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Characterization of genome-wide transcriptional changes in liver and adipose tissues of ZDF (fa/fa) rats fed R-α-lipoic acid by next-generation sequencing

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Submitted 4 September 2013; accepted in final form 1 October 2013

Pashaj A, Yi X, Xia M, Canny S, Riethoven JJM, Moreau R. Characterization of genome-wide transcriptional changes in liver and adipose tissues of ZDF (fa/fa) rats fed R-α-lipoic acid by next-generation sequencing. Physiol Genomics 45: 1136–1143, 2013. First published October 8, 2013; doi:10.1152/physiolgenomics.00138.2013.—We report on the characterization of lipogenic tissue transcriptional networks that support physiological responses of obese rats to a lipid-lowering bioactive food compound, R-α-lipoic acid (LA). Nine-week-old male Zucker diabetic fatty (fa/fa) rats were fed a Chow diet supplemented with 3 g LA per kg diet or pair fed for 2 wk. At the end of the trial, high-quality RNA was extracted from the liver and epididymal fat and subjected to transcriptome analysis by RNA-Seq technology. Results showed a substantially higher number of differentially expressed genes [DEG, false discovery rate adjusted P ≤ 0.05 and absolute log2 (fold change) ≥ 1] in the liver (110 genes) vs. epididymal fat (10 genes). Most epididymal fat DEG were also differentially expressed in liver and shared directionality of change. Gene Ontology (GO) analysis of these transcripts revealed significant enrichment of GO categories related to immune response, stress response, lipid metabolism, and carboxylic acid metabolic processes. Of interest, interferon-related genes involved in defense against microorganisms and innate immune response were induced by LA. Lipid metabolism-related transcript changes observed in LA-fed animals included downregulation of lipogenic genes (Pnopala3, Pnopala5, Elov6, Acly, Gpam, and Aacs) and concomitant upregulation of short-, medium-, and long-chain fatty acid metabolic processes (Acct1, Acct2, Acctf2, and Crtal). Transcriptional changes were accompanied by the lowering of abdominal adiposity and blood triacylglycerol levels. We conclude that LA dietary supplementation induces prominent gene expression changes in liver in support of significant improvement of whole-body lipid status.

Studies have documented a strong association between NAFLD and traditional and nontraditional risk factors for cardiovascular disease (CVD) (40, 41). Accordingly, patients with NAFLD have an increased prevalence and incidence of CVD (5, 26). It is believed that NAFLD and CVD share common risk factors, such as visceral obesity, insulin resistance, hyperglycemia, hypertension, and dyslipidemia. Consequently, the remediation of NAFLD is expected to positively impact the number and severity of cardiovascular events.

Modulation of hepatic triacylglycerol (TG) content and hepatic VLDL secretion is key to produce effective treatments for NAFLD and CVD. Despite aggressive LDL-cholesterol lowering by statin therapy, approximately two-thirds of all CVD events remain. These residual events appear to be independent of LDL-cholesterol. Statin drugs do reduce postprandial lipemia, but they do not affect TG levels sufficiently to be of clinical relevance in hypertriglyceridemic conditions (8, 13, 44). Heightened production of TG-rich VLDL in liver is the root cause of hypertriglyceridemia for 16% of Americans (30). Using Zucker diabetic fatty (ZDF, fa/fa) rats, a well-accepted model of obesity-mediated hypertriglyceridemia, we showed previously that diet supplementation with R-α-lipoic acid (LA) prevents the rapid rise in plasma VLDL-TG that predictably occurs between week 7 and week 8 of age by downregulating key enzymes of TG synthesis (7). As one of the most influential processes in biological systems, gene expression must drive and reflect tissue pathophysiological processes during hypertriglyceridemia and therapy-induced transcriptional changes central to its remediation. However, no genome-wide study has been conducted to capture global changes in transcriptional regulatory gene networks induced by LA in the setting of dyslipidemia.

Herein we present an investigation of the regulatory gene networks that support physiological responses of the obese rat to LA, a lipid-lowering bioactive food compound, by characterizing transcriptomic changes across lipogenic tissues. LA has been used therapeutically in diabetic neuropathy and retinopathy (1, 6, 19, 33, 36, 38, 46, 49–52), but its TG-lowering properties have only recently been recognized, first in laboratory animals (7, 22, 43, 47) and then in humans (27, 48). LA is a naturally occurring, essential, dithiol-containing cofactor synthesized enzymatically from octanoate in most prokaryotic and eukaryotic micro-organisms as well as plant and animal mitochondria (15). The presence of an asymmetric carbon yields two enantiomers, R-LA and S-LA, during organic syn-
thesis. Only the naturally occurring R isomer of LA is bound covalently to the epsilon amino group of lysine residues of mitochondrial multienzyme complexes. Although de novo synthesis supplies all LA needed for its function of mitochondrial cofactor, it is also absorbed from foods (leafy green vegetables and red meats) and dietary supplements. We employed RNA-Seq to quantify the abundance of RNA transcripts in the liver and epididymal fat pad collected from ZDF (fa/fa) rats fed ± LA. Results show a marked transcriptional response to LA in liver compared with epididymal fat. New candidate genes were identified as regulatory points of immune response and long-, medium-, and short-chain fatty acid metabolism, suggesting a putative role of these genes in the progression and mitigation of hepatosteatosis and dyslipidemia.

