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Trans-10, *cis*-12-conjugated linoleic acid alters hepatic gene expression in a polygenic obese line of mice displaying hepatic lipidosis ☆

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Abstract

The *trans*-10, *cis*-12 isomer of conjugated linoleic acid (CLA) causes a rapid reduction of body and adipose mass in mice. In addition to changes in adipose tissue, numerous studies have reported alterations in hepatic lipid metabolism. Livers of CLA-fed mice gain mass, partly due to lipid accumulation; however, the precise molecular mechanisms are unknown. To elucidate these mechanisms, we examined fatty acid composition and gene expression profiles of livers from a polygenic obese line of mice fed 1% *trans*-10, *cis*-12-CLA for 14 days. Analysis of gene expression data led to the identification of 1393 genes differentially expressed in the liver of CLA-fed male mice at a nominal *P* value of .01, and 775 were considered significant using a false discovery rate (FDR) threshold of .05. While surprisingly few genes in lipid metabolism were impacted, pathway analysis found that protein kinase A (PKA) and cyclic adenosine monophosphate (cAMP) pathways signaling pathways were affected by CLA treatment and 98 of the 775 genes were found to be regulated by hepatocyte nuclear factor 4α, a transcription factor important in controlling liver metabolic status.

Keywords: Gene expression; Mice; Liver; Conjugated linoleic acid; Obesity; Hepatic steatosis; Fatty liver

1. Introduction

Conjugated linoleic acid (CLA) is a term that refers to positional and geometrical isomers of linoleic acid (LA) [1]. Previous mouse studies have shown that the *trans*-10, *cis*-12 isomer of CLA has mostly delipidative effects [2-5], while the *cis*-9, *trans*-11 isomer has mostly anticarcinogenic effects [6]. The *trans*-10, *cis*-12-CLA isomer elicits delipidative effects not only in mice, but also in rats, chicks, pigs and humans [7-9]. Effects of CLA on obesity vary between and within species; in mice, CLA reduces fat-body mass by 50% to 70% [8]. These changes in adipose and body mass may be caused by reduced size and number of adipocytes [10-13], differences in energy expenditure [14-17] and/or feed intake [18]. In addition to changes in adipose tissue, increases in size, cytoplasmic vacuolization and fatty acid oxidation in liver of CLA-fed mice have been reported [4,7,8,19]. Much of the increased liver mass may be explained by accumulation of lipids [19,20] derived from mobilization of delipidated adipose tissue stores [21,22].

Attempts have been made to elucidate the molecular mechanisms of action of CLA. To date, four microarray studies using gene expression profiling to examine the effects of CLA have been reported [10,19,23,24]. Regulatory pathways involved in development, signal transduction and fatty acid metabolism affected by CLA have been identified. In a previous study [19], we reported

Abbreviations: acox1, acetyl CoA oxidase 1; agpat2, 1-acylglycerol-3-phosphate *O*-acyltransferase 2; bcl6, B-cell leukemia/lymphoma 6; cAMP, cyclic adenosine monophosphate; CLA, conjugated linoleic acid; cyp1a1, cytochrome P450, family 1, subfamily a, polypeptide 1; cyp27a1, cytochrome P450, family 27, subfamily a, polypeptide 1; dgat2, diacylglycerol *O*-acyltransferase 2; egfr, epidermal growth factor receptor; FDR, false discovery rate; fasn, fatty acid synthase; HNF-4α, hepatocyte nuclear factor 4α transcription factor; igfbp2, insulin-like growth factor binding protein 2; LA, linoleic acid; me, malic enzyme; NF-κB, nuclear factor κB; pdcd8, programmed cell death 8; PKA, protein kinase A; ppargc1, peroxisome proliferative activated receptor, gamma, coactivator 1 alpha; SCD1, stearoyl CoA desaturase 1; slc10a1, solute carrier family 10 (sodium/bile acid cotransporter family), member 1; socs6, suppressor of cytokine signaling 6; srebp2, sterol regulatory element binding factor 2; stat, signal transducer and activator of transcription; Tie2, endothelial cell receptor tyrosine kinase; tspyl4, testis-specific Y-like 4.

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microarray data from adipose tissue of a polygenic obese line of mice fed 1% *trans*-10, *cis*-12-CLA. It was concluded that the delipidative effects of CLA occur through a pleiotropic reduction in fatty acid and triglyceride translocation and storage, decreased glucose availability and increased fatty acid oxidation. In that experiment, adipose mass was 30% less in the epididymal depot of CLA-fed mice, 27% less in the mesenteric depot and 58% less in brown adipose tissue after 14 days of CLA treatment [19]. In that same study, we found that livers of the CLA-fed mice accumulated more fat and mass than the livers from LA-fed mice. Here we report gene expression and fatty acid composition data from livers of these male mice that were fed *trans*-10, *cis*-12-CLA for 14 days [19]. Based on these data, it was concluded that CLA is associated with differential regulation of many genes in the murine liver, particularly those regulated by the hepatocyte nuclear factor 4α transcription factor (HNF-4α), which is thought to be important in controlling the metabolic status of the liver [25].

2. Materials and methods

2.1. Mice

M16 mice were maintained according to an approved North Carolina State University's Institutional Animal Care and Use Committee protocol. The M16 line is a moderately obese line that was selected for 3- to 6-week postweaning gain [26].

