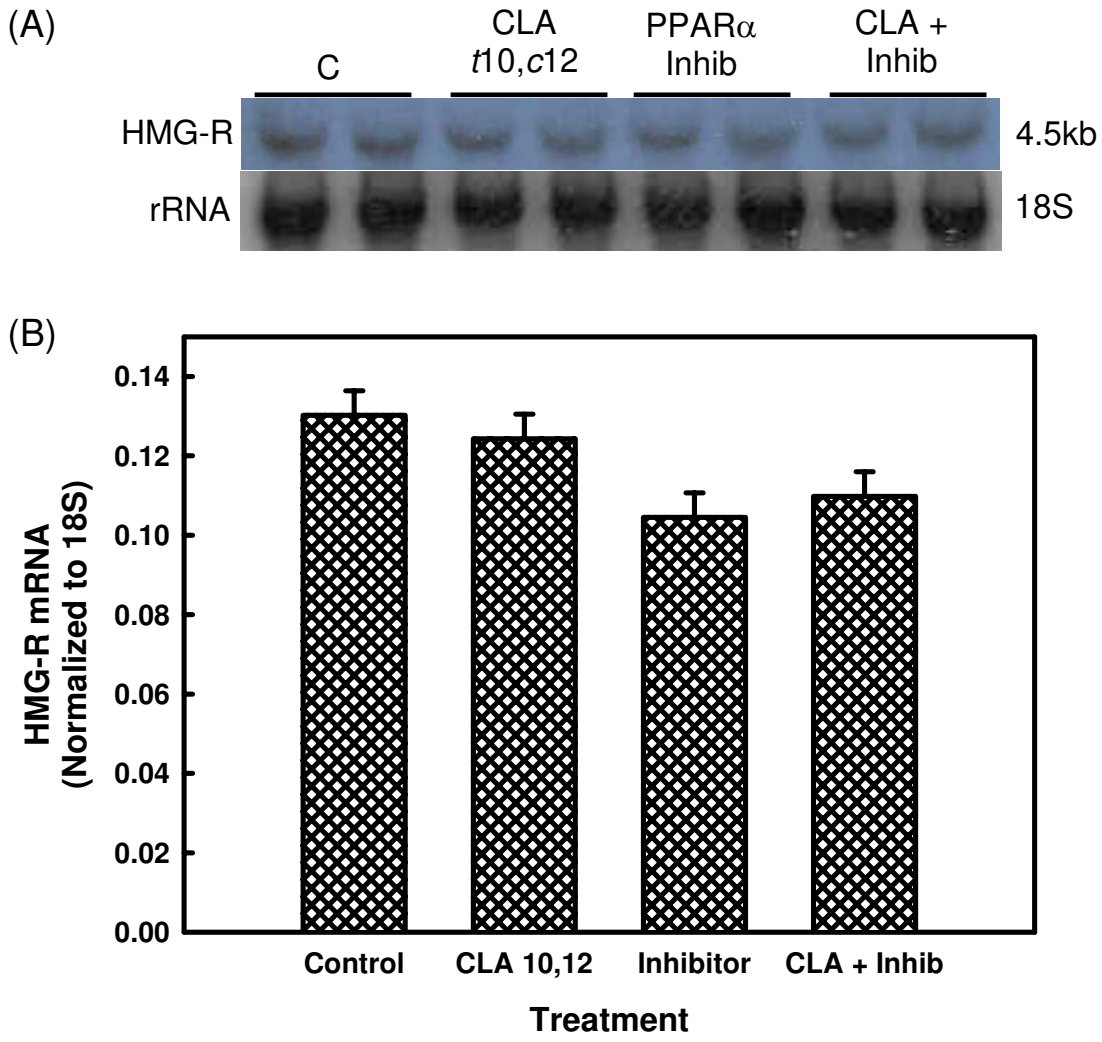

Figure 4-19. Effect of MK886 on HMG-R mRNA response to *trans*-10, *cis*-12 CLA in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Inhib) vs. (CLA + CLA+I), P = 0.9; Contrast 2: CLA vs. CLA+I, P = 0.1; Contrast 3: Control vs. Inhib, P = 0.02.

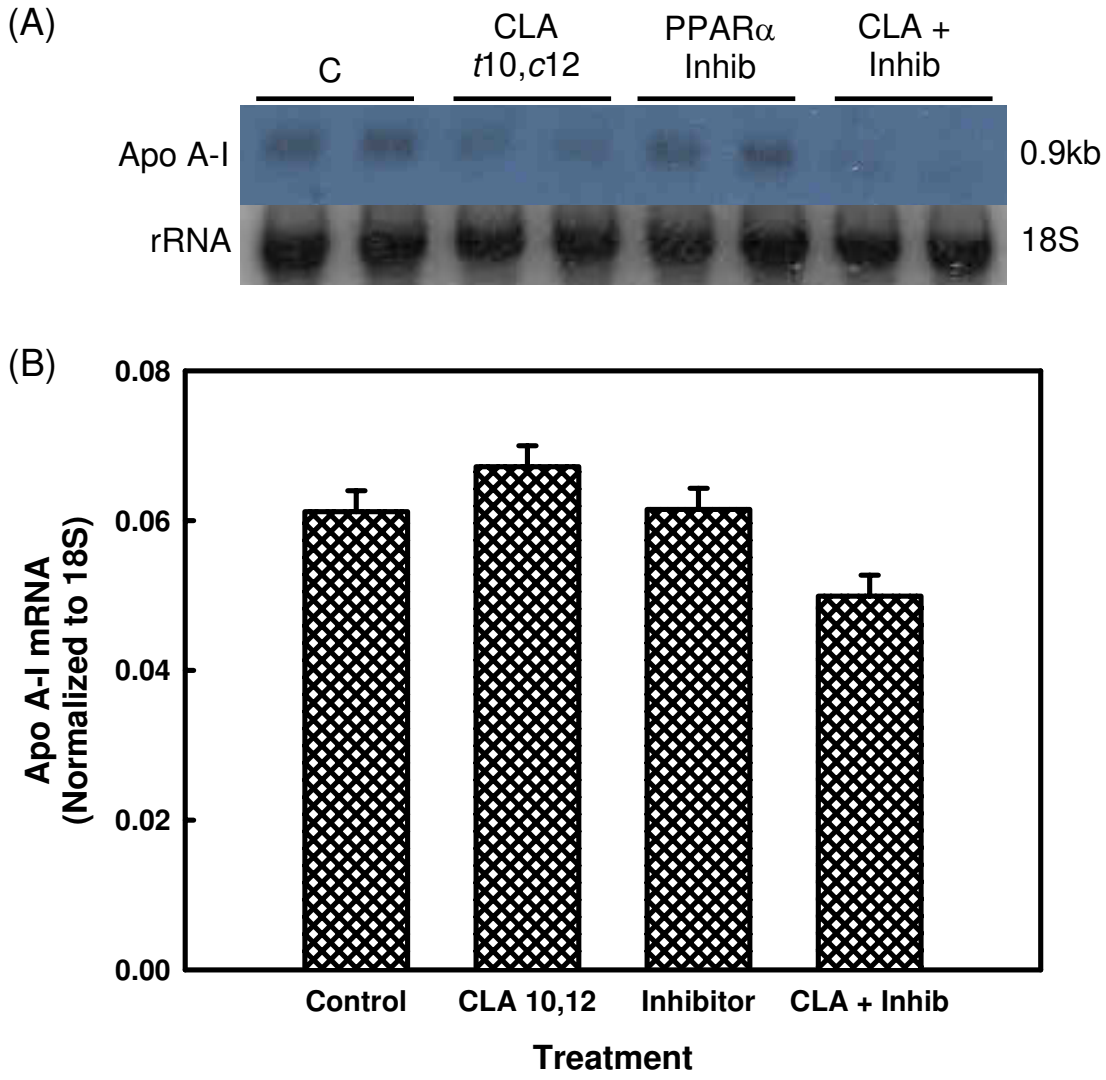

Figure 4-20. Effect of MK886 on Apo A-I mRNA response to *trans*-10, *cis*-12 CLA in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Inhib) vs. (CLA + CLA+I), P = 0.4; Contrast 2: CLA vs. CLA+I, P = 0.002; Contrast 3: Control vs. Inhib, P = 0.9.

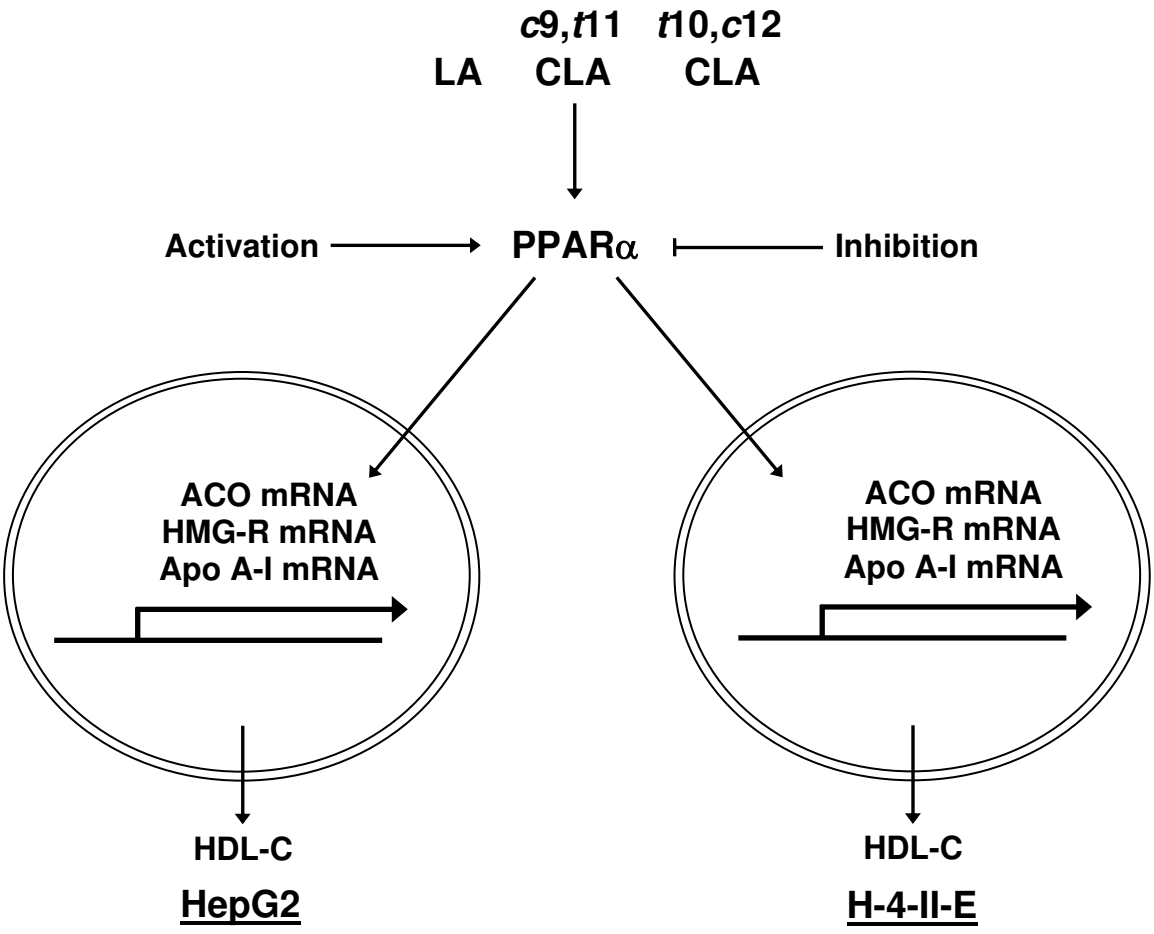

Figure 4-21. Regulation of lipid metabolizing genes and HDL cholesterol production by CLA. In HepG2 cells, ACO, HMG-R, and Apo A-I gene expression were up-regulated by CLA isomers as compared to LA, with the greatest induction seen with *t*10,*c*12 CLA. Activation of PPARα increased basal expression of HMG-R and Apo A-I mRNA. HDL cholesterol production was decreased by *c*9,*t*11 CLA. In H-4-II-E cells, all of the fatty acids increased expression of ACO mRNA and decreased expression of Apo A-I mRNA. On average, CLA isomers increased HMG-R gene expression. Activation of PPARα increased basal expression of ACO mRNA. Inhibition of PPARα decreased basal expression of HMG-R mRNA and attenuated *t*10,*c*12 CLA-effects on Apo A-I gene expression.

CHAPTER 5
EFFECTS OF *CIS* AND *TRANS* ISOMERS OF OCTADECENOIC ACID ON LIPID METABOLIZING GENES AND HIGH-DENSITY LIPOPROTEIN CHOLESTEROL PRODUCTION IN CULTURED HUMAN AND RAT HEPATOCYTES

Introduction

Trans-fatty acids are geometrical isomers of unsaturated fatty acids (FA) that assume a saturated fat-like configuration that differs from that of their *cis* counterparts. The predominant source of *trans* fats in the human diet is hydrogenated oils, such as margarine and partially hydrogenated soybean oil, commonly found in baked goods and deep fat-fried fast foods (Hu et al., 2001). Metabolic studies in several species have shown that *trans*-FA can negatively alter the lipid profile to a greater extent than saturated fats, because they not only increase the concentration of small, dense low-density lipoprotein (LDL) cholesterol (Mauger et al., 2003), but also decrease high-density lipoprotein (HDL) cholesterol concentration in some studies (Judd et al., 1994; de Roos et al., 2003). Additionally, epidemiological evidence has reported *trans*-FA intake to be associated with increased risk for cardiovascular disease (Ascherio et al., 1999). Few studies, however, have examined the role that individual *trans*-FA may have in modulating lipid metabolism. As has been reported with other fatty acids, it is possible that *cis* and *trans* isomers of octadecenoic acid may also have differential effects on lipid metabolism.

The objective of this study was to examine the short term effects of *cis* and *trans* isomers of octadecenoic acid on lipid metabolizing gene expression and HDL cholesterol production in HepG2 (human) and H-4-II-E (rat) hepatoma cell lines. Based on both

dietary and *in vitro* studies of lipid metabolism, we hypothesized that the different *cis* and *trans* isomers may have differing effects on acyl-CoA oxidase (ACO), 3-hydroxy, 3-methylglutaryl CoA reductase (HMG-R), and apolipoprotein A-I (Apo A-I) gene expression. Also, because several fatty acids and their derivatives are known ligands for peroxisome proliferator-activated receptors (PPAR), we hypothesized that these fatty acids may act on lipid metabolizing genes through activation of PPARα in the liver.

Materials and Methods

Materials

Polystyrene tissue culture dishes (100 x 20 mm) were purchased from Corning (Corning Glass Works, Corning, NY). The antibiotic/antimycotic (ABAM), sodium pyruvate, fatty acid-free bovine serum albumin (BSA), *cis*-vaccenic acid (c11), *trans*-vaccenic acid (t11), WY 14,643, and MK886 were from Sigma Chemical Co. (St. Louis, MO). Minimum Essential Medium (MEM), phenol red-free MEM, Hanks Balanced Salt Solution (HBSS) and TriZol reagent were from GIBCO BRL (Carlsbad, CA). The fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA). Oleic (c9) and elaidic (t9) acids were from Cayman Chemicals (Ann Arbor, MI). BioTrans nylon membrane and [α-^{32}P]deoxycytidine triphosphate (SA 3000 Ci/nmol) were from MP Biolomedicals (Atlanta, GA). The Enzyme Color Solution, Reacting Solution, and HDL Calibrator were from Wako Diagnostics (Richmond, VA).

Cell Culture and Treatment

HepG2 (ATCC # HB-8065; Manassas, VA) and H-4-II-E (ATCC # CRL-1548; Manassas, VA) cells were cultured and fatty acids were complexed as described in chapter 3. To investigate the effects of supplemental octadecenoic fatty acids of differing bond position and orientation on hepatic gene expression and cholesterol synthesis,

HepG2 and H-4-II-E cells were treated with oleic (c9; *cis*-9,18:1), elaidic (t9; *trans*-9, 18:1), *cis*-vaccenic (c11; *cis*-11, 18:1) or *trans*-vaccenic (*trans*-11; 18:1) acids (100 μM). Sub-confluent cells were incubated with serum-free medium alone (Control) or with appropriate treatments (listed above) complexed with BSA, for a period of 24 h. Cells were then rinsed twice with 10 mL HBSS. The remaining cell monolayer was then lysed in 3 mL TriZol reagent, and stored at -80°C for subsequent mRNA analysis. The same FA treatments were repeated, using phenol red-free MEM. After incubation, conditioned media were collected and stored at -20^{o}C until lipid extraction and HDL cholesterol analysis.

To investigate whether FA effects on gene expression involves PPARα activation, confluent HepG2 and H-4-II-E cells were treated with the appropriate FA (100 μM), the PPARα agonist WY 14,643 (10 μM), or a combination of FA and WY 14,643. In the HepG2 cells, c11 was used, as this FA lead to the greatest responses in gene expression, whereas in the H-4-II-E cells, t11 was used for the same reason. Additional sets of culture dishes were incubated with FA alone, the PPARα inhibitor MK886 (10 μM; Kehrer et al., 2001), or a combination of FA and MK886. After 24 h of incubation, cells were washed twice with 10 mL HBSS, lysed with TriZol, and stored at -80^{o}C until mRNA analysis.

RNA Isolation and Analysis

Total cellular RNA was isolated from cells using TriZol reagent according to the manufacturer's instructions. Ten micrograms of total RNA was fractioned in a 1.0% agarose formaldehyde gel following previously described protocols (Ing et al., 1996) using the MOPS buffer (Fisher Scientific, Pittsburgh, PA) and transferred to a Biotrans

nylon membrane by downward capillary transfer in 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) using the TurboBlotting system (Schleicher and Schuel, Keene, NH). Nylon membranes were cross-linked by exposure to a UV light source for 90 sec and baked at 80°C for 1 h. Membranes were incubated for 2 h at 50°C in ultrasensitive hybridization buffer (ULTRAhyb; Ambion, Austin, TX) followed by an overnight incubation at 50°C in the same ULTRAhyb solution containing the ^{32}P-labeled ACO, HMG-R, and Apo A-I cDNA probes. Probes were generated by RT-PCR for ACO (forward 5'-CCGGAGCTGCTTACACACAT-3'; reverse 5'-GGTCATACGTGGCTGT GGTT-3'), HMG-R (forward 5'-TCCTTGGTGATGGGAGCTTGTTGTG-3'; reverse 5'-TGCGAACCCTTCAGATGTTTCGAGC-3'), human Apo A-I (forward 5'-AAGACA GCGGCAGAGACTAT-3'; reverse 5'-ATCTCCTCCTGCCACTTCTT-3'), and rat Apo A-I (forward 5'-AAGGACAGCGGCAGAGACTA-3'; reverse 5'-CCACAACCTTTAG ATGCCTT-3'). The sizes and sequences of these cDNA probes were verified by DNA sequencing prior to their use in Northern blot analysis. Filters were sequentially washed in 2X SSC (1X= 0.15 M sodium chloride, 0.015 M sodium citrate)-0.1% SDS and in 0.1x SSC-0.1% SDS two times each at 50°C and then exposed to X-ray film to detect radiolabeled bands. Equal loading of total RNA for each experimental sample was verified by comparison to 18S rRNA ethidium bromide staining.

Lipid Extraction

Total lipids were extracted from conditioned media as described by Bligh and Dyer (1959), with modifications. For each sample, 2 mL of conditioned media was aliquotted into a 20 mL glass screw-top vial. Fourteen mL of chloroform:methanol (2:1, v/v) was then added and the vials were vortexed for 5 minutes. The vials were then centrifuged at 1700 rpm for 5 min. The bottom lipid-containing chloroform layer was transferred to a

clean, dry, pre-weighed vial, placed in a 37°C water bath, and dried under nitrogen gas. Dry samples were placed in a 50°C oven for 10 minutes and placed in a desiccator to cool to room temperature. Samples were weighed, and lipid weight was determined by difference. The sample was resuspended in chloroform and stored at -20°C until HDL cholesterol analysis.

HDL Cholesterol Assay

Lipid extracts from conditioned media were analyzed using a commercially available L-Type HDL-C kit, following the manufacturer's directions. Briefly, using a 96-well plate, 3 µL of sample was pipetted into each well. Two hundred seventy µL of Enzyme Color Solution (R1) was added, and the plate was incubated for 5 minutes at 37°C. Ninety µL of Reacting Solution (R2) was then added, and the plate was incubated another 5 minutes at 37°C. The absorbance at 600 nm was measured using the SpectraMax 340 PC microplate reader (Molecular Devices, Sunnyvale, CA), and the concentration of HDL cholesterol in each samples was calculated by plotting against a standard curve.

Statistical Analysis

All hybridization signals as measured by densitometry were evaluated by least squares analysis of variance (ANOVA) using the General Linear Model (GLM) procedure of the SAS software package (SAS Institute Inc, Cary, NC). In each experiment, treatments were run in duplicate, and the whole experiment was also duplicated, giving n=4 plates per treatment. The general model for mRNA analysis included experiment, treatment, and experiment x treatment interaction. In mRNA analyses, densitometric values for target genes were expressed as ratios of target gene densitometric values over the corresponding 18S rRNA densitometric values. For HDL

cholesterol concentration, the sources of variation included experiment, treatment, experiment x treatment interaction, and plate (experiment x treatment). The plate, nested within experiment and treatment, was considered a random variable, and therefore the plate variance was used as an error term to test the effects of experiment, treatment, and experiment x treatment interaction. Treatment means were further compared using preplanned orthogonal contrasts. These contrasts were control vs. fat treatment (c9, t9, c11, t11), double bonds in position 9 (c9, t9) vs. 11(c11, t11), c9 vs. t9, and c11 vs. t11. For all responses, the two cell lines were analyzed separately.

Results

Effects of Octadecenoic Acids on HepG2 Cells

In HepG2 cells, incubation with monounsaturated FA with bonds in the 11 position increased (+14%; P = 0.005) ACO mRNA expression as compared to MUFA with bonds in the 9 position. This effect was primarily due to the increase (+16%; P = 0.01) in ACO gene expression from c11 as compared to t11 (Figure 5-1). Steady-state levels of HMG-R mRNA were unaffected (P = 0.6) by any FA treatment (Figure 5-2). No differences were detected in Apo A-I gene expression due to double bond position (P = 0.2) or c9 as compared to t9 (P = 0.3). However, the c11 isomer up-regulated (+19%; P = 0.04) Apo A-I gene expression as compared to the t11 isomer (Figure 5-3). None of the FA studied had effects (P = 0.4) on HDL cholesterol production by HepG2 cells (Figure 5-4).

Effects of Octadecenoic Acids on H-4-II-E Cells

On average, incubation of H-4-II-E cells with FA up-regulated (+10%; P = 0.01) ACO mRNA expression as compared to control. Additionally, the c9 isomer increased (+11%; P = 0.02) ACO mRNA levels as compared to t9, and the t11 isomer increased

(+10%; P = 0.03) gene expression as compared to c11 (Figure 5-5). The t11 FA isomer increased (+21%; P = 0.005) HMG-R gene expression as compared to the c11 isomer (Figure 5-6). As compared to control, incubation with FA decreased (-10%; P = 0.002) Apo A-I mRNA concentrations, although there were no differences among the FA studied (Figure 5-7). High-density lipoprotein cholesterol production by H-4-II-E cells was unaffected (P = 0.3) by any FA studied (Figure 5-8).

Role of PPARα in Vaccenic Acid-Induced Effects on Gene Expression

Incubation of HepG2 cells with 10 μm WY 14,643, a specific PPARα agonist, had no effect on either basal (P = 0.06) or c11-induced (P = 0.3) ACO mRNA expression (Figure 5-9). Basal levels of HMG-R mRNA were increased (+16%; P = 0.05) by use of the PPARα agonist, but there was no effect (P = 0.4) on c11-induced gene expression (Figure 5-10). Co-incubation with WY 14,643 enhanced (+33%; P = 0.007) the effect of c11 on Apo A-I mRNA concentration (Figure 5-11). In HepG2 cells, incubation with MK886, a specific PPARα inhibitor, had no effects (P > 0.2) on basal or c11-induced expression of any of the genes studied (ACO, Figure 5-12; HMG-R, Figure 5-13; Apo A-I, Figure 5-14).

In H-4-II-E cells, incubation with WY 14,643 enhanced (+52%; P = 0.001) the effect of t11 on ACO gene expression (Figure 5-15). A similar effect was seen in HMG-R mRNA levels, with activation of PPARα increasing (+44%; P = 0.05) t11-induced gene expression (Figure 5-16). Activation of PPARα enhanced (+55%; P = 0.002) the effect of t11 on Apo A-I gene expression, as compared to t11 alone (Figure 5-17). Incubation with MK886 decreased (-55%; P = 0.0007) the basal level and attenuated (-169%; P < 0.0001) t11-induced ACO mRNA levels in H-4-II-E cells (Figure

5-18). Inhibition of PPARα had no effects ($P > 0.1$) on basal or t11-induced HMG-R or Apo A-I gene expression (HMG-R, Figure 5-19; Apo A-I, Figure 5-20).

Discussion

Dietary *trans* fatty acids have been implicated as potent negative factors in the development of numerous disease states, including dyslipidemia and cardiovascular disease. These changes may be mediated, in part, by the effects of fats on lipid metabolism; however, these effects may be different for different isomers. In this study, we investigated the effects of *cis* and *trans* isomers of octadecenoic acid in human and rat hepatoma cell lines. In HepG2 (human) cells, MUFA with the double bond in the 11 position increased ACO mRNA expression as compared to those FA with the bond in the 9 position. In particular, *cis*-vaccenic acid (c11) increased ACO gene expression. In contrast, in the H-4-II-E (rat) cells, all FA up-regulated ACO mRNA concentrations, with oleic acid (c9) and *trans*-vaccenic acid (t11) increasing gene expression as compared to elaidic acid (t9) and *cis*-vaccenic acid (c11), respectively. In contrast with our findings in gene expression, elaidic acid was shown to be a better substrate than oleic acid for fat oxidation, particularly peroxisomal oxidation, in rat hepatocytes (Guzman et al., 1999). However, in a human dietary study, supplementation with *trans*-FA increased and *cis*-FA decreased fat oxidation (as measured by indirect calorimetry) as compared to a saturated fat control diet (Lovejoy et al., 2002). Additionally, in INS-1 cells, similar effects were seen as in H-4-II-E cells, with palmitate oxidation increasing when cells were incubated with oleic, elaidic, *cis*-vaccenic or *trans*-vaccenic acids. Though gene expression was not measured, *trans*-vaccenic acid increased oxidation to a greater extent than its *cis* counterpart (Alstrup et al., 2004).