MATERIALS AND METHODS

Animals, diets, and tissue sampling. All procedures were approved by the Institutional Animal Care and Use Committee, University of Nebraska–Lincoln. Eight obese male Zucker rats (ZDF/GmiCrl-fa/fa, 7 wk old) were purchased from Charles River Laboratories (Wilmington, MA). On the basis of a pilot trial, the feeding study was designed as a 2 wk intervention trial where LA (3 g/kg diet; MAK Wood, Grafton, WI) is administered in the diet after blood TG have become elevated. This situation most resembles the human situation where individuals seek treatment after hyperglycemia is diagnosed. Upon arrival, rats were acclimated for 2 wk in individual cages in a controlled environment (ambient temperature 22 ± 2°C, 12:12 h light-dark cycle) with free access to food (Purina 5008: 26.8% calories from protein, 16.7% from fat, 56.4% from carbohydrates; Dyets, Bethlehem, PA) and water. At 9 wk of age, the rats were randomly assigned to one of two treatments (Purina 5008 +LA or Purina 5008 pair-feeding) for 2 wk (n = 4 replicates). Throughout the trial, animals were given 2-day feeding rations between 1 and 3 PM and provided MilliQ water to drink. Food and water intake as well as body weight were recorded every other day. Three-hour fasted blood was collected at the beginning and end of the trial in EDTA-coated tubes, and plasma was obtained by centrifugation at 12,000 g for 1 min and stored at −80°C. Plasma TG concentration was measured as a marker of dyslipidemia with the Serum Triglyceride Determination kit (Sigma-Aldrich, St. Louis, MO). At the end of the trial, rats (11 wk of age) were anesthetized with inhalant isoflurane, and samples of liver and epididymal fat were quickly removed, frozen in liquid N2, and stored at −80°C. The entire liver and abdominal adipose tissue (epididymal + mesenteric + omental + retroperitoneal fat) were removed and weighed.

RNA-Seq and bioinformatics. RNA-Seq was performed to measure gene expression in the liver and epididymal fat of 4 replicate ZDF (fa/fa) rats fed ± LA for 2 wk once TG had become elevated. Total RNA was isolated from tissue with TRIzol (Life Technologies, Gaithersburg, MD) and treated with DNase I (Qiagen, Valencia, CA), and RNA was isolated from tissue with TRIzol (Life Technologies, Gaithersburg, MD). Complementary DNA sequencing library was prepared from the total RNA with the mRNA-Seq Sample Preparation kit (Illumina, San Diego, CA). Briefly, polyadenylated RNA was isolated from 4 μg total RNA by Sera-Mag Magnetic Oligo-dT beads (ThermoFisher Scientific, Waltham, MA). Purified mRNA was fragmented, annealed to high concentrations of random hexamers, and reverse transcribed. Following second-strand cDNA synthesis, end repair, and A-tailing, Oligo adapters complementary to sequencing primers were ligated to cDNA fragment ends. Resultant cDNA libraries were size fractionated on an agarose gel, and 200 bp fragments were excised and then amplified by 15 cycles of polymerase chain reaction (PCR). Clusters were generated from the cDNA sequencing library on the surface of a flowcell in the Cluster Station (Illumina) by so-called bridge amplification. The flowcell was used to perform 100 cycles of sequencing-by-synthesis chemistry in the Genome Analyzer IIx, producing an average of 15 million reads per sample and ~65 million reads per tissue-feed combination.

To determine the quality of the replicates we performed a least-square simple linear regression between the replicates within each of the four tissue-feed combinations liver/−LA, liver/+LA, adipose/−LA, and adipose/+LA, respectively. We calculated the R2 statistic (0.807 ≤ R2 ≤ 0.995, median 0.971) and slope (0.644 ≤ b ≤ 1.18, median 0.897), which provides measures of goodness-of-fit and correlation, respectively, using the regress function in MATLAB (version 8.1.0.604 (R2013a), The MathWorks). Full ranges of R2 and b per tissue-feed combination are provided in the Supplemental Data.1 For use in all further analyses, the Rattus norvegicus genome and gene models were downloaded from Illumina’s iGenomes (NCBI build RGSC 3.4), gene functional descriptions and Gene Ontology to gene mappings from the Rat Genome Database (build 3.4 Aug 2013 updates, Ref. 25), and the Gene Ontology itself from the Gene Ontology Consortium (release Aug 2013, Ref. 2).