2.2. Diet composition and tissue sampling

Animals were fed purified AIN93G pellets (Harlan Teklad, Madison, WI, USA) formulated with either 1% *trans*-10, *cis*-12-CLA or 1% LA as a treatment control. The *trans*-10, *cis*-12-CLA was kindly donated by BASF (Ludwigshafen, Germany), and LA was purchased from Nu-Chek-Prep (Elysian, MN, USA). Dietary *trans*-10, *cis*-12-CLA and LA content were analyzed, confirming 92% and 99% purity, respectively. All animals were fed the LA diet for 7 days and then given *ad libitum* access to their assigned diet (either CLA or LA) for 14 days. A total of 36 nine-week-old male mice were used for this experiment, with 18 mice receiving each diet. Mice in the fed state were euthanized by CO_2 asphyxiation, and liver samples collected, weighed and stored at −80°C.

2.3. Fatty acid composition of tissues

Lipids were extracted from liver samples of eight mice per treatment group, and fatty acids were quantified by gas–liquid chromatography as previously described [27]. Dietary treatment effects (LA vs. CLA) were analyzed according to a completely randomized design using the general linear models procedure of SAS (SAS Institute, Cary, NC, USA). Differences were considered significant when $P<.05$.

2.4. RNA Extractions

Total RNA was isolated from liver using TriReagent (Sigma, St. Louis, MO, USA) and further purified with Qiagen RNeasy Mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol with modifications previously described [28].

2.5. Microarrays

Agilent (Palo Alto, CA, USA) G4121A mouse oligo microarray slides containing more than 20,000 probes were used for this experiment. Pooled total RNA was labeled with cyanine 3 (Cy3)- and cyanine 5 (Cy5)-labeled dCTP using the Agilent Fluorescent Direct Label kit following the manufacturer's protocol. Pooled samples were used to reduce costs of the experiment. For pooling, equal amounts of total RNA from four or five different mice were combined for labeling. Because there were 36 mice from each treatment group, four biological replicates were represented on the microarrays. Four microarrays were hybridized using RNA pooled from the 36 animals (18 CLA-fed, 18 LA-fed). Microarrays were hybridized for 17 h and washed as previously described [19]. Slides were scanned with an Agilent G2565BA microarray scanner.

2.6. Microarray data collection and analysis

Microarray data were collected and analyzed as previously described [19]. Briefly, microarray data extraction was performed using Agilent G2567AA Feature Extraction software, following Agilent's direct labeling protocol. Rosetta Resolver version 3.2, build 3.2.2.0.33 (Rosetta Biosoftware, Kirkland, WA, USA), was used to analyze data as previously described [19]. Intensity plots were produced and genes were identified as candidate genes if $P<.01$ using the Rosetta Resolver error model [29]. Raw data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus in accordance with "minimum information about microarray experiments" guidelines and given accession numbers GSM272840, GSM272907, GSM272908, GSM272909 and GSE10865. *P* values from Rosetta Resolver were converted to false discovery rate (FDR) *P* values [30].

2.7. Real-time RT-PCR

Real-time RT-PCR primers were designed to assay 12 genes and the ribosomal protein S18 housekeeping gene using Beacon Designer 7 (PREMIER Biosoft International, Palo Alto, CA, USA) or selected from primers previously designed [23] (Table 1). PCR products were sequenced to confirm their identity. Genes targeted for quantitative real-time RT-PCR verification were selected based on their involvement in signal transduction pathways (*socs6* and *stat5b*) or because they had greater than twofold differential expression based on microarray results (*bcl6*, *pdcd8*, *tspyl4*). Others were selected because they had previously been studied in the liver of female mice fed CLA (*acox1*, *egfr*, *fasn*, *igfbp2*, *me*) [23] or are important components of metabolic pathways (*agpat2* and *dgat2*).

A total of 500 ng RNA was reverse transcribed using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Foster City, CA, USA) for each mouse. Real-time RT-PCR reactions for 12 target genes and the S18 housekeeping gene were performed using Applied Biosystems Power SYBR Green PCR Master Mix in a BioRad iCycler (Hercules, CA, USA) with minor modifications. Fluorescein was added at a final concentration of 10 nM as the reference dye. Cycling conditions were as follows: 95°C for 7 min, 60 cycles of 95°C for 30 s, appropriate annealing temperature (Table 1) for 30 s, 72.0°C for 30 s, followed by 72°C for 5 min, 95°C for 1 min, 55.0°C for 1 min, followed by a melt curve analysis of 80 cycles of 10 s at 55°C with a 0.5°C increase every cycle. SYBR Green gene expression data were collected from two replicates of each sample and three replicates of each standard curve point. Replicate samples were averaged and analyzed using the Pfaffl [31] method to quantify expression ratios between the CLA and LA cycle threshold values. Determination of statistical significance was done by ANOVA (SAS Inc., Cary, NC, USA). The Relative Expression Software Tool [32] calculated fold changes adjusted for differences in PCR amplification efficiencies.