As numerous studies have shown effects of trans-FA on cholesterol, we examined the mechanisms by which these fats may modulate cholesterol production. One potential gene involved is HMG-R, the rate limiting enzyme in cholesterol synthesis. In HepG2 cells, HMG-R mRNA expression was unaffected by any FA studied. In the rat cells, however, *trans*-vaccenic (t11) acid increased gene expression as compared to *cis*-vaccenic (c11) acid. Though evidence suggests that FA can affect cholesterol production, few studies have examined the role of HMG-R in this response. In support of our findings, dietary oleic acid had no effect on HMG-R activity in Golden Syrian hamsters (Kurushima et al., 1995a; Kurushima et al., 1995b). These studies, however did not examine the effects of *trans*-FA. A recent study in mice showed no effects of dietary trans 18:1 fatty acids on HMG-R gene expression (Cassagno et al., 2005).

Another factor involved in normal lipoprotein profiles and metabolism is Apo A-I, the predominant apolipoprotein associated with HDL cholesterol. Dietary *trans*-FA have the ability to modulate plasma lipids, and may act, in part, by their effects on apolipoproteins. The effects of *trans*-FA on cholesterol production have been examined extensively, but results seem to depend on the model used. In HepG2 cells, *cis*-vaccenic acid (c11) increased Apo A-I mRNA levels as compared to *trans*-vaccenic acid (t11). These changes in gene expression did not correlate with HDL cholesterol production, as none of the FA treatments had any effects. In H-4-II-E cells, treatment with all of the FA decreased Apo A-I mRNA concentration, although there were no differences among the FA studied. As with the human cells, these changes in gene expression did not affect HDL cholesterol production, as concentrations were not different among the treatments. In contrast with our findings, HDL cholesterol was decreased by *trans*-FA in two human

dietary studies (Mensink and Katan, 1990; Tholstrup et al., 2006), although in the second study, the saturated to monounsaturated fat ratio of the diets may have played a significant role. In monkeys, dietary elaidic acid decreased Apo A-I and HDL cholesterol as compared to a saturated fat diet (Khosla et al., 1997). In HepG2 cells, oleic acid had no effects on Apo A-I or HDL cholesterol production (Dashti and Wolfbower, 1987), whereas elaidic acid may increase HDL cholesterol (Dashti et al., 2000). Numerous studies, however, support our findings. In hamsters, dietary oleic acid had no effects on HDL cholesterol production as compared to saturated fats (Kurushima et al., 1995b), LA (Kurushima et al., 1995a; Nicolosi et al., 2004), or *trans*-FA (Nicolosi et al., 1998). When comparing vaccenic and elaidic acids, no effects on HDL cholesterol production were seen (Meijer et al., 2001). Serum and liver cholesterol concentrations were not different in Wistar rats fed diets high in *cis*-FA, *trans*-FA or saturated FA (Colandre et al, 2003). A recent study in mice fed 3% of dietary energy as *trans* 18:1 FA showed no changes in total or HDL cholesterol (Cassagno et al., 2005). In two human studies, HDL cholesterol was unaffected by diets rich in *trans*-FA as compared with those high in *cis*-FA (Judd et al., 1994; Lovejoy et al., 2002).

As FA and their derivatives have been identified as potential ligands for PPARs, we investigated the possibility that FA effects in the two cell lines may be mediated by PPARα. In HepG2 cells, incubation with WY 14,643, a selective PPARα agonist, increased basal expression of HMG-R mRNA and enhanced c11-induced Apo A-I mRNA expression. Activation of PPARα had no effects on basal or c11-induced ACO gene expression. Inhibition of PPARα by MK886 had no effects on the three genes studied. In contrast with the effects seen in HepG2 cells, activation of PPARα enhanced

the effects of t11 on ACO, HMG-R, and Apo A-I gene expression in the H-4-II-E cells. Inhibition of PPARα decreased the basal levels and attenuated the effects of *trans*-vaccenic acid on ACO gene expression. 3-hydroxy, 3-methylglutaryl CoA reductase and Apo A-I mRNA concentrations were unaffected by inhibition of PPARα. Although ACO is an established PPARα responsive gene (Tugwood, et al., 1992), species differences do exist. It is questionable whether the PPAR response element of human ACO is active (Woodyatt et al., 1999). Dietary studies have shown that rodents are responsive to the effects of PPARα activation, but non-rodent species, such as primates and guinea pigs, are resistant or unresponsive to some of the negative effects (Bentley et al., 1993; Cattley et al., 1998). In a comprehensive analysis of gene expression in human and rat hepatoma cells by microarray analysis, only rat ACO mRNA was responsive to WY 14,643 (Vanden Heuvel et al., 2003). Other genes that may be differentially regulated in human and rat liver include cytosolic aspartate aminotransferase (Tomkiewicz et al., 2004), peroxisomal 3-oxoacyl-CoA thiolase (Lawrence et al., 2001), and catalase (Ammerschlaeger et al., 2004). Additionally, different PPARα agonists may regulate lipid metabolism in a compound-dependent manner. A recent study by Duez and coworkers (2005) showed that, in mice, fenofibrate and gemfibrozil, both stimulate ACO mRNA expression, but only fenofibrate greatly induces Apo A-I gene expression.

Summary

In HepG2 cells, only treatment with the *cis*-11 fatty acid up-regulated ACO and Apo A-I mRNA expression. 3-hydroxy, 3-methylglutaryl CoA reductase steady-state mRNA concentrations were unaffected by treatment with any *cis* or *trans* MUFAs. High-density lipoprotein cholesterol production was unchanged by any FA studied. Activation of PPARα increased basal concentrations of HMG-R mRNA and enhanced

c11-induced Apo A-I gene expression, but no effects of PPARα inhibition were seen on any gene studied in HepG2 cells. These results are consistent with the low levels of endogenous PPARα expression in this cell line (Hsu et al., 2001).

In H-4-II-E cells, incubation with *cis* and *trans* FA increased ACO mRNA and decreased Apo A-I mRNA levels, but there were no differences among FA effects on either gene. The *trans*-11 isomer increased HMG-R mRNA expression as compared to the *cis*-11 isomer. None of the FA studied effected HDL cholesterol production by H-4-II-E cells. Selective activation of PPARα in H-4-II-E cells enhanced t11-induced expression of ACO, HMG-R and Apo A-I gene transcripts. Inhibition of PPARα decreased basal expression and attenuated t11-induced ACO mRNA concentrations. However, no effects were seen on HMG-R or Apo A-I mRNA. These results indicate that t11-induced ACO gene expression may be mediated by PPARα in the H-4-II-E cells, whereas effects on HMG-R and Apo A-I genes may be independent of PPARα. As responses to FA and PPARα activation and inhibition were different in the human and rat cells lines, net effects are likely species specific.

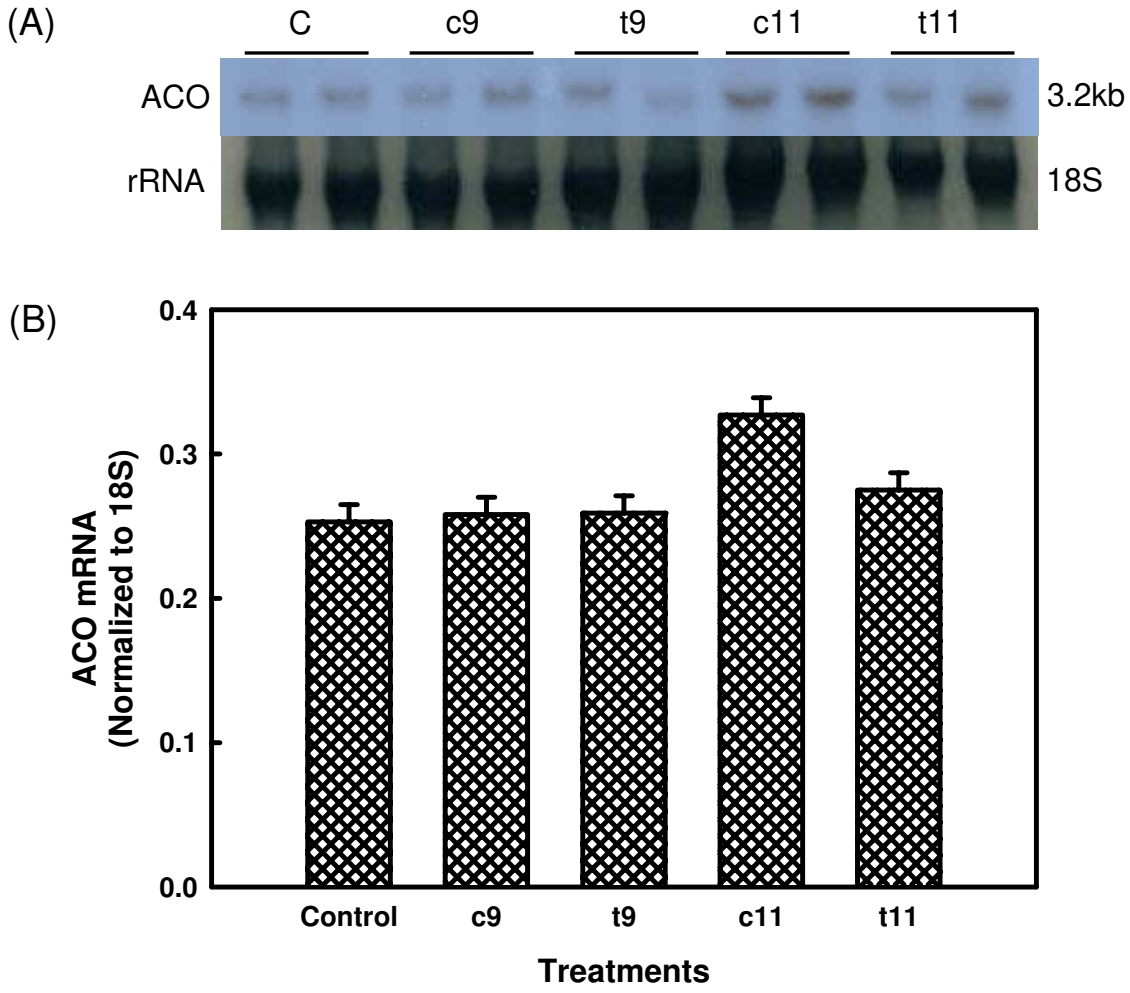

Figure 5-1. Effect of *cis* and *trans* isomers of octadecenoic acid on ACO mRNA expression in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (c9 + t9 + c11 + t11), P = 0.06; Contrast 2: (c9 + t9) vs. (c11 + t11), P = 0.005; Contrast 3: c9 vs. t9, P = 0.98; Contrast 4: c11 vs. t11, P = 0.01.

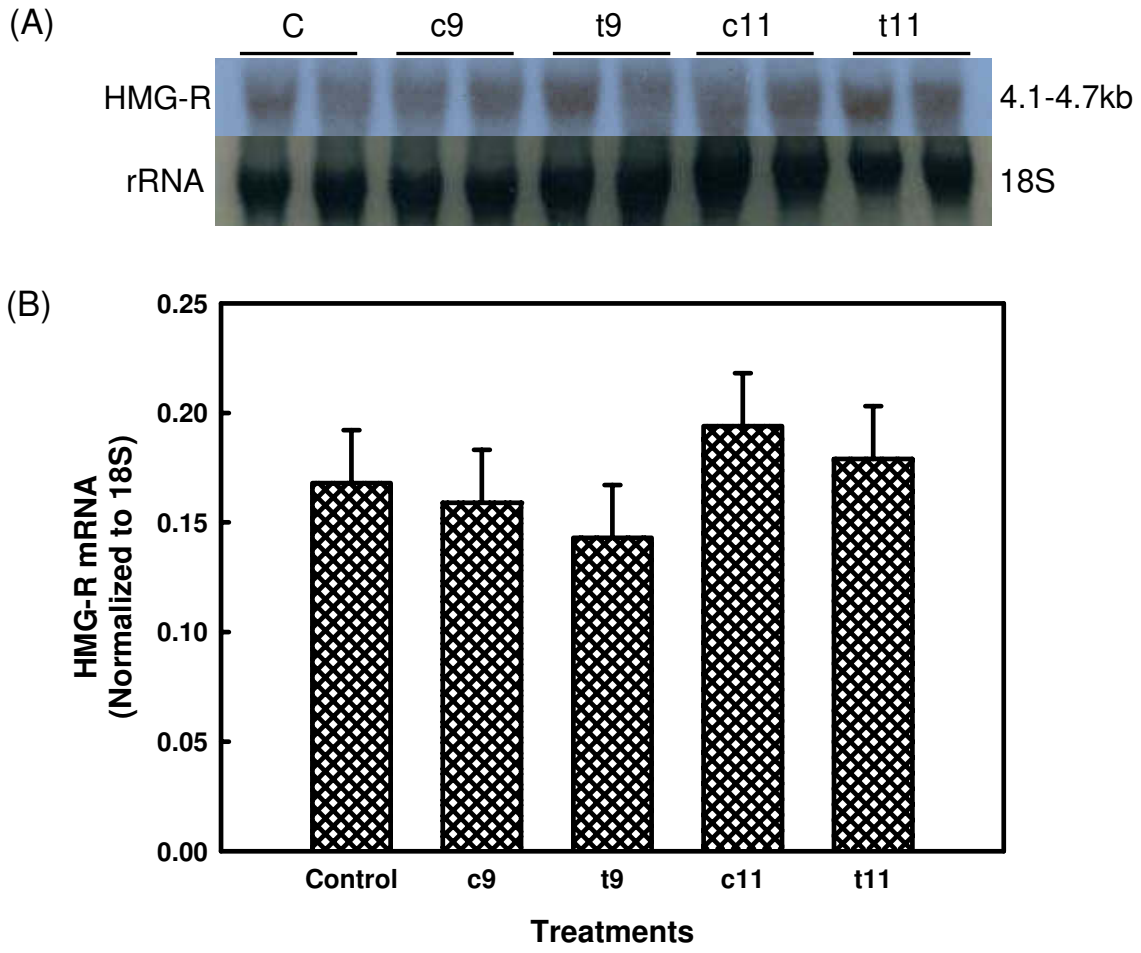

Figure 5-2. Effect of *cis* and *trans* isomers of octadecenoic acid on HMG-R mRNA expression in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (c9 + t9 + c11 + t11), P = 0.99; Contrast 2: (c9 + t9) vs. (c11 + t11), P = 0.2; Contrast 3: c9 vs. t9, P = 0.7; Contrast 4: c11 vs. t11, P = 0.7.

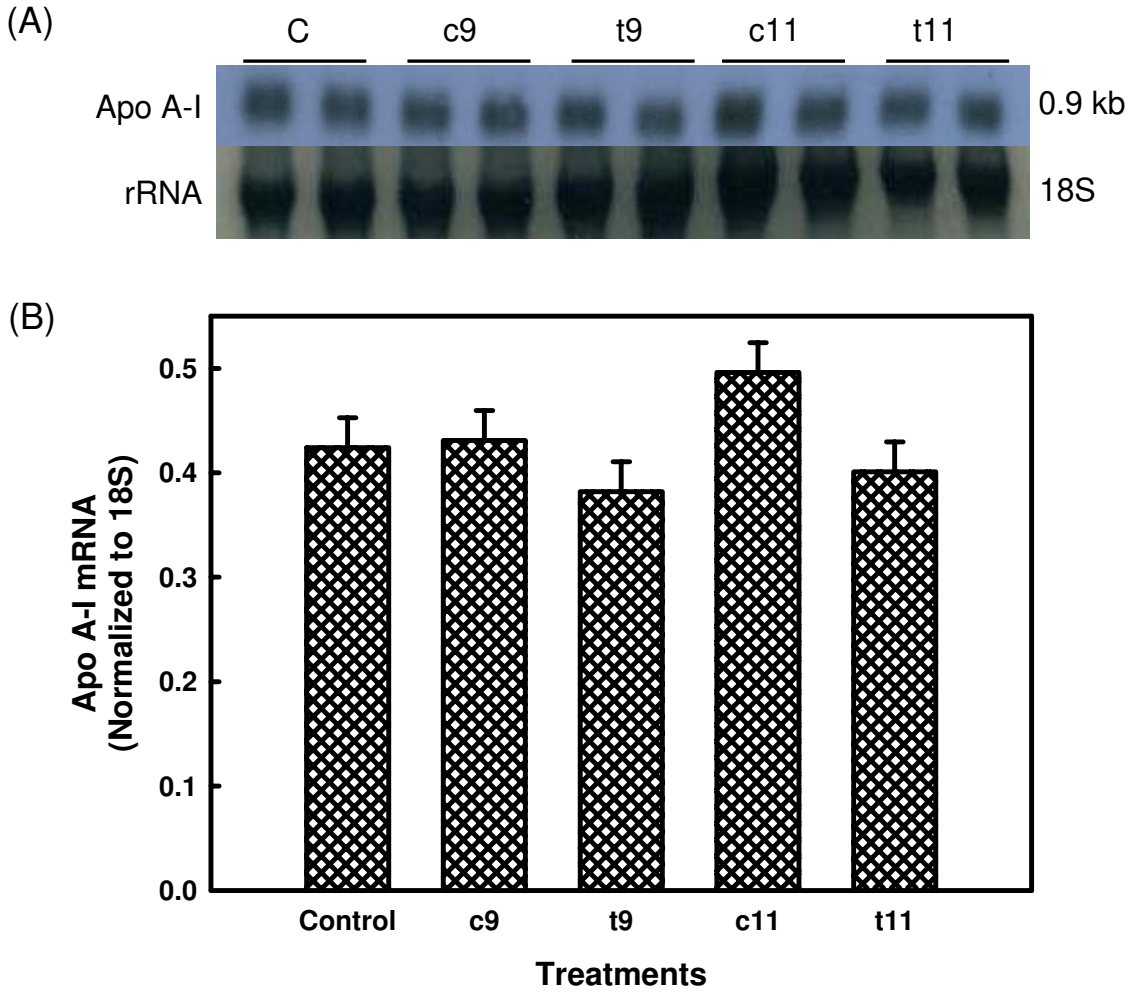

Figure 5-3. Effect of *cis* and *trans* isomers of octadecenoic acid on Apo A-I mRNA expression in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (c9 + t9 + c11 + t11), P = 0.9; Contrast 2: (c9 + t9) vs. (c11 + t11), P = 0.2; Contrast 3: c9 vs. t9, P = 0.3; Contrast 4: c11 vs. t11, P = 0.04.

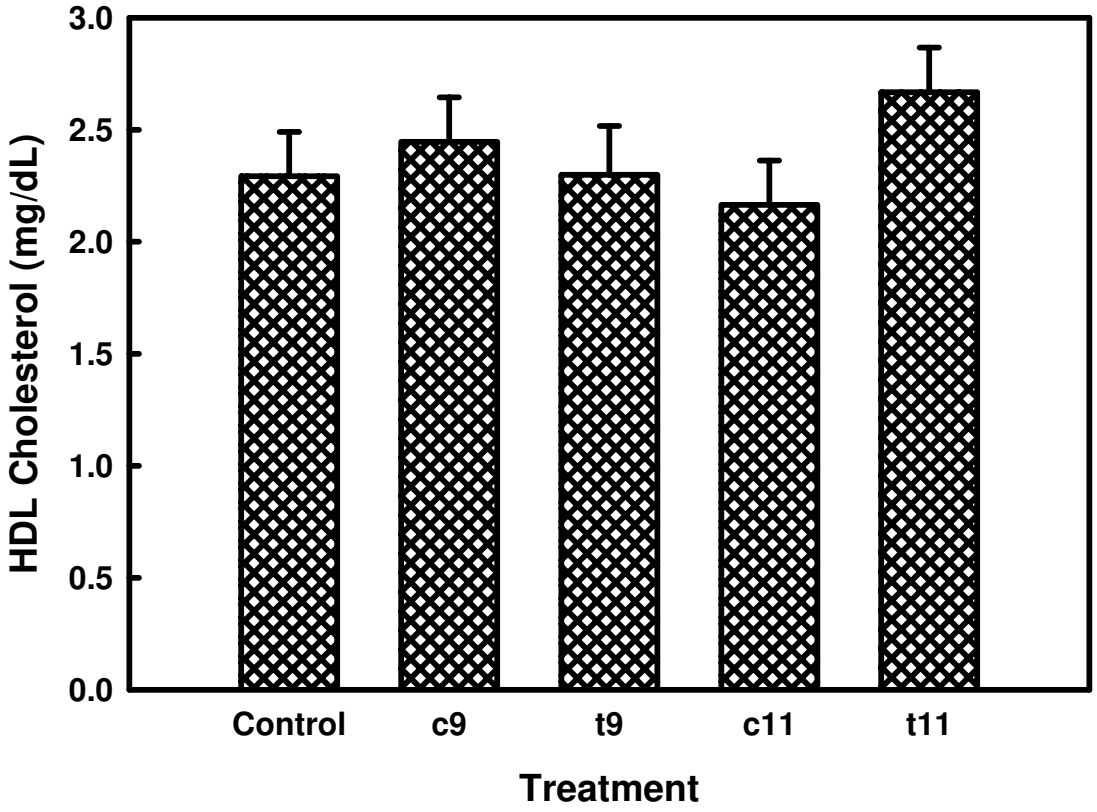

Figure 5-4. Effects of *cis* and *trans* isomers of octadecenoic acid on HDL cholesterol production by HepG2 cells. Data represents least square means ± SEM calculated over two experiments. To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (c9 + t9 + c11 + t11), P = 0.7; Contrast 2: (c9 + t9) vs. (c11 + t11), P = 0.8; Contrast 3: c9 vs. t9, P = 0.6; Contrast 4: c11 vs. t11, P = 0.09.

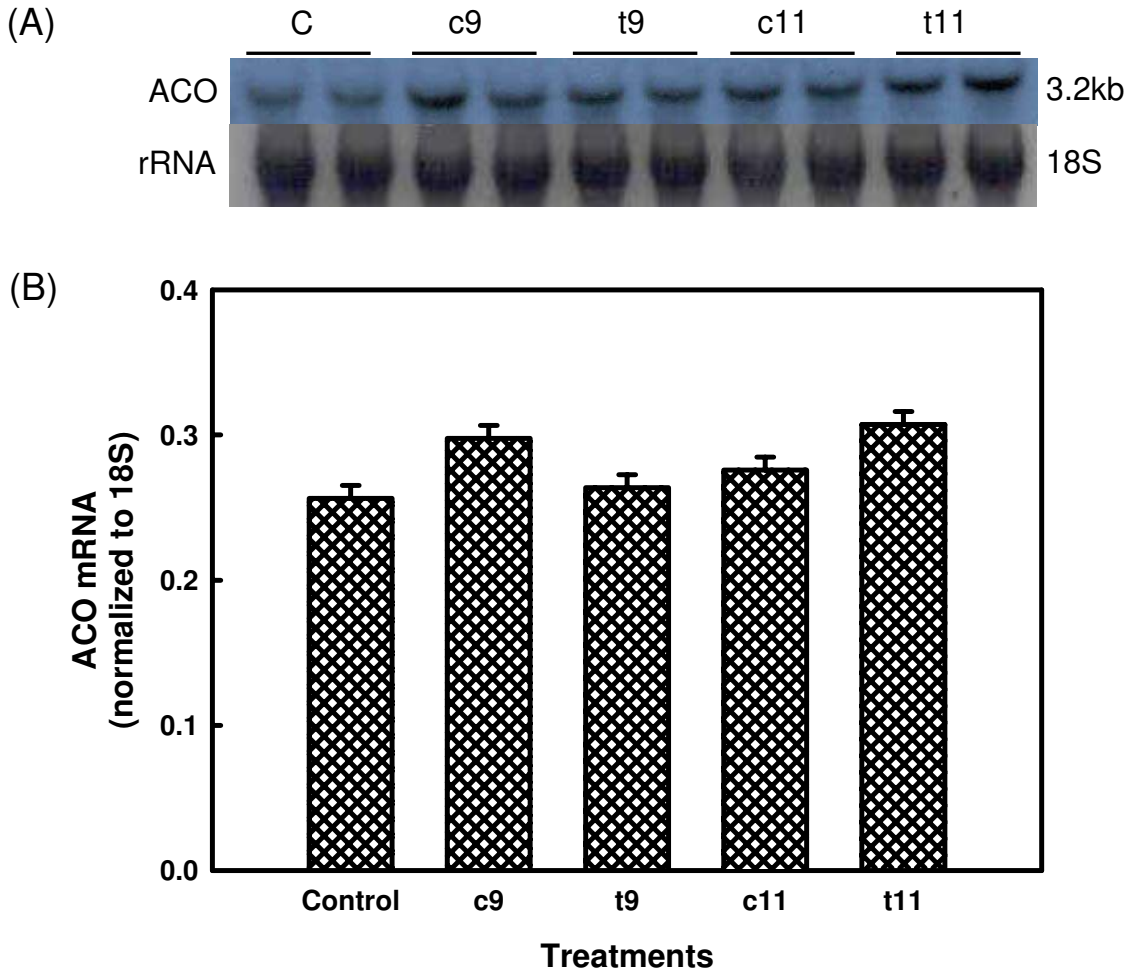

Figure 5-5. Effect of *cis* and *trans* isomers of octadecenoic acid on ACO mRNA expression in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (c9 + t9 + c11 + t11), P = 0.01; Contrast 2: (c9 + t9) vs. (c11 + t11), P = 0.3; Contrast 3: c9 vs. t9, P = 0.02; Contrast 4: c11 vs. t11, P = 0.03.

