ORIGINAL ARTICLE

Elaidic Acid Increases Hepatic Lipogenesis by Mediating Sterol Regulatory Element Binding Protein-1c Activity in HuH-7 Cells

Fei Shao · David A. Ford

Abstract The liver is the major organ responsible for lipid biosynthesis. Sterol regulatory element-binding proteins (SREBP) are major transcription factors that regulate the expression of genes regulating fatty acid and cholesterol biosynthesis. Here we show that elaidic acid upregulates hepatic *de-novo* fatty acid and cholesterol synthesis in HuH-7 cells. To define the molecular mechanism involved in this unique regulation on hepatic lipogenesis, luciferase reporter gene assays were performed in HEK293 cells to compare the regulation of sterol regulatory element (SRE) that is present in SREBP-target promoter by elaidic acid and oleic acid. The results show that elaidic acid potently induced SRE-luciferase activity, whereas oleic acid inhibited this activity. Furthermore, elaidic acid increased SREBP-1c mRNA, while oleic acid did not alter it. Oleic acid inhibited mature form of SREBP-1 protein level, while elaidic acid did not show inhibitory effects. In addition, elaidic acid was also found to increase several selected lipogenic genes that are involved in fatty acids and sterol synthesis. These data demonstrate a unique role of elaidic acid, the most abundant *trans* fatty acid, in modulating hepatic lipogenesis.

Keywords *Trans* fatty acid · Sterol regulatory element binding protein · Sterol regulatory element · Hepatic lipogenesis

Abbreviations

ACC	Acetyl CoA carboxylase
CE	Cholesteryl ester
ESI	Electrospray ionization
ER	Endoplasmic reticulum
FA	Fatty acid
FAS	Fatty acid synthase
GC	Gas chromatography
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HMG	Hydroxy-2-methylglutaryl
MS	Mass spectrometry
NASH	Non-alcoholic steatohepatitis
ONPG	*ortho*-Nitrophenyl-β-galactoside
PFB	Pentafluorobenzyl
PtdCho	Phosphatidylcholine
SCD	Stearoyl CoA desaturase
SRE	Sterol regulatory element
SREBP	SRE binding protein
SCAP	SREBP cleavage-activating protein
TLC	Thin layer chromatography
TAG	Triacylglycerols

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Introduction

The liver plays a critical role in regulating lipids. It is the central organ for synthesis of various lipids including fatty acids and cholesterol [1]. Dietary *trans* fats have been associated with non-alcoholic steatohepatitis (NASH), which can lead to progressive cirrhosis and liver cancer [2]. Increased hepatic *de-novo* lipogenesis is one of the mechanisms that leads to NASH [3]. Therefore, in this study, we investigated the effect of elaidic acid on hepatic lipogenesis and examined the underlying mechanism.

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Sterol regulatory element-binding proteins (SREBP) are a family of transcription factors including SREBP-1a, SREBP-1c, and SREBP-2, which regulate gene expression of key enzymes involved in hepatic fatty acid and cholesterol biosynthesis [4–6]. Sterol regulatory elements (SRE) are *cis*-acting promoter elements present in genes that perform key steps in lipid synthetic pathways. Among the three SREBP isoforms, SREBP-1c is mainly responsible for regulating genes involved in hepatic fatty acid biosynthesis, whereas SREBP-2 is predominantly involved in the regulation of cholesterol biosynthetic genes. All SREBP family members are synthesized as inactive precursor proteins that are embedded in endoplasmic reticulum (ER) membrane and processed to liberate the N-terminal, which then translocate from ER to Golgi and eventually enter the nucleus to act as transcription factors [7]. Within the nucleus, SREBP binds to the sterol regulatory elements (SRE) that are found in the promoter region of the genes involved in lipogenic pathways to increase transcription of the target genes [8]. Interestingly, SRE is also present in the promoter region of SREBP genes, thereby regulating their own gene expression [9]. SREBP target genes are functionally categorized into two groups. The first group includes lipogenic genes such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), stearoyl CoA desaturase (SCD) and glycerol-3-phosphate acyltransferase. The other group contains cholesterol biosynthetic genes such as 3-hydroxy-2-methylglutaryl-CoA (HMG-CoA) synthase, HMG-CoA reductase and farnesyl diphosphate synthase.

Notably, all three SREBP members induce a severe fatty liver phenotype in transgenic mice with abundant accumulation of triglycerides and cholesterol [10–12]. SREBP proteolytic processing is highly regulated by two ER membrane proteins, the SREBP cleavage-activating protein (SCAP), and the insulin-inducing gene (Insig) [13]. When cells are depleted of sterols, SCAP escorts SREBP from ER to Golgi. Within the Golgi, SREBP precursors are processed by two proteases, site-1-protease and site-2-protease, to release the N-terminal mature SREBP to enter the nucleus to act as transcription factors [14, 15]. Conversely, when the cells have plenty of sterols, SCAP binds to cholesterol, induces conformational changes, binds to Insig, and thereby retaining SREBP on ER membrane. SREBP family members also have SRE present in the promoter region of their genes, thereby forming a positive feedback regulation by itself [16].

Current understanding of the regulation of different SREBP isoforms by fatty acids have focused on *cis* unsaturated fatty acids and saturated fatty acids [9, 17–19]. Hepatic SREBP-1c has been implicated in regulating lipogenic genes. Oleic, linoleic and arachidonic acids have been shown to down-regulate mRNA levels of SREBP-1

and to inhibit the proteolytic processing of SREBP in cultured cells and rodent liver tissues [9, 17, 18]. However, the effects of *trans* fatty acids on SREBP remain to be elucidated. Therefore, the present studies were performed to investigate the effect of elaidic acid on hepatic lipogenesis using the human hepatoma cell line HuH-7 cells. Results show that in comparison to oleic acid, elaidic acid increases hepatic lipogenesis including fatty acids and cholesterol synthesis compared to blank control. The mechanism responsible for elaidic acid mediated hepatic lipogenesis was shown to be via SREBP-1c processing, SRE element activation and subsequent lipogenic gene and protein expression.