Table 1
Real-time RT-PCR primers

Primer	Accession number	Primer sequence forward	Primer sequence reverse	Product length (bp)	Annealing temperature (°C)	PCR Efficiency
acox1[a]	NM_015729	5′-GGTGGGTGGTATGGTGTCGTAC-3′	5′-CAAAGACCTTAACGGTCACGTAGTG-3′	278	56.0	79.8%
agpat2	NM_026212	5′-TTCGTTCGGTCCTTC-3′	5′-CGCTTAGGGAGTATTTC-3′	146	54.0	89.7%
bcl6	NM_009744	5′-TAATCTCGTGAACAGGTC-3′	5′-GCTAGAATCCGAATACTC-3′	196	56.0	106.7%
dgat2	NM_026384	5′-CGTTGGCTGGTAACTTCC-3′	5′-CCACGATGATGATAGCATTG-3′	132	60.0	93.8%
egfr	NM_007912	5′-AATGTCTGCCACCTATGC-3′	5′-ATTTGGAAGAAACTGGAAGG-3′	176	56.0	85.1%
fasn[a]	NM_007988	5′-CTGAAGAGCCTGGAAGATCGG-3′	5′-CCCTCCCGTACACTCACTCGT-3′	365	56.0	83.1%
igfbp2	NM_008342	5′-AGACGCTACGCTGCTATC-3′	5′-CTGCTACCACCTCCCAAC-3′	196	59.6	79.9%
me[a]	NM_008615	5′-AGCAGTGCTACAAGGTGACCAA-3′	5′-CTCCAGGGAACACGTAGGAATT-3′	129	56.0	81.5%
pdcd8	NM_012019	5′-GGAGCAGAGGTGAAGAGTAGAAC-3′	5′-CCGCCGATAACTGTAATTGACTTG-3′	104	60.0	102%
s18	NM_011296	5′-ACCCACGACAGTACAAGATCC-3′	5′-AGCCTCTCCAGGTCCTCAC-3′	124	56.0	96.5%
socs6	NM_018821	5′-AATGGTAGTATGCTGGTCAG-3′	5′-TTTCTACAGGCAAATCTTATGG-3′	108	56.3	118.7%
stat5b	NM_011489	5′-TGATGGCGTGATGGAAGTATTG-3′	5′-CCGTCTGGCTTGTTGATGAG-3′	117	59.9	110.2%
tspyl4	NM_030203	5′-AGGAGGGAAAGAGGAGAC-3′	5′-TTGGATGATGAAACTTCTGC-3′	181	56.0	112.1%

All primers were designed using Beacon Designer 7 unless otherwise stated. Annealing temperatures were empirically determined.
[a] Designed by Rasooly et al. [23].

2.8. Pathway and network analysis

GeneGo's MetaCore software suite (St. Joseph, MI, USA) was used to further interrogate the differentially expressed genes from the experiment. Gene abbreviations for the 775 genes exceeding the FDR threshold of $P<.05$ and their fold changes were uploaded into the MetaCore workflow to identify potential pathways or networks associated with CLA treatment. The most relevant signaling or metabolic pathways and networks were identified using the "enrichment analysis–pathway maps" option. In addition, networks were built using network building tools provided in the software. The "analyze network (transcription factors)" tool built networks connecting differentially expressed gene targets of transcription factors.

3. Results

3.1. Fat content and fatty acid composition of liver

The average amount of fat in the CLA and LA livers was 8.54 and 5.34 g, respectively, per 100 g of wet tissue, indicating accumulation of 62.5% more fat in the CLA-fed livers ($P<.002$; Table 2). As we reported previously [19], there was a 33% increase in liver weight from mice fed the CLA diet for 14 days. Accumulation of fat accounted for approximately 20% of this increase. Because feeding CLA increased hepatic lipid levels, we examined the fatty acid profile. The *trans*-10, *cis*-12-CLA isomer (18:2) was only present in livers of mice fed the CLA-supplemented diet (Table 2). The C18:0/C18:1 ratio was significantly different ($P<.05$) between the livers of mice fed CLA and LA.

3.2. Hepatic gene expression and pathway analysis

Microarrays were used to identify genes differentially expressed due to CLA treatment. Analysis of gene expression data identified 1393 differentially expressed genes at a nominal *P* value of .01. After FDR correction, 775 genes were differentially expressed ($P<.05$; a list of these genes can be found with the GEO accession GSE10865). Quantitative real-time RT-PCR assays designed for 12 genes and a housekeeping gene (Table 1) were used to validate our results or to compare with results reported in a similar study [23] in which CLA was fed and liver gene expression examined. Of the 12 target genes evaluated (Table 3), results from one gene disagreed between techniques indicating 92% of the genes tested had results congruent between microarray and RT-PCR assays. The result for *stat5b* was likely a false positive from the microarray, in which the more sensitive real-time PCR method indicated no statistical differential expression for this gene.

Our list of 775 differentially expressed genes was imported into GeneGo software to identify pathways affected when CLA is added to the diet. Five pathways were identified that may be affected by CLA treatment ($P<.03$) (Table 4). These pathways involve cell

Table 2
Fat content and fatty acid composition of liver tissue from obese mice fed LA or CLA for 14 days[a]

Parameter	LA	CLA	SEM	*P* value
	g/100 g wet tissue			
Total fat	5.34	8.54	0.58	.002
	g/100 g fatty acids			
C14:0	0.52	0.68	0.05	.061
C16:0	22.67	24.77	0.70	.051
C18:0	6.37	5.19	0.38	.046
C16:1(n9)	2.71	2.60	0.29	.786
C18:1(n9)	23.72	31.97	2.56	.039
C18:2(n6)	23.01	20.40	1.45	.224
C18:2 (CLA)	nd	0.66	0.02	<.0001
C18:3(n6)	0.39	0.23	0.04	.014
C18:3(n3)	0.86	1.02	0.06	.089
C20:1(n9)	0.50	0.63	0.06	.178
C20:2(n6)	0.37	0.63	0.02	<.0001
C20:3(n3)	1.19	0.60	0.06	<.0001
C20:4(n6)	10.73	5.79	0.64	<.0001
C22:6(n3)	4.77	3.30	0.35	.009
	Ratio			
C16:0/C16:1	8.99	10.51	1.17	.372
C18:0/C18:1	0.31	0.18	0.04	.045
Saturated/unsaturated[b]	0.43	0.45	0.02	.465

[a] Values are least square means of $n=8$ mice per treatment.
[b] (C14:0+C16:0+C18:0)/(C16:1+C18:1+C18:2+C18:3+C20:1+C20:2+C20:3+C20:4+C22:6).