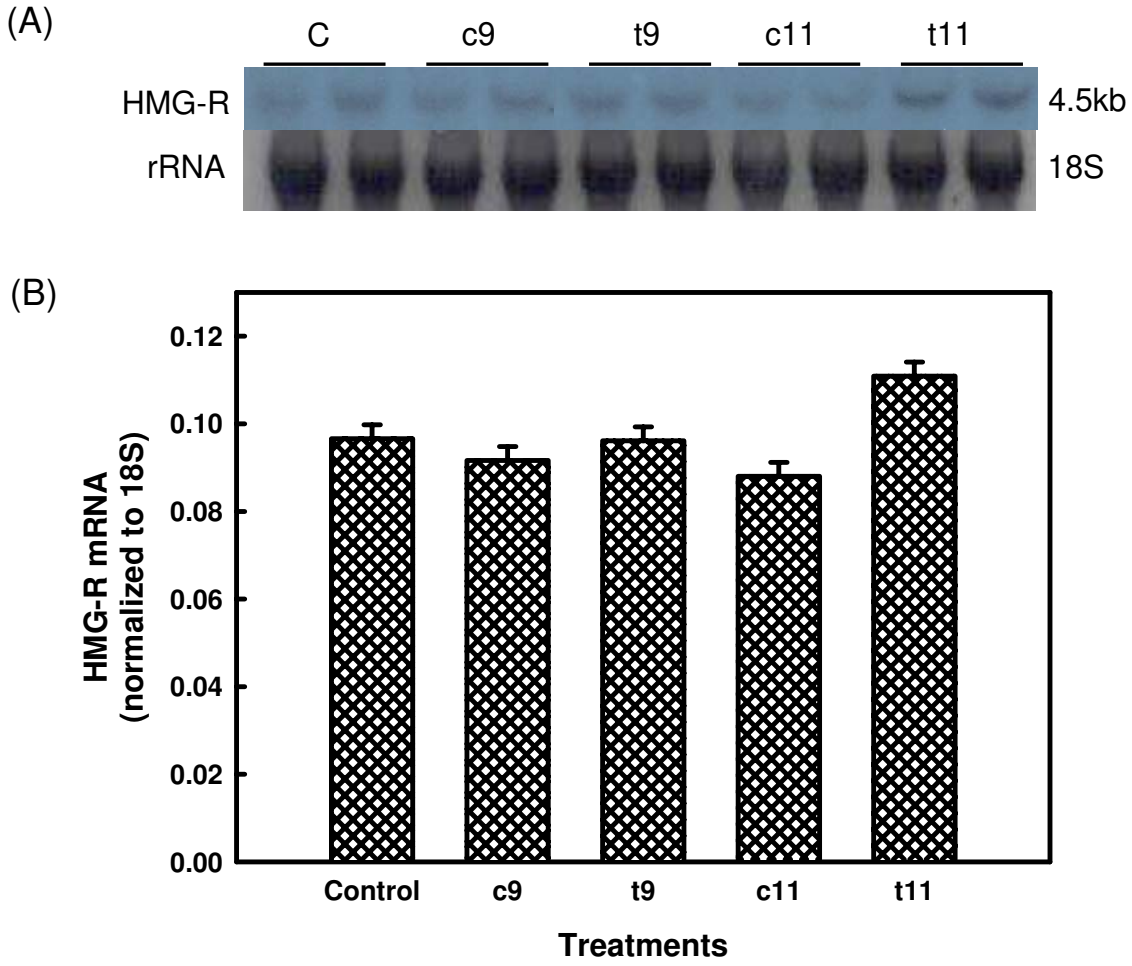

Figure 5-6. Effect of *cis* and *trans* isomers of octadecenoic acid on HMG-R mRNA expression in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (c9 + t9 + c11 + t11), P = 0.99; Contrast 2: (c9 + t9) vs. (c11 + t11), P = 0.1; Contrast 3: c9 vs. t9, P = 0.4; Contrast 4: c11 vs. t11, P = 0.005.

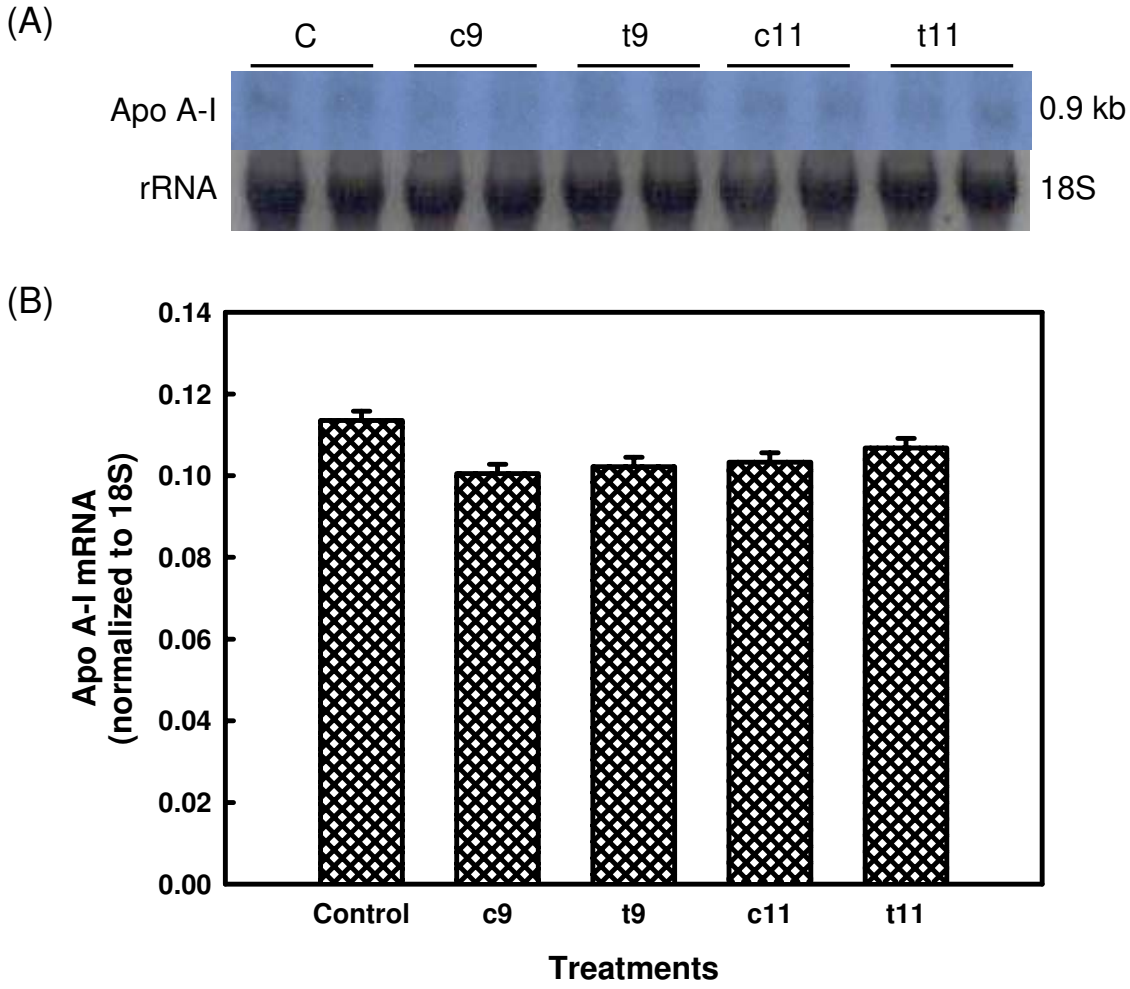

Figure 5-7. Effect of *cis* and *trans* isomers of octadecenoic acid on Apo A-I mRNA expression in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (c9 + t9 + c11 + t11), P = 0.002; Contrast 2: (c9 + t9) vs. (c11 + t11), P = 0.1; Contrast 3: c9 vs. t9, P = 0.6; Contrast 4: c11 vs. t11, P = 0.3.

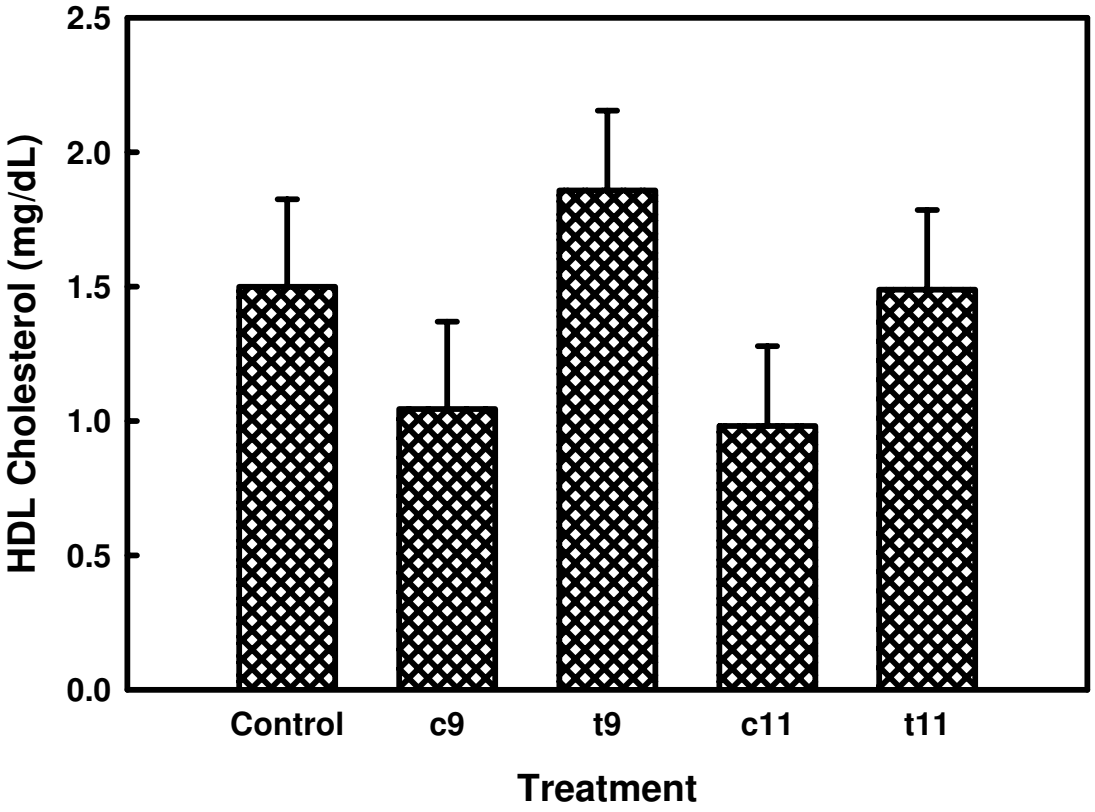

Figure 5-8. Effects of *cis* and *trans* isomers of octadecenoic acid on HDL cholesterol production in H-4-II-E cells. Data represents least square means ± SEM calculated over two experiments. To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: Control vs. (c9 + t9 + c11 + t11), P = 0.7; Contrast 2: (c9 + t9) vs. (c11 + t11), P = 0.5; Contrast 3: c9 vs. t9, P = 0.09; Contrast 4: c11 vs. t11, P = 0.2.

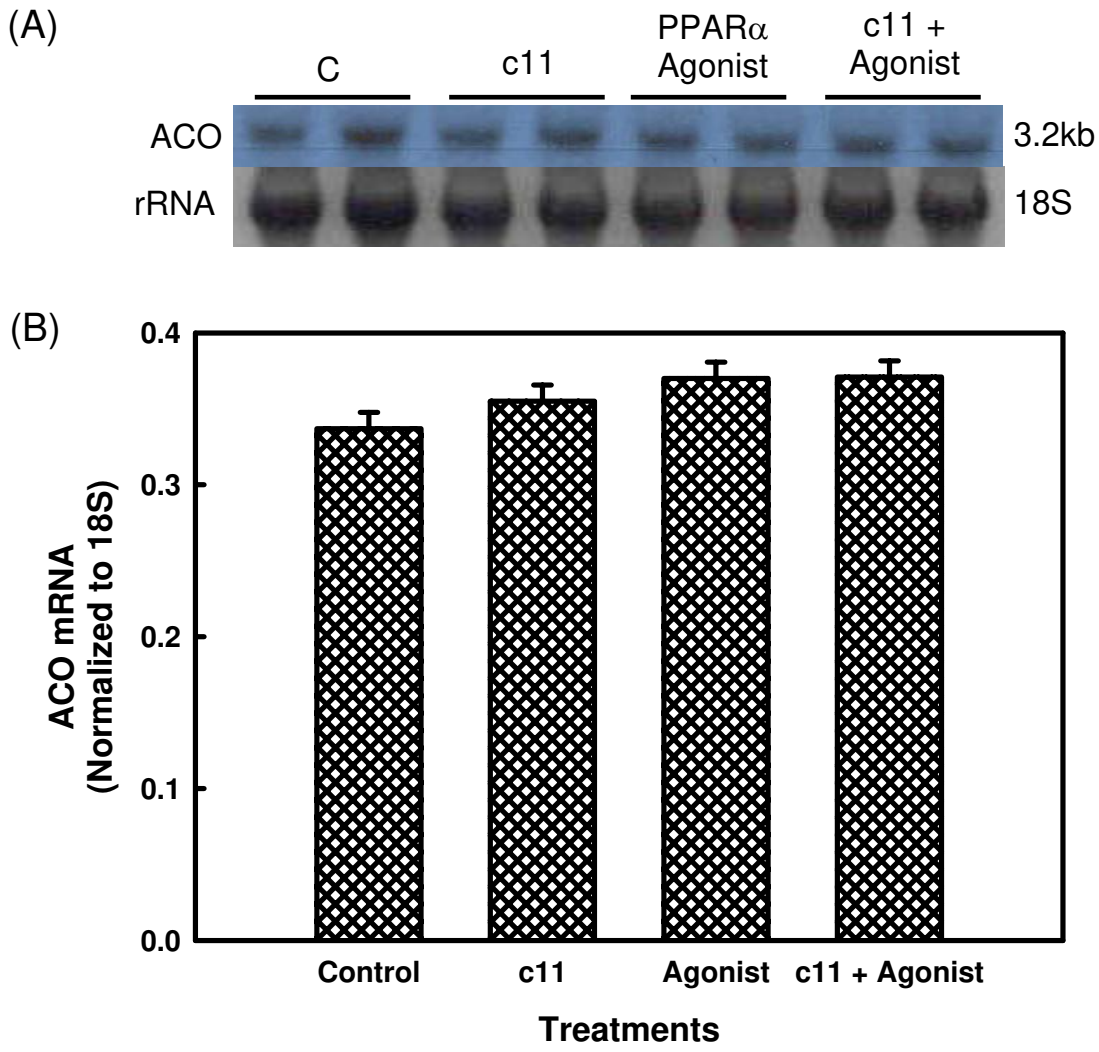

Figure 5-9. Effect of WY 14,643 on ACO mRNA response to *cis*-vaccenic acid in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (c11 + c11+A), P = 0.4; Contrast 2: c11 vs. c11+A, P = 0.3; Contrast 3: Control vs. Agonist, P = 0.06.

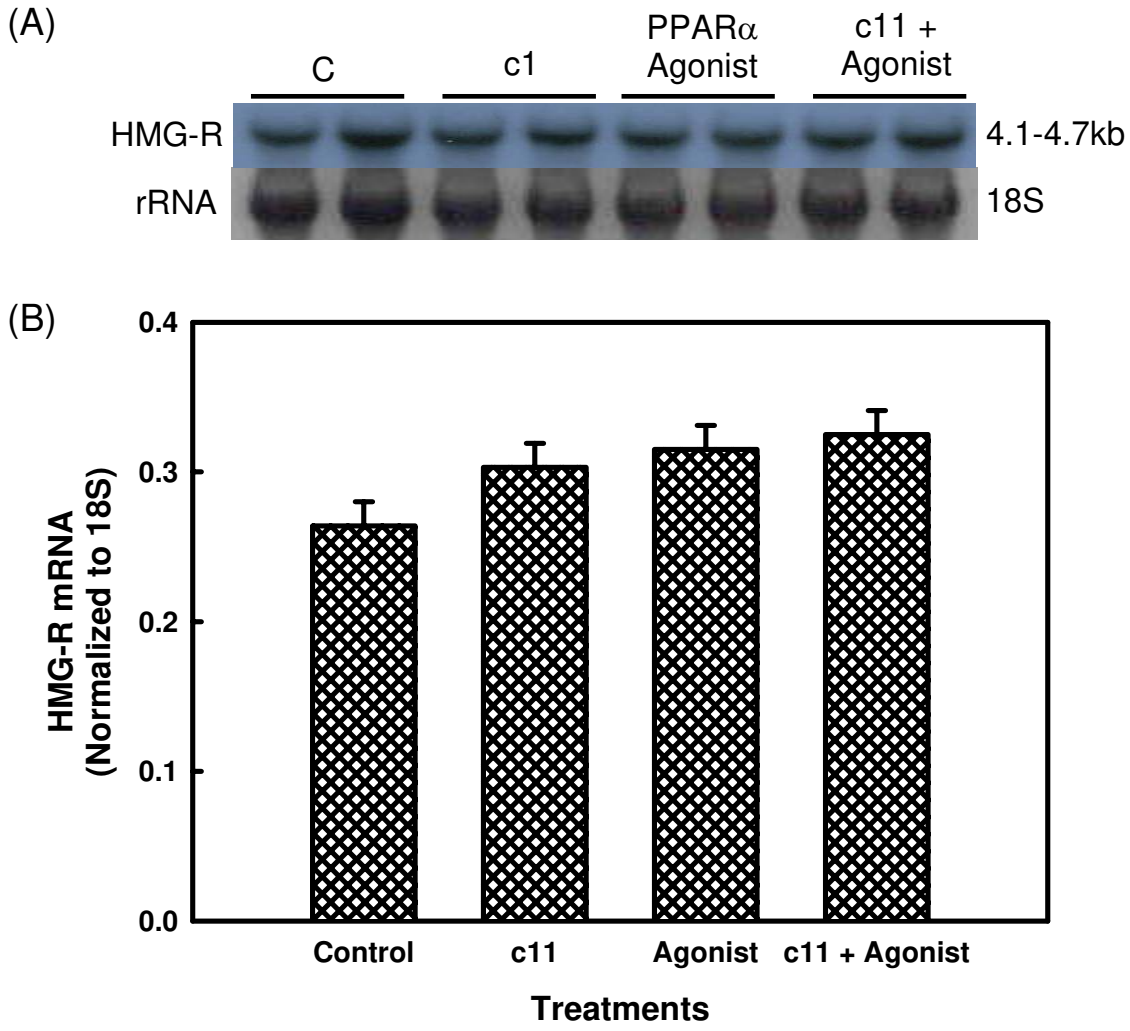

Figure 5-10. Effect of WY 14,643 on HMG-R mRNA response to *cis*-vaccenic acid in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (c11 + c11+A), P = 0.2; Contrast 2: c11 vs. c11+A, P = 0.4; Contrast 3: Control vs. Agonist, P = 0.05.

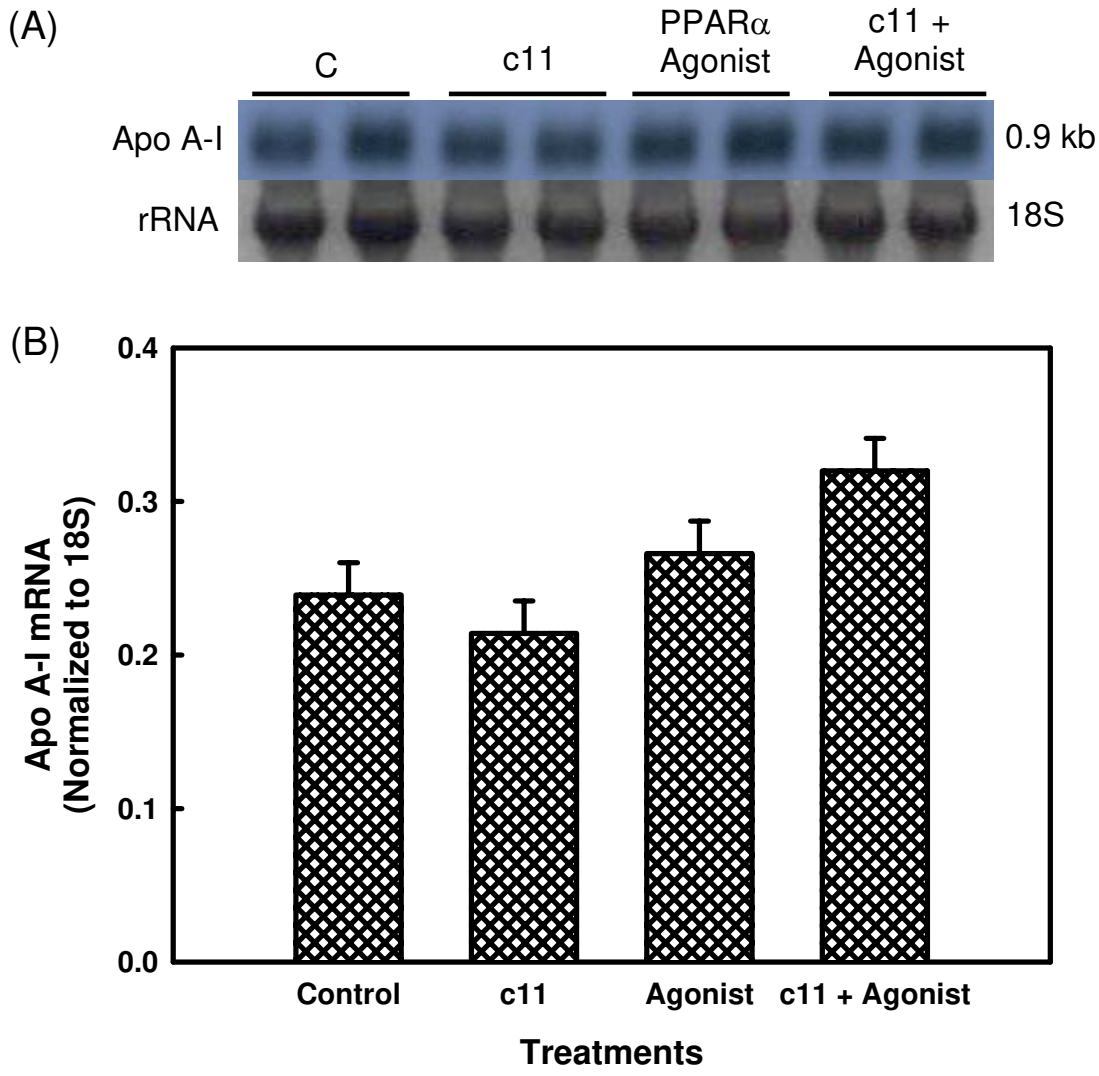

Figure 5-11. Effect of WY 14,643 on Apo A-I mRNA response to *cis*-vaccenic acid in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (c11 + c11+A), P = 0.5; Contrast 2: c11 vs. c11+A, P = 0.007; Contrast 3: Control vs. Agonist, P = 0.4.

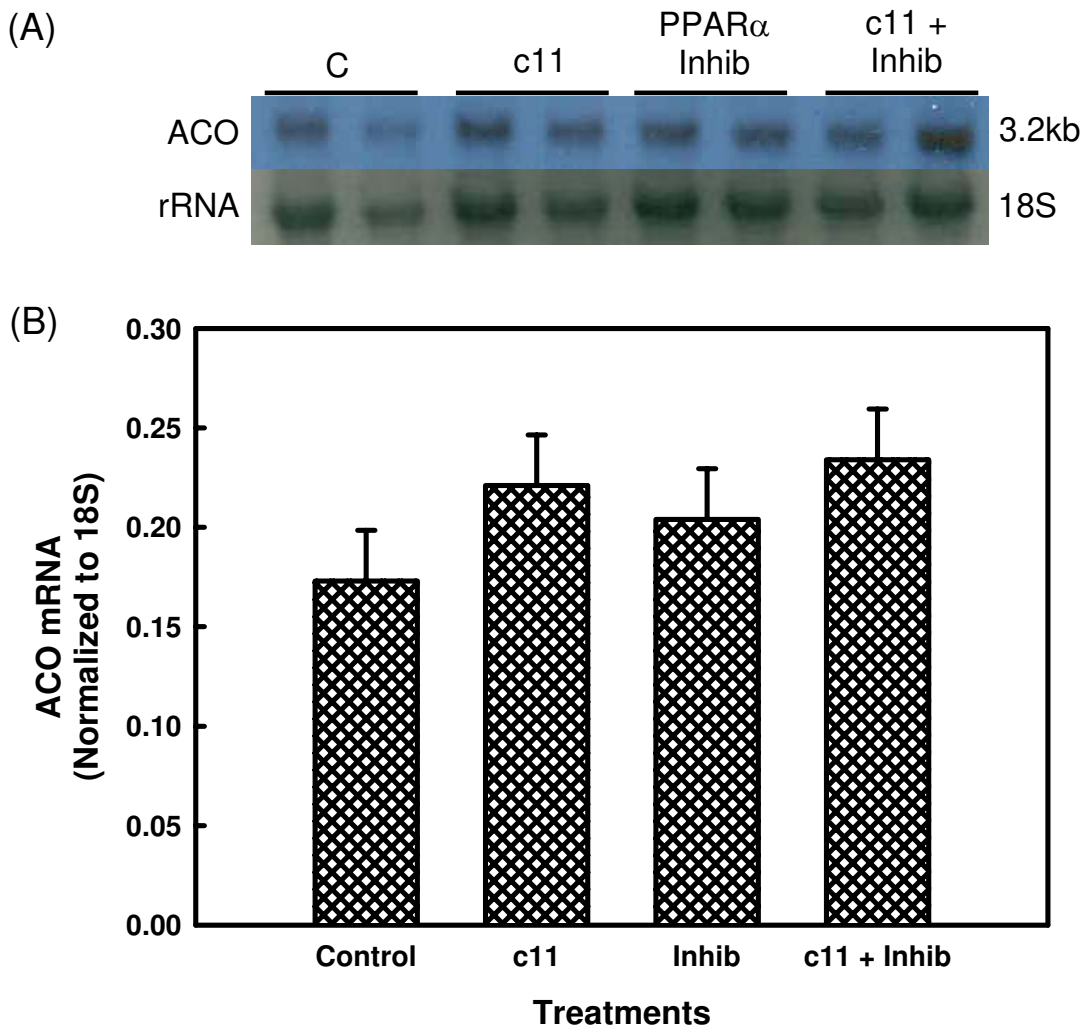

Figure 5-12. Effect of MK886 on ACO mRNA response to *cis*-vaccenic acid in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). Contrast 1: (Control + Inhib) vs. (c11 + c11+I), P = 0.2; Contrast 2: c11 vs. c11+I, P = 0.7; Contrast 3: Control vs. Inhib, P = 0.4.