Materials and Methods

Fatty Acid-Bovine Serum Albumin (BSA) Conjugate

Fatty acids were purchased from NuChek Prep (Waterville, MN). Fatty acid free BSA was purchased from MP Biomedicals. Fatty acids were made into fatty acid-BSA conjugate solution (5 mM). Briefly, fatty acids were saponified with NaOH at 70 °C for 30 min and then mixed with 6 % fatty acid free BSA dissolved in PBS. 5 mM stock solutions were stored in aliquots at −20 °C [20]. These fatty acid-BSA conjugates were also routinely analyzed by GC with flame ionization detection of their acid methanolysis products [21] to confirm the concentration and identity of the purity of the fatty acid molecular species. These fatty acid-BSA conjugates were dissolved in cell culture media to desired concentrations immediately prior to use in experiments.

Analysis of Lipid *de Novo* Synthesis with [14C]-Acetate

HuH-7 cells were incubated with 100 μM of indicated fatty acids for 18 h. Media was then replaced with DMEM containing sodium [14C]-acetate (0.5 μCi, specific activity 55 μCi/μmol). After 6 h of sodium [14C]-acetate labeling, cells were washed with PBS and scraped off the plate in PBS. Lipids were then extracted from cell lysates by a modified Bligh & Dyer procedure [22]. Lipid samples were resuspended in chloroform and stored under nitrogen in −80 °C until further analysis.

Thin Layer Chromatography

Lipid samples were applied to silica gel 60 plates for thin layer chromatography (TLC) separation using a solvent system comprised of petroleum ether:ethyl ether:acetic acid (80:20:1). Separated lipids were visualized using 0.05 % primuline dissolved in 80 % acetone. Lipid classes

were identified using authentic lipid standards run in separate lanes on the TLC plates. At the end of thin layer chromatography, individual lipid classes were identified by their co-migration of standards, and these regions of silica were scraped from the TLC plate and collected for scintillation counting. The relative amounts of lipids were determined using dpm from scintillation counting and normalized to protein content of each sample determined from Bio-Rad protein assay.

Lipidomics Analysis of HuH-7 Cells with Fatty Acid Pre-Labeling Followed by Sodium Acetate Treatment

HuH-7 cells were grown to 95 % confluence in DMEM medium containing 10 % FBS. Cells were then treated with 100 μM of [13,13,14,14-d_4]-elaidic acid, [17,17,18,18,18-d_5]-oleic acid, palmitic acid or no fatty acid addition negative control in DMEM supplemented with 0.2 % BSA for 18 h. Cells were either collected at the end of 18 h of fatty acid incubation or the medium was changed to DMEM containing sodium acetate (the same molecular concentration as that was used in sodium [^{14}C]-acetate labeling experiment), and after 6 h of sodium acetate incubation, cells were collected. Cell suspensions were subjected to Bligh & Dyer lipid extraction [22] in the presence of specific internal standards for specific lipid classes. Lipidomics analysis was conducted using electrospray ionization mass spectrometry (ESI–MS) by direct infusion shotgun lipidomics techniques [23] using a ThermoFisher Quantum Ultra triple quadrupole instrument. Cholesteryl esters (CE) were analyzed using neutral loss scan of m/z 368.5, and quantitated by comparing to internal standard (17:0-CE) [24]. Triacylglycerols (TAG) were analyzed by shotgun lipidomics employing TAG fingerprinting and quantified by comparison with internal standard (tri-17:1 TAG) [25].

Quantification of Free Cholesterol Using Electrospray Ionization Mass Spectrometry

Free cholesterol was derivatized to its cholesteryl acetate derivative to aid in ionization under ESI–MS conditions [24]. Product ion (PI) m/z 83 scans for the acetylated cholesterol derivative were acquired over 3–5 min with a scan rate of 0.5 scans/s. Quantification of free cholesterol was achieved by comparing the ion intensity of cholesteryl acetate (m/z 451) to the ion intensity of internal standard d_4-cholesteryl acetate (m/z 455).

GC–MS Analysis of Free Fatty Acids

To quantitate cellular levels of free fatty acids of HuH-7 cells, pentafluorobenzyl (PFB) bromide was used to make PFB esters that were subsequently analyzed by gas chromatography-mass spectrometry (GC–MS) [26]. Briefly, 100 μl lipid extract of cell samples obtained from Bligh & Dyer extraction was dried down and resuspended in a mixture of 95 μl acetonitrile, 5 μl PFB bromide and 2.5 μl diisopropylethylamine at 45 °C for 1 h. At the end of the reaction, samples were dried down under nitrogen and resuspended in approximately 250 μl ethyl acetate for GC–MS analyses. Quantitation of fatty acids was achieved by comparing the ion intensities of individual fatty acids to the internal standard. (17:0 fatty acid).

Real-Time Reverse-Transcription Pcr of Lipogenic Genes

HuH-7 cells were grown to 95 % confluence before treatment in growing media. Cells were treated with either elaidic acid, oleic acid, palmitic acid or BSA (blank control) for 24 h. At the end of treatment, cells were washed twice with cold PBS before being collected with Trizol reagent (Invitrogen). RNA extraction from cells was done following the Trizol Invitrogen RNA isolation kit instructions. cDNA were generated from 5 μg of DNase1-treated RNA using SuperScript III and random hexamers (Invitrogen). Real-time PCR was done with SYBR Green reagent (Roche), using a LightCycler 480 real-time PCR detection system (Roche). Quantification of mRNA levels was done by normalizing values to GAPDH and calculated using comparative C_T method.

Immunoblot Analysis of HuH-7 Cells

Nuclear extracts of HuH-7 cells were obtained following the optimized Invitrogen nuclear extraction protocol. Briefly, cells were collected in PBS by scraping from culture dishes. After washing cells once with PBS, the cell pellet was collected after centrifugation and resuspended in 500 μl of hypotonic buffer by pipetting up and down several times. Cell suspension was incubated on ice for 15 min followed by addition of 25 μl detergent (IGEPAL, Sigma I8896) and vortexing for 10 s. The cell homogenate was centrifuged for 10 min at 800×g at 4 °C, and the nuclear pellet was resuspended in 50 μl RIPA extraction buffer for 30 min on ice with vortexing at 10 min intervals. The nuclear suspension was then centrifuged for 30 min at 14,000×g at 4 °C, and the supernatant nuclear fraction was subsequently collected. Protein concentration was measured using a BCA protein assay kit (Pierce). Immunoblot analysis was done with SREBP-1 antibody (Santa Cruz Biotechnology, sc-8984).