Table 3
Microarray and real-time RT-PCR results comparison

Gene name	Microarray results[a]		Real-time PCR results[b]	
	P value	Fold change[c]	*P* value	Fold change
Genes with >2-fold change				
bcl6	2.06×10^{-8}	2.12	.038	2.21
pdcd8	4.64×10^{-16}	2.19	.037	1.23
tspyl4	7.90×10^{-13}	0.42	.0096	0.58
Signal transduction genes				
socs6	.0044	0.79	.003	0.54
stat5b	.0020	1.24	.273	NA[d]
Metabolism genes				
acox1	.3748	0.83	.010	0.53
agpat2	.29	1.34	.41	NA[d]
dgat2	.37	1.20	.52	NA[d]
egfr	.0019	0.71	.008	0.38
fasn	.2588	1.17	.190	NA[d]
igfbp2	.1252	0.74	.020	0.42
me	.100	1.26	.141	NA[d]

All *P* values are nominal *P* values.
[a] Results based on hybridization of 4 two-color microarrays using 36 samples, pooled based on dietary treatment into four groups of nine individuals.
[b] Results based on C_t values calculated in duplicate for 36 individual samples.
[c] A fold change >1 indicates an up-regulation of that gene in the CLA samples as compared to the LA samples; a fold change <1 indicates a down-regulation of that gene in the CLA samples as compared to the LA samples.
[d] Not applicable because the *P* value indicated the gene was not differentially expressed between treatment groups.

Table 4
GeneGo pathway and process maps containing significant numbers of differentially expressed genes

GeneGo Map	Cell process	*P* value	Objects*
Signal transduction, PKA signaling	Protein kinase cascade, G protein-coupled receptor protein signaling pathway, second messenger-mediated signaling	7.16×10^{-5}	8/23
Muscle contraction, GPCRs in regulation of smooth muscle tone	G protein-coupled receptor protein signaling pathway	2.56×10^{-3}	10/54
Signal transduction, cAMP signaling	G protein-coupled receptor protein signaling pathway, second messenger-mediated signaling	4.32×10^{-3}	7/32
Development of angiopoietin-Tie2 signaling	Response to extracellular stimulus	6.16×10^{-3}	7/34
Immune response, antigen presentation by MHC Class I	Immune response	6.18×10^{-3}	6/26

* The numerator indicates the number of differentially expressed genes identified in the experiment and the denominator indicates total number of objects in the GeneGo map.

Table 5
Effects of dietary CLA vs. LA on expression of hepatic genes associated with lipid metabolism pathways[a] after 14 days of feeding