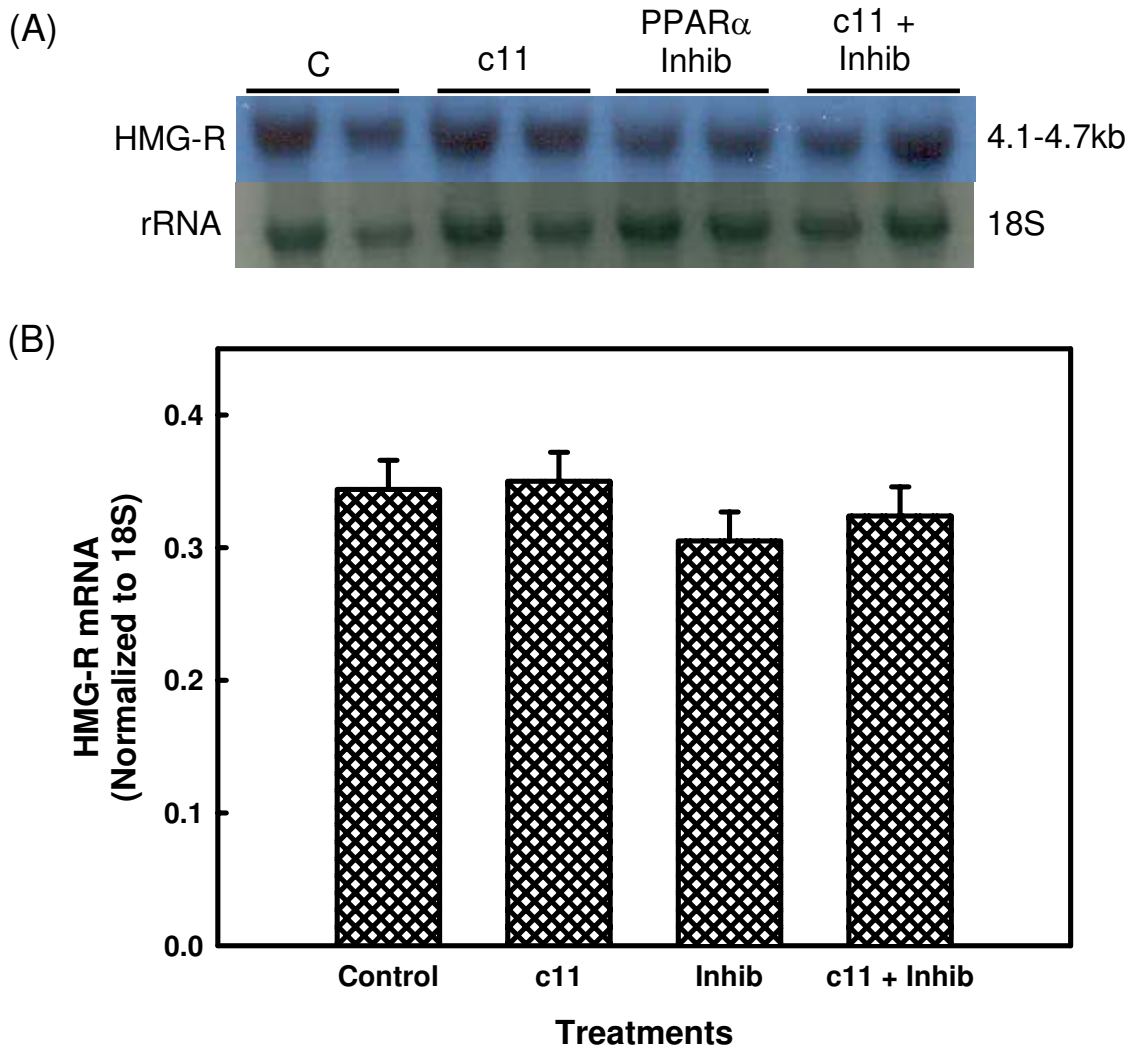

Figure 5-13. Effect of MK886 on HMG-R mRNA response to *cis*-vaccenic acid in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). Contrast 1: (Control + Inhib) vs. (c11 + c11+I), P = 0.6; Contrast 2: c11 vs. c11+I, P = 0.4; Contrast 3: Control vs. Inhib, P = 0.2.

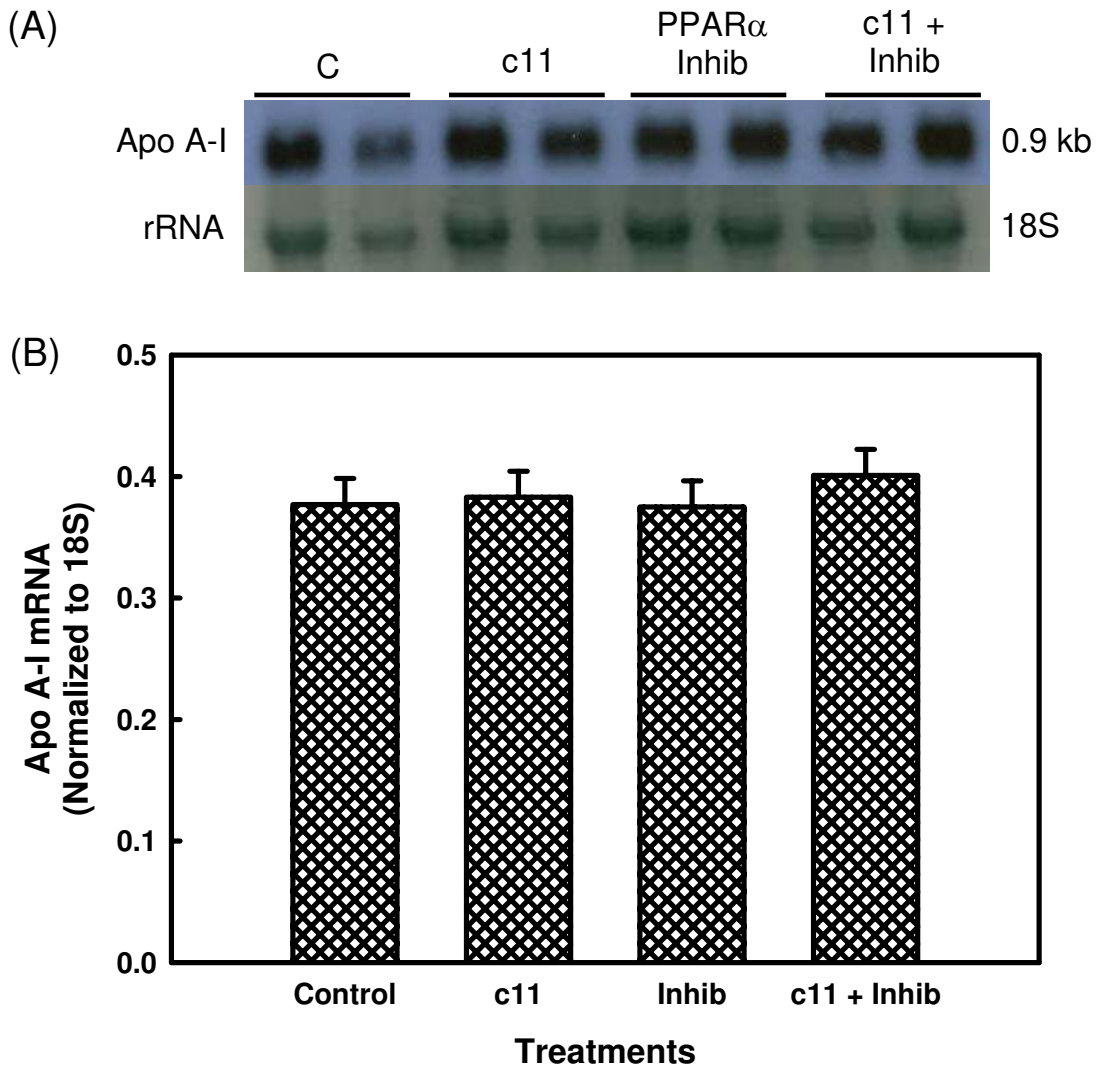

Figure 5-14. Effect of MK886 on Apo A-I mRNA response to *cis*-vaccenic acid in HepG2 cells. Ten micrograms of total cellular RNA isolated from control and treated HepG2 cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). Contrast 1: (Control + Inhib) vs. (c11 + c11+I), P = 0.5; Contrast 2: c11 vs. c11+I, P = 0.6; Contrast 3: Control vs. Inhib, P = 0.9.

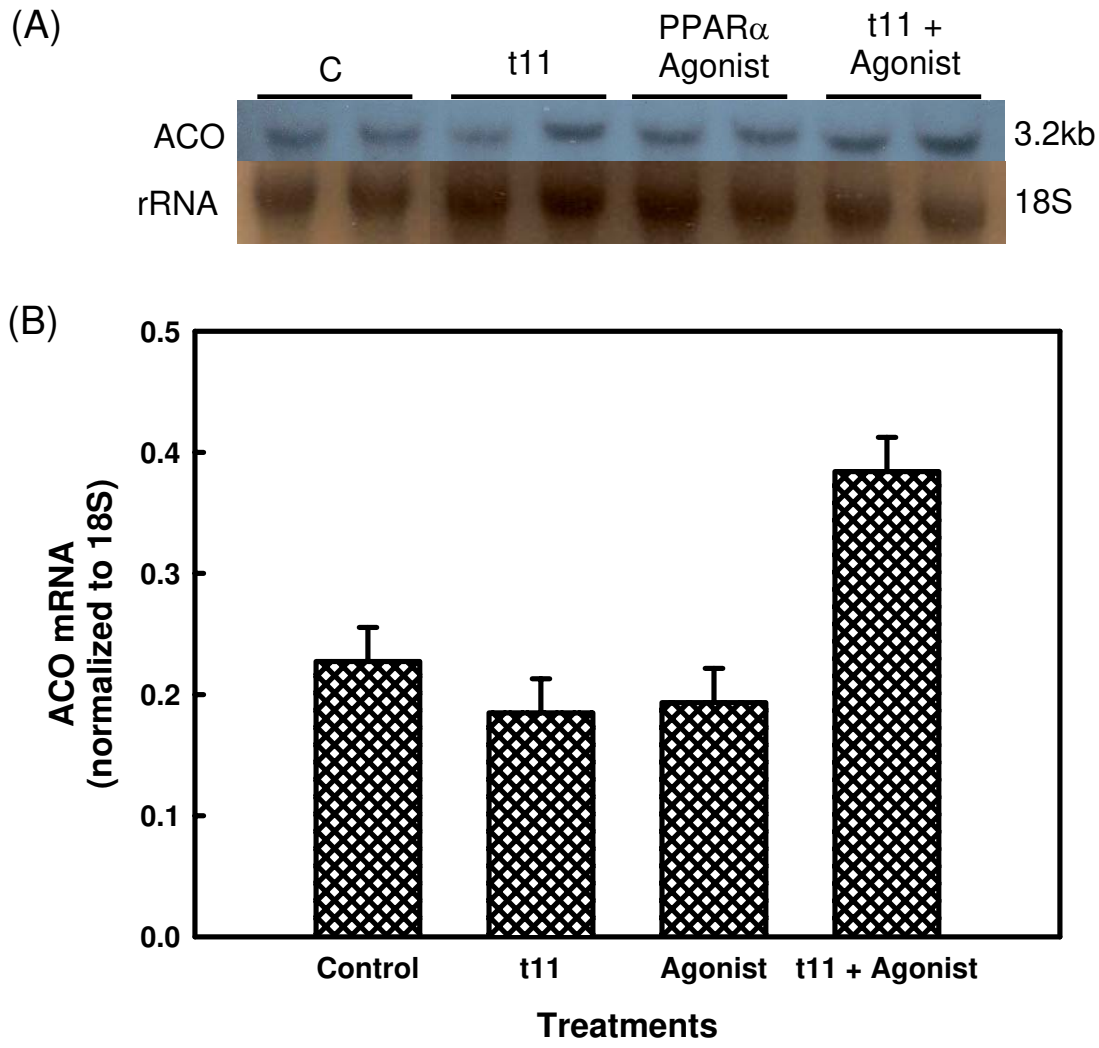

Figure 5-15. Effect of WY 14,643 on ACO mRNA response to *trans*-vaccenic acid in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (t11 + t11+A), P = 0.03; Contrast 2: t11 vs. t11+A, P = 0.001; Contrast 3: Control vs. Agonist, P = 0.4.

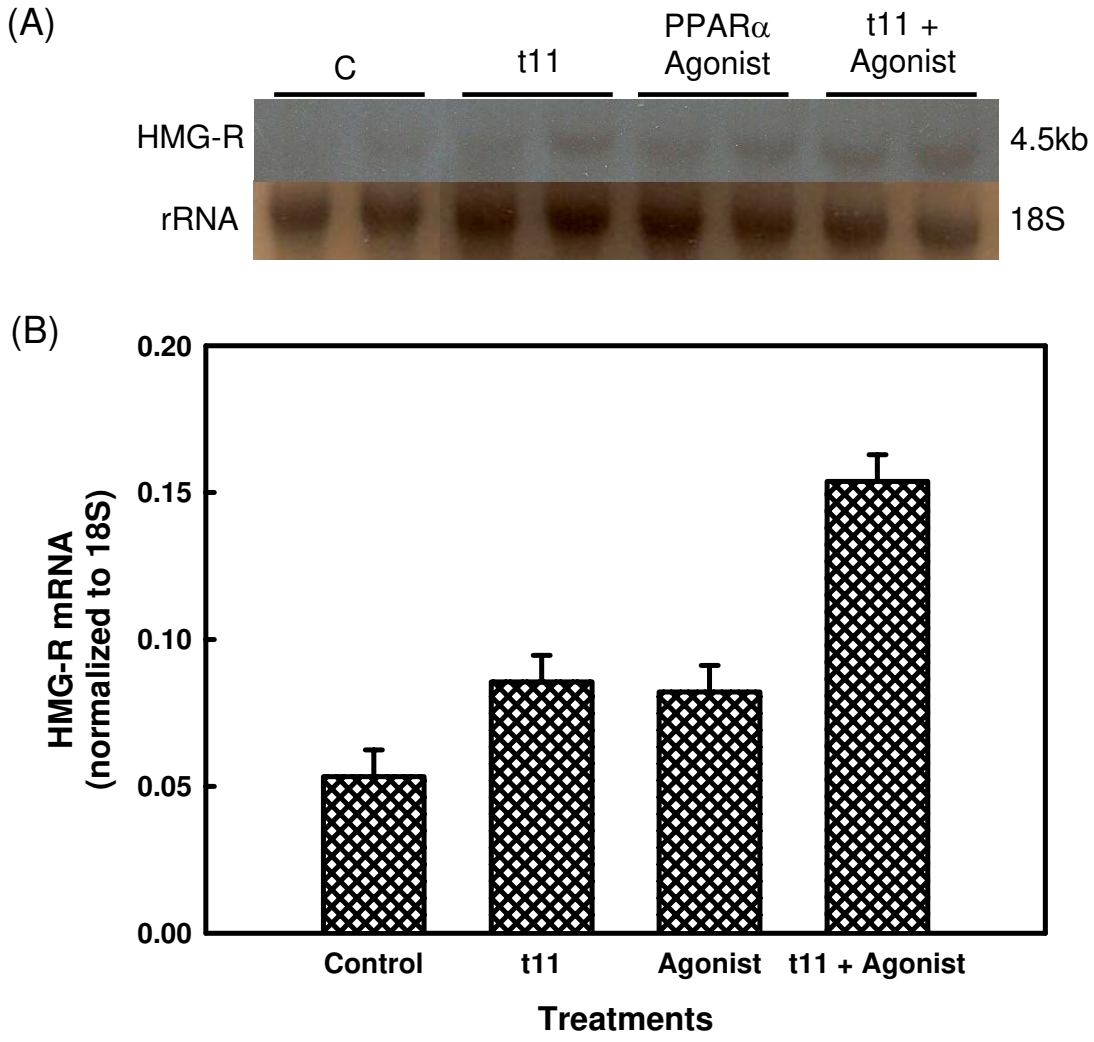

Figure 5-16. Effect of WY 14,643 on HMG-R mRNA response to *trans*-vaccenic acid in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (t11 + t11+A), P = 0.0005; Contrast 2: t11 vs. t11+A, P = 0.0007; Contrast 3: Control vs. Agonist, P = 0.06.

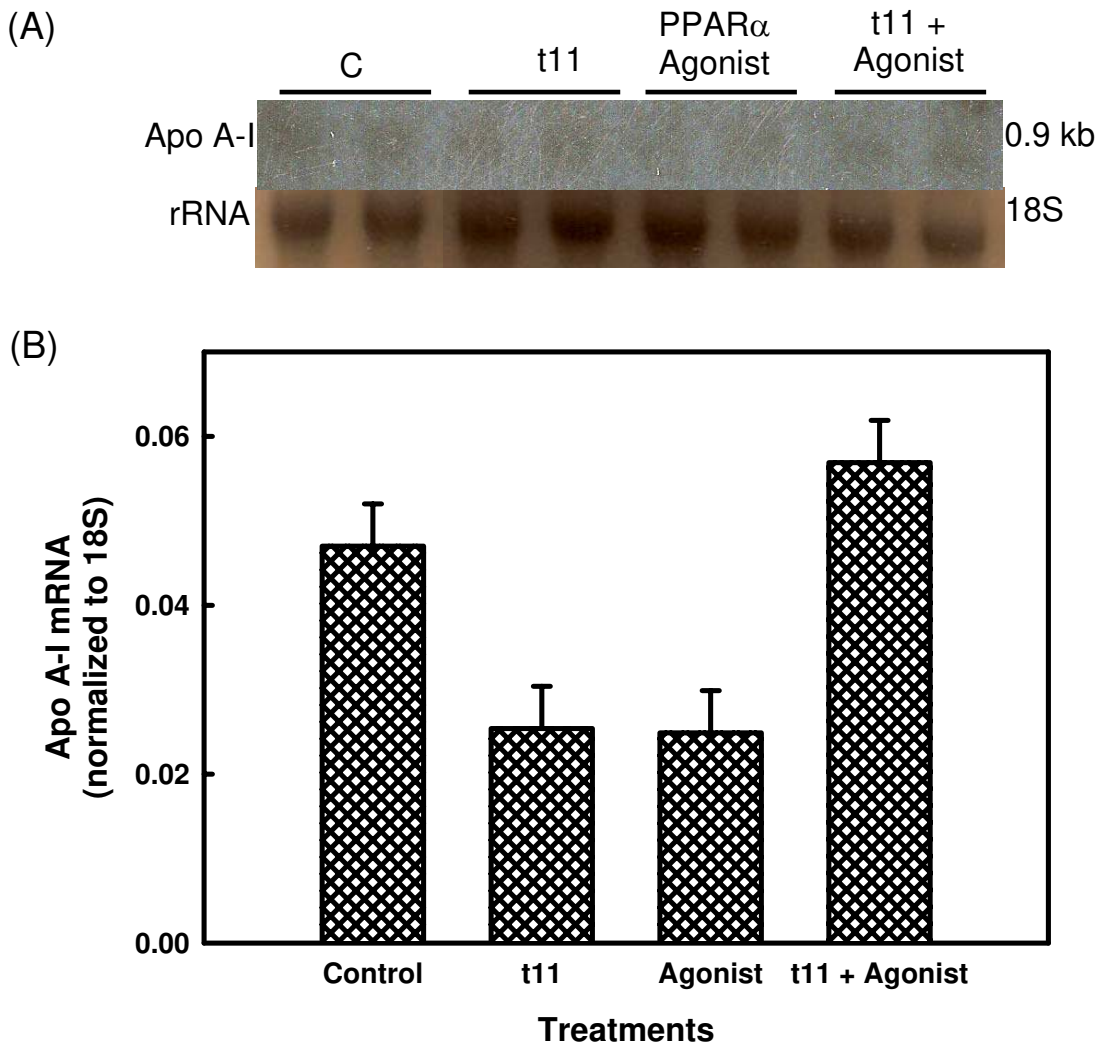

Figure 5-17. Effect of WY 14,643 on Apo A-I mRNA response to *trans*-vaccenic acid in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Agonist) vs. (t11 + t11+A), P = 0.3; Contrast 2: t11 vs. t11+A, P = 0.002; Contrast 3: Control vs. Agonist, P = 0.01.

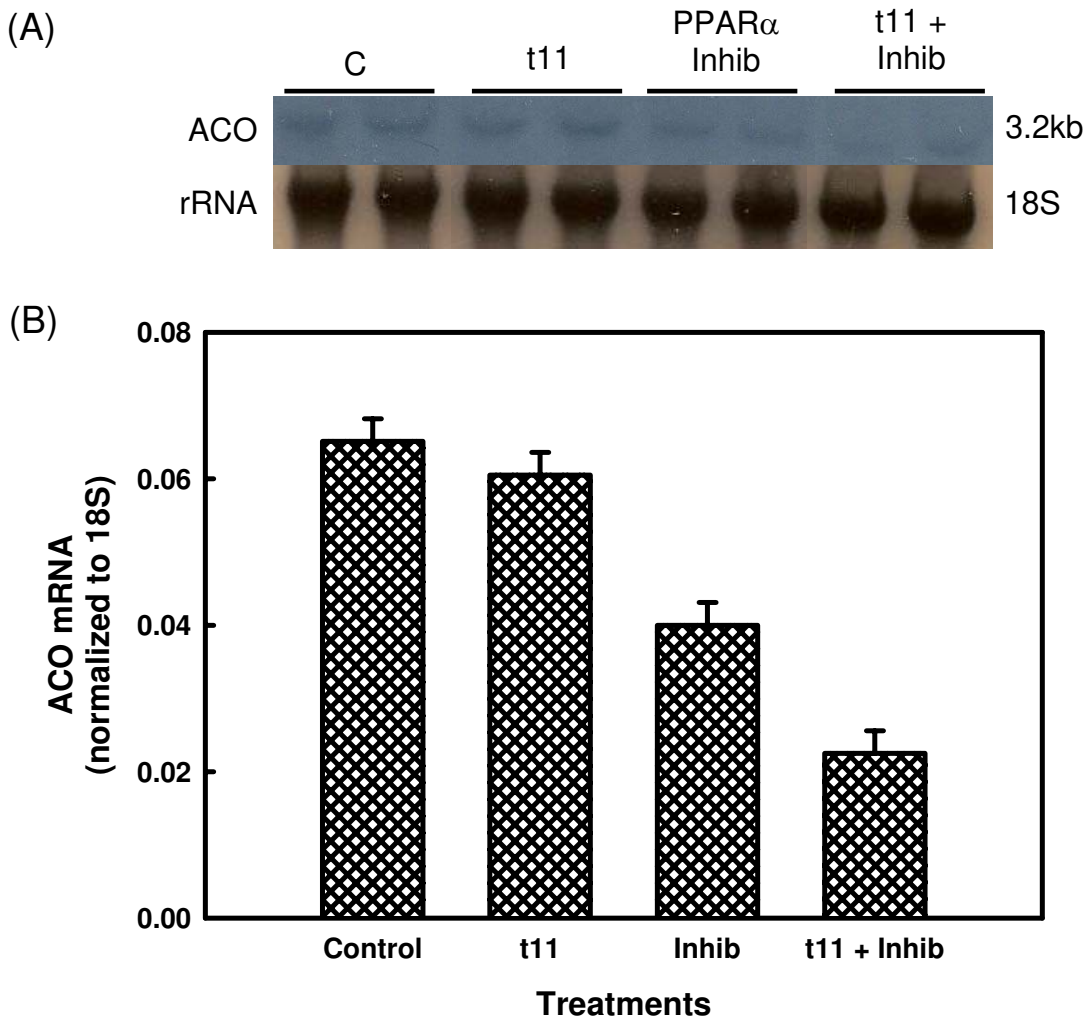

Figure 5-18. Effect of MK886 on ACO mRNA response to *trans*-vaccenic acid in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Inhib) vs. (t11 + t11+I), $P = 0.004$; Contrast 2: t11 vs. t11+I, $P < 0.0001$; Contrast 3: Control vs. Inhib, $P = 0.0007$.

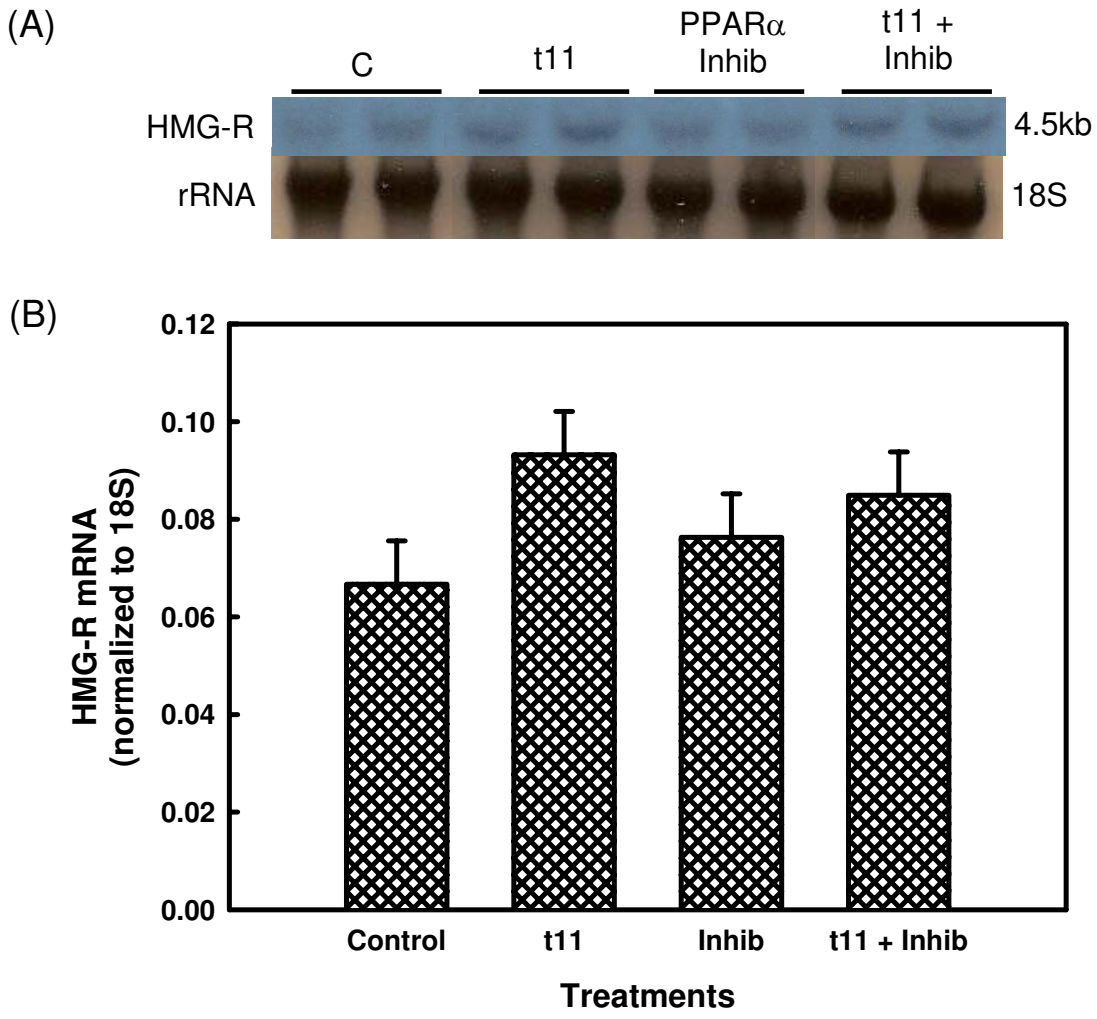

Figure 5-19. Effect of MK886 on HMG-R mRNA response to *trans*-vaccenic acid in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Inhib) vs. (t11 + t11+I), P = 0.08; Contrast 2: t11 vs. t11+I, P = 0.5; Contrast 3: Control vs. Inhib, P = 0.5.