The bowtie (version 2.1.0, Ref. 24) and tophat (version 2.0.8, Ref. 21) packages were used to map the RNA sequence reads from the liver and epididymal fat tissues under the two feed regimes to the genome and to determine the expression quantity of known transcripts in each tissue. The cufflinks package (42) was run to calculate expression changes and associated q values (false discovery rate adjusted P values) for each gene, between: liver +LA and −LA, and epididymal fat +LA and −LA. We further classified genes as being significantly differentially expressed when the following conditions were met: q ≤ 0.05 and | log2 (fold change) | ≥ 1. The output files of cuffdiff were further annotated (in-house Perl script) by adding gene functional descriptions and GO classifications (Supplemental Data).

Gene Ontology (GO) term enrichment and fold enrichment or depletion for gene lists of significantly up- and downregulated genes in adipose and liver tissue were determined. For each GO term, fold-enrichment is the observed number of genes in the gene list divided by the expected number of genes, given the number of genes in the gene list associated with GO terms compared with the number of genes in the R. norvegicus genome associated with the same GO terms (20). Fold-depletion is 1/fold-enrichment. P values were calculated with the MATLAB hypergeometric probability density function hypgepdf and subsequently adjusted by the Benjamini and Hochberg multiple test correction method (4). Fold-enrichment values were determined significant if their adjusted P value ≤ 0.05. A condensed significant GO term list was created by selecting top-level ancestor GO terms from the significant GO term list.

The loci of genes on both the forward and reverse strand of the R. norvegicus genome were plotted on each chromosome and significantly up and downregulated genes in liver tissue were plotted in red and green, respectively, using an in-house MATLAB® script that is available upon request.

The raw transcriptome sequence files for liver +LA, liver −LA, adipose +LA, and adipose −LA have been uploaded, together with gene expression result files, to NCBI’s Gene Expression Omnibus under sequence number GSE50424.

Quantitative real-time PCR. RNA isolated from the liver and epididymal fat was analyzed by quantitative real-time PCR (qRT-PCR) to confirm the transcriptional changes obtained by RNA-Seq. For that purpose, we selected three genes significantly upregulated in liver [acyl-CoA thioesterase (Acot2), acyl-CoA synthetase family member 2 (Acas2), and carnitine O-acetyltransferase (Crtat)] and three genes significantly downregulated in liver [acetocacyl-CoA synthetase (Acsc), ATP-citrate synthase (Acly), and protein-like phospholipase D domain containing 3 (or adiponutrin) (Pnpla3)]. We also quantified the mRNA levels of three significantly upregulated genes

1 The online version of this article contains supplemental material.
[interferon regulatory factor 7 (Irf7), interferon-induced GTP-binding protein Mxl (Mx1), and schlaflon 3 (Slf3)] and three significantly downregulated genes [acyl-CoA carboxylase 1 (Acaca), serpin peptidase inhibitor clade A member 12 (SerpinA12), and adiponutrin (Ppia)] in epididymal fat. The selected genes included short and long genes (mean cDNA size of 3,438 bp ± 1,531, SD) to account for the inherent propensity of next-generation sequencing to produce more reads mapping to longer genes than shorter ones with the same expression level. Total RNA was reverse transcribed with oligo(dT) and random primers using Bio-Rad iScript (Bio-Rad Laboratories, Hercules, CA). qRT-PCR was performed with a Bio-Rad CFX96 and SYBR Green supermix according to the manufacturer’s instructions. Amplification confirmation was verified by melt curve analysis and agarose gel electrophoresis. PCR efficiencies were assessed with serial dilutions of the template (0.001, 0.01, 0.1, 1, 10, and 100 ng cDNA/reaction) and 0.3 μM of each primer, and plotting quantification cycle vs. log amount of template. PCR efficiencies between target genes and housekeeping genes were comparable, thus unknown amounts of target in the sample were determined relative to cyclophilin A (Ppia) and 60S acidic ribosomal protein P0 (Rplp0). Primers were synthesized by Eurofins MWG Operon (Huntsville, AL). Primers sequences are shown in Table 1.

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Statistical analysis. Morphometric, biochemical, and qRT-PCR data were analyzed by unpaired two-tailed Student t-test with Welch’s correction to the 5% significance level. Hierarchical clustering on genes and animals was performed using the MATLAB clustergram function.

RESULTS

Animal response to LA feeding. Feeding LA to 9 wk old ZDF rats for 2 wk significantly lowered abdominal fat mass (−1.9 g/rat, P < 0.013, n = 4) compared with pair-fed animals (Table 2). Initial and final body weights did not differ between treatments, nor did weight gain, food intake, and liver weight at the end of the trial (Table 2). No overt anatomical differences or gross pathologies were noted