Effects of Conjugated Linoleic Acids on Lipid Metabolizing Genes and High-Density Lipoprotein Cholesterol Production in Human Hepatocytes

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ABSTRACT

Conjugated linoleic acids (CLA) are known to reduce body fat and plasma lipids in animal models. This study examined the short-term effects of 2 biologically active isomers of CLA (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) on lipid-metabolizing genes and high-density lipoprotein (HDL) cholesterol production in cultured HepG2 cells. Steady-state levels of acyl CoA oxidase (ACO); 3-hydroxy, 3-methylglutaryl CoA reductase (HMG-R); and apolipoprotein A-I (Apo A-I) mRNA were examined after 24-hour incubation in the absence (control) or presence of 100 μm each of linoleic acid (LA), *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA. Concentrations of ACO and HMG-R mRNA transcripts were increased in HepG2 cells treated with *trans*-10, *cis*-12 CLA. Incubation with MK886, a specific PPARα inhibitor, had minimal effect on basal or CLA-induced gene expression in HepG2 cells. The *cis*-9, *trans*-11, but not *trans*-10, *cis*-12, CLA decreased HDL cholesterol concentration in cell-conditioned media. There was no apparent relationship between Apo A-I and HDL cholesterol responses to fatty acids. Results indicate that CLA may control peroxisomal oxidation of fatty acids through up-regulation of hepatic ACO gene expression. The physiological relevance of CLA effect on HMG-R mRNA content in the liver is yet to be determined.

INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term describing a mixture of positional and geometric dienoic isomers of linoleic acid (LA). To date, up to 17 CLA isomers have been described, with double bonds ranging in position from carbons 6 and 8 to carbons 12 and 14.[1] Unlike naturally occurring unsaturated fatty acids, the double bonds in CLA occur on adjacent carbons and are not separated by a methylene group. Essentially all *cis*- and *trans*- isomeric

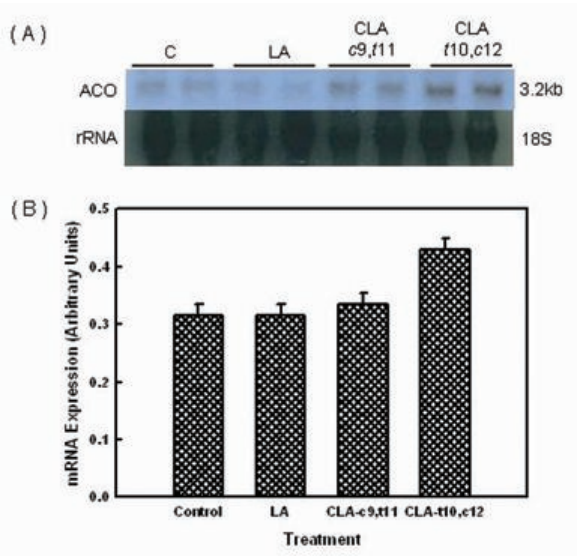

Figure 1. Effect of conjugated linoleic acid (CLA) on acyl CoA oxidase (ACO) mRNA content in HepG2 cells. Representative Northern blot (A) and densitometric values (B) calculated over 2 independent experiments (n = 4 for each treatment). Means without a common letter differ ($P<0.05$).

conformations of CLA have been identified in food. However, the predominant CLA in ruminant fats is the *cis*-9, *trans*-11 isomer, which accounts for more than 80% of total CLA isomers in dairy products and 75% of those in beef fats.[2] This isomer originates from CLA produced by rumen bacteria as an intermediate in the biohydrogenation of LA or from tissue synthesis of CLA by Δ^9-desaturase conversion of *trans*-11 18:1 fatty acid[1]. Because mammals do not possess Δ^{12}-desaturase they cannot convert *trans*-10 octadecenoic acid to *trans*-10, *cis*-12 CLA. Consequently, the *trans*-10, *cis*-12 CLA reported in ruminant tissues likely originates solely from *trans*-10, *cis*-12 that is absorbed from the gastrointestinal tract.[3] Recent studies have shown that the 2 main isoforms of CLA can have different effects on metabolism and cell function and may act through different signaling pathways.[4]

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that regulate multiple physiological processes, including inflammation, development, and lipid metabolism.[5] Tissue distribution of PPARα and PPARγ gene transcripts indicates that they play roles in fatty acid catabolism[6,7] and adipogenesis,[8,9] respectively. Much less is known about the function and regulation of PPAR∂, although it is highly expressed in the brain, colon, and skin.[10-12]

The objective of this study was to examine the short-term effects of the 2 biologically active isomers of CLA on lipid-metabolizing gene expression and high-density lipoprotein (HDL) cholesterol production in HepG2 cells.