Gene abbreviation	Gene name	Fold change[b]	Nominal *P* value
acaa1a	Acetyl-coenzyme A acyltransferase 1A	1.45	.22
acaa2	Acetyl-coenzyme A acyltransferase 2	0.92	.44
acaca	Acetyl-coenzyme A carboxylase alpha	1.06	.69
acacb	Acetyl-coenzyme A carboxylase beta	1.00	.98
acad8	Acyl-coenzyme A dehydrogenase family, member 8	0.89	.33
acad9	Acyl-coenzyme A dehydrogenase family, member 9	0.97	.80
acad10	Acyl-coenzyme A dehydrogenase family, member 10	0.99	.91
acadl	Acyl-coenzyme A dehydrogenase, long-chain	0.91	.66
acadm	Acyl-coenzyme A dehydrogenase, medium chain	1.10	.61
acadsb	Acyl-coenzyme A dehydrogenase, short chain	1.18	.51
acadvl	Acyl-coenzyme A dehydrogenase, very long chain	1.06	.62
acat1	Acetyl-coenzyme A acetyltransferase 1	0.79	.40
acat2	Acetyl-coenzyme A acetyltransferase 3	1.14	.46
acox1	Acyl-coenzyme A oxidase 1, palmitoyl	0.83	.37
acox3	Acyl-coenzyme A oxidase 3, pristanoyl	1.00	.99
acyl	ATP-citrate lyase	1.05	.78
adh1	Alcohol dehydrogenase 1 (Class I)	1.10	.44
adh4	Alcohol dehydrogenase 4 (Class II), pi polypeptide	1.00	.93
adh5	Alcohol dehydrogenase 5 (Class III), chi polypeptide	0.84	.19
adh7	*Alcohol dehydrogenase 7 (Class IV), mu or sigma polypeptide*	*1.27*	*.002*
agpat1	1-Acylglycerol-3-phosphate *O*-acyltransferase 1	0.85	.09
agpat2	1-Acylglycerol-3-phosphate *O*-acyltransferase 2	1.34	.29
agpat3	1-Acylglycerol-3-phosphate *O*-acyltransferase 3	1.24	.19
agpat4	1-Acylglycerol-3-phosphate *O*-acyltransferase 1	1.37	.06
agpat5	1-Acylglycerol-3-phosphate *O*-acyltransferase 5	1.16	.58
agpat6	1-Acylglycerol-3-phosphate *O*-acyltransferase 6	1.92	.16
akr1b3	Aldo-keto reductase family 1, member B3	1.35	.17
akr1b7	Aldo-keto reductase family 1, member B7	0.73	.12
akr1b8	Aldo-keto reductase family 1, member B8	1.63	.52
akr1c6	Aldo-keto reductase family 1, member C6	0.82	.12
akr1d1	Aldo-keto reductase family 1, member D1	0.98	.90
akr1e1	Aldo-keto reductase family 1, member E1	0.79	.38
aldh1b1	Aldehyde dehydrogenase 1 family, member B1	1.20	.51
aldh2	*Aldehyde dehydrogenase 2, mitochondrial*	*1.34*	*.03*
aldh3a2	Aldehyde dehydrogenase family 3, subfamily A2	1.05	.66
aldh7a1	Aldehyde dehydrogenase family 7, member A1	0.88	.16
aldh9a1	Aldehyde dehydrogenase 9, subfamily A1	1.24	.45
bdh1	3-Hydroxybutyrate dehydrogenase, type 1	1.09	.21
cel	Carboxyl ester lipase	1.09	.42
chka	Choline kinase alpha	1.11	.60
chkb	Choline kinase beta	0.94	.57
cpt1a	Carnitine palmitoyltransferase 1a, liver	0.92	.64
cpt1b	Carnitine palmitoyltransferase 1b, muscle	1.16	.14
cpt1c	Carnitine palmitoyltransferase 1c	1.12	.54
cpt2	Carnitine palmitoyltransferase 2	0.99	.95
cyp27a1	*Cytochrome P450, family 27, subfamily a, polypeptide 1*	*0.71*	*.00008*
cyp4a10	Cytochrome P450, family 4, subfamily a, polypeptide 10	1.12	.24
cyp4a14	*Cytochrome P450, family 4, subfamily a, polypeptide 14*	*1.25*	*.04*
cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1	0.98	.85
dak	*Dihydroxyacetone kinase 2 homolog (yeast)*	*1.40*	*.05*
dci	*Dodecenoyl-coenzyme A delta isomerase (3,2 trans-enoyl-coenyme A isomerase)*	*1.40*	*.00002*
dgat1	Diacylglycerol *O*-acyltransferase 1	1.46	.15
dgat2	Diacylglycerol *O*-acyltransferase 2	1.20	.37
dgka	Diacylglycerol kinase, alpha	0.82	.38
dgkb	Diacylglycerol kinase, beta	0.98	.93
dgkg	Diacylglycerol kinase, gamma	1.00	1.00
dgkq	Diacylglycerol kinase, theta	1.11	.06
dgkz	Diacylglycerol kinase zeta	1.17	.38
echs1	Enoyl coenzyme A hydratase, short chain, 1, mitochondrial	0.77	.25
fasn	Fatty acid synthase	1.18	.26
gcdh	Glutaryl-coenzyme A dehydrogenase	1.10	.25
gk2	Glycerol kinase 2	1.09	.70
gla	Galactosidase, alpha	1.02	.91
glb1	*Galactosidase, beta 1*	*0.79*	*.004*
gpam	Glycerol-3-phosphate acyltransferase, mitochondrial	1.07	.62
gpd1	Glycerol-3-phosphate dehydrogenase	1.60	.12
gyk	Glycerol kinase, transcript variant 1	1.20	.12
hadha	Hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase/enoyl-coenzyme A hydratase (trifunctional protein), alpha subunit	1.03	.74
hadhb	*Hydroxyacyl-coenzyme A dehydrogenase/ 3-ketoacyl-coenzyme A thiolase/ enoyl-coenzyme A hydratase (trifunctional protein), beta subunit*	*1.25*	*.01*
hmgcl	3-Hydroxy-3-methylglutaryl-coenzyme A lyase	1.27	.08
hmgcs1	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1	1.00	.98
hmgcs2	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 2	1.15	.20
hsd17b4	Hydroxysteroid (17-beta) dehydrogenase 4	0.96	.71
hsd3b7	*Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7*	*0.71*	*.04*
idh1	Isocitrate dehydrogenase 1	0.98	.83
idh2	Isocitrate dehydrogenase 2	1.31	.45
idh3a	Isocitrate dehydrogenase 3a	1.00	.96
idh3b	Isocitrate dehydrogenase 3b	1.20	.58
idh3g	Isocitrate dehydrogenase 3g	0.77	.07
lipc	Lipase, hepatic	0.84	.20
lipf	Lipase, gastric	0.89	.42
lipg	Lipase, endothelial	0.95	.53
lpl	Lipoprotein lipase	0.88	.35
me1	Malic enzyme 1	1.26	.10
me2	Malic enzyme 2	1.15	.22
me3	*Malic enzyme 3*	*1.31*	*.03*
oxct1	*3-Oxoacid CoA transferase 1*	*1.24*	*.04*
oxct2a	3-Oxoacid CoA transferase 2A	1.14	.10
peci	Peroxisomal delta3, delta2-enoyl-coenzyme A isomerase	1.13	.30
pnliprp2	*Pancreatic lipase-related protein 2*	*1.51*	*.03*
pnpla3	Patatin-like phospholipase domain containing 3	1.24	.18
ppap2a	*Phosphatidic acid phosphatase 2a*	*0.76*	*.04*
ppap2b	Phosphatidic acid phosphatase type 2b	0.88	.47
ppap2c	Phosphatidic acid phosphatase type 2c	0.89	.59
rdh11	Retinol dehydrogenase 11	0.81	.17
rdh12	Retinol dehydrogenase 12	1.07	.15
rdh13	Retinol dehydrogenase 13 (all-*trans* and 9-*cis*)	0.98	.83
rdh14	Retinol dehydrogenase 14 (all-*trans* and 9-*cis*)	0.90	.39
slc27a5	Solute carrier family 27 (fatty acid transporter), member 5	0.99	.92
soat1	Sterol *O*-acyltransferase 1	1.05	.83
soat2	Sterol *O*-acyltransferase 2	1.02	.92
ugcgl1	UDP-Glucose ceramide glucosyltransferase-like 1	1.64	.17

[a] Genes included in this table were selected from the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/pathways.html) pathways associated with lipid metabolism. This included the fatty acid biosynthesis, bile acid synthesis, glycerophospholipid metabolism, glycerolipid metabolism, and synthesis and degradation of ketone body pathways.
[b] CLA/LA.

signaling cascades, while more traditional metabolic pathways were unaffected (Table 5).

4. Discussion

In this study, we examined differential gene expression using microarrays and real-time PCR in the livers of mice fed either CLA or LA. We found that livers of CLA fed mice weighed more and contained more lipid. Conversion of saturated fatty acids to their corresponding monounsaturated fatty acids is catalyzed by stearoyl CoA desaturase 1 (SCD1); hence, comparing ratios of unsaturated to monounsaturated fatty acids may provide an indication of enzymatic activity [33]. Some groups have shown SCD1 levels to be reduced [34,35], while others have found levels to be unaffected [23,36] or increased [37] in murine liver when mice were fed CLA. In our evaluation of liver samples, the 16:0/16:1 ratio was unchanged (P=.372), while the ratio of 18:0/18:1 was decreased (P=.045). Therefore we cannot determine whether SCD activity was affected by CLA treatment based solely on fatty acid ratios. In liver, the 18:0/18:1 ratio was decreased and the 16:0/16:1 ratio was unchanged; however, in adipose tissue, we found the opposite, with 16:0/16:1 increased and 18:0/18:1 unchanged in these same mice [19]. It is possible that hepatic steatosis was caused by accretion of fatty acids originating from delipidated adipose tissue during the 14 days of CLA treatment [21,22]. Putative translocation of fatty acids from adipose to liver tissues could have masked changes in the ratios of saturated to unsaturated fatty acids resulting from *de novo* hepatic synthesis. Although we did not measure circulating nonesterified fatty acid concentration or composition, Oikawa et al. [38] observed an attenuation of CLA-induced hepatic lipidosis *via* arachidonic acid and this effect was not associated with altered NEFA concentration.

Using GeneGo, we were able to identify biological pathways that appear to be affected when CLA is added to the diet. The five identified pathways involve cell signaling cascades, while more traditional metabolic pathways appear to be unaffected (Table 5). These results support use of microarrays, because initially we hypothesized that metabolic pathways such as fatty acid and glycerol lipid synthesis and/or degradation would be affected. However, these pathways were not significantly affected, with most of the genes associated with these pathways being unchanged. While carbon flux through these hepatic pathways may well be altered by dietary CLA feeding, our data would suggest that changes in flux, by and large, are not mediated by changes in gene expression of enzymes in the pathways.

CLA treatment appears to affect the protein kinase A (PKA) signal transduction pathway containing a total of 23 objects, such as proteins and genes, as defined by GeneGo (Table 4). Eight of these objects were differentially expressed in our experiment, with four genes down-regulated in the livers of the CLA-fed mice and four genes up-regulated. Many of these same genes are found in the cyclic adenosine monophosphate (cAMP) signaling pathway, which regulates PKA and was another pathway significantly affected by CLA treatment (Table 4). Down-regulation of these four genes may indicate that less cAMP is available in the liver, which may lead to less degradation of stored glycogen [39]. Additional evidence supporting CLA's effect on

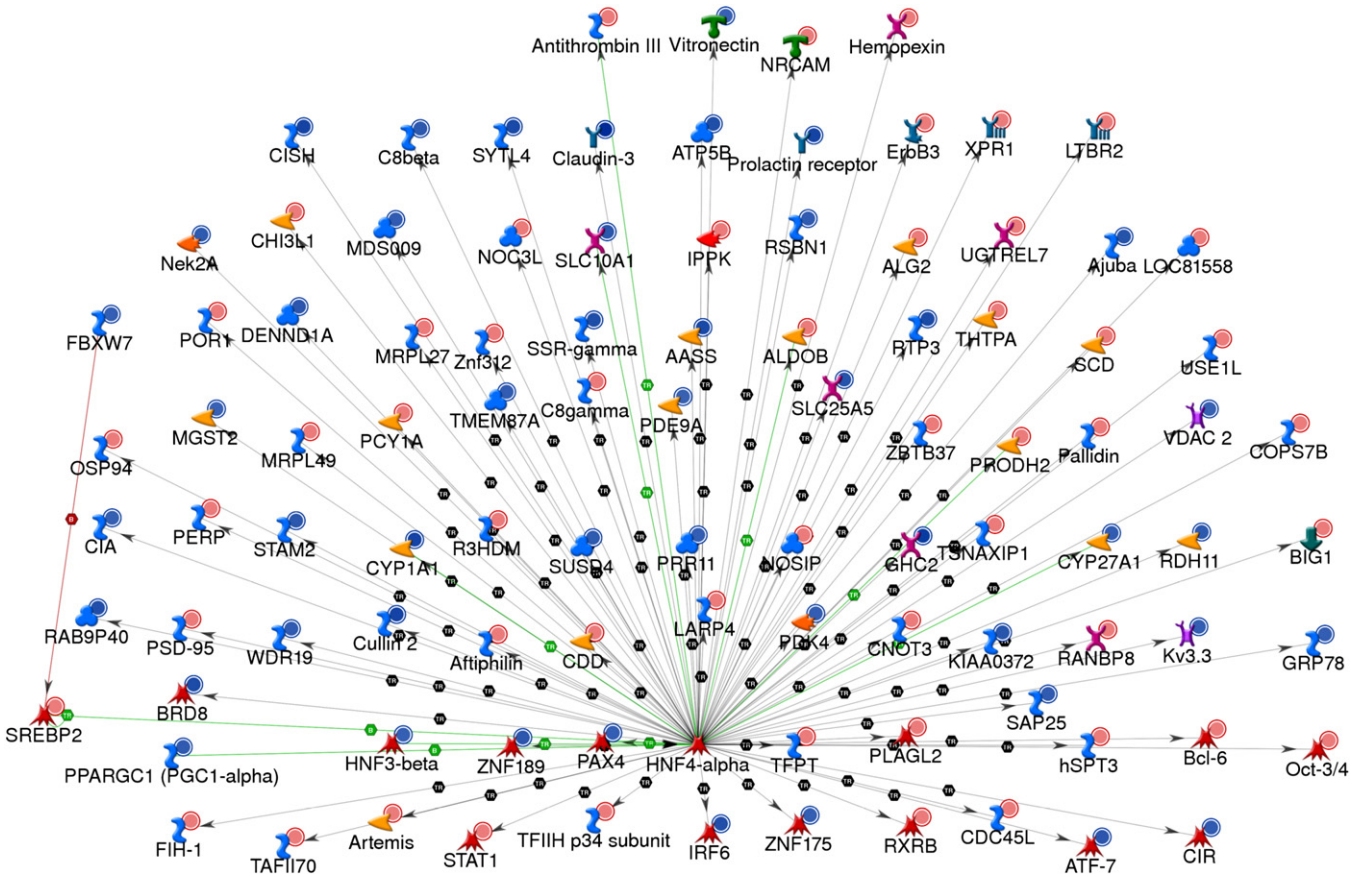

Fig. 1. GeneGo transcription factor network built for HNF-4α. Networks built around transcription factors using the differentially expressed gene list identified a common transcription factor, HNF-4α, which regulates gene expression for 98 of the 775 differentially expressed genes identified in the CLA–LA experimental comparison. Gene symbols in red indicate genes that are up-regulated in the CLA-fed mouse livers and those in blue indicate down-regulated genes in CLA-fed livers. For complete figure symbol legend, go to http://ftp.genego.com/files/MC_legend.pdf.

the cAMP/PKA signaling pathway was shown in rat cardiac gene expression, in which Tappia et al. [40] suggest that abnormalities in this signaling pathway are modulated by dietary CLA.

The G protein-coupled receptor protein signaling pathway associated with smooth muscle contraction was also affected by CLA treatment. This pathway contains a total of 54 objects, with 10 of those being differentially expressed in our experiment (Table 4). Genes in this pathway include many of the same genes in the cAMP and PKA signaling pathways discussed above. This includes adenylate cyclase, whose transcript was down-regulated in the CLA-treated livers and whose gene product normally increases levels of cAMP, which in turn, activates PKA [41]. CLA was previously shown to affect vascular smooth muscle cells through inhibition of nuclear factor κB (NF-κB) activation *via* a peroxisome proliferator-activated receptor γ-dependent mechanism, preventing an inflammatory response and development of atherosclerosis [42].

An immune response pathway was affected in our CLA experiment (Table 4). This pathway, involving antigen presentation by major histocompatibility complex Class I proteins, contains six out of 26 objects differentially expressed when CLA was fed. CLA isomers have been shown to have anti-inflammatory properties [43], and affected genes in this pathway were generally down-regulated, adding further evidence of CLA's anti-inflammatory effects.

The angiopoietin-Tie2 signaling pathway was also affected by CLA treatment. This pathway contains 34 objects, seven of which were differentially expressed in our experiment (Table 4). Angiopoietin down-regulates NF-κB by binding to endothelial cell receptor tyrosine kinase (Tie2), which eventually results in failure of NF-κB activation. Wunderlich et al. [44] reported that repression of NF-κB in the liver of mice fed a high-fat diet was associated with accumulation of lipid in hepatocytes, similar to what was observed in the liver of mice fed CLA. CLA modulates NF-κB activation in macrophages [45] and may have a similar effect in hepatocytes.

A study similar to our study was conducted feeding 0.5% *trans*-10, *cis*-12-CLA to female mice [23]. Rasooly et al. [23] targeted genes involved in fatty acid oxidation and we used several of the primers they reported [23] to directly compare real-time PCR results (Table 3). They found a tendency toward differential expression of acetyl CoA oxidase 1 (*acox1*), while we found significant down-regulation of this gene. Rasooly et al. [23] found down-regulation of epidermal growth factor receptor (*egfr*) in their CLA-fed mice and our results support that finding. Insulin-like growth factor binding protein 2 (*igfbp2*) was differentially expressed in both studies [23], while fatty acid synthase (*fasn*) was not differentially expressed in our real-time PCR data, but was in their study. We found no differential expression of malic enzyme (*me*), while their microarray result was unconfirmed using real-time PCR. Therefore, it is most likely that *me* is not differentially expressed in liver when *trans*-10, *cis*-12-CLA is fed. The few discrepancies between these studies may be due to gender differences because we examined males and they [23] examined females. Clodfelter et al. [46] reported extensive gender gene expression differences in murine liver.

Networks built around transcription factors using the differentially expressed gene list identified a common transcription factor, hepatocyte nuclear factor 4α (HNF-4α), which regulates gene expression for 98 of the 775 genes (Fig. 1). This transcription factor regulates a vast number of genes in hepatocytes and pancreatic islets, and mutations within HNF-4α cause the type 1 form of maturity-onset diabetes of the young [47]. HNF-4α is an extensive controller of liver function, and previous studies showed that conditional knockout of this gene in the mouse liver [25] produces offspring that accumulate lipid in the liver, similar to what is seen when CLA is fed. In addition, pathway analysis indicated that the PKA pathway was affected in this experiment and Viollet et al. [48] reported that PKA phosphorylates HNF-4α, modulating the DNA-binding activity of this important hepatic transcription factor.

Holloway et al. [49] examined murine liver-specific HNF4α deficiency and found lack of this transcription factor has a greater impact on gene expression in males. They suggest HNF-4α regulates gender-specific gene expression in the liver using mechanisms active in males and primarily inactive in females [49]. We only examined males which may explain why we identified genes regulated by HNF-4α as being differentially expressed.

Co-enzyme A esters of long-chain fatty acids modulate the transcriptional activity of HNF-4α based on chain length and degree of saturation [50], but no data exist on specific effects of different CLA isomers. Khan and Vanden Heuvel [50] hypothesized that different CLA isomers act as peroxisome proliferator-activated receptor

Table 6
Significant HNF-4α-regulated genes involved in inflammation or fatty acid metabolism

Gene symbol	Gene name	Fold change	*P* value	Biological process	Function
cish	Cytokine inducible SH2-containing protein	0.51	.00114	Inflammation	Suppressor of cytokine signaling, negatively regulates Jak/STAT pathway
stam2	Signal transducing adaptor molecule 2	0.65	5.09×10^{-7}	Inflammation	Downstream signaling of cytokine receptors
stat1	Signal transducer and activator of transcription 1	1.48	.00002	Inflammation	Mediates interferon signaling, cell proliferation
bcl6	B-Cell leukemia/lymphoma 6	2.12	2.06×10^{-8}	Inflammation	Transcriptional repressor, regulates B-cell differentiation and inflammation
cyp1a1	Cytochrome P450, family 1, subfamily a, polypeptide 1	0.51	8.21×10^{-7}	Fatty acid metabolism	NADPH-dependent electron transport oxidizing fatty acids
sytl4	Synaptotagmin-like 4	0.53	.00007	Fatty acid metabolism	Exocytosis and docking of insulin granules to plasma membrane
slc10a1	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	0.65	2.97×10^{-6}	Fatty acid metabolism	Bile acid transport
ppargc1	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	0.69	.00018	Fatty acid metabolism	Coactivator of HNF-4, apolipoprotein induction and export, bile acid transport
cyp27a1	Cytochrome P450, family 27, subfamily a, polypeptide 1	0.71	.00008	Fatty acid metabolism	Converts cholesterol to bile acids
srebp2	Sterol regulatory element binding factor 2	1.41	.00021	Fatty acid metabolism	Binds regulatory elements in cholesterol biosynthesis genes
pcy1a	Phosphate cytidylyltransferase 1, choline, alpha isoform	1.50	.00019	Fatty acid metabolism	Phosphatidylcholine synthesis
rxrb	Retinoid X receptor beta	1.51	1.57×10^{-6}	Fatty acid metabolism	Regulator of PPAR signaling pathway controlling fatty acid metabolism
scd1	Stearoyl-coenzyme A desaturase 1	1.82	.00102	Fatty acid metabolism	Desaturates fatty acids
noc3l	Nucleolar complex associated 3 homolog	1.96	.00003	Fatty acid metabolism	Adipocyte differentiation

ligands, suggesting that specific nuclear receptors might recognize distinct fatty acid structures leading to different phenotypes, perhaps explaining the unique effects of CLA feeding.

Analysis of the amount of fat in our liver samples indicated that only 20% of the increase in liver mass was due to increase in fat. Some of the weight accretion may be due to cell proliferation through inhibition of HNF-4α. Lazarevich et al. [51] showed that loss of HNF-4α led to cell proliferation and dedifferentiation in hepatocellular carcinomas. Support for HNF-4α inhibition is found in examination of transcript levels for the genes affected by CLA treatment. Examination of putative functions of the 98 genes regulated by HNF-4α found four to be involved in inflammation and 10 to be involved in fatty acid and bile acid metabolism (Table 6). The sodium bile acid cotransporter, *slc10a1*, was down-regulated in the CLA-fed mice, and this gene is directly activated by HNF-4α [52]. HNF-4α also stimulates two cytochrome P450 genes (*cyp27a1* and *cyp1a1*) [53,54], which were down-regulated in the CLA-fed livers. It appears that bile acid metabolism and transport may be affected in these samples, which was previously found in murine liver accumulating fat after mitochondrial β-oxidation was inhibited [55]. *Hnf4α* was not differentially expressed ($P=.11$) in our study, but the HNF-4α coactivator, peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (*ppargc1*, also known as *pgc-1α*), was down-regulated in the CLA livers and sterol regulatory element binding factor 2 (*srebp2*), a competitive inhibitor of PPARGC1 recruitment to HNF-4α [56], was up-regulated. Therefore, lack of HNF-4α activation, and not differential gene expression of this gene, may be responsible for some of the effects on gene expression we observed.

Previously [19], we used microarrays to identify differentially expressed genes in adipose tissue from these mice after 5 and 14 days of CLA feeding and herein have reported differential expression in the livers after 14 days of CLA treatment. Use of microarrays is one approach to studying the effects of dietary supplementation on gene expression and may provide insight into the major responses that occur. In this study, we have begun to elucidate some of the molecular mechanisms of CLA's effects on the male murine liver, realizing we did not capture the entire picture because we did not measure gene product activity. A comparison of affected GeneGo pathways across the adipose and liver tissues finds that different pathways and genes are differentially expressed. Therefore, the effect of feeding CLA to a polygenic obese line of mice does not appear to be a simple case where one metabolic pathway is up-regulated in one tissue and down-regulated in the other tissue and continued research is needed to clarify the effects of CLA treatment.

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