Luciferase Assay

HEK293 cells were co-transfected with SRE-luciferase plasmid and β-galactosidase plasmid using the calcium

phosphate transfection method. After 16 h of transfection, the medium was replaced with DMEM containing 100 μM of different fatty acids or a blank control as indicated in the presence or absence of 10 μg/ml of cholesterol and 1 μg/ml of hydroxycholesterol for 48 h. At the end of incubation, luciferase activity was detected using the luciferase assay system (Promega), and β-galactosidase activity was measured with *ortho*-nitrophenyl-β-galactoside (ONPG) (Sigma). Luc Activity is presented as luciferase activity after normalized to β-galactosidase activity.

HuH-7 cells were transfected with SRE-luciferase plasmid and β-galactosidase plasmid using the lipofectamine transfection method. Fatty acid treatment and measurement of luciferase activity and β-galactosidase activity were conducted as described above for measurements in HEK293 cells.

Statistical Analysis

All statistical test were performed using Prism software, and a value of $p < 0.05$ was considered significant. ANOVA with Newman-Keuls *post-hoc* test was used to determine the level of significance between different treatment groups.

Results

Disparate Regulation of Hepatic *de-Novo* Lipogenesis by Elaidic Acid and Oleic Acid in HuH-7 Cells

To examine the effect of elaidic acid on hepatic *de-novo* lipogenesis, HuH-7 cells were incubated with 100 μM of indicated fatty acids for 18 h followed by 6 h of [14C]-acetate labeling. As shown in Fig. 1a, b cells that were pretreated with elaidic acid had higher levels of *de-novo* synthesis of free fatty acids and free cholesterol compared to control treatment over 6 h of [14C]-acetate labeling. In contrast, treatment with either palmitic acid or oleic acid did not significantly alter *de-novo* synthesis of fatty acid or free cholesterol. As for incorporation of [14C]-acetate into complex lipids, Fig. 1c, d showed that *de-novo* synthesis of both cholesteryl esters and triglycerides was increased with either elaidic acid or palmitic acid pretreatment over 6 h. In contrast, oleic acid pretreatment did not alter *de-novo* synthesis of cholesteryl esters or triglycerides. These data suggested that elaidic acid stimulated hepatic lipid biosynthesis in HuH-7 cells including fatty acids, free cholesterol, cholesteryl esters and triglycerides, which is distinct from its corresponding *cis* fatty acid (oleic acid). Furthermore these data show that elaidic acid is more potent than palmitic acid in inducing lipogenesis in HuH-7 cells, which may imply some unique roles of elaidic acid in regulating hepatic lipogenesis pathways.

HuH-7 Cell Labeling Study Using Deuterated Fatty Acids

[14C]-acetate labeling assay demonstrated the impact of elaidic acid on hepatic *de-novo* lipogenesis over the selected labeling time interval, however, it did not provide information on the effect of the different treatments on changes of cellular lipid mass. Therefore, $[13,13,14,14-d_4]$-elaidic acid and $[17,17,18,18,18-d_5]$-oleic acid were used to follow their incorporation into different lipid species as well as examine changes in the amount of lipid classes under conditions similar to that used for *de-novo* lipogenesis study with [14C]-acetate. In this labeling experiment, HuH-7 cells were treated with 100 μM of $[13,13,14,14-d_4]$-elaidic acid and $[17,17,18,18,18-d_5]$-oleic acid, palmitic acid or blank control in DMEM supplemented with 0.2 % BSA for 18 h. Figure 2 shows the regulation of five different lipid classes by indicated fatty acids incubation. Total cellular levels of cholesteryl esters, triglycerides and phosphatidylcholine (PtdCho) were increased after elaidic acid treatment. No significant changes of total fatty acids or free cholesterol levels were observed with elaidic acid treatment. On the other hand, oleic acid treatment caused reduction of fatty acid levels, whereas palmitic acid treatment resulted in increased triglyceride levels. These results in addition to those from the experiments employing [14C]-acetate labeling suggested that elaidic acid is the most potent fatty acid among the fatty acids we tested in inducing CE, TAG and PtdCho levels in HuH-7 cells. Mass spectrometry lipidomics analysis provided more detailed information on lipid composition of each lipid class and the incorporation of deuterated labeled fatty acid. As shown in Fig. 3a, major cholesteryl esters including 16:1-CE, 16:0-CE, 18:1-CE and 18:0-CE were present for each fatty acid treatment, and substantial incorporation of deuterated labeled fatty acid was observed as $[d_4]$-18:1-CE (elaidic) and $[d_5]$-18:1-CE (oleic). Deuterium label was also found to be incorporated into $[d_4]$-16:1-CE and $[d_5]$-16:1-CE, suggesting that the original deuterated labeled fatty acids were likely β-oxidized prior to incorporation into CE pools. Data shown in Fig. 3b reveals the major fatty acid composition of triglycerides with different fatty acid treatments, which was obtained by using mass spectrometry neutral loss fingerprinting analysis for each fatty acid [25]. $[d_4]$-Elaidic acid is incorporated into triglycerides at levels about two-fold higher than that of $[d_5]$-oleic acid. The predominant molecular species of phosphatidylcholine are shown in Fig. 3c. Two of the most abundant natural PtdCho molecular species, 16:0/18:1-PtdCho and 18:0/18:1-PtdCho were decreased with elaidic acid or oleic acid treatment. However, $[d_4]$-elaidic acid predominantly was incorporated into the 18:1/$[d_4]$-18:1 molecular species, while $[d_5]$-oleic acid was distributed about equally between