Based on both dietary and in vitro studies of lipid metabolism, we hypothesized that the 2 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

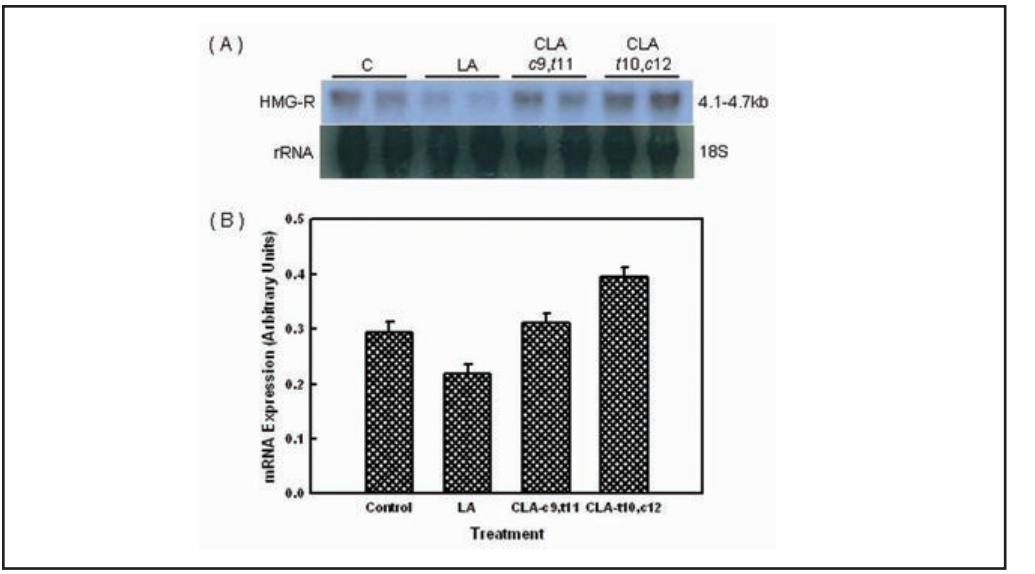

Figure 2. Effect of conjugated linoleic acid (CLA) on 3-hydroxy, 3-methylglutaryl CoA reductase (HMG-R) mRNA content in HepG2 cells. Representative Northern blot (A) and densitometric values (B) calculated over 2 experiments (n=4 for each treatment). Means without a common letter differ ($P<0.05$).

expression. Also, because several fatty acids and their derivatives are known ligands for PPARs,[13] 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 mm × 20 mm) were purchased from Glass Works (Corning, NY). The antibiotic/antimycotic (ABAM), sodium pyruvate, fatty acid-free bovine serum albumin (BSA), 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) cells were resuspended in 10 mL of growth medium (MEM, containing 2.2 g/L sodium bicarbonate, 1.0 mM sodium pyruvate, 1% ABAM and 10% FBS) and incubated at 37°C in a humidified atmosphere consisting of 95% O_2 and 5% CO_2. Cultures were replenished with fresh medium every 2 days until cells were approximately 90% confluent. Cells then were washed twice in HBSS and cultured in a fresh serum-free medium containing appropriate fatty acid treatment. At preparation of treatments, fatty acids were mixed with serum-free medium containing 33 mg/mL fatty acid-free BSA to a concentration of 1 mM. This mixture was incubated for 2 hours at 37°C to allow complexation of the fatty acids with BSA and then further