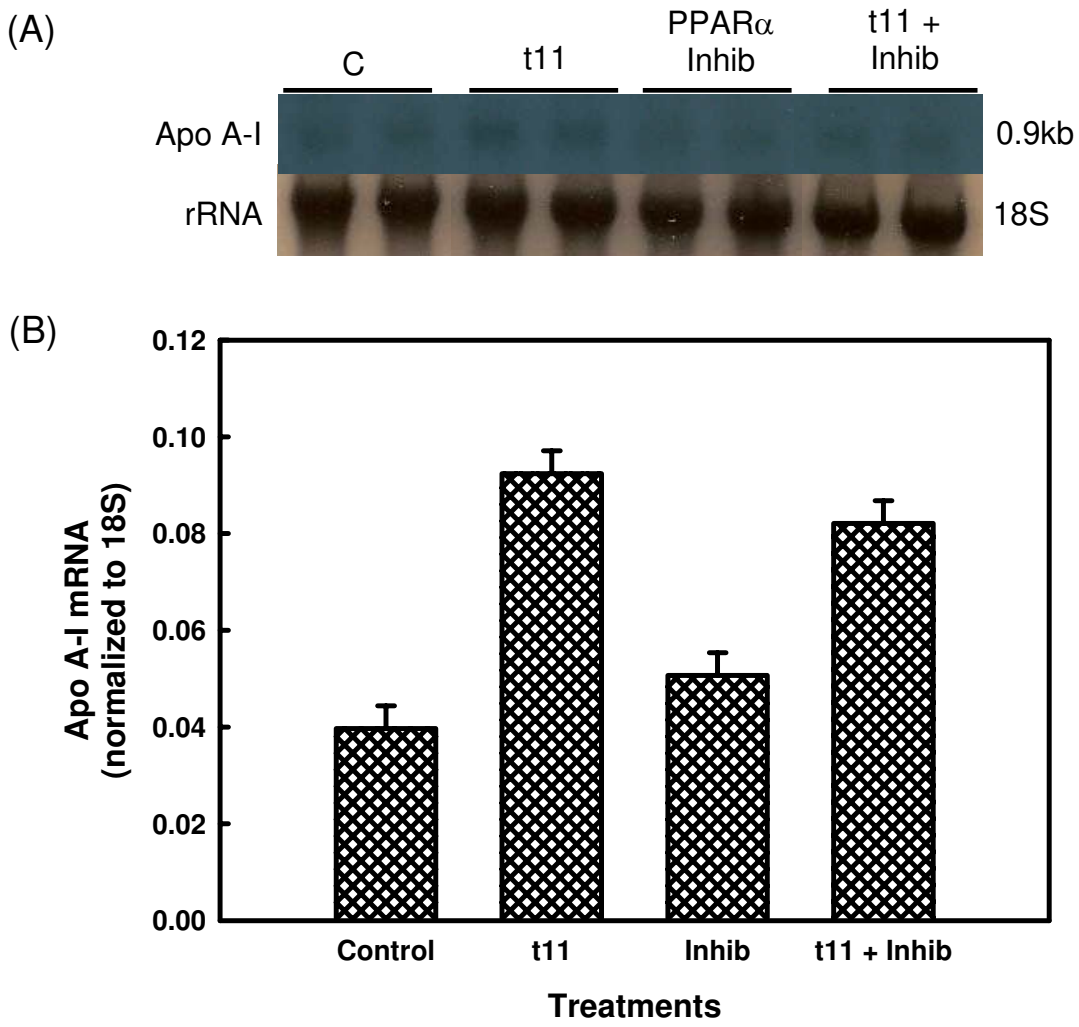

Figure 5-20. Effect of MK886 on Apo A-I mRNA response to *trans*-vaccenic acid in H-4-II-E cells. Ten micrograms of total cellular RNA isolated from control and treated H-4-II-E cells were subjected to Northern blot analysis, and resulting densitometric values were analyzed by the GLM procedure of SAS. A) A representative Northern blot. B) Means ± SEM calculated over two experiments (n = 4 for each treatment). To further examine treatment effects, means were separated using orthogonal contrasts. Contrast 1: (Control + Inhib) vs. (t11 + t11+I), $P < 0.0001$; Contrast 2: t11 vs. t11+I, $P = 0.2$; Contrast 3: Control vs. Inhib, $P = 0.1$.

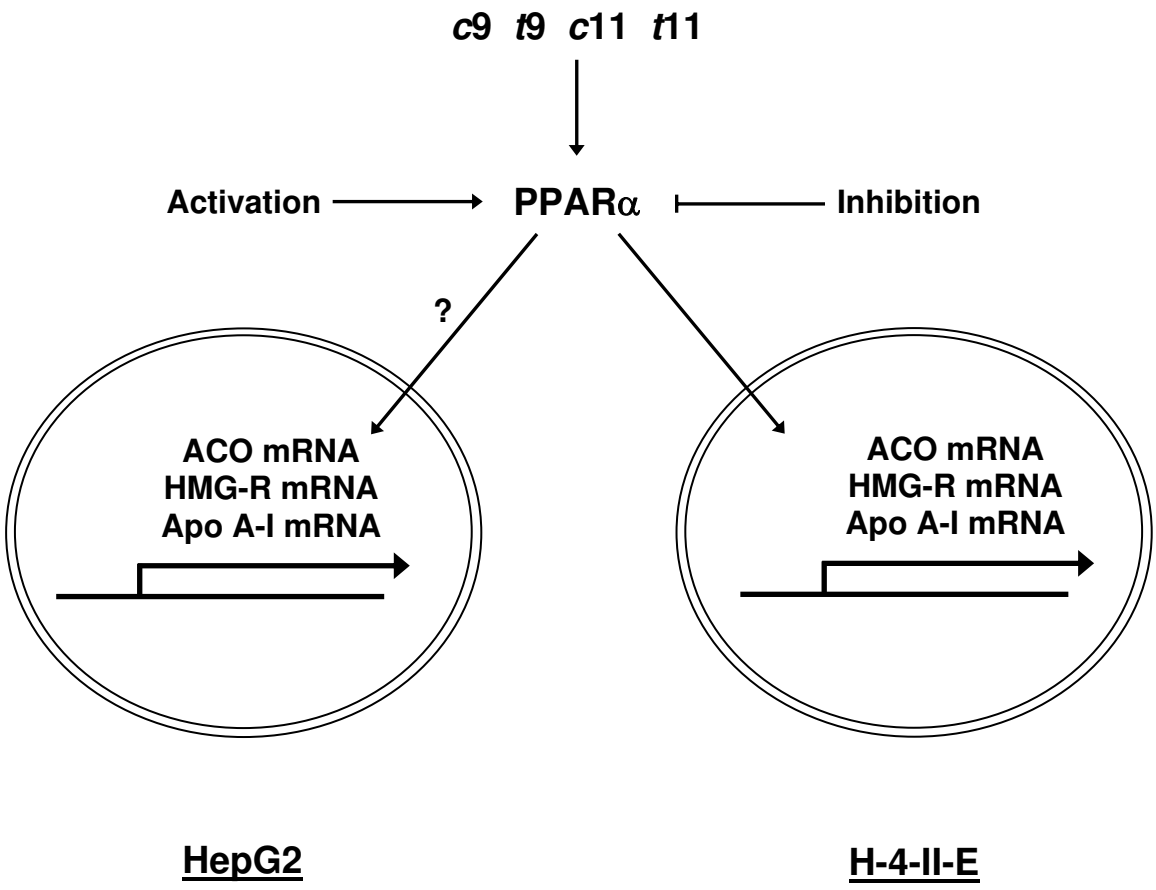

Figure 5-21. Regulation of lipid metabolizing genes and HDL cholesterol production by *cis* and *trans* octadecenoic fatty acids. In HepG2 cells, *cis*-vaccenic acid (c11) up-regulated ACO and Apo A-I gene expression. Activation of PPARα increased basal expression of HMG-R mRNA and enhanced c11-induced Apo A-I gene expression. In H-4-II-E cells, all fatty acids studied increased ACO and Apo A-I gene expression to the same extent. *Trans*-vaccenic acid (t11) increased HMG-R mRNA concentrations. Activation of PPARα enhanced the effects of t11 on ACO, HMG-R and Apo A-I gene expression. Inhibition of PPARα decreased basal expression and attenuated t11-induced ACO mRNA levels.

CHAPTER 6
GENERAL DISCUSSION

Dietary fat has been implicated as a major factor in many areas of health and disease. Though historically considered primarily as an energy source and cell membrane constituent, health effects are more likely due to fatty acid effects on gene expression and the subsequent effects on metabolism. However, it has been suggested by numerous studies that all fatty acids (FA) may not have the same effects. In these studies, both human and rat hepatoma cells were used as models, as it also has been suggested that species differences exist in fatty acid metabolism (Bergen and Mersmann, 2005).

In the first experiment, we examined the role of fatty acids with differing saturation and bond position on genes involved in β-oxidation, HDL cholesterol synthesis, and high-density lipoprotein (HDL) cholesterol secretion into the culture media. In HepG2 (human) cells, acyl CoA oxidase (ACO) mRNA expression was unaffected by stearic (ST), linoleic (LA), linolenic (LNA), or eicosapentaenoic (EPA) acids. In contrast, in the H-4-II-E (rat) cells, ACO mRNA expression was induced by ST. Consistent with our findings in rat cells, pigs fed a tallow-based diet high in saturated fat had an increased concentration of ACO mRNA as compared to fish oil-fed pigs (Ding et al., 2003). Other studies, however, have reported up-regulation of ACO mRNA in rat liver by dietary polyunsaturated fatty acids (PUFA) as well as saturated fats (Berthou et al., 1995). In HepG2 cells, it has been reported that PUFAs of differing saturation and length can regulate ACO mRNA in a dose-dependent and differential manner (Rise and Galli, 1999).

In a human retinoblastoma cell line, low concentrations of supplemental n-3 PUFA increased ACO mRNA, whereas high concentrations of the FA decreased it (Langelier et al., 2003).

In numerous animal models and human dietary and epidemiological studies, fatty acids have been demonstrated to have the ability to modulate serum cholesterol concentrations in both a positive and negative manner. 3-hydroxy, 3-methylglutaryl CoA reductase (HMG-R) is the rate limiting enzyme in cholesterol synthesis, and its inhibition is the target of the statin class of drugs, used in the treatment of hyperlipidemias. If select dietary FA stimulate HMG-R expression, then they may be important FA to limit consumption of in order to prevent elevation of blood cholesterol concentrations in humans. In this study, we showed that in HepG2 cells, HMG-R mRNA was up-regulated by ST as compared to the PUFAs, whereas in the H-4-II-E cells, it was up-regulated by both ST and EPA. Consistent with our findings in rodent cells, in Reuber H35 rat hepatoma cells, a cell line closely related to H-4-II-E cells, incubation with either saturated fats or PUFAs increased HMG-R enzyme activity (Garcia-Pelayo et al., 2003). Enzyme activity of HMG-R has also been shown to be increased in mice fed a diet high in PUFAs (Kuan and Dupont, 1989). 3-hydroxy, 3-methylglutaryl CoA reductase mRNA was increased to a greater extent in C3H mice fed a PUFA diet than in mice fed a saturated fat diet (Cheema and Agellon, 1999).

Apolipoprotein A-I (Apo A-I) is the predominant lipoprotein associated with HDL cholesterol and is essential for its normal metabolism. Deletion of the Apo A-I gene in humans results in very low blood concentrations of HDL cholesterol and premature coronary artery disease (Schaefer et al., 1982). Dietary fat has the ability to modulate

plasma lipids, and may act, in part, by effects on apolipoproteins. In this study, we determined that, in HepG2 cells, Apo A-I mRNA was up-regulated by all FA. However, no change was detected in HDL cholesterol concentration in the culture media. This is supported by a study by Dashti and coworkers (2002) in which HDL concentration was not different between linoleic acid (LA) and saturated fat-treated HepG2 cells. In Golden-Syrian hamsters, an effective model for human diet and blood lipid interactions, feeding canola and soybean oils (unsaturated FA) increased Apo A-I mRNA expression as compared to a butter-containing diet, though HDL concentrations were lowered in the hamsters fed diets containing unsaturated as compared to butter fats (Dorfman et al., 2005). In the H-4-II-E cells, ST increased Apo A-I mRNA concentration to the greatest extent. In contrast to current findings, Sprague-Dawley rats fed diets high in saturated fat or PUFAs showed no differences in Apo A-I mRNA levels (Hatahet et al., 2003). However, the saturated fat diet contained primarily palmitic acid, not stearic acid, as in this study.

Numerous beneficial physiological effects have been attributed to conjugated linoleic acid (CLA), though these effects may be both isomer and species specific. Therefore, in the second experiment, we examined the effects of the rwo biologically active CLA isomers on key regulatory genes of lipid metabolism. In HepG2 cells, ACO mRNA expression was up-regulated by CLA as compared to LA-treated cells, with an increase additionally seen with incubation of *trans*-10, *cis*-12 CLA as compared with *cis*-9, *trans*-11 CLA. In contrast, all FA increased ACO mRNA as compared to control in H-4-II-E cells, but there were no differences among the FA studied. Several animal and cell models have also reported similar effects. Feeding mice a mix of (Peters et al.,

2001) or individual CLA isomers (Warren et al., 2003; Degrace et al., 2004) resulted in an increased expression of ACO mRNA as compared to control animals. In two studies, the increases detected in ACO mRNA levels in CLA-fed mice also coincided with increases in enzyme activity (Takahashi et al., 2003; Ide, 2005). In FaO cells, a rat hepatoma cell line derived from H4IIEC3 cells (Bayly et al., 1993), ACO gene expression was increased with 200 μM *cis*-9, *trans*-11 CLA. This effect was not seen, however, with lower concentrations of CLA (Moya-Camarena et al., 1999). In a recent study using a hamster model, ACO activity was increased by *trans*-10, *cis*-12 CLA as compared to control or *cis*-9, *trans*-11 CLA (Macarulla et al., 2005). Together with our findings, these studies suggest a role for CLA in increasing liver peroxisomal β-oxidation in the liver.

When we examined CLA effects on genes involved in cholesterol synthesis in HepG2 cells, CLA isomers increased HMG-R mRNA transcript as compared to the parent molecule, LA. Between the CLA isomers, *trans*-10, *cis*-12 CLA up-regulated HMG-R mRNA concentrations as compared with the *cis*-9, *trans*-11 isomer. This differed in the H-4-II-E cells, where, on average, CLA increased gene expression as compared to LA, but no differences were seen between the two CLA isomers. Although the effects of saturated fats and PUFA on HMG-R gene expression and enzyme activity have been examined, few studies have explored the role of CLA. In a recent study, HMG-R activity was decreased in rats fed diacylglycerol-enriched structured lipids containing CLA as compared to those fed lipids without CLA or those fed corn oil (Kim et al., 2006). Though this differs from our findings, gene expression was not measured,

and as with other target genes, it would not be surprising if species and model-specific differences exist.

In general, CLA-induced changes in the blood lipid profile observed in various models are conflicting. In our study, Apo A-I gene transcript was increased by the CLA isomers as compared to LA, and by *trans*-10, *cis*-12 CLA as compared to *cis*-9, *trans*-11 CLA in HepG2 cells. High-density lipoprotein cholesterol production in HepG2 cells was decreased by the CLA isomers as compared to LA, with the effect predominantly derived from the decrease due to *cis*-9, *trans*-11 CLA. In H-4-II-E cells, steady-state levels of Apo A-I mRNA was decreased by LA and the CLA isomers; however, there were no differences among the FA studied. These decreases in gene expression did not result in increased HDL cholesterol production, as levels were not different among the treatments. The different responses seen in the two cell lines is reflected by conflicting responses in other species studied. Mice supplemented with *cis*-9, *trans*-11 or *trans*-10, *cis*-12 CLA showed no differences in Apo A-I mRNA concentrations in control or CLA-fed animals (Warren et al., 2003). In contrast, in apo-E deficient mice, dietary *trans*-10, *cis*-12 CLA decreased plasma Apo A-I levels as compared to *cis*-9, *trans*-11 CLA (Arbones-Mainar et al., 2006). Similar to the decrease in HDL cholesterol production in HepG2 cells, rabbits fed a mixture of CLA isomers showed an increase in total serum cholesterol and a decrease in HDL cholesterol (Kritchevsky et al., 2000). Additionally, in the Syrian Golden hamster, diets containing *trans*-10, *cis*-12 CLA increased HDL cholesterol as compared with LA or *cis*-9, *trans*-11 CLA diets (Mitchell et al., 2005). However, some studies in rat models have shown no effect of dietary CLA on serum HDL cholesterol (Kloss et al., 2005). In a human dietary study, both Apo A-I

gene transcript and HDL cholesterol were decreased by consumption of CLA-enriched butter as compared to pre-supplement levels (Desroches et al., 2005).

In the third experiment, we examined the effects of *cis* and *trans* isomers of octadecenoic acid on lipid metabolism. Dietary *trans*-FA have been implicated as potent negative factors in the development of numerous disease states, including dyslipidemia and cardiovascular disease. These changes may be mediated, in part, by the effects of fats on lipid metabolism; however, these effects may be different for different isomers. In HepG2 cells, MUFAs with the double bond in the 11 position increased ACO mRNA expression as compared to those FA with the bond in the 9 position. In particular, *cis*-vaccenic acid (c11) increased ACO gene expression. In contrast, in the H-4-II-E cells, all FA up-regulated ACO mRNA concentrations, with oleic acid (c9) and *trans*-vaccenic acid (t11) increasing gene expression as compared to elaidic acid (t9) and *cis*-vaccenic acid (c11), respectively. In contrast with our findings in gene expression, elaidic acid was shown to be a better substrate than oleic acid for fat oxidation, particularly peroxisomal oxidation, in rat hepatocytes (Guzman et al., 1999). However, in a human dietary study, supplementation with *trans*-FA increased and *cis*-FA decreased fat oxidation (as measured by indirect calorimetry) as compared to a saturated fat control diet (Lovejoy et al., 2002). Additionally, in INS-1 cells, similar effects were seen as in H-4-II-E cells, with palmitate oxidation increasing with oleic, elaidic, *cis*-vaccenic and *trans*-vaccenic acids. Though gene expression was not measured, *trans*-vaccenic acid increased oxidation to a greater extent than its *cis* counterpart (Alstrup et al., 2004).

In HepG2 cells, HMG-R mRNA expression was unaffected by any of the FA studied. In the rat cells, however, *trans*-vaccenic (t11) acid increased gene expression as

compared to *cis*-vaccenic (c11) acid. Though evidence suggests that FA can affect cholesterol production, few studies have examined the role of HMG-R in this response. In support of our findings, dietary oleic acid had no effect on HMG-R activity in Golden Syrian hamsters (Kurushima et al., 1995a; Kurushima et al., 1995b). These studies, however did not examine the effects of *trans*-FA. A recent study in mice showed no effects of dietary *trans* 18:1 fatty acids on HMG-R gene expression (Cassagno et al., 2005).

Dietary *trans*-FA have the ability to modulate plasma lipids, and may act, in part, by effects on apolipoproteins. The effects of *trans*-FA on cholesterol production have been examined extensively, but results seem to depend on the model used. In HepG2 cells, *cis*-vaccenic acid (c11) increased Apo A-I mRNA levels as compared to *trans*-vaccenic acid (t11). These changes in gene expression did not correlate with HDL cholesterol production, as none of the FA treatments had any effects. In H-4-II-E cells, treatment with all of the monoenes decreased Apo A-I mRNA concentration, although there were no differences among the monoenes studied. As with the human cells, these changes in gene expression did not affect HDL cholesterol production, as concentrations were not different among the treatments. In contrast with our findings, HDL cholesterol concentration was decreased by consumption of *trans*-FA in two human studies (Mensink and Katan, 1990; Tholstrup et al., 2006), although in the second study, the saturated to monounsaturated fat ratio of the diets may have played a significant role. In monkeys, dietary elaidic acid decreased Apo A-I and HDL cholesterol as compared to a saturated fat diet (Khosla et al., 1997). In HepG2 cells, oleic acid had no effects on Apo A-I or HDL cholesterol production (Dashti and Wolfbower, 1987), whereas elaidic acid may

increase HDL cholesterol (Dashti et al., 2000). Numerous studies, however, support our findings. In hamsters, dietary oleic acid had no effects on HDL cholesterol production as compared to saturated fats (Kurushima et al., 1995b), LA (Kurushima et al., 1995a; Nicolosi et al., 2004), or *trans*-FA (Nicolosi et al., 1998). When comparing vaccenic and elaidic acids, no effects on HDL cholesterol production were seen (Meijer et al., 2001). A recent study in mice fed 3% of dietary energy as *trans* 18:1 fatty acids reported no change in total or HDL cholesterol (Cassagno et al., 2005). In two human studies, blood HDL cholesterol concentration was unaffected by diets rich in *trans*-FA as compared with those high in *cis*-FA (Judd et al., 1994; Lovejoy et al., 2002).

Fatty acids and their derivatives have been identified as potential ligands for peroxisome proliferator-activated receptors (PPAR). Therefore, we tested the hypothesis that fatty acid effects on gene expression may be mediated by PPARα. In the three experiments, activation of PPARα by WY 14,643 or inhibition by MK886 had marginal effects on basal or fatty acid-induced gene expression in HepG2 cells. These results are consistent with the low levels of endogenous PPARα expression in this cell line (Hsu et al., 2001). In contrast, activation of PPARα consistently increased basal expression and enhanced ST- and *trans*-vaccenic-induced ACO gene expression in H-4-II-E cells. Although ACO is an established PPARα responsive gene (Tugwood, et al., 1992), species differences do exist. It is questionable whether the PPAR response element of human ACO is active (Woodyatt et al., 1999). Dietary studies have shown that rodents are responsive to the effects of PPARα activation, but non-rodent species, such as primates and guinea pigs, are resistant or unresponsive to some of the negative effects (Bentley et al., 1993; Cattley et al., 1998). In a comprehensive analysis of gene expression in human

and rat hepatoma cells by microarray analysis, only rat ACO mRNA was responsive to WY 14,643 (Vanden Heuvel et al., 2003). Other genes that may be differentially regulated in human and rat liver include cytosolic aspartate aminotransferase (Tomkiewicz et al., 2004), peroxisomal 3-oxoacyl-CoA thiolase (Lawrence et al., 2001), and catalase (Ammerschlaeger et al., 2004). Consistent with our findings in each experiment with the rat cell line, Apo A-I gene expression was not different from controls in mice fed fenofibrate, a potent PPARα activator (Warren et al., 2003). However, different PPARα agonists may regulate lipid metabolism in a compound-dependent, as well as species-dependent, manner. A recent study by Duez and coworkers (2005) showed that, in mice, fenofibrate and gemfibrozil, both stimulate ACO mRNA expression, but only fenofibrate greatly induces Apo A-I gene expression. Together with our findings, these results indicate that fatty acids may differentially regulate specific lipid metabolizing genes in the liver through a PPARα-dependent mechanism. However, due to different responses in the human and rat hepatoma cell lines, the net effects are likely species specific.

APPENDIX

LS MEANS AND P-VALUES FOR ANALYSIS OF FATTY ACID EFFECTS ON LIPID-METABOLIZING GENES AND HDL CHOLESTEROL PRODUCTION IN HEPG2 AND H-4-II-E CELLS

Table A-1. Effects of n-3 and n-6 FA on lipid-metabolizing genes and HDL cholesterol production in HepG2 cells.

Response	LS Means						P-value		
	Control	ST	LA	LNA	EPA	SEM	Exp	Trt	E x T
ACO	0.477	0.551	0.432	0.498	0.412	0.048	0.40	0.33	0.32
HMG-R	0.613	0.754	0.600	0.575	0.564	0.043	0.03	0.06	0.27
Apo A-I	0.396	0.458	0.447	0.464	0.500	0.029	0.002	0.23	0.02
HDL, mg/dL	2.85	2.43	3.30	3.15	3.36	0.802	0.27	0.90	0.35

Table A-2. Effects of n-3 and n-6 FA on lipid-metabolizing genes and HDL cholesterol production in H-4-II-E cells.

Response	LS Means						P-value		
	Control	ST	LA	LNA	EPA	SEM	Exp	Trt	E x T
ACO	0.077	0.109	0.084	0.089	0.069	0.007	0.21	0.02	0.90
HMG-R	0.160	0.201	0.115	0.111	0.218	0.011	0.15	0.0001	0.99
Apo A-I	0.043	0.059	0.036	0.028	0.041	0.003	0.31	0.0002	0.22
HDL, mg/dL	6.33	3.20	1.61	13.15	2.08	1.01	0.05	0.04	0.03

Table A-3. Effects of WY 14,643 on mRNA responses to ST in HepG2 cells.

Response	LS Means					P-value		
	Control	ST	Agonist	ST + A	SEM	Exp	Trt	E x T
ACO	0.227	0.245	0.229	0.226	0.005	0.0001	0.11	0.56
HMG-R	0.251	0.274	0.190	0.248	0.007	0.002	0.0001	0.90
Apo A-I	0.179	0.192	0.162	0.168	0.018	0.24	0.65	0.76

Table A-4. Effects of MK886 on mRNA responses to ST in HepG2 cells.

Response	LS Means					P-value		
	Control	ST	Inhibitor	ST + I	SEM	Exp	Trt	E x T
ACO	0.157	0.260	0.286	0.289	0.033	0.39	0.07	0.03
HMG-R	0.231	0.301	0.365	0.338	0.029	0.01	0.05	0.15
Apo A-I	0.319	0.437	0.417	0.458	0.025	0.003	0.02	0.18

Table A-5. Effects of WY 14,643 on mRNA responses to ST in H-4-II-E cells.

Response	LS Means					P-value		
	Control	ST	Agonist	ST + A	SEM	Exp	Trt	E x T
ACO	0.039	0.043	0.050	0.069	0.003	0.01	0.0006	0.002
HMG-R	0.074	0.080	0.051	0.062	0.005	0.002	0.01	0.03
Apo A-I	0.019	0.030	0.022	0.042	0.002	0.0008	0.0001	0.0001

Table A-6. Effects of MK886 on mRNA responses to ST in H-4-II-E cells.

Response	LS Means					P-value		
	Control	ST	Inhibitor	ST + I	SEM	Exp	Trt	E x T
ACO	0.237	0.258	0.211	0.201	0.008	0.0004	0.005	0.01
HMG-R	0.068	0.101	0.096	0.100	0.005	0.0009	0.003	0.09
Apo A-I	0.028	0.040	0.014	0.028	0.003	0.002	0.003	0.16

Table A-7. Effects of CLA on lipid-metabolizing genes and HDL cholesterol production in HepG2 cells.

Response	LS Means					P-value		
	Control	LA	c9, t11 CLA	t10, c12 CLA	SEM	Exp	Trt	E x T
ACO	0.316	0.316	0.335	0.430	0.020	0.0001	0.01	0.07
HMG-R	0.295	0.219	0.311	0.396	0.018	0.54	0.0008	0.16
Apo A-I	0.338	0.287	0.317	0.406	0.023	0.006	0.04	0.01
HDL, mg/dL	2.62	2.76	2.05	2.64	0.151	0.002	0.08	0.03

Table A-8. Effects of CLA on lipid-metabolizing genes and HDL cholesterol production in H-4-II-E cells.

Response	LS Means					P-value		
	Control	LA	c9, t11 CLA	t10, c12 CLA	SEM	Exp	Trt	E x T
ACO	0.161	0.202	0.201	0.223	0.007	0.0007	0.002	0.28
HMG-R	0.072	0.058	0.101	0.087	0.006	0.003	0.005	0.14
HMG-R	0.056	0.040	0.047	0.041	0.003	0.0001	0.03	0.02
HDL, mg/dL	2.46	11.43	8.87	1.30	3.28	0.22	0.65	0.65

Table A-9. Effects of WY 14,643 on mRNA responses to *trans*-10, *cis*-12 CLA in HepG2 cells.

Response	LS Means					P-value		
	Control	CLA	Agonist	CLA + A	SEM	Exp	Trt	E x T
ACO	0.156	0.227	0.181	0.227	0.018	0.0001	0.06	0.99
HMG-R	0.273	0.365	0.354	0.391	0.019	0.0001	0.01	0.30
Apo A-I	0.249	0.398	0.400	0.395	0.013	0.0001	0.0001	0.0001

Table A-10. Effects of MK886 on mRNA responses to *trans*-10, *cis*-12 CLA in HepG2 cells.

Response	LS Means					P-value		
	Control	CLA	Inhibitor	CLA + I	SEM	Exp	Trt	E x T
ACO	0.260	0.266	0.252	0.273	0.017	0.002	0.84	0.26
HMG-R	0.483	0.529	0.487	0.534	0.013	0.0001	0.04	0.26
Apo A-I	0.539	0.449	0.515	0.471	0.022	0.30	0.07	0.43

Table A-11. Effects of WY 14,643 on mRNA responses to *trans*-10, *cis*-12 CLA in H-4-II-E cells.

Response	LS Means					P-value		
	Control	CLA	Agonist	CLA + A	SEM	Exp	Trt	E x T
ACO	0.076	0.113	0.101	0.089	0.006	0.85	0.01	0.99
HMG-R	0.051	0.058	0.050	0.050	0.004	0.007	0.45	0.15
Apo A-I	0.69	0.56	0.67	0.66	0.004	0.0006	0.17	0.12

Table A-12. Effects of MK886 on mRNA responses to *trans*-10, *cis*-12 CLA in H-4-II-E cells.

Response	LS Means					P-value		
	Control	CLA	Inhibitor	CLA + I	SEM	Exp	Trt	E x T
ACO	0.119	0.160	0.136	0.175	0.010	0.003	0.02	0.93
HMG-R	0.130	0.124	0.105	0.110	0.006	0.0002	0.06	0.09
Apo A-I	0.061	0.067	0.061	0.050	0.003	0.0001	0.01	0.01

Table A-13. Effects of *cis* and *trans* isomers of octadecenoic acid on lipid-metabolizing genes and HDL cholesterol production in HepG2 cells.

Response	LS Means						P-value		
	Control	c9	t9	c11	t11	SEM	Exp	Trt	E x T
ACO	0.253	0.258	0.259	0.327	0.275	0.012	0.0001	0.007	0.14
HMG-R	0.168	0.159	0.143	0.194	0.179	0.024	0.0004	0.64	0.28
Apo A-I	0.424	0.431	0.382	0.496	0.401	0.029	0.001	0.13	0.40
HDL, mg/dL	2.29	2.45	2.30	2.16	2.67	0.21	0.002	0.37	0.15

Table A-14. Effects of *cis* and *trans* isomers of octadecenoic acid on lipid-metabolizing genes and HDL cholesterol production in H-4-II-E cells.

Response	LS Means						P-value		
	Control	c9	t9	c11	t11	SEM	Exp	Trt	E x T
ACO	0.256	0.298	0.264	0.276	0.307	0.009	0.0003	0.01	0.004
HMG-R	0.097	0.092	0.096	0.088	0.111	0.003	0.74	0.005	0.38
Apo A-I	0.113	0.100	0.102	0.103	0.107	0.002	0.88	0.02	0.09
HDL, mg/dL	1.50	1.04	1.86	0.98	1.50	0.33	0.42	0.26	0.99

Table A-15. Effects of WY 14,643 on mRNA responses to *cis*-vaccenic acid in HepG2 cells.

Response	LS Means					P-value		
	Control	c11	Agonist	c11 + A	SEM	Exp	Trt	E x T
ACO	0.337	0.355	0.370	0.371	0.011	0.0001	0.16	0.18
HMG-R	0.264	0.303	0.315	0.325	0.016	0.0001	0.11	0.08
Apo A-I	0.239	0.214	0.266	0.320	0.021	0.002	0.04	0.11

Table A-16. Effects of MK886 on mRNA responses to *cis*-vaccenic acid in HepG2 cells.

Response	LS Means					P-value		
	Control	c11	Inhibitor	c11 + I	SEM	Exp	Trt	E x T
ACO	0.173	0.221	0.204	0.234	0.025	0.07	0.41	0.61
HMG-R	0.344	0.350	0.305	0.324	0.022	0.005	0.50	0.77
Apo A-I	0.377	0.383	0.375	0.401	0.021	0.005	0.83	0.78

Table A-17. Effects of WY 14,643 on mRNA responses to *trans*-vaccenic acid in H-4-II-E cells.

Response	LS Means					P-value		
	Control	t11	Agonist	t11 + A	SEM	Exp	Trt	E x T
ACO	0.227	0.185	0.193	0.384	0.028	0.82	0.003	0.55
HMG-R	0.053	0.085	0.082	0.154	0.009	0.68	0.0003	0.44
Apo A-I	0.047	0.025	0.025	0.057	0.005	0.40	0.004	0.66

Table A-18. Effects of MK886 on mRNA responses to *trans*-vaccenic acid in H-4-II-E cells.

Response	LS Means					P-value		
	Control	t11	Inhibitor	t11 + I	SEM	Exp	Trt	E x T
ACO	0.065	0.061	0.042	0.023	0.003	0.14	0.0001	0.97
HMG-R	0.067	0.093	0.076	0.085	0.009	0.89	0.25	0.56
Apo A-I	0.040	0.092	0.051	0.082	0.005	0.01	0.0001	0.002

LIST OF REFERENCES

Albert CM, Campos H, Stampfer MJ, Ridker PM, Manson JE, Willett WC, Ma J. Blood levels of long-chain n-3 fatty acids and the risk of sudden death. N Engl J Med. 2002;346:1113-8.

Allmann DW, Gibson DM. Fatty acid synthesis during early linoleic acid deficiency in the mouse. J Lipid Res. 1965;6:51-62.

Alstrup KK, Brock B, Hermansen K. Long-Term exposure of INS-1 cells to cis and trans fatty acids influences insulin release and fatty acid oxidation differentially. Metabolism. 2004;53:1158-65.

American Heart Association. AHA dietary guidelines, revision 2000: a statement for healthcare professionals from the nutrition committee of the American Heart Association. Circulation. 2000;102:2284-99.

Ammerschlaeger M, Beigel J, Klein KU, Mueller SO. Characterization of the species-specificity of peroxisome proliferators in rat and human hepatocytes. Toxicol Sci. 2004;78:229-40.

Arbones-Mainar JM, Navarro MA, Acin S, Guzman MA, Arnal C, Surra JC, Carnicer R, Roche HM, Osada J. *Trans*-10, *cis*-12- and *cis*-9, *trans*-11 conjugated linoleic acid isomers selectively modify HDL-apolipoprotein composition in apolipoprotein E knockout mice. J Nutr. 2006;136:353-9.

Aro A, Jauhiainen M, Partanen R, Salminen I, Mutanen M. Stearic acid, trans fatty acids, and dairy fat: effects on serum and lipoprotein lipids, apolipoproteins, lipoprotein(a), and lipid transfer proteins in healthy subjects. Am J Clin Nutr. 1997;65:1419-26.

Ascherio A, Katan MB, Zock PL, Stampfer MJ, Willett WC. *Trans* fatty acids and coronary heart disease. N Engl J Med. 1999;340:1994-8.

Ascherio A, Rimm EB, Giovannucci EL, Spiegelman D, Stampfer M, Willett WC. Dietary fat and risk of coronary heart disease in men: cohort follow up study in the United States. BMJ. 1996;313:84-90.

Astrup A, Buemann B, Flint A, Raben A. Low-fat diets and energy balance: how does the evidence stand in 2002? Proc Nutr Soc. 2002;61:299-309.

Astrup A, Ryan L, Grunwald GK. The role of dietary fat in body fatness: evidence from a preliminary meta-analysis of ad libitum low-fat dietary intervention studies. Br J Nutr. 2000;83: S25-32.

Auboeuf D, Rieusset J, Fajas L, Vallier P, Frering V, Riou JP, Staels B, Auwerx J, Laville M, Vidal H. Tissue distribution and quantification of the expressin of mRNAs of peroxisome proliferator-activated receptors and liver X receptor α in humans. Diabetes 1997;46:1319-27.

Baer DJ, Judd JT, Clevidence BA, Tracy RP. Dietary fatty acids affect plasma markers of inflammation in healthy men fed a controlled diet: a randomized crossover study. Am J Clin Nutr. 2004;79:969-73.

Bang HO, Dyerberg J, Sinclair HM. The composition of Eskimo food in northwest Greenland. Am J Clin Nutr. 1980;33:2657-61.

Barak Y, Liao D, He W, Ong ES, Nelson MC, Olefsky JM, Boland R, Evans RM. Effects of peroxisome proliferator-activated receptor δ on placentation, adiposity, and colorectal cancer. Proc Natl Acad Sci USA. 2002;99:303–8.

Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, Chien KR, Koder A, Evans RM. PPARγ is required for placental, cardiac, and adipose tissue development. Mol Cell. 1999;4:585-95.

Bartlett K, Eaton S. Mitochondrial β-oxidation. Eur J Biochem. 2004;271:462-9.

Baumgard LH, Matitashvili E, Corl BA, Dwyer DA, Bauman DE. Trans-10, cis-12 conjugated linoleic acid decreases lipogenic rates and expression of genes involved in milk lipid synthesis in dairy cows. J Dairy Sci. 2002;85:2155-63.

Bayly AC, French NJ, Dive C, Roberts RA. Non-genotoxic hepatocarcinogenesis in vitro: the FaO hepatoma line responds to peroxisome proliferators and retains the ability to undergo apoptosis. J Cell Sci. 1993;104:307-15.

Belury MA. Dietary conjugated linoleic acid in health: physiological effects and mechanisms of action. Annu Rev Nutr. 2002;22:505-31.

Belury MA, Kempa-Steczko A. Conjugated linoleic acid modulates hepatic lipid composition in mice. Lipids. 1997;32:199-204.

Belury MA, Vanden Huvel JP. Modulation of diabetes by conjugated linoleic acid. In: Yurawecz MP, Mossoba MM, Kramer JKG, Pariza MW. Nelson G, editors. Advances in Conjugated Linoleic Acid Research, Volume 1. Champaign (IL): AOCS Press; 1999. p. 180-200.

Benatti P, Peluso G, Nicolai R, Calvani M. Polyunsaturated fatty acids: biochemical, nutritional and epigenetic properties. J Am Col Nutr. 2004;23:281-302.

Benito P, Nelson G, Kelley D, Bartolini G, Schmidt P, Simon V. Effect of conjugated linoleic acid on platelet function, platelet fatty acid composition, and blood coagulation in humans. Lipids. 2001;36:221-7.

Bentley P, Calder I, Elcombe C, Grasso P, Stringer D, Weigand H-J. Hepatic peroxisome proliferation in rodents and its significance for humans. Food Chem Toxicol. 1993;31:857-907.

Bergen WG, Mersmann HJ. Comparative aspects of lipid metabolism: impact on contemporary research and use of animal models. J Nutr. 2005;135:2499-2502.

Berger J, Moller DE. The mechanism of action of PPARs. Annu Rev Med. 2002;53:409-35.

Berthou L, Saladin R, Yaqoob P, Branellec D, Calder P, Fruchart JC, Denefle P, Auwerx J, Staels B. Regulation of rat liver apolipoprotein A-I, apolipoprotein A-II and acyl-coenzyme A oxidase gene expression by fibrates and dietary fatty acids. Eur J Biochem. 1995;232:179-87.

Bieber LL. Carnitine. Ann Rev Biochem. 1988;57:261-83.

Billman GE, Kang JX, Leaf A. Prevention of ischemia-induced cardiac sudden death by n-3 polyunsaturated fatty acids in dogs. Lipids. 1997;32:1161-8.

Billman GE, Kang JX, Leaf A. Prevention of sudden cardiac death by dietary pure omega-3 polyunsaturated fatty acids in dogs. Circualtion. 1999;99:2452-7.

Blankson H, Stakkestad JA, Fagertun H, Thom E, Wadstein J, Gudmundsen O. Conjugated linoleic acid reduces body fat mass in overweight and obese humans. J Nutr. 2000;130:2943-8.

Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959;37:911-7.

Brady RO, Gurin S. Biosynthesis of fatty acids by cell-free or water-soluble enzyme systems. J Biol Chem. 1952;199:421-31.

Braissant O, Foufelle F, Scotto C, Dauca M, Wahli W. Differential expression of peroxisome proliferatoe-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and –gamma in the adult rat. Endocrinology. 1996;137:354-66.

Bray GA, Lovejoy JC, Smith SR, DeLany JP, LeFevre M, Hwang D, Ryan DH, York DA. The influence of different fats and fatty acids on obesity, insulin resistance and inflammation. J Nutr. 2002;132:2488-91.

Bray GA, Paeratakul S, Popkin BM. Dietary fat and obesity: a review of animal, clinical and epidemiological studies. Physiol Behav. 2004;83:549-55.

Bray GA, Popkin BM. Dietary fat intake does affect obesity! Am J Clin Nutr. 1998;68:1157-73.

Bremer J. Carnitine in intermediary metabolism. Reversible acetylation of carnitine by mitochondria. J Biol Chem. 1962;237:2228-31.

Brown JM, McIntosh M. Conjugated linoleic acid in humans: regulation of adiposity and insulin sensitivity. J Nutr. 2003;133:3041-6.

Bucher HC, Hengstler P, Schindler C, Meier G. n-3 polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials. Am J Med. 2002;112:298-304.

Bugaut M, Myher JJ, Kuksis A, Hoffman AGD. An examination of the stereochemical course of acylation of 2-monoacylglycerols by rat intestinal villus cells using [2H3] palmitic acid. Biochim Boiphys Acta. 1984;792:254-69.

Burant CF, Sreenan S, Hirano K, Tai TA, Lohmiller J, Lukens J, Davidson NO, Ross S, Graves RA. Troglitazone action is independent of adipose tissue. J Clin Invest. 1997;100:2900-8.

Burr GO, Burr MM. A new deficiency disease produced by the rigid exclusion of fat from the diet. J Biol Chem. 1929;82:345-67.

Carlier H, Bernard A, Caselli C. Digestion and absorption of polyunsaturated fatty acids. Reprod Nutr Dev. 1991;125:S62-8.

Cartwright IJ, Plonne D, Higgins JA. Intracellular events in the assembly of chylomicrons in rabbit enterocytes. J Lipid Res. 2000;41:1728-39.

Cassagno N, Palos-Pinto A, Costet P, Breilh D, Darmon M, Berard AM. Low amounts of trans 18:1 fatty acids elevate plasma triacylglycerols but not cholesterol and later the cellular defense to oxidative stress in mice. B J Nutr. 2005;94:346-52.

Cattley RC, DeLucaJ, Elcombe C, Fenner-Crisp P, Lake BG, Marsman DS, Pastoor TA, PoppJA, Robindon DE, Schwetz B, Tugwood J, Wahli W. Do peroxisome proliferating compounds pose a hepatocarcinogenic hazard to humans? Reg Toxicol Pharmacol. 1998;27:47-60.

Chao L, Marcus-Samuels B, Mason MM, Moitra J, Vinson C, Arioglu E, Gavrilova O, Reitman ML. Adipose tissue is required for the antidiabetic, but not for the hypolipidemic, effect of thiazolidinediones. J Clin Invest. 2000;106:1221-8.

Cheema SK, Agellon LB. Metabolism of cholesterol is altered in the liver of C3H mice fed fats enriched with different C-18 fatty acids. J Nutr. 1999;129:1718-24.

Cheng L, Ding G, Qin Q, Xiao Y, Woods D, Chen YE, Yang Q. Peroxisome proliferator-activated receptor delta activates fatty acid oxidation in cultured neonatal and adult cardiomyocytes. Biochem Biophys Res Commun. 2004;313:277-86.

Chin S, Liu W, Storkson J, Ha Y, Pariza M. Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens. J Food Comp Anal. 1992;5:185-97.

Chin S, Storkson J, Albright K, Cook M, Pariza M. Conjugated linoleic acid is a growth factor for rats as shown by enhanced weight gain and improved feed efficiency. J Nutr. 1994;124:2344-9.

Chinetti G, Griglio S, Antonucci M, Torra IP, Delerive P, Majd Z, Fruchart JC, Chapman J, Najib J, Staels B. Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte derived macrophages. J Biol Chem. 1998;273:25573-80.

Chirala SS, Wakil SJ. Structure and function of animal fatty acid synthase. Lipids. 2004;39:1045-53.

Christensen JH, Gustenhoff P, Korup E, Aaroe J, Toft E, Moller J, Rasmussen K, Dyerberg J, Schmidt EB. Effect of fish oil on heart rate variability in survivors of myocardial infarction: a double blind randomized controlled trial. BMJ 1996;312:677-8.

Christie WW. Lipid Analysis, 3rd edition. Oily Press, Bridgewater, UK. 2003.

Clarke SD, Jump DB. Dietary polyunsaturated fatty acid regulation of gene transcription. Annu Rev Nutr. 1994;14:83-98.

Colandre ME, Diez RS, Bernal CA. Metabolic effects of *trans* fatty acids on an experimental dietary model. Brit J Nutr. 2003;89:631-8.

Coleman RA. Hepatic sn-glycerol-3-phosphate acyltransferases: effect of monoacylglycerol analogs on mitochondrial and microsomal activities. Biochim Biophys Acta. 1988;963:367-74.

Coleman RA, Haynes EB. Hepatic monoacylglycerol acyltransferase activity. Characterization of an activity associated with the suckling period in rats. J Biol Chem. 1984;259:8934-8.

Cowles RL, Lee JY, Gallaher DD, Stuefer-Powell CL, Carr TP. Dietary stearic acid alters gallbladder bile acid composition in hamsters fed cereal-based diets. J Nutr. 2002;132:3119-22.

Cunnane SC, Anderson MJ. Pure linoleate deficiency in the rat: influence on growth, accumulation of n-6 polyunsaturates, and [1-14C]linoleate oxidation. J Nutr. 1997;127:146-52.

D'Agnolo G, Rosenfeld IS, Vagelos PR. Multiple forms of beta-keto-acyl carrier protein synthetase in Escherichia coli. J Biol Chem. 1975;250:5289-94.

Dakin H. Oxidation and reduction in the animal body. London (UK): Longmans Greene; 1912.

Dang AQ, Kemp K, Faas FH, Carter WJ. Effects of dietary fats on fatty acid composition and delta 5 desaturase in normal and diabetic rats. Lipids. 1989;24:882-9.

Dashti N, Feng Q, Franklin FA. Long-term effects of cis and trans monounsaturated (18:1) and saturated (16:0) fatty acids on the synthesis and secretion of apolipoprotein A-I- and apolipoprotein B-containing lipoproteins in HepG2 cells. J Lipid Res. 2000;41:1980-90.

Dashti N, Feng Q, Freeman MR, Gandhi M, Franklin FA. Trans polyunsaturated fatty acids have more adverse effects than saturated fatty acids on the concentration and composition of lipoproteins secreted by human hepatoma HepG2 cells. J Nutr. 2002;132:2651-9.

Dashti N, Wolfbauer G. Secretion of lipids, apolipoproteins, and lipoproteins by human hepatoma cell line, HepG2: effects of oleic acid and insulin. J Lipid Res. 1987;28:423-36.

de Deckere E, van Amelsvoort J, McNeill G, Jones P. Effects of conjugated linoleic acid (CLA) isomers on lipid levels and peroxisome proliferation in the hamster. Brit J Nutr. 1999;82:309-17.

Degrace P, Demizieux L, Gresti J, Chardigny JM, Sebedio JL, Clouet P. Association of liver steatosis with lipid oversecretion and hypotriglyceridemia in C57BL/6J mice fed trans-10, cis-12 linoleic acid. FEBS Lett. 2003;546:335-9.

Degrace P, Demizieux L, Gresti J, Chardigny JM, Sebedio JL, Clouet P. Hepatic steatosis is not due to impaired fatty acid oxidation capacities in C57BL/6J mice fed the conjugated trans-10,cis-12-isomer of linoleic acid. J Nutr. 2004;134:861-7.

Delaplanque B, Richard JL, Jacotot B. Influence of diet on the plasma levels and distribution of apo A-I-containing lipoprotein particles. Prog Lipid Res. 1991;30:159-70.

de Roos NM, Schouten EG, Katan MB. Trans fatty acids, HDL-cholesterol, and cardiovascular disease. Effects of dietary changes on vascular sensitivity. Eur J Med Res. 2003;8:355-7.

Desroches S, Chouinard PY, Galibois I, Corneau L, Delisle J, Lamarche B, Couture P, Bergeron N. Lack of effect of dietary conjugated linoleic acids naturally incorporated into butter on the lipid profile and body composition of overweight and obese men. Am J Clin Nutr. 2005;82:309-19.

Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev. 1999;20:649-88.

Dietschy JM. Dietary fatty acids and the regulation of plasma low density lipoprotein cholesterol cholesterol concentration. J. Nutr. 1998;128:S444-8.

Dietschy JM, Sallee VL, Wilson FA. Unstirred water layers and absorption across the intestinal mucosa. Gastroenterology. 1971;61:932-4.

Dietschy JM, Wollett LA, Spady DK. The interaction of dietary cholesterol and specific fatty acids in the regulation of LDL receptor activity and and plasma LDL-cholesterol concentrations. Ann NY Acad Sci. 1993;676:11-26.

Ding ST, Lapillonne A, Heird WC, Mersmann HJ. Dietary fat has minimal effects on fatty acid metabolism transcript concentrations in pigs. J Anim Sci. 2003;81:423-31.

DiRusso CC, Black PN. Long-chain fatty acid transport in bacteria and yeast. Paradigms for defining the mechanism underlying this protein-mediated process. Mol Cell Biochem. 1999;192:31-52.

Doleck TA. Epidemiological evidence of relationships between dietary polyunsaturated fatty acids and mortality in the multiple risk factor intervention trial. Proc Soc Exp Biol Med. 1992;200:177-82.

Dorfman SE, Wang S, Vega-Lopez S, Jauhiainen M, Lichtenstein AH. Dietary fatty acids and cholesterol differentially modulate HDL cholesterol metabolism in Golden-Syrian hamsters. J Nutr. 2005;135:492-8.

Dreyer C, Keller H, Mahfoudi A, Laudet V, Krey G, Wahli W. Control of the peroxisomal β-oxidation pathway by a novel family of nuclear hormone receptors. Cell. 1992;68:879–87.

Duez H, Lefebvre B, Poulain P, Torra IP, Percevault F, Luc G, Peters JM, Gonzalez FJ, Gineste R, Helleboid S, Dzavik V, Fruchart JC, Fievet C, Lefebvre P, Staels B. Regulation of human apoA-I by gemfibrozil and fenofibrate through selective peroxisome proliferator-activated receptor alpha modulation. Arterioscler Thromb Vasc Biol. 2005;25:585-91.

Dugan MER, Aalhus JL, Schaefer AL, Kramer JKG. The effect of conjugated linoleic acid on fat to lean repartitioning and feed conversion in pigs. Can J Anim Sci. 1997;77:723-5.

Eaton S. Control of mitochondrial β-oxidation flux. Prog Lipid Res. 2002;41:197-239.

Eaton S, Bartlett K, Pourfarzam M. Mammalian mitochondrial beta-oxidation. Biochem J. 1996;320:345-57.

Erkkila AT, Lehto S, Pyorala K, Uusitupa MI. n-3 fatty acids and 5-y risks of death and cardiovascualr disease events in patients with coronary artery disease. Am J Clin Nutr. 2003;78:65-71.

Esrey KL, Joseph L, Grover SA. Relationship between dietary intake and coronart heart disease mortality: lipid research clinics prevalence follow-up study. J Clin Epidemiol. 1996;49:211-6.

Evans M, Pariza M, Park Y, Curtis L, Kubler B, McIntosh M. Trans-10cis-12 conjugated linoleic acid reduces triglyceride content while differentially affecting peroxisome proliferator-activated receptor-γ2 and aP2 expression in 3T3-L1 preadipocytes. Lipids. 2000;36:1223-32.

Ferdinandusse S, Denis S, Mooijer PA, Zhang Z, Reddy JK, Spector AA, Wanders RJ. Identification of the peroxisomal beta-oxidation enzymes involved in the biosynthesis of docosahexaenoic acid. J Lipid Res. 2001;42:1987-95.

Ferguson JC, Mackay N, McNicol GP. Effect of feeding fat on fibrinolysis, Stypven time, and platelet aggregation in Africans, Asians, and Europeans. J Clin Pathol. 1970;23:580-5.

Finck BN, Han X, Courtois M, Aimond F, Nerbonne JM, Kovacs A, Gross RW, Kelly, DP. A critical role for PPAR alpha-mediated lipotoxicity in the pathogenesis of diabetic cardiomyopathy: modulation by dietary fat content. Proc Natl Acad Sci. 2003;100:1226-31.

Finck BN, Lehman JJ, Leone TC, Welch MJ, Bennett MJ, Kovacs A, Han X, Gross RW, Kozak R, Lopaschuk GD, Kelly DP. The cardiac phenotype induced by PPAR alpha overexpression mimics that caused by diabetes mellitus. J Clin Invest. 2002;109:121-30.

Fogerty A, Ford G, Svoronos D. Octadeca-9-11-dienoic acid in foodstuff and in the lipids of human blood and breast milk. Nutr Reports Int. 1988;38:937-44.

Food and Nutrition Board. Dietary reference intakes for energy, carbohydrates, fiber, fat, fatty acids, cholesterol, protein, and amino acids (macronutrients). Washington, DC (USA): National Academy Press; 2005.

Foreyt JP, Poston WS. Consensus view on the role of dietary fat and obesity. Am J Med. 2002;113:60S-62S.

Forman BM, Chan J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ. Proc Natl Acad Sci USA. 1997;94:4312-7.

Fournier B, Smeitink JAM, Dorland L, Berger R, Sausubray JM, Poll-The BT. Peroxisomal disorders: a review. J Inher Metab Dis. 1994;17:470-86.

Frayn KN. Non-esterified fatty acid metabolism and postprandial lipaemia. Atherosclerosis. 1998;141:S41-6.

Freedman MR, King J, Kennedy E. Popular diets: a scientific review. Obes Res. 2001;9:1S-40S.

Fritz IB, Yue KTN. Long-chain carnitine acyltransferase and the role of acylcarnitine derivatives in the catalytic increase of fatty acid oxidation induced by carnitine. J Lipid Res. 1963;4:279-88.

Frohnert BI, Bernlohr DA. Regulation of fatty acid transporters in mammalian cells. Prog Lipid Res. 2000;39:83-107.

Fruchart JC. Peroxisome proliferator-activated receptor-alpha activation and high-density lipoprotein metabolism. Am J Cardiol. 2001;88:24N-29N.

Fuentes F, Lopez-Miranda J, Sanchez E, Sanchez F, Paez J, Paz-Rojas E, Marin C, Gomez P, Jimenez-Pereperez J, Ordovas JM, Perez-Jimenez F. Mediterranean and low-fat diets improve endothelial function in hypercholesterolemic men. Ann Intern Med. 2001;134:1115-9.

Fumeron F, Brigant L, Ollivier V, de Prost D, Driss F, Darcet P, Bard JM, Parra HJ, Fruchart JC, Apfelbaum M. n-3 polyunsaturated fatty acids raise low-density lipoproteins, high-density lipoprotein 2, and plasminogen-activator inhibitor in healthy young men. Am J Clin Nutr. 1991;54:118-22.

Garcia-Pelayo MC, Garcia-Peregrin E, Martinez-Cayuela M. Modification of phospholipid fatty acid composition in Reuber H35 hepatoma cells: effect on HMG-CoA reductase activity. J Cell Biochem. 2003;90:568-91.

Gardner CD, Kramer HC. Monounsaturated versus polyunsaturated dietary fat and serum lipids: a meta-analysis. Arterioscler Thromb Vasc Biol. 1995;15:1918-27.

Gaullier J-M, Berven G, Blankson H, Gudmundsen O. Clinical trials support a preference for using CLA preparations enriched with two isomers rather than four isomers in human studies. Lipids. 2002;37:1019-25.

Gaullier J-M, Halse J, Hoye K, Kristiansen K, Fagertun H, Vik H, Gudmundsen O. Supplementation with conjugated linoleic acid for 24 months is well tolerated by and reduces body fat mass in healthy overweight humans. J Nutr. 2005;135:778-84.

Gavino V, Gavino G, LeBlanc M, Tuchweber B. An isomeric mixture of conjugated linoleic acids but not pure cis-9, trans-11-octadecodienoic acid affects bodyweight gain and plasme lipids in hamsters. J Nutr. 2000;130:27-9.

Ginsberg HN. Lipoprotein physiology. Endocrinol Metab Clin North Am. 1998;27:503-19.

Glomset JA. The plasma lecithin:cholesterol acyl-transferase reaction. J Lipid Res. 1958;9:155-67.

Gottlicher M, Widmark E, Li Q, Gustafsson JA. Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. Proc Natl Aca Sci USA. 1992;89:4653-7.

Griinari JM, Bauman DE. Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk ruminants. In: Yurawecz MP, Mossoba MM, Kramer JKG, Pariza MW, Nelson G, editors. Advances in Conjugated Linoleic Acid Research, Volume 1. Champaign (IL): AOCS Press; 1999. p. 180-200.

Griinari JM, Corl BA, Lacy SH, Chouinard PY, Nurmela KVV, Bauman DE. Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by delta-9-desaturase. J Nutr. 2000;130:2285-91

Grundy SM, Bilheimer D, Blackburn D, Brown WV, Kwiterovich PO, Mattson F, Schonfeld G, Weidman WH. Rationale of the diet-heart statement of the American Heart Association. Report of Nutrition Committee. Circulation. 1982;65:A839-54.

Grundy SM, Cleeman JI, Merz CNB, Brewer HB, Clark LT, Hunninghake DB, Pasternak RC, Smith SC, Stone NJ. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. Arterioscler Thromb Vasc Biol. 2004;24:e149-61.

Grynberg A, Demaison L. Fatty acid oxidation in the heart. J Cardiovasc Pharmacol. 1996;28:S11-7.

Gurr MI, Harwood JL, Frayn KN. Lipid Boichemistry. 5th ed. Oxford (UK): Blackwell Science; 2002.

Guzman M, Klein W, del Pulgar TG, Geelen MJ. Metabolism of trans fatty acids by hepatocytes. Lipids. 1999;34:381-6.

Ha Y, Grimm N, Pariza M. Anticarcinogens from fried ground beef: heat altered derivatives of linoleic acid. Carcinogenesis. 1987;8:1881-7.

Ha Y, Storkson J, Pariza M. Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by conjugated derivatives of linoleic acid. Cancer Res. 1990;50:1097-101.

Hamosh M. Rat lingual lipase. Factors affecting enzyme activity and secretion. Am J Physiol. 1978;235:E416-21.

Hamosh M, Scow RO. Lingual lipase and its role in the digestion of dietary lipid. J Clin Invest. 1973;52:88-95.

Hansen AE, Haggard ME, Boelsche AN, Adam DJD, Wiese HF. Essential fatty acids in infant nutrition III. Clinical manifestations of linoleic acid deficiency. J Nutr. 1958;66:564-70.

Harris WS. Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. J Lipid Res. 1989;30:785-807.

Harris WS. Fatty acids and serum lipoproteins: human studies. Am J Clin Nutr. 1997;65:S1645-54.

Harris WS, Dujovne CA, Zucker M, Johnson B. Effects of a low saturated fat, low cholesterol fish oil supplement in hypertriglyceridemic patients. Ann Intern Med. 1988;109:465-70.

Hatahet W, Cole L, Kudchodkar BJ, Fungwe TV. Dietary fats differentially modulate the expression of lecithin:cholesterol acyltransferase, apoprotein-A1 and scavenger receptor b1 in rats. J Nutr. 2003;133:689-94.

He W, Barak Y, Hevener A, Olson P, Liao D, Le J, Nelson M, Ong E, Olefsky JM, Evans RM. Adipose-specific peroxisome proliferator-activated receptor γ knockout causes insulin resistance in fat and liver but not in muscle. Proc Natl Acad Sci USA. 2003;100:15712-7.

Heini AF, Weinsier RL. Divergent trends in obesity and fat intake patterns: the American paradox. Am J Med. 1997;102:259-64.

Hertz J, Bishara-Shieban J, Bar-Tana B. Mode of action of peroxisome proliferators as hypolipidemic drugs, suppression of apolipoprotein C-III. J Biol Chem. 1995;270:13470-5.

Hiltunen JK, Qin YM. β-oxidation – strategies for the metabolism of a wide variety of acyl-CoA esters. Biochim Biophys Acta. 2000;1484:117-28.

Hirai A, Terano T, Tamura Y, Yoshida S. Eicosapentaenoic acid and adult diseases in Japan: epidemiological and clinical aspects. J Intern Med. 1989;225:69-75.

Holman RT. Polyunsaturated fatty acid profiles in human disease. In: Bazan NG, Paoletti R, Iacono JM, editors. New Trends in Nutrition, Lipid Research and Cardiovascular Diseases. New York (NY): Alan R Liss; 1981. p. 25-42.

Holman RT, Johnson SB, Hatch TF. A case of human linolenic acid deficiency involving neurological abnormalities. Am J Clin Nutr. 1982;35:617-23.

Holman RT, Mahfouz MM. Cis and trans-octadecenoic acids as precursors of polyunsaturated acids. Prog Lipid Res. 1980;20:151-6.

Hontecillas R, Wannemeulher MJ, Zimmerman DR, Hutto DL, Wilson JH. Nutritional regulation of porcine bacterial-induced colitis by conjugated linoleic acid. J Nutr. 2002;132:2019-27.

House RL, Cassady JP, Eisen EJ, McIntosh MK, Odle J. Conjugated linoleic acid evokes de-lipidation through the regulation of genes controlling lipid metabolism in adipose and liver tissue. Obes Rev. 2005;6:247-58.

Houseknecht K, Vanden Huevel J, Moya-Camarena S, Portocarrero C, Peck L, Nickel K, Belury M. Dietary conjugated linoleic acid normalizes impaired glucose tolerance in Zucker diabetic fatty fa/fa rat. Biochem Biophys Res Commun. 1998;244:678-82.

Hsu MH, Savas U, Griffin KJ, Johnson EF. Identification of peroxisome proliferator-responsive human genes by elevated expression of the peroxisome proliferator-activated receptor α in HepG2 cells. J Biol Chem. 2001;276:27950-8.

Hu FB, Manson JE, Willett WC. Types of dietary fat and risk of coronary heart disease: a critical review. J Am Coll Nutr. 2001;20:5-19.

Hu FB, Stampfer MJ, Manson JE, AscherioA, Colditz GA, Speizer FE, Hennekens CH, Willett WC. Dietary saturated fat and their food sources in relation to the risk of coronary heat disease in women. Am J Clin Nutr. 1999;70:1001-8.

Hyun J, Kothari H, Herm E, Mortensen J, Treadwell CR, Vahouny GV. Purificatoin and properties of pancreatic juice cholesterol esterase. J Biol Chem. 1969;244:1937-45.

Iacono JM, Dougherty RM. Lack of effect of linoleic acid on the high-density lipoprotein-cholesterol fraction of plasma lipoproteins. Am J Clin Nutr 1991;53:660-4.

Ide T. Interaction of fish oil and conjugated linoleic acid in affecting hepatic activity of lipogenic enzymes and gene expression in liver and adipose tissue. Diabetes. 2005;54:412-23.

Ikeda I, Sasaki E, Yasunami H, Nomiyama S, Nakayama, M, Sugano M, Imaizumi K, Yazawa K. Digestion and lymphatic transport of eicosapentaenoic and docosahexaenoic acids given in the form of triacylglycerol, free acid and ethyl ester in rats. Biochim Biophys Acta. 1995;1259:297-304.

Ing NH, Spencer TE, Bazer FW. Estrogen enhances endometrial estrogen receptor gene expression by a posttranscriptional mechanism in the ovariectomized ewe. Biol Reprod. 1996;54:591-9.

Innis SM. Essential fatty acids in growth and development. Prog Lipid Res. 1991;30:39-103.

Inoue I, Shino K, Noji S, Awata T, Katayama S. Expression of peroxisome proliferator-activated receptor alpha (PPARα) in primary culture of human vascular endothelial cells. Biochem Biophys Res Commun. 1998;246:370-4.

Iso H, Sato S, Umemura U, Kudo M, Koike K, Kitamura A, Imano H, Okamura T, Naito Y, Shimamoto T. Linoleic acid, other fatty acids, and the risk of stroke. Stroke. 2002;33:2086-93.

Issemann I, Green S. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature. 1990;347:645-50.

IUPAC-IUB Commission on Biochemical Nomenclature (CNB) the nomenclature of lipids: recommendations 1976. Eur J Biochem. 1977;79:11-21.

Jackson RL, Kashyap ML, Barnhart RL, Allen C, Hogg E, Glueck CJ. Influence of polyunsaturated and saturated fats on plasmal lipids and lipoproteins in man. Am J Clin Nutr. 1984;39:589-97.

Jackson SM, Parhami F, Xi XP, Berliner JA, Hsueh WA, Law RE, Demer LL. Peroxisome proliferator-activated receptor activators target human endothelial cells to inhibit leukocyte-endothelial cell interaction. Arterioscler Thromb Vasc Biol. 1999;19:2094-104.

Jeffrey RW, Hellerstedt WL, French SA, Baxter JE. A randomized controlled trial of counseling for fat restriction versus calorie restriction in the treatment of obesity. Int J Obes Relat Metab Disord. 1995;19:132-7.

Jensen RG, DeJong FA, Clark RM. Determination of lipase specificity. Lipids. 1983;18:239-52.

Jiang J, Wolk A, Vessby B. Relation between the intake of milk fat and the occurrence of conjugated linoleic acid in human adipose tissue. Am J Clin Nutr. 1999;70:21-7.

Joshi AK, Witkowski A, Smith S. The malonyl/acetyl transfrease and β-ketoacyl synthase domains of the animal fatty acid synthase can cooperate with the acyl carrier protein domain of either subunit. Biochem. 1998;37:2515-23.

Judd JT, Clevidence BA, Muesing RA, Wittes J, Sunkin ME, Podczasy JJ. Dietary trans fatty acids: effects of plasma lipids and lipoproteins on healthy men and women. Am J Clin Nutr. 1994;59:861-8.

Jump DB, Clarke SD, Thelen A, Limatta M, Ren B, Badin M. Dietary polyunsaturated fatty acid regulation of gene transcription. Prog Lipid Res. 1996;35:227-41.

Jump DB, Clarke SD. Regulation of gene expression by dietary fat. Annu Rev Nutr. 1999;19:63-90.

Kang JX, Leaf A. Protective effects of free polyunsaturated fatty acids on arrhythmias induced by lysophosphatidylcholine or palmitoylcarnitine in neonatal rat cardiac myocytes. Eur J Pharmacol. 1996;297:97-106.

Katan MB, Zock PL. Trans fatty acids and their effects on lipoproteins in humans. Annu Nutr Rev. 1995;15:473-93.

Keelan M, Burdick S, Wirzba B, Thompson AB. Characterization of lipid intake into rabbit jejunal brush border membrane vesicles. Can J Physiol Pharmacol. 1992;70:1128-33.

Kehrer JP, Biswal SS, La E, Thuillier P, Datta K, Fischer SM, Vanden Heuvel JP. Inhibition of peroxisome-proliferator-activated receptor (PPAR)α by MK886. Biochem J. 2001;356:899-906.

Kelley DS, Bartolini GL, Warren JM, Simon VA, Mackey AE, Erickson KL. Contrasting effects of t10,c12- and c9,t11-conjugated linoleic acid isomers on the fatty acid profiles of mouse liver lipids. Lipids. 2004;39:135-41.

Kelly FD, Sinclair AJ, Mann NJ, Turner AH, Abedin L, Li D. A stearic acid-rich diet improves thrombogenic and atherogenic risk factor profiles in healthy males. Eur J Clin Nutr. 2001;55:88-96.

Kepler CR, Hirons KP, McNeill JJ, Tove SB. Intermediates and products of the biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*. J Biol Chem. 1966;241:1350-4.

Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B, Wahli W. Peroxisome proliferator-activated receptor α mediates the adaptive response to fasting. J Clin Invest. 1999;103:1489–98.

Keys A, Menotti A, Karvonen MJ, Aravanis C, Blackburn H, Buzina R, Djordjevic BS, Dontas AS, Fidanza F, Keys MH. The diet and 15-year death rate in the seven countries study. Am J Epidemiol. 1986;124:903-15.

Khosla P, Hajri T, Pronczuk A, Hayes KC. Replacing dietary palmitic acid with elaidic acid (t-C18:1 delta9) depresses HDL and increases CETP activity in cebus monkeys. J Nutr. 1997;127:531S-6.

Kim HJ, Lee KT, Lee MK, Jeon SM, Choi MS. Diacylglycerol-enriched structured lipids containing CLA and capric acid alter body fat mass and lipid metabolism in rats. Ann Nutr Metab. 2006;50:219-228.

Klenk E, Mohrhauer H. Studies on the metabolism of polyenoic fatty acids in the rat. Hoppe Seylers Z Physiol Chem. 1960;320:218-32.

Kliewer SA, Forman BM, Blumberg B, Ong ES, Borgmeyer U, Mangelsdorf DJ, Umesono K, Evans RM. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. Proc Natl Acad Sci USA. 1994;91:7355–9.

Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, Lehmann JM. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptora α and γ. Proc Natl Acad Sci USA. 1997;94:4318-23.

Kloss R, Linscheid J, Johnson A, Lawson B, Edwards K, Linder T, Stocker K, Petitte J, Kern M. Effects of conjugated linoleic acid supplementation on blood lipids and adiposity of rats fed diets rich in saturated versus unsaturated fat. Pharmacol Res. 2005;51:503-7.

Knoop F. Der Abbau aromatischer Fettsauren im Tierkorper. Beir. Chem. Physiol. Pathol. 1904;6:150-62.

Knowles JR. The mechanism of biotin-containing enzymes. Ann Rev Biochem. 1989;58:195-221.

Krey G, Braissant O, L'Horset F, Kalkhoven E, Perroud M, Parker MG, Wahli W. Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. Mol Endocrinol. 1997;11:779-91.

Kris-Etherton PM, Yu S. Individual fatty acids on plasma lipids and lipoproteins in human studies. Am. J. Clin. Nutr. 1997;65:S1628-44.

Kritchevsky D, Tepper SA, Wright S, Tso P, Czarnecki SK. Influence of conjugated linoleic acid (CLA) on establishment and progression of atherosclerosis in rabbits. J. Am. Coll. Nutr. 2000;19:S472-7.

Kritchevsky D, Tepper SA, Wright S, Czarnecki SK, Wilson TA, Nicolosi RJ. Conjugated linoleic acid isomer effects in atherosclerosis: growth and regression of lesions. Lipids. 2004;39:611-6.

Kromhout D, Bosschieter EB, de Lezenne Coulander C. The inverse relationship between fish consumption and 20-year mortality from coronary heart disease. Engl J Med. 1985;312:1205-9.

Kromhout D, Menotti A, Bloemberg B, Aravanis C, Blackburn H, Buzina R, Dontas AS, Fidanza F, Giaipaoli S, Jansen A, Karvonen M, Katan M, Nissinen A, Nedeljkovic S, Pekkanen J, Pekkarinen M, Punsar S, Rasanen L, Simic B, Toshima H. Dietary saturated and *trans* fatty acids and cholesterol and 25-year mortality from coronary heart disease: the Seven Countries Study. Prevent Med. 1995;24:308-15.

Kuan SI, Dupont J. Dietary fat and cholesterol effects on cholesterol metabolism in CBA/J and C57BR/cdJ mice. J Nutr. 1989;119:349-55.

Kunau WH, Dommes P. Degradation of unsaturated fatty acids. Identification of intermediates in the degradation of *cis*-4-decenoly-CoA by extracts of beef-liver mitochondria. Eur J Biochem. 1978;91:533-44.

Kunau WH, Dommes V, Schulz H. β-oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: a century of continued progress. Prog Lipid Res. 1995;34:267-342.

Kurushima H, Hayashi K, Toyota Y, Kambe M, Kajiyama G. Comparison of hypocholesterolemic effects induced by dietary linoleic acid and oleic acid in hamsters. Atherosclerosis. 1995;114:213-21.

Kurushima H, Hayashi K, Shingu T, Kuga Y, Ohtani H, Okura Y, Tanaka K, Yasunobu Y, Nomura K, Kajiyama G. Opposite effects on cholesterol metabolism and their mechanisms induced by dietary oleic acid and palmitic acid in hamsters. Biochim Biophys Acta. 1995;1258:251-6.

Lambe KG, Woodyatt NJ, Macdonald N, Chevalier S, Roberts RA. Species differences in sequence and activity of the peroxisome proliferator response element (PPRE) within the acyl CoA oxidase gene promoter. Toxicol Lett. 1999;110:119-27.

Langelier B, Furet JP, Perruchot MH, Alessandri JM. Docosahexaenoic acid membrane content and mRNA expression of acyl-CoA oxidase and of peroxisome proliferator-activated receptor-delta are modulated in Y79 retinoblastoma cells differently by low and high doses of alpha-linolenic acid. J Neurosci Res. 2003;74:134-41.

Larsen TM, Toubro S, Astrup A. Efficacy and safety of dietary supplements containing CLA for the treatment of obesity: evidence from animal and human studies. J Lipid Res. 2003;44:2234-41.

Larsen TM, Toubro S, Astrup A. Conjugated linoleic acid supplementation for 1 y does not prevent weight or body fat regain. Am J Clin Nutr. 2006;83:606-12.

Lawrence JW, Li Y, Chen S, DeLuca JG, Berger JP, Umbenhauer DR, Moller DE, Zhou G. Differential gene regulation in human versus rodent hepatocytes by peroxisome proliferator-activated receptor (PPAR) alpha. PPAR alpha fails to induce peroxisome proliferation-associated genes in human cells independently of the level of receptor expresson. J Biol Chem. 2001;276:31521-7.

Lazarow PB, de Duve C. A fatty acyl-CoA oxidizing system in rat liver peroxisomes; enhancement by clofibrate, a hypolipidemic drug. Proc Natl Acad Sci USA. 1976;73:2043-6.

Leaf A, Kang JX. Dietary n-3 fatty acids in the prevention of lethal cardiac arrhythmias. Curr Opin Lipidol. 1997;8:4-6.

Leblond CP, Bennett, G. In: Brinkley BR, Porter KR, editors. International Cell Biology. New York (NY): Rockerfeller Univ Press; 1977. p. 326-36.

Lee KN, Kritchevsky D, Pariza MW. Conjugated linoleic acid and atherosclerosis in rabbits. Atherosclerosis. 1994;108:19-25.

Lee KN, Pariza MW, Ntambi JM. Conjugated linoleic acid decreases hepatic stearoyl-CoA desaturase mRNA expression. Biochem Biophys Res Commun. 1998;248:817-21.

Lehmann JM, Lenhard JM, Oliver BB, Ringold GM, Kliewer SA. Peroxisome proliferator-activated receptors α and γ are activated by indomethacin and other non-steroidal anti-inflammatory drugs. J Biol Chem. 1997;272:3406-10.

Lehner R, Kuksis A, Itabashi Y. Stereospecificity of monoacylglycerol and diacylglycerol acyltransferases from rat intestine as determined by chiral phase high-performance liquid chromatography. Lipids. 1993;28:29-34.

Lehner R, Kuksis A. Biosynthesis of triacylglycerols. Prog Lipid Res. 1996;35:169-201.

Leibowitz MD, Fievet C, Hennuyer N, Peinado-Onsurbe J, Duez H, Bergera J, Cullinan CA, Sparrow CP, Baffic J, Berger GD, Santini C, Marquis RW, Tolman RL, Smith RG, Moller DE, Auwerx J. Activation of PPARdelta alters lipid metabolism in db/db mice. FEBS Lett. 2000;473:333-6.

Lemaiter RN, King IB, Mozaffarian D, Kuller LH, Tracy RP, Siscovick DS. n-3 polyunsaturated fatty acids, fatal ischemic heart disease, and nonfatal myocardial infarction in olfer adults: the Cardiovascular Health Study. Am J Clin Nutr. 2003;77:319-25.

Li L, Beauchamp MC, Renier G. Peroxisome proliferator-activated receptor alpha and gamma agonists upregulate human macrophage lipoprotein lipase expression. Atherosclerosis. 2002;165:101-10.

Lin H, Boylston T, Chang M, Luedecke L, Schultz T. Survey of the conjugated linoleic acid contents of dairy products. J Dairy Sci. 1995;78:2358-65.

Lovejoy JC, Smith SR, Champagne CM, Most MM, Lefevre M, DeLany JP, Denkins YM, Rood JC, Veldhuis J, Bray GA. Effects of diets enriched in saturated (palmitic), monounsaturated (oleic), or trans (elaidic) fatty acids on insulin sensitivity and substrate oxidation in healthy adults. Diabetes Care. 2002;25:1283-8.

Macarulla MT, Fernandez-Quintela A, Zabala A, Navarro V, Echevarria E, Churruca I, Rodriguez VM, Portillo MP. Effects of conjugated linoleic acid on liver composition and fatty acid oxidation are isomer-dependent in hamster. Nutrition. 2005;21:512-9.

Mahley RW, Bennett BD, Morre DJ, Gray ME, Thistlethwaist W, LeQuire VS. Lipoproteins associated with the Golgi apparatus isolated from epithelial cells of rat small intestine. Lab Invest. 1971;25:435-43.

Malmendier CL, Delcroix C. Effects of of fenofibrate on high and low density lipoprotein metabolism in heterozygous familial hypercholesterolemia. Atherosclerosis. 1985;55:161-9.

Mannaerts GP, van Veldhoven PP, Casteels M. Peroxisomal lipid degradation via beta- and alpha-oxidation in mammals. Cell Biochem Biophys. 2000;32:73-87.

Martin G, Schoonjans K, Lefebvre AM, Staels B, Auwerx J. Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARα and PPARγ activators. J Biol Chem. 1997;272:28210-7.

Mata P, Alonso R, Lopez-Farre A, Ordovas JM, Lahoz C, Garces C, Caramelo C, Codoceo R, Blazquez E, de Oya M. Effect of dietary fat saturation on LDL oxidation and monocyte adhesion to human endothelial cells in vitro. Arterioscler Thromb Vasc Biol. 1996;16:1347-55.

Mata P, Alvarezsala LA, Ubio MJ, Nuno J, de Oya M. Effects of long-term monounsaturated-enriched diets on lipoproteins in healthy men and women. Am J Clin Nutr. 1992;55:846-50.

Mattson FH, Volpenhein RA. Rearrangement of glyceride fatty acids during digestion and absorption. J Biol Chem. 1962;237:53-5.

Mattson FH, Volpenhein RA. The digestion and absorption of triglycerides. J Biol Chem. 1964;239:2772-7.

Mauger J-F, Lichtenstein AH, Ausman LM, Jalbert SM, Jauhiainen M, Ehnholm C, Lamarche B. Effect of different forms of dietary hydrogenated fats on LDL particle size. Am J Clin Nutr. 2003;78:370-5.

Mead JF. Progress in the Chemistry of Fats and Other Lipids. Volume IX. 1968. p. 159-92.

Medina EA, Horn WF, Keim NL, Havel PJ, Benito P, Kelley DS, Nelson GJ, Erickson KL. Conjugated linoleic acid supplementation in humans. Effects on circulating leptin concentrations and appetite. Lipids. 2000;35:783-88.

Meijer GW, van Tol A, van Berkel TJ, Weststrate JA. Effect of dietary elaidic versus vaccenic acid on blood and liver lipids in the hamster. Atherosclerosis. 2001;157:31-40.

Mellies MJ, Stein EA, Khoury P, Lamkin G, Glueck CJ. Effects of fenofibrate on lipids, lipoproteins and apolipoproteins in 33 subjects with primary hypercholesterolaemia. Atherosclerosis. 1987;63:57-64.

Mensink RP, Katan MB. Effect of dietary trans fatty acids on high-density and low-density lipoprotein cholesterol levels in healthy subjects. N Engl J Med. 1990;323:439-45.

Meyer JH, Jones RS. Canine pancreatic responses to intestinally perfused fat and products of fat digestion. Am J Physiol. 1974;226:1178-87.

Miller C, Park Y, Pariza M, Cook M. Feeding conjugated linoleic acid to animals partially overcomes catabolic responses due to endotoxin injection. Biochem. Biophys Res Commun. 1994;198:1107-12.

Mitchell PL, Langille MA, Currie DL, McLeod RS. Effect of conjugated linoleic acid isomers on lipoproteins and atherosclerosis in the Syrian Golden hamster. Biochim Biophys Acta. 2005;1734:269-76.

Mitropoulos KA, Miller GJ, Martin JC, Reeves BE, Cooper J. Dietary fat induces changes in factor VII coagulant activity through effects on plasma free stearic acid concentration. Arterioscler Thromb. 1994;14:214-22.

Miyata KS, McCaw SE, Marcus SL, Rachubinski RA, Capone JP. The peroxisome proliferator-activated receptor interacts with the retinoid X receptor in vivo. Gene. 1994;148:327-30.

Moon YA, Shah NA, Mohapatra S, Warrington JA, Horton JD. Identification of a mammalian long chain fatty acyl elongase regulated by sterol regulatory element-binding proteins. J Biol Chem. 2001;276:45358-66.

Motojima K, Passilly P, Peters JM, Gonzales FJ, Latruffe N. Expression of putative fatty acid transporter genes are regulated by peroxisome proliferator-activated receptor alpha and gamma activators in a tissue- and inducer-specific manner. J Biol Chem. 1998;273:16710-4.

Moya-Camarena SY, Vanden Heuvel JP, Blanchard SG, Leesnitzer LA, Belury MA. Conjugated linoleic acid is a potent naturally occurring ligand and activator of PPARalpha. J Lipid Res. 1999;40:1426-33.

Mozaffarian D, Pischon T, Hankinson SE, Rifai N, Joshipura K, Willett WC, Rimm EB. Dietary intake of trans fatty acids and systemic inflammation in women. Am J Clin Nutr. 2004;79:606-12.

Mu H, Hoy C-E. The digestion of dietary triacylglycerols. Prog Lipid Res. 2004;43:105-33.

Muoio DM, MacLean PS, Lang DB, Li S, Houmard JA, Way JM, Winegar DA, Corton JC, Dohm GL, Kraus WE. Fatty acid homeostasis and induction of lipid regulatory genes in skeletal muscles of peroxisome proliferators-activated receptor α knock-out mice: evidence for compensatory regulation by PPARδ. J Biol Chem. 2002;277:26089-97.

Nagata YJ, Chen J, Cooper AD. Role of low density lipoprotein receptor-dependent and -independent sites in the binding and uptake of chylomicron remnants in rat liver. J Biol Chem. 1988;263:15151-8.

Nagy L, Tontonoz P, Alvarez JGA, Chen H, Evans RM. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARγ. Cell. 1998;93:229-40.

Nawa T, Nawa MT, Cai Y, Zhang C, Uchimura I, Narumi S, Numano F, Kitajima S. Repression of TNF-alpha-induced E-selectin expression by PPAR activators: involvement of transcriptional repressor LRF-1/ATF3. BiochemS Biophys Res Commun. 2000;275:406-11.

Nestel P, Noakes M, Belling B. Plasma lipoprotein and Lp[a] changes with substitution of elaidic acid for oleic acind in the diet. J Lipid Res. 1992;33:1029-36.

Nicolosi RJ, Wilson TA, Rogers EJ, Kritchevsky D. Effects of specific fatty acids (8:0, 14:0, cis-18:1, trans-18:1) on plasma lipoproteins, early atherogenic potential, and LDL oxidative properties in the hamster. J Lipid Res. 1998;39:1972-80.

Nicolosi RJ, Woolfrey B, Wilson TA, Scollin P, Handelman G, Fisher R. Decreased aortic early atherosclerosis and associated risk factors in hypercholesterolemic hamsters fed a high- or mid-oleic acid oil compared to a high-linoleic acid oil. J Nutr Biochem. 2004;15:540-7.

Nielsen SJ, Siega-Riz AM, Popkin BM. Trends in energy intake in US between 1977 and 1996: similar shifts seen across age groups. Obes Res. 2002;10:370-8.

Nolte RT, Wisely GB, Westin S, Cobb JE, Lambert MH, Kurokawa R, Rosenfeld MG, Willson TM, Glass CK, Milburn MV. Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. Nature. 1998;395:137-43.

Ockner RK, Hughes FB, Isselbacher KJ. Very low density lipoproteins in intestinal lymph: role in triglyceride and cholesterol transport during fat absorption. J Clin Invest. 1969;48:2367-73.

Oliver WR, Shenk JL, Snaith MR, Russell CS, Plunket KD, Bodkin NL, Lewis MC, Winegar DA, Sznaidman ML, Lambert MH, Xu HE, Sternbach DD, Kliewer SA, Hansen BC, Willson TM. A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. Proc Natl Acad Sci USA. 2001;98:5306-11.

Oomen CM, Feskens EJ, Rasanen L, Fidanza F, Nissinen AM, Menotti A, Kok FJ, Kromhout D. Fish consumption and coronary heart disease mortality in Finland, Italy, and the Netherlands. Am J Epidemiol. 2000;151:999-1006.

Palmer CN, Hsu MH, Griffin KJ, Raucy JL, Johnson EF. Peroxisome proliferator-activated receptor expression in human liver. Mol Pharmacol. 1998;53:14-22.

Pande SV. A mitochondrial carnitine acylcarnitine translocase system. Proc Natl Acad Sci USA. 1975;72:883-7.

Pariza MW, Hargraves WA. A beef-derived mutagenesis modulator inhibits initation of mouse epidermal tumors by 7,12-dimethylbenz[a]anthracene. Carcinogenesis. 1985;6:591-3.

Pariza MW, Park Y, Cook ME. Mechanisms of action of conjugated linoleic acid: evidence and speculation. Proc Soc Exptl Biol Med. 2000;241:8-13.

Park Y, Albright K, Liu W, Storkson JM, Cook ME, Pariza MW. Effect of conjugated linoleic acid on body composition in mice. Lipids. 1997;32:853-8.

Park Y, Albright KJ, Storkson JM, Liu W, Cook ME, Pariza MW. Changes in body composition in mice during feeding and withdrawl of conjugated linoleic acid. Lipids. 2001;34:243-8.

Park Y, McGuire MK, Behr R, McGuire MA, Evans MA, Shultz TD. High-fat dairy product consumption decreases delta 9c,11t-18:2 (rumenic acid) and total lipid concentrations of human milk. Lipids. 1999a;34:543-9.

Park Y, Storkson JM, Albright KJ, Liu W, Pariza MW. Evidence that the *trans*-10, *cis*-12 isomer of conjugated linoleic acid induces body composition changes in mice. Lipids. 1999b;34:235-41.

Parker A, Engel PC. Preliminary evidence for the existence of specific functional assemblies between enzymes of the beta-oxidation pathway and the respiratory chain. Biochem J. 2000;345:429-35.

Parkinson AJ, Cruz AL, Heyward WL, Bulkow LR, Hall D, Barstaed L, Connor WE. Elevated concentrations of placma omega-3 polyunsaturated fatty acids among Alaskan Eskimos. Am J Clin Nutr. 1994;59:384-8.

Pasceri V, Wu H, Willerson J, Yeh ETH. Modulation of vascular inflammation in vitro and in vivo by peroxisome proliferator-activated receptor γ activators. Circulation. 2000;101:235-8.

Patton JS. Gastrointestinal lipid digestion. In: Johnston LR, editor. Physiology of the Gastrointestinal Tract. New York (NY): Raven Press; 1981. p. 1123-46.

Pedersen JI, Ringstad J, Almendingen K, Haugen TS, Stensvold I, Thelle DS. Adipose tissue fatty acids and risk of myocardial infarction – a case control study. Eur J Clin Nutr. 2000;54:618-25.

Peters JM, Lee SS, Li W, Ward JM, Gavrilova O, Everett C, Reitman ML, Hudson LD, Gonzalez FJ. Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor β(δ). Mol Cell Biol. 2000;20:5119–28.

Peters JM, Park Y, Gonzalez FJ, Pariza MW. Influence of conjugated linoleic acid on body composition and target gene expression in peroxisome proliferator-activated receptor alpha-null mice. Biochim Biophys Acta. 2001;1533:233-42.

Pollard MR, Gunstone FD, James AT, Morris LJ. Desaturation of positional and geometric isomers of monoenoic fatty acids by microsomal preparations from rat liver. Lipids. 1980;15:306-14.

Posner BM, Cobb JL, Belanger AJ, Cupples LA, D'Agostino RB, Stokes J. Dietary lipid predictors of coronary heart disease in men. The Framingham Study. Arch Intern Med. 1991;151:1181-7.

Pownall HJ, Pao Q, Massey JB. Acyl chain and headgroup specificity of human lecithin:cholesterol acyltransferase. J Biol Chem. 1985;260:2146-52.

Qiu X, Hong H, MacKenzie SL. Identification of a Delta 4 fatty acid desaturase from Thraustochytrium sp. involved in the biosynthesis of docosahexanoic acid by heterologous expression in Saccharomyces cerevisiae and Brassica juncea. J Biol Chem. 2001;276:31561-6.

Ramsay RR, Tubbs PK. The mechanism of fatty acid uptake by heart mitochondria: an acylcarnitine-carnitine exchange. FEBS Lett. 1975;54:21-5.

Reddy JK, Hashimoto T. Peroxisomal β-oxidation and peroxisome proliferator-activated receptor α: an adaptive metabolic system. Annu Rev Nutr. 2001;21:193-230.

Reddy KS, Katan MB. Diet, nutrition and the prevention of hypertension and cardiovascular diseases. Pub Health Nutr. 2004;7:167-86.

Redgrave TG, Small DM. Quantitation of the transfer of surface phospholipid of chylomicrons to the high-density lipoprotein fraction during the catabolism of chylomicrons in the rat. J Clin Invest. 1979;64:162-71.

Ren B, Thelen AP, Peters JM, Gonzales FJ, Jump DB. Polyunsaturated fatty acid suppression of hepatic fatty acid synthase and S14 gene expression does not require peroxisome proliferator-activated receptor α. J Biol Chem. 1997;272:26827-32.

Rickert R, Steinhart H, Fristche J, Sehat N, Yurawecz MP, Mossoba MM, Roach JAG, Eulitz K, Ku Y, Kramer JKG. Enhanced resolution of conjugated linoleic acid isomers by tandem-column silver ion high performance liquid chromatography. J High Resolut Chromatogr. 1999;22:144-8.

Riemersma RA, Wood DA, Butler S, Elton RA, Oliver M, Salo M, Nikkari T, Vartiainen E, Puska P, Gey F. Linoleic acid content in adipose tissue and coronary heart disease. Br Med J. 1986;292:1423-7.

Rise P, Galli C. Arachidonic and docosahexaenoic acids differentially affect the expression of fatty acyl-CoA oxidase, protein kinase C and lipid peroxidation in HepG2 cells. Prostaglandins Leukot Essent Fatty Acids. 1999;60:367-70.

Riserus U, Smedman A, Basu S, Vessby B. Metabolic effects of conjugated linoleic acid in humans: the Swedish experience. Am J Clin Nutr. 2004;79:S1146-8.

Ritzenthaler KL, McGuire MK, Falen R, Shultz TD, Dasgupta N, McGuire MA. Estimation of conjugated linoleic acid intake by written dietary assessment methodologies underestimates actual intake evaluated by food duplicatd methodology. J Nutr. 2001;131:1548-54.

Rizek RL, Friend B, Page L. Fat in today's food supply – level of use and source. J Am Oil Chem Soc. 1974;51:244-50.

Rodriguez JC, Gil-Gomez G, Hegardt FG, Haro D. Peroxisome proliferator-activated receptor mediates induction of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by fatty acids. J Biol Chem. 1994;269:18767-72.

Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM. Transcriptional regulation of adipogenesis. Genes Dev. 2000;14:1293–1307.

Ross SR, Graves RA, Spiegelman BM. Targeted expression of a toxin gene to adipose tissue: transgenic mice resistant to obesity. Genes Dev. 1993;7:1318-24.

Ryder JW, Portocarrero CP, Song XM, Cui L, Yu M, Conbatsiaris T, Galuska D, Bauman DE, Barbano DM, Charron MJ, Zierath JR, KL. Isomer-specific anti-diabetic properties of conjugated linoleic acid. Improved glucose tolerance, skeletal muscle insulin action, and UCP-2 gene expression. Diabetes. 2001;50:1149-57.

Sabesin SM, Frase S. Electron microscopic studies of the assembly, intracellular transport, and secretion of chylomicrons by rat intestine. J Lipid Res. 1977;19:496-511.

Sampath H, Ntambi JM. Polyunsaturated fatty acid regulation of genes of lipid metabolism. Annu Rev Nutr. 2005;25:317-40.

Sanders TAB. High- versus low-fat diets in human diseases. Curr Opin Clin Nutr Metab Care. 2003;6:151-5.

Santora JE, Palmquist DL, Roehrig KL. *Trans*-vaccenic acid is desaturated to conjugated linoleic acid in mice. J Nutr. 2000;130:208-15.

Schaefer EJ, Kay LL, Zech LA, Brewer HB Jr. Tangier disease. High-density lipoprotein deficiency due to defective metabolism of an abnormal apolipoprotein A-I (ApoA-I Tangier). J Clin Invest. 1982;70:934-45.

Scheuerbrandt G, Bloch K. Unsaturated fatty acids in microorganisms. J Biol Chem. 1962;237:2064-8.

Schmidt EB, Dyerberg J. Omega-3 fatty acids. Current status in cardiovascular medicine. Drugs. 1994;47:405-24.

Schoonjans K, Staels B, Auwerx J. Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. J Lipid Res. 1996;37:907-25.

Schoonjans K, Watanabe M, Suzuki H, Mahfoudi A, Krey G, Wahli W, Grimaldi P, Staels B, Yamamoto T, Auwerx J. Induction of acyl-coenzyme A synthetase gene by fibrates and fatty acids is mediated by a peroxisome proliferator response element in the C promoter. J Biol Chem. 1995;270:19269-76.

Schroepfer GJ, Bloch K. The stereospecific conversion of stearic acid to oleic acid. J Biol Chem. 1965;240:54-63.

Sethi S, Gibney MJ, Williams CM. Postprandial lipoprotein metabolism. Nutr Res Rev. 1993;6:161-83.

Seubert W, Podack ER. Mechanisms and physiological roles of fatty acid chain elongation in microsomes and mitochondria. Mol Cell Biochem. 1973;1:29-40.

Shacter E, Chock PB, Rhee SG, Stadtman E. Cyclic cascades and metabolic regulation. In: Boyer PD, Krebs EG, editors. The Enzymes. San Diego (CA): Academic Press; 1986. pp. 23-25.

Shepherd J, Packard CJ, Patsch JR, Grotto AM, Taunton OD. Effects of dietary polyunsaturated and saturated fat on the properties of high-density lipoproteins and the metabolism of apoliprotein A-I. Am J Clin Invest. 1978;61:1582-92.

Sher T, Yi HF, McBride OW, Gonzales FJ. cDNA cloning, chromosomal mapping and functional characterization of the human peroxisome proliferator-activated receptor. Biochem. 1993;32:5598-604.

Simon JA, Hodgkins ML, Browner WS, Neuhaus JM, Bernert JT, Hulley SB. Serum fatty acids and the risk of coronary heart disease. Am J Epidemiol. 1995;142:469-76.

Simopoulos AP. Essential fatty acids in health and chronic disesase. Am J Clin Nutr. 1999;70:S560-9.

Simopoulos AP, Leaf A, Salem N. Workshop statement on the essentiality of and recommended dietary intakes for omegs-6 and omega-3 fatty acids. Prost Leuk Ess Fatty Acids. 2000;63:119-21.

Simpson HC, Barker K, Carter RD, Cassels E, Mann JI. Low dietary intake of linoleic acid predisposes to myocardial infarction. Br Med J. 1982;285:683-4.

Sinclair HM. Essential fatty acids – an historical perspective. Biochem Soc Trans. 1992;18:756-61.

Singh I, Moser AB, Goldfischer S, Moser H. Lignoceric acid is oxidized in the peroxisome: implications for the Zellweger cerebro-hepato-renal syndrome and adrenoleukodystrophy. Proc Acad Natl Sci USA. 1984;81:4203-7.

Siscovick DS, Raghunathan TE, King I, Weinmann S, Wicklund KG, Albright J, Bovbjerg V, Arbogast P, Smith H, Kushi LH. Dietary intake and cell membrane levels of long-chain n-3 polyunsaturated fatty acid and the risk of primary cardiac arrest. JAMA. 1995;274:1363-7.

Sisk MB, Hausman DB, Martin RJ, Azain MJ. Dietary conjugated linoleic acid reduces adiposity in lean but not obese Zucker rats. J Nutr. 2001;131:1668-74.

Smedman A, Vessby B. Conjugated linoleic acid supplementation in humans – metabolic effects. Lipids. 2001;36:773-81.

Smith A. Oxford Dictionary of Biochemistry and Molecualr Biology, 2nd ed. Oxford (UK): Oxford University Press; 2000.

Smith S. The animal fatty acid synthase: one gene, one polypeptide, seven enzymes. FASEB J. 1994;8:1248-59.

Sood V, Colleran K, Burge MR. Thiszolidinediones: a comparative review of approved uses. Diabetes Technol Ther. 2000;2:429-40.

Sprecher H. Biochemistry of essential fatty acids. Prog Lipid Res. 1981;20:13-22.

Sprecher H, Luthria DL, Mohammed BS, Baykousheva SP. Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. J Lipid Res. 1995;36:2471-7.

Staels B, Koening W, Habib A, Merval R, Lebret M, Torra IP, Delerive P, Fadel A, Chinetti G, Fruchart JC, Najib J, Maclouf J, Tedgui A. Activation of human aortic smooth muscle cells is inhibited by PPARα but not by PPARγ activators. Nature. 1998;393:790-3.

Staels B, Vu-Dac N, Kosykh V, Saladin R, Fruchart JC, Dallongeville J, Auwerx J. Fibrates down-regulate apolipoprotein C-III expression independent of induction of peroxisomal acyl-CoA oxidase. J Clin Invest. 1995;95:705-12.

Stangl GI, Muller H, Kirchgessner M. Conjugated linoleic acid effects on circulating hormones, metabolites and lipoproteins, and its proportion in fasting serum and erythrocyte membranes of swine. Eur J Nutr. 1999;38:271-7.

Stoffel W, Caesar H. Metabolism of unsaturated fatty acids. V. On the beta-oxidation of mono- and polyene-fatty acids. Mechanism of enzymatic reactions of delta-2-cis-enoyl-CoA compounds. Hoppe Seylers Z Physiol Chem. 1965;341:76-83.

Stritmatter P, Spatz L, Corcoran D, Rogers MJ, Setlow B, Redline B. Purification and properties of reat liver microsomal stearyl coenzyme A desaturase. Proc Natl Acad Sci USA. 1974;71:4565-9.

Sundram K, Ismail A, Hays KC, Jeyamalar R, Pathmanathan R. Trans (elaidic) fatty acids adversely affect the lipoprotein profile relative to specific saturated fatty acids in humans. J Nutr. 1997;127:S514-20.

Suneja SK, Osei P, Cook L, Nagi MN, Cinti DL. Enzyme site-specific changes in hepatic microsomal fatty acid chain elongation in streptozotocin-induced diabetic rats. Biochim Biophys Acta. 1990;1042:81-5.

Szymczyk B, Pisulewski P, Szczurek W, Hanczakowski P. The effects of feeding conjugated linoleic acid (CLA) on rat growth performance, serum lipoproteins and subsequent lipid composition of selected rat tissues. J Sci Food Agric. 2000;80:1553-8.

Takahashi Y, Kushiro M, Shinohara K, Ide T. Activity and mRNA levels of enzymes involved in hepatic fatty acid synthesis and oxidation in mice fed conjugated linoleic acid. Biochim Biophys Acta. 2003;1631:265-73.

Tall AR. Plasma cholesterol ester transfer protein. J Lipid Res. 1993;34:1255-74.

Tanaka T, Yamamoto J, Iwasaki S, Asaba H, Hamura H, Ikeda Y, Watanabe M, Magoori K, Ioka RX, Tachibana K, Watanabe Y, Uchiyama Y, Sumi K, Iguchi H, Ito S, Doi T, Hamakubo T, Naito M, Auwerx J, Yanagisawa M, Kodama T, Sakai J. Activation of peroxisome proliferator-activated receptor delta induces fatty acid beta-oxidation in skeletal muscle and attenuates metabolic syndrome. Proc Natl Acad Sci USA. 2003;100:15924-9.

Terpstra AH. Effect of conjugated linoleic acid on body composition and plasma lipids in humans: an overview of the literature. Am J Clin Nutr. 2004;79:352-61.

Thiel-Cooper RL, Parrish FC, Sparks JC, Weigand BR, Ewan RC. Conjugated linoleic acid changes swine performance and carcass composition. J Anim Sci. 2001;79:1821-8.

Tholstrup T, Raff M, Basu S, Nonboe P, Sejrsen K, Straarup EM. Effects of butter high in ruminant trans and monounsaturated fatty acids on lipoproteins, incorporation of fatty acids into lipid classes, plasma C-reactive protein, oxidative stress, hemostatic variables, and insulin in healthy young men. Am J Clin Nutr. 2006:83:237-43.

Tollefson JH, Ravnik S, Albers JJ. Isolatin and characterization of a phospholipids transfer protein (LTP-II) from human plasma. J Lipid Res. 1988;29:1593-1602.

Tomkiewicz C, Muzeau F, Edgar AD, Barouki R, Aggerbeck M. Opposite regulation of the rat and human cytosolic aspartate aminotransferase genes by fibrates. Biochem Pharmacol. 2004;67:213-25.

Tordjman K, Bernal-Mizrachi C, Zemany L, Weng S, Feng C, Zhang F, Leone TC, Coleman T, Kelly DP, Semenkovich CF. PPARalpha deficiency reduces insulin resistance and atherosclerosis in apoE-null mice. J Clin Invest. 2001;107:1025-34.

Torra IP, Chinetti G, Duval C, Fruchart JC, Staels B. Peroxisome proliferator-activated receptors: from transcriptional control to clinical practice. Curr Opin Lipidol. 2001;3:245-54.

Truitt A, McNeill G, Vanderhoek JY. Antiplatelet effects of conjugated linoleic acid isomers. Biochim Boiphys Acta. 1999;1438:239-46.

Tsuboyama-Kasaoka N, Takahashi M, Tanemura K, Him H-J, Tange T, Okuyama H, Kasai M, Ikemoto S, Ezaki O. Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. Diabetes. 2000;49:1534-42.

Tugwood JD, Issemann I, Anderson RG, Bundell KR, McPheat WL, Green S. The mouse peroxisome proliferator activated receptor recognizes a response element in the 5' flanking sequence of the rat acyl CoA oxidase gene. EMBO J. 1992;11:433-9.

Turpeinen AM, Mutanen M, Aro A, Salminen I, Basu S, Palmquist DL, Griinari JM. Bioconversion of vaccenic acid to conjugated linoleic acid in humans. Am J Clin Nutr. 2002;76:504-10.

Utermann G. The mysteries of lipoprotein (a). Science. 1989;246:904-10.

van Deenen LLM, deHaas GH. Hydrolysis of synthetic mixed phosphatides by phospholipase A from human pancreas. Biochim Biophys Acta. 1963;67:295-306.

Vanden Heuvel JP, Kreder D, Belda B, Hannon DB, Nugent CA, Burns KA, Taylor MJ. Comprehensive analysis of gene expression in rat and human hepatoma cells exposed to the peroxisome proliferator WY14,643. Toxicol Appl Pharmacol. 2003;188:185-98.

Vogel RA, Corretti MC, Plotnick GD. The postprandial effect of components of the Mediterranean diet on endothelial function. J Am Coll Cardiol. 2000;36:1455-60.

Vosper H, Patel L, Graham TL, Khoudoli GA, Hill A, Macphee CH, Pinto I, Smith SA, Suckling KE, Wolf CR, Palmer CN. The peroxisome proliferator-activated receptor delta promotes lipid accumulation in human macrophages. J Biol Chem. 2001;276:44258-65.

Wahle KW, Heys SD, Rotondo D. Conjugated linoleic acids: are they beneficial or detrimental to health? Prog Lipid Res. 2004;43:553-87.

Wahli W, Martinez E. Superfamily of steroid nuclear receptors: positive and negative regulators of gene expression. FASEB J. 1991;5:2243-9.

Wakil SJ. Enzymatic synthesis of fatty acids. Comp Biochem Physiol. 1962;4:123-58

Wakil SJ, Pugh EL, Sauer F. The mechanism of fatty acid synthesis. Proc Natl Acad Sci USA. 1964;52:106-14.

Wakil SJ, Titcheher EB, Gibson DM. Evidence for the participation of biotin in the enzymic synthesis of fatty acids. Biochim Biophys Acta. 1958;29:225-6.

Wang M, Briggs MR. HDL: the metabolism, function, and therapeutic importance. Chem Rev. 2004;104:119-37.

Wang Y, Jones PJ. Dietary conjugated linoleic acid and body composition. Am J Clin Nutr. 2004;79:S1153-8.

Warren JM, Simon VA, Bartolini G, Erickson KL, Mackey BE, Kelley DS. Trans-10, cis-12 CLA increases liver and decreases adipose tissue lipids in mice: possible roles of specific lipid metabolism genes. Lipids. 2003;38:497-504.

Way JM, Harrington WW, Brown KK, Gottschalk WK, Sundseth SS, Mansfield TA, Ramachandran RK, Wilson TM, Kliewer SA. Comprehensive messenger ribonucleic acid profiling reveals the peroxisome proliferator-activated receptor gamma activation has coordinate effects on gene expressionin multiple insulin-sensitive tissues. Endocrinology. 2001;142:1269-77.

West DB, York B. Dietary fat, genetic predisposition, and obesity: lessons from animal models. Am J Clin Nutr. 1998;67:S505-12.

Westhuyzen J. The oxidation hypothesis of atherosclerosis: an update. Ann Clin Lab Sci. 1997;27:1-10.

Willett WC, Ascherio A. Trans fatty acids: are the effects only marginal? Am J Public Health. 1994;84:722-4.

Wilson FA, Sallee VL, Dietschy JM. Unstirred water layers in intestine: rate determinant of fatty acid absorption from micellar solutions. Science. 1971;174:1031-3.

Woodyatt NJ, Lambe KG, Myers KA, Tugwood JD, Roberts RA. The peroxisome proliferator (PP) response element upstream of the human acyl CoA oxidase gene is inactive among a sample human population: significance for species differences in response to PPs. Carcinogenesis. 1999;20:369-72.

World Health Report 2002; Reducing Risks to health, promoting healthy life. 2002.

Xu HE, Lambert MH, Montana VG, Parks DJ, Blanchard SG, Brown PJ, Sternbach DD, Lehmann JM, Wisely GB, Willson TM, Kliewer SA Milburn MV. Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. Mol Cell. 1999;3:397-403.

BIOGRAPHICAL SKETCH

Elizabeth Sarah Greene was born in Turnersville, New Jersey in 1980. She is the daughter of Dr. David and Hilary Johnson. She graduated with a Bachelor of Science degree in microbiology and cell science from the University of Florida in May 2002. After graduation, she began work on her Doctor of Philosophy degree under the guidance of Dr. Lokenga Badinga in the Department of Animal Sciences, at the University of Florida. Her research focused on the effects of fatty acids on lipid metabolism in human and rat liver. After graduating, Elizabeth plans to move to Texas with her husband and pursue certification in Health Education.