Fig. 1 *De-novo* lipid synthesis analyses in HuH-7 cells using [^{14}C]-acetate incorporation into different lipid species. HuH-7 cells were sequentially incubated with 100 μM of indicated fatty acids for 18 h and then [^{14}C]-acetate. After 6 h of incubation, cells were washed with PBS and the incorporating radiolabel into indicated lipid classes was determined as described in "Materials and Methods". *FA* fatty acid, *CE* cholesterol esters, *TAG* triacylglycerols. Values represent the mean ± SEM for three independent experiments. *$p < 0.05$ and **$p < 0.01$, respectively, compared to control blank treatment
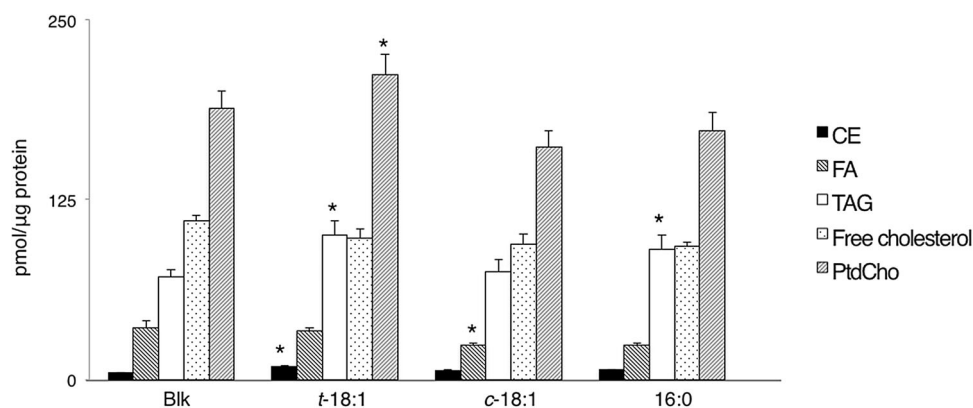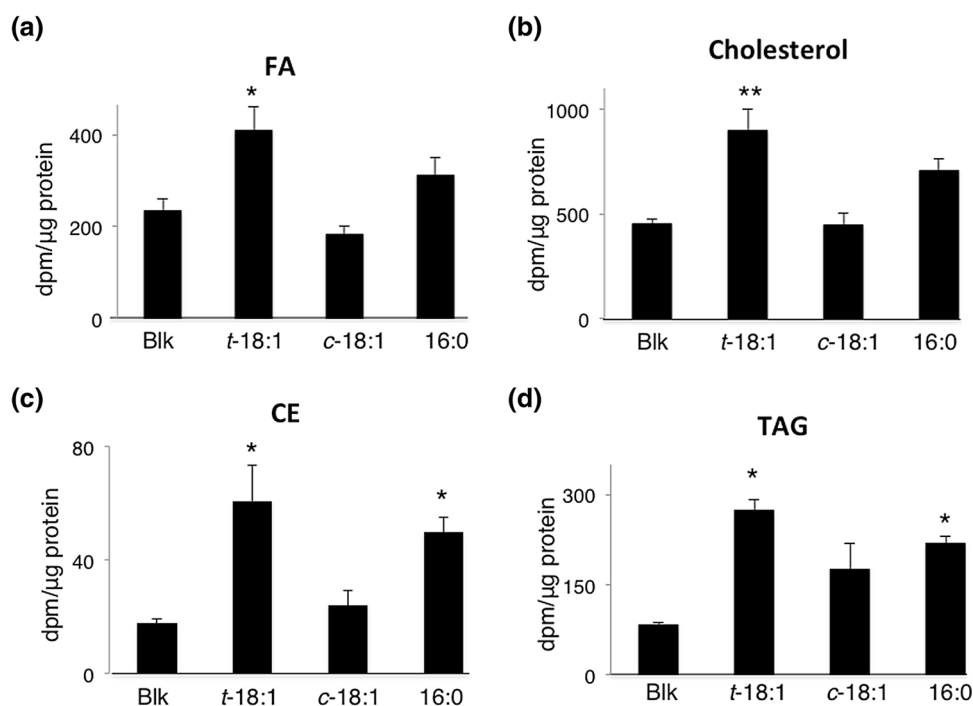

Fig. 2 Cellular lipid mass of total cholesteryl esters (CE), fatty acids (FA), triacylglycerols (TAG), free cholesterol and phosphatidylcholine (PtdCho) in HuH-7 cells treated with indicated fatty acids for 18 h. HuH-7 cells were treated with 100 μM of 13,13,14,14-d_4 elaidic acid, 17,17,18,18,18-d_5 oleic acid, palmitic acid or blank control in DMEM supplemented with 0.2 % BSA for 18 h. Cells were collected at the end of 18 h of fatty acid for Bligh & Dyer lipid extraction after addition of specific internal standard for individual lipid species. Quantification of each molecular species was performed using electrospray ionization mass spectrometry (ESI–MS) as described in "Materials and Methods". Values were mean ± SEM from three independent experiments. *$p < 0.05$ compared to control blank treatment for each lipid species

16:0/[d_5]-18:1 and 18:1/[d_5]-18:1 phosphatidylcholine molecular species. In the free fatty acid pools of these cells fatty acids were greatly altered by the treatments with [d_4]-elaidic acid and [d_5]-oleic acid. As shown in Fig. 3d, both elaidic acid and oleic acid treatments lead to lower levels of natural 16:0 (palmitic acid) compared to blank control. However, the higher levels of [d_4]-elaidic acid and [d_4]-palmitelaidic acid (16:1) compared to that of [d_5]-oleic acid and [d_5]-palmitoleic acid (16:1) contribute to the relatively higher levels of total fatty acids with [d_4]-elaidic acid treatment.