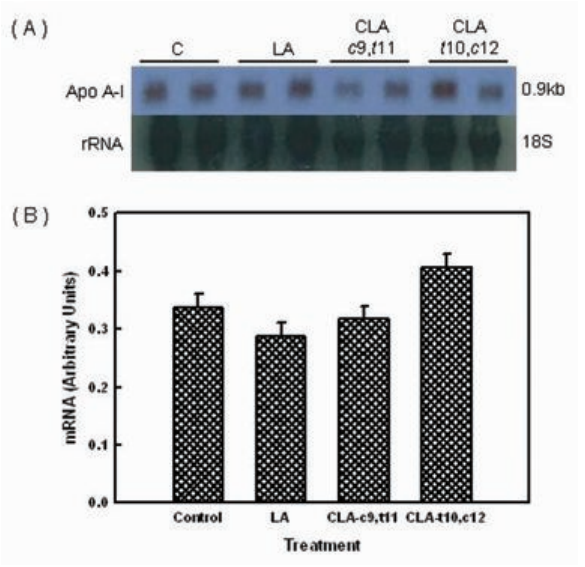

Figure 3. Effect of conjugated linoleic acid (CLA) on apolipoprotein A-I (Apo A-I) mRNA content in HepG2 cells. Representative Northern blot (A) and densitometric values (B) calculated over 2 experiments (n=4 for each treatment). Means without a common letter differ ($P<0.05$).

diluted in culture medium to a final treatment concentration of 100 µM of fatty acids.

To examine the effects of supplemental CLA on hepatic gene expression and cholesterol synthesis, HepG2 cells were treated with LA, *cis*-9, *trans*-11 CLA, or *trans*-10, *cis*-12 CLA (100 µm) for 24 hours. Cells were then rinsed twice with 10 mL HBSS and the remaining cell monolayer was lysed in 3 mL TriZol reagent, and stored at -80°C for subsequent mRNA analysis. The same fatty acid treatments were repeated using phenol red-free MEM for HDL-cholesterol analysis. After incubation, conditioned media were collected and stored at -20°C until lipid extraction and HDL cholesterol analysis.

To test whether CLA effects on gene expression involve PPARα activation, confluent HepG2 cells were treated with the *trans*-10, *cis*-12 CLA isomer (100 µM), the PPARα antagonist MK886 (10 µm), or a combination of CLA and MK886. After 24 hours of incubation, cells were washed twice with 10 mL HBSS, lysed with TriZol, and stored at -80°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 were fractioned in a 1.0% agarose formaldehyde gel 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 an ultraviolet light source for 90 seconds and baked at 80°C for 1 hour. Membranes were incubated for 2 hours 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.

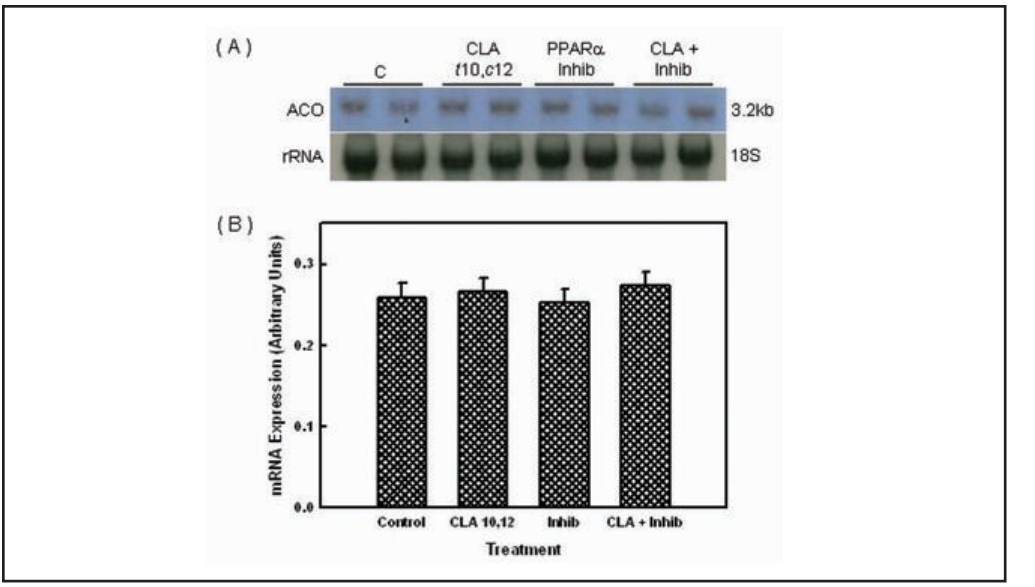

Figure 4. Effect of MK886 on acyl CoA oxidase (ACO) mRNA response to trans-10, cis-12 CLA in HepG2 cells. Representative Northern blot (A) and densitometric values (B) calculated over 2 independent experiments (n=4 for each treatment). Treatment means were not statistically different.

Complementary DNA Probes were generated by RT-PCR using primers designed from human ACO (forward: 5'-CCG GAG CTG CTT ACA CAC AT-3'; reverse: 5'-GGT CAT ACG TGG CTG TGG TT-3'), human HMG-R (forward: 5'-TCC TTG GTG ATG GGA GCT TGT TGT G-3'; reverse: 5'-TGC GAA CCC TTC AGA TGT TTG GAC C-3'), and human Apo A-I (forward: 5'-AAG ACA GCG GCA GAG ACT AT-3'; reverse: 5'-ATC TCC TCC TGC CAC TTC TT-3') sequences. The sizes and sequences of these cDNA probes were verified by DNA sequencing before their use in Northern blot analyses. 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 2 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.[14] Briefly, 2 mL of cell-conditioned media was aliquotted into a 20 mL glass screw-top vial. Fourteen milliliters of chloroform:methanol (2:1, v/v) were then added and the vials were vortexed for 5 minutes. The vials were centrifuged at 1700 rpm for 5 minutes. The bottom lipid-containing chloroform layer was transferred to a clean, dry, preweighed 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 cell-conditioned media were analyzed using a commer-

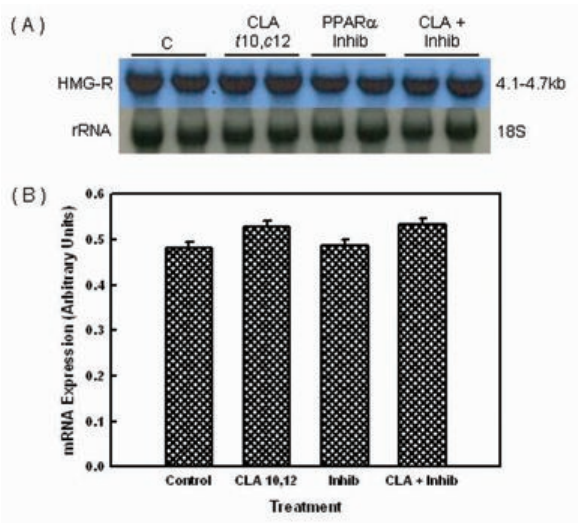

Figure 5. Effect of MK886 on 3-hydroxy, 3-methylglutaryl CoA reductase (HMG-R) mRNA response to trans-10, cis-12 CLA in HepG2 cells. Representative Northern blot (A) and densitometric values (B) calculated over 2 independent experiments (n=4 for each treatment). Means without a common letter differ ($P<0.05$).

cially available L-Type HDL-C kit (Wako Diagnostics, Richmond, VA) following the manufacturer's instructions. Briefly, using a 96-well plate, 3 µL of sample were pipetted into each well. Two hundred seventy microliters of Enzyme Color Solution (R1) were added, and the plate incubated for 5 minutes at 37°C. Ninety microliters of Reacting Solution (R2) were then added, and the plate incubated for another 5 minutes to 37°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 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 mRNA analyses, densitometric values for target genes were expressed as ratios of target gene densitometric values over the corresponding 18S rRNA densitometric values. Treatment means were compared using the PDIFF option of SAS and considered different at $P<0.05$.

RESULTS

Concentrations of ACO (Figure 1) and HMG-R (Figure 2) mRNA transcripts were increased in HepG2 cells treated with *trans*-10, *cis*-12 CLA. Neither CLA isomer had detectable effect on Apo A-I mRNA abundance in HepG2 cells (Figure 3). Co-incubation with MK886, a specific PPARα inhibitor, had minimal effect on basal or CLA-induced gene expression in HepG2 cells (Figures 4 through 6).

Treatment of HepG2 cells with *cis*-9, *trans*-11 CLA decreased ($P<0.05$) HDL-cholesterol production (Figure 7). The *trans*-10, *cis*-12 CLA isomer had no detectable effect on HDL-cholesterol production. There was no apparent rela-

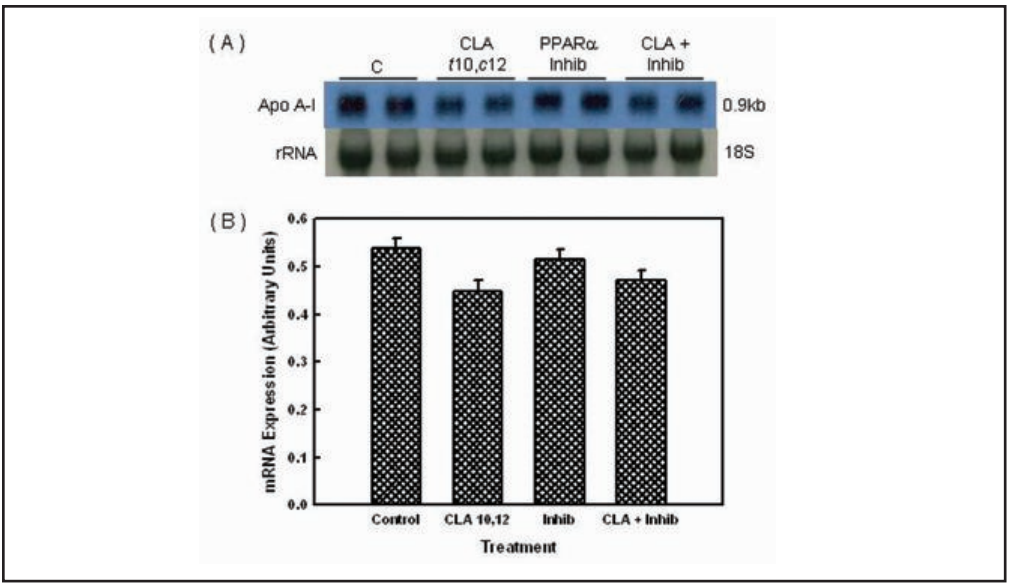

Figure 6. Effect of MK886 on apolipoprotein A-I (Apo A-I) mRNA response to trans-10, cis-12 CLA in HepG2 cells. Representative Northern blot (A) and densitometric values (B) calculated over 2 experiments (n=4 for each treatment). Means without a common letter differ ($P<0.05$).

tionship between Apo A-I mRNA and HDL-cholesterol production in cultured HepG2 cells.

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. In the present study, concentrations of ACO and HMG-R mRNA transcripts were increased in HepG2 cells treated with *trans*-10, *cis*-12 CLA. Results are consistent with previous reports that a mixture or individual CLA isomers increased ACO gene expression in mice.[15-17] In 2 studies, the increases in ACO mRNA contents in CLA-fed mice also coincided with increases in enzyme activity.[18,19] In FaO cells, a rat hepatoma cell line derived from H2IIEC3 cells, ACO gene expression was increased with 200 µm *cis*-9, *trans*-11 CLA,[20] but this effect was not detected with lower concentrations of CLA.[21] These findings collectively suggest a role for CLA in the control of hepatic peroxisomal oxidation of fatty acids.

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. In this study, CLA induced HMG-R gene expression in cultured human hepatocytes. Although the effects of saturated and polyunsaturated fats on HMG-R gene expression and enzyme activity have been examined previously, 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 without CLA or corn oil fed rats.[22] In the latter study, 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 posttranscrip-

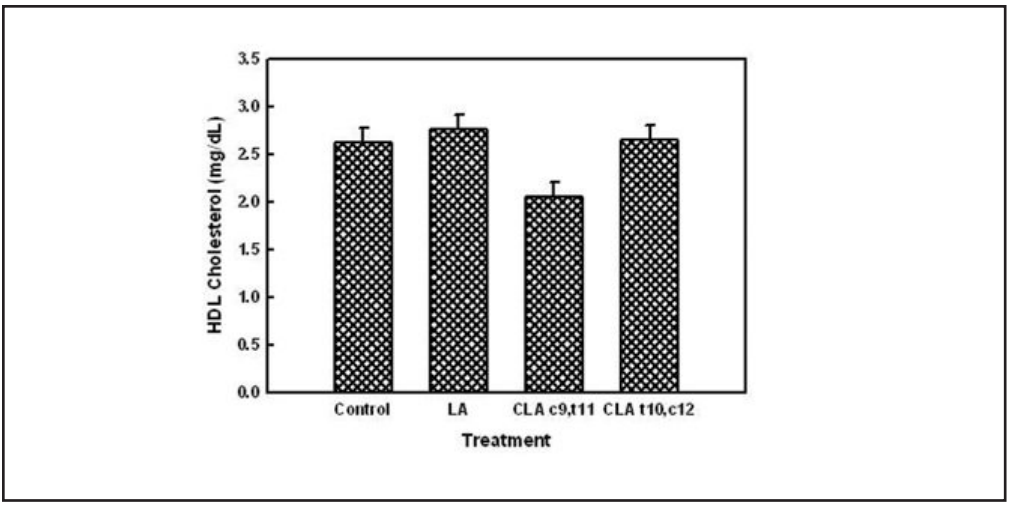

Figure 7. Effect of conjugated linoleic acid (CLA) on HDL-cholesterol production in HepG2 cells. Data represent least squares means ± standard error of the mean (SEM) calculated over 2 experiments. Means without a common letter differ ($P<0.05$).

tional regulation of the HMG-R enzyme.

Another factor involved in normal lipoprotein profile and metabolism is Apo A-I, the predominant apolipoprotein associated with HDL cholesterol. Dietary fats are known to modulate plasma lipids, and may act, in part, by effects on apolipoproteins. In the present study, treatment of HepG2 cells with CLA had minimal effects on steady-state Apo A-I mRNA concentration and HDL-cholesterol production. There was no apparent relationship between Apo A-I mRNA content and HDL cholesterol production in cultured human hepatocytes. Results are consistent with a previous report that mice fed with *cis*-6, *trans*-11 or *trans*-10, *cis*-12 CLA showed no differences in Apo A-I mRNA concentrations between control or CLA-fed groups.[15] Conversely, 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.[23] In a human dietary study, both Apo A-I gene transcript and HDL cholesterol were decreased by CLA-enriched butter as compared with presupplement levels.[24] These observations further underline species differences in Apo A-I gene expression and suggest that in vivo regulation of HDL-cholesterol synthesis may involve factors that are not controlled by in vitro models.

Fatty acids elicit several physiological effects through alteration of the activity and/or synthesis of peroxisome proliferator-activated receptors (PPAR). As several CLA isomers have been identified as high-affinity ligands and activators of PPARα,[21] we investigated the possibility that CLA effects on HepG2 cells may be mediated by PPARα. Inhibition of PPARα had no detectable effects on any of the genes studied in HepG2 cells. These findings contrast the rat data,[25] and suggest that PPARα responsiveness may vary between human and rodent hepatocytes. In fact, dietary studies have shown that rodents are responsive to PPARα activation, but nonrodent species, such as primates and guinea pigs, are resistant or unresponsive to PPARα activation.[26,27]

CONCLUSION

In summary, the observation that supplemental *trans*-10, *cis*-12 CLA increased steady-state concentration of ACO A-I mRNA in HepG2 cells suggests a role for this CLA isomer in the control of peroxisomal oxidation of fatty acids in the liver. Consistent with the low level of endogenous expression of PPARs in the human hepatocyte, the PPARα inhibitor had minimal effects on basal and CLA-stimulated gene expression in cultured HepG2 cells. Additional studies are needed to determine the cellular and molecular mechanisms by which *trans*-10, *cis*-12 CLA modulates ACO and HMG-R gene expression in human hepatocytes.

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