Additional studies were performed following treatments with fatty acids under conditions shown in Figs. 2 and 3 with an additional 6 h incubation with unlabeled acetate at an equimolar concentration to that used in radiolabeled acetate labeling experiments. Data shown in Fig. 4 represent the change in free fatty acid and cholesterol following this additional 6 h incubation. As shown in Fig. 4a, oleic acid treatment resulted in a decreased total fatty acid level during 6 h of acetate treatment, whereas both elaidic acid and palmitic acid preincubation led to slight increases in the total fatty acid level after 6 h of acetate treatment. Free cholesterol levels were
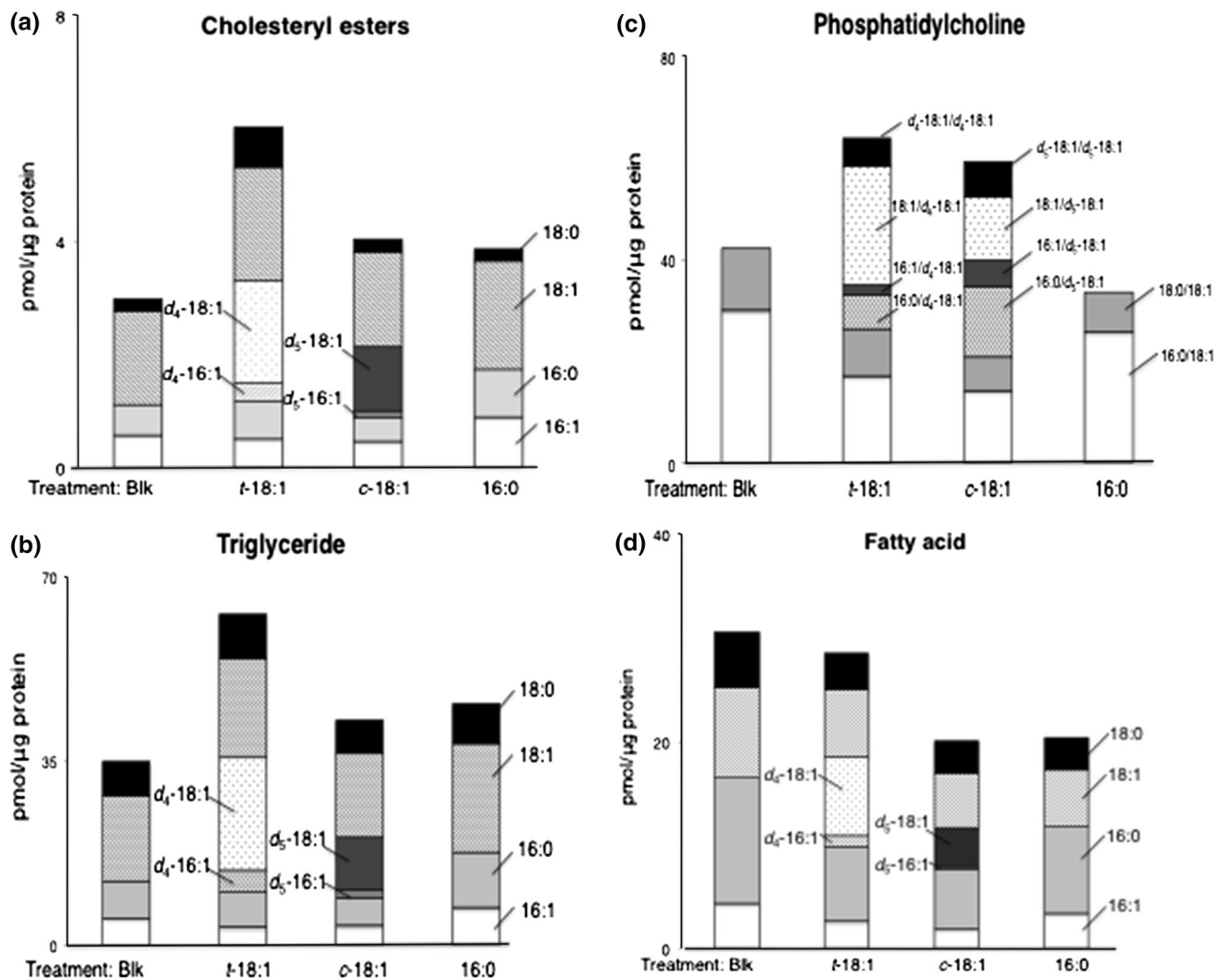
Fig. 3 Regulation of major cellular lipid species levels by different fatty acids and incorporation of deuterium into individual lipid species including cholesteryl esters (**a**), triglyceride (**b**), phosphatidylcholine (**c**) and fatty acids (**d**). HuH-7 cells were treated with 100 μM of 13,13,14,14-d_4 elaidic acid, 17,17,18,18,18-d_5 oleic acid, palmitic acid or blank control in DMEM supplemented with 0.2 % BSA for 18 h. Cells were collected at the end of treatment for Bligh & Dyer lipid extraction after addition of specific internal standard for individual lipid species. Lipidomics analysis and quantification of each lipid species were performed using electrospray ionization mass spectrometry (ESI–MS) as described in "Materials and Methods".

Values were mean obtained from three independent experiments. Briefly, **a** indicated cholesteryl ester species were quantified using neutral loss scan of m/z 368 by comparing to the internal standard 17:0-CE; **b** incorporation of indicated fatty acids into triglyceride was quantified using neutral loss fingerprinting method; **c** phosphatidylcholine was quantified with neutral loss scan of m/z 59.1 using di-20:0-PtdCho as an internal standard; **d** major fatty acids species were quantified with GC–MS after converting to the pentafluorobenzyl (PFB) bromide derivative. Data were presented as pmol per μg protein based on means values from three independent experiments

increased with all three fatty acid treatments compared to blank control treatment (Fig. 4b). During 6 h of acetate treatment, cells that were pretreated with elaidic acid, oleic acid and palmitic acid had increased free cholesterol up to 16.6, 2.9 or 10.0 pmol/μg protein respectively.

Elaidic Acid Induce Sterol Regulatory Element (SRE) Activity

Next, the effect of elaidic acid on SREBP promoter activity was investigated using an *in vitro* luciferase reporter

analysis. An SRE-luciferase reporter and β-galactosidase plasmid were introduced into HuH-7 cells by lipofectamine transfection. Data shown in Fig. 5a show modest increases in SRE luciferase activity in cells treated with 100 and 150 μM elaidic acid in the presence of sterols (10 μg/ml cholesterol + 1 μg/ml hydroxycholesterol) for 48 h while this activity was decreased in cells treated with the same concentrations of oleic acid. Additional assays examined the SRE promoter activity in HEK293 cells that were transfected with the same plasmids but in this case using the calcium phosphate transfection method. These cells
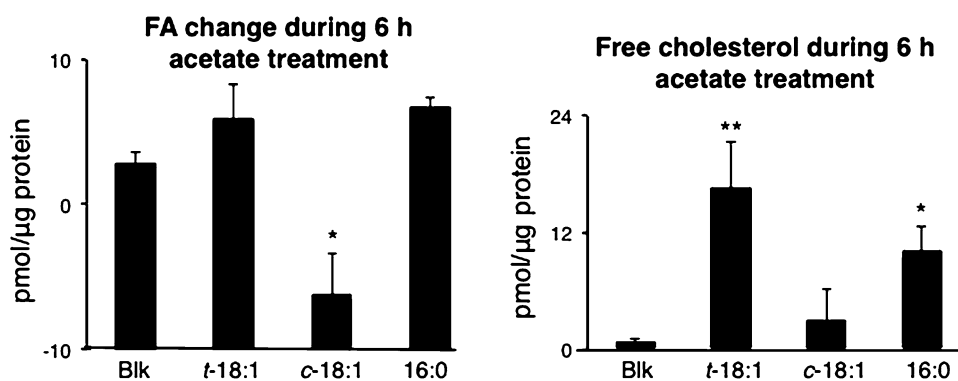
Fig. 4 Changes of fatty acid and free cholesterol levels over 6 h of acetate treatment in HuH-7 cells. HuH-7 cells were treated with 100 μM of 13,13,14,14-d_4 elaidic acid, 17,17,18,18,18-d_5 oleic acid, palmitic acid or blank control in DMEM supplemented with 0.2 % BSA for 18 h. Cells were either collected at the end of 18 h of fatty acid incubation or medium was changed to DMEM containing sodium acetate (the same molecular concentration as that was used in sodium [^{14}C]-acetate labeling experiment). After 6 h of sodium acetate were subsequently incubated with indicated *trans* fatty incubation, cells were collected for Bligh & Dyer lipid extraction after addition of specific internal standard for different lipid species. Values are presented as means ± SEM calculated by subtracting the mean values of lipid mass before acetate treatment from each value of lipid mass after acetate treatment. Data were based on three independent experiments. *$p < 0.05$ and **$p < 0.01$ respectively compared to blank control treatment

were subsequently incubated with indicated *trans* fatty acids and *cis* fatty acids at a concentration of 100 μM for 48 h. Results of luciferase activity after normalization to β-galactosidase activity demonstrate that elaidic acid (9*t*-18:1) was able to induce SRE activity in the presence or absence of sterols, whereas all other fatty acids being tested including oleic acid (9*c*-18:1), *trans* and *cis* vaccenic acid (11*t*-18:1 and 11*c*-18:1), *trans* and *cis* linoleic acid (t-18:2 and c-18:1) all displayed reduced SRE activity (Fig. 5b). Interestingly, *trans* vaccenic acid (11*t*-18:1), the most abundant ruminant derived *trans* fat, also slightly increased SRE activity albeit to a lesser extent than elaidic acid. Further studies showed that within a lower concentration range from 5 to 50 μM, elaidic acid in the presence of sterol induced SRE activity in a dose-dependent manner, while oleic acid, its corresponding *cis* fatty acid, decreased the activity with increasing concentration as shown in Fig. 5c. These results suggest that the difference in the double bond configuration renders elaidic acid drastically different in regulating SRE activity, which implies that elaidic acid and oleic acid disparately modulate SREBP activity.

Elaidic Acid Induces Lipogenic Genes in Hepatocytes

To further elucidate the underlying mechanism of distinct effects of *trans* fatty acid versus *cis* fatty acid on hepatic lipogenesis pathway, we designed real-time PCR experiments to examine whether elaidic acid differentially induces key genes responsible for hepatic lipogenesis compared to oleic acid and palmitic acid. HuH-7 cells were treated with elaidic acid, oleic acid or palmitic acid at 100 μM for 24 h. Quantitative real-time PCR analyses revealed that elaidic acid upregulated mRNA levels of stearoyl-CoA desaturase-1 (SCD1), fatty acid synthase (FAS), 3-hydroxy-2-methylglutaryl-CoA reductase (HMGR) and acetyl-CoA carboxylase (ACC) to approximately 1.5- to 2-fold compared to blank control treatment. In contrast, oleic acid did not significantly alter the transcription of these genes except for a reduction of SCD1. Palmitic acid had no significant effects on the mRNA levels of these selected genes. (Fig. 6).

Elaidic Acid Induces SREBP-1 mRNA Levels

To investigate the effect of elaidic acid on SREBP proteolytic processing, HuH-7 cells were treated with elaidic acid or oleic acid at 100 μM for 24 h. SREBP-1 was detected by immunoblot analysis of cellular nuclear fraction. Consistent with previous studies, oleic acid decreased SREBP-1 levels of its nuclear and mature form (Fig. 7). In contrast, the inhibitory effect on SREBP-1 mature form was not observed with elaidic acid treatment.

Regulation of SREBP-1 can be at two different levels, one at the proteolytic processing level, and the other at the transcriptional level. SRE is present in the promoter region of the SREBP-1 gene, and mature SREBP-1 binds to the SRE site to have a self-regulation circuit. To examine if elaidic acid alters SREBP-1c at the transcriptional level, HuH-7 cells incubated with 100 μM of elaidic acid or oleic acid for 24 h were examined with quantitative real-time rt PCR for SREBP-1c. As shown in Fig. 8, elaidic acid increased SREBP-1c mRNA level about 1.5-fold compared to blank control treatment, whereas oleic acid did not significantly alter SREBP-1c mRNA levels.
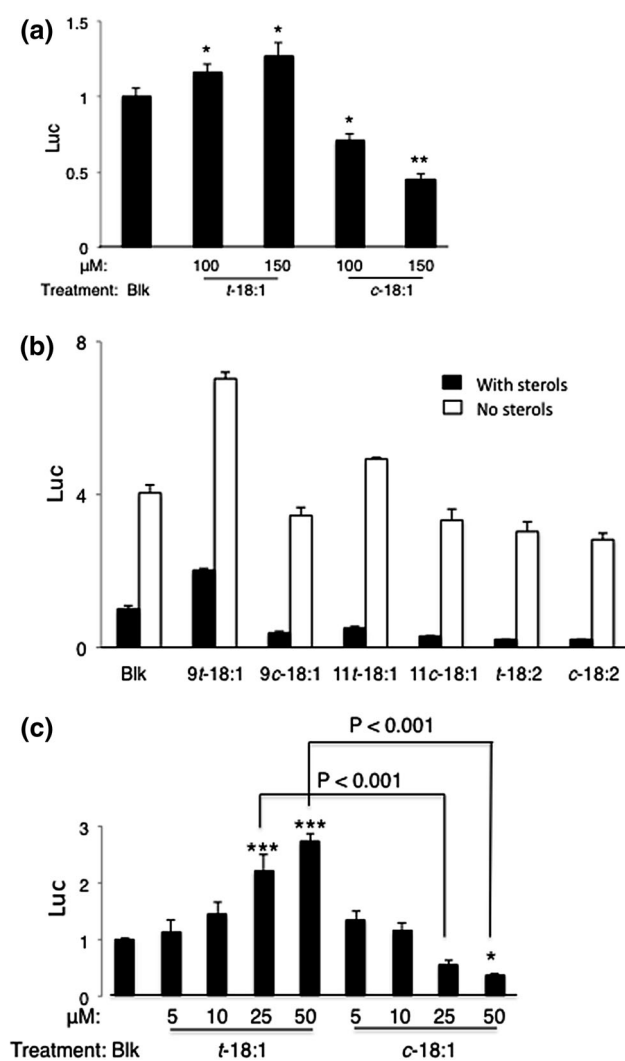
Fig. 5 Luciferase assay with HuH-7 and HEK293 cells. Either HuH-7 or HEK293 cells were simultaneously transfected with plasmid containing SRE upstream of luciferase reporter gene and β-galactosidase plasmid. Luc Activity is the luciferase activity normalized to β-galactosidase activity. In **a**, HuH-7 cells were incubated with indicated concentrations of either elaidic or oleic acid for 48 h in the presence of sterols before luciferase reporter assay. In **b**, cells were incubated with 100 μM of indicated fatty acids for 48 h with the presence or absence of sterols before luciferase reporter assay. In **c**, cells were incubated with indicated concentrations of either elaidic acid or oleic acid in the presence of sterols for 48 h before luciferase reporter assay. Values are mean ± SEM for three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, respectively, compared to control blank treatment. *Brackets* indicate other statistically significant differences in **c**

Discussion

Data herein demonstrate a unique role of elaidic acid in hepatic lipogenesis through upregulating the SREBP-1 pathway. It was surprising that the administration of elaidic acid would cause more fatty acids and cholesterol synthesis in hepatocytes, which seems paradoxical to the current

understanding of negative regulation of lipid synthesis by end products. Previous studies have shown that *trans* fatty acids are poor substrates for enzymatic incorporation into triglyceride compared to their *cis* analogues [27]. Further studies are needed to investigate the molecular mechanism underlying this disparate regulation of SREBP-1 by elaidic acid such as in vivo studies.

The investigation was initiated by testing the impact of elaidic acid on hepatic *de-novo* lipogenesis compared to oleic acid and palmitic acid. The most striking evidence from this experiment was that elaidic acid induced *de-novo* synthesis of all four major lipids including fatty acids, cholesterol, cholesteryl esters and triglyceride (Fig. 1), which implied that elaidic acid may modulate lipogenic gene expression during the 18 h incubation. To further investigate the regulation of total cellular lipids by different fatty acids, the deuterated fatty acid labeling study was designed in parallel with the *de-novo* lipid synthesis assay. Cells that were treated with elaidic acid resulted in increased amount of total CE, TAG and PtdCho levels (Fig. 2), which can at least be partially explained by the increased *de-novo* synthesis of these lipid classes over 6 h of [^{14}C]-acetate labeling (Fig. 1c, d). Changes in cellular levels of fatty acids and free cholesterol over 6 h of acetate treatment demonstrated that elaidic acid treatment increased these two lipid classes (Fig. 4a, b), which is similar to the increased TAG and free cholesterol synthesis observed from the [^{14}C]-acetate *de-novo* lipid synthesis assay (Fig. 1a, b). As fatty acids and free cholesterol are the precursors for synthesis of CE and TAG, the increased levels of CE and TAG also reflect the *de-novo* synthesis of fatty acids and cholesterol.

Previous studies on fatty acids regulating SREBP activity have focused on *cis* polyunsaturated fatty acids and saturated fatty acids. Generally, polyunsaturated fatty acids and oleic acid are considered as negative regulators of hepatic lipogenesis by suppressing both SREBP-1 proteolytic processing and gene transcription [9, 17]. Moreover, additive or synergistic inhibition of SREBP was observed when *cis* unsaturated fatty acids and saturated fatty acids are administered together with sterols. In contrast to current understanding about the inhibitory effect of *cis* unsaturated fatty acids on SREBP activity, our results demonstrate a unique role of elaidic acid in regulating SREBP-1 mediated hepatic lipogenesis. Notably, elaidic acid significantly induced SRE-luciferase activity even in the presence of sterols, which suggested that elaidic acid was able to antagonize the inhibition of SRE-activity by sterols (Fig. 5a). With a single difference in the double bond configuration, elaidic acid had drastic different effects on SREBP-1 expression compared to oleic acid. Elaidic acid induced SREBP-1c mRNA levels up to about 1.5-fold, while oleic acid did not alter it. Oleic acid significantly
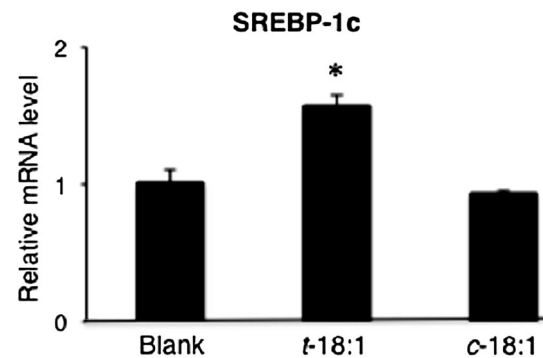
Fig. 6 Real-time PCR of HuH-7 cells SRE target genes. HuH-7 cells were treated with 100 μM of indicated fatty acids for 24 h. Cells were collected at the end of the treatment for RNA isolation with Trizol. rDNA were generated and real-time PCR was done with SYBR Green reagent as described in "Materials and Methods". mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). $*p < 0.05$ and $**p < 0.01$ compared to blank control treatment
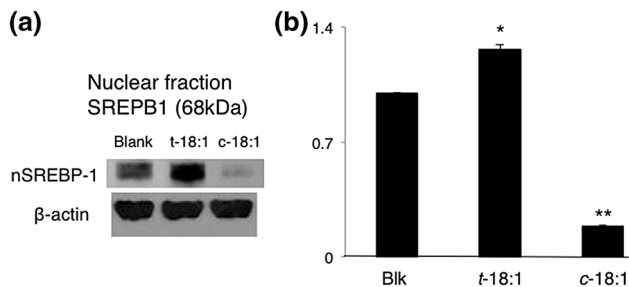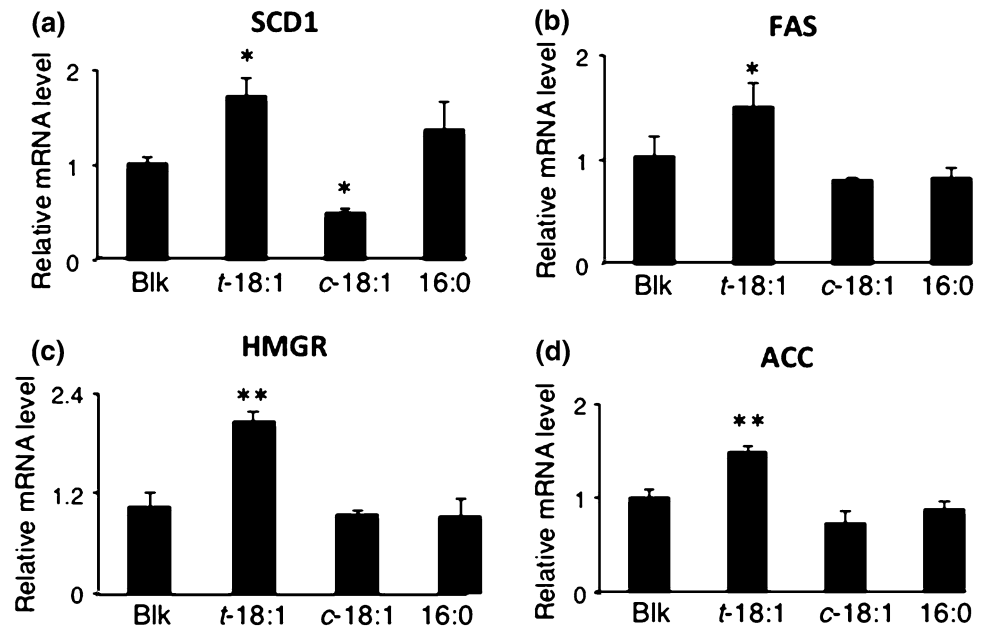
Fig. 7 SREBP-1 immunoblot analysis of HuH-7 cells nuclear fraction. HuH-7 cells were treated with 100 μM of elaidic acid or oleic acid for 24 h. Nuclear fraction was obtained for western blot of SREBP-1. **a** Representative western blot of SREBP1 from nuclear fraction of HuH-7 cell. **b** Densitometry analysis for western blot of SREBP1 normalized to β-actin. Values are means ± SEM for three different western blots. $*p < 0.05$ and $**p < 0.01$, respectively, compared to blank control treatment

Fig. 8 Real-time PCR analysis of SREBP-1c in HuH-7 cells. HuH-7 cells were treated with 100 μM of elaidic acid or oleic acid for 24 h. mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). $*p < 0.05$ compared to blank control

reduced the nuclear abundance of SREBP-1 protein, whereas elaidic acid did not have the same inhibitory effect. Taken together, compared to oleic acid, elaidic acid does not inhibit the maturation of SREBP that is cleaved and contains the N-terminus nuclear localization sequence enabling it to be targeted to the nucleus [6, 7].

Furthermore, elaidic acid was found to be able to induce SREBP target genes including SCD1, FAS and ACC (Fig. 6), which are all lipogenic genes regulated by SREBP-1. Interestingly, Minville–Walz's group demonstrated that elaidic acid increases both mRNA and protein levels of SCD1 in human aortic smooth muscle cells [28]. In these studies it is stated that SCD1 is not modulated via an SREBP-1 pathway (the data was not shown), and

suggest that in the smooth muscle cell system SCD1 may be regulated via LXR since others have shown increases in SCD1 activity in SREBP-1C$^{-/-}$ mice [29]. It is quite possible that the difference underlying the present study with this previous study may be due to the lipogenic role of liver as well as differences in experimental design (e.g., the previously described studies involved treating smooth muscle cells with fatty acids for 48 h). Additionally, HMG CoA reductase, target gene of SREBP-2, was also induced by elaidic acid treatment, which demonstrated that the induction effect of elaidic acid might not be limited to SREBP-1. However, further investigations are required to study the effect of elaidic acid on SREBP-2. Hepatic lipogenesis provides lipids for VLDL formation, which is the precursor of LDL. Considering that *trans* fats

consumption leads to increased LDL cholesterol levels, our findings may partially explain this effect through increased hepatic lipogenesis. Nevertheless, further studies should be conducted to confirm the effects in vivo.

The studies herein focused on comparisons of the industrialized *trans* fatty acid, elaidic acid to the *cis* fatty acid, oleic acid. While epidemiological evidence shows that increased ingestion of *trans* fatty acid is associated with cardiovascular risk, it is important to note that it is less likely that *trans* fatty acids from ruminants are associated with cardiovascular risk. In fact the predominant *trans* fat of ruminants, vaccenic acid, is converted to the protective fatty acid, 9*t*,11*c* conjugated linoleic acid. The relationship of ruminant *trans* fatty acids has recently been reviewed [30].

In conclusion, the present study demonstrates that elaidic acid upregulates hepatic *de-novo* fatty acids and cholesterol synthesis pathway in HuH-7 cells. Biochemical analyses further reveal that elaidic acid induces hepatic lipogenesis by increasing SREBP-1c gene transcription and downstream lipogenic genes. With increased awareness of cardiovascular risks with dietary *trans* fats, the levels of *trans* fats in the diet and plasma of humans are decreasing. Although, total *trans* fats in human plasma have been shown to reach similar levels to those examined in the cell culture studies described herein (e.g., 10–100 µM) [31], it will be important in future studies to examine accelerated hepatic lipogenesis elicited by elaidic acid in an *in-vivo* model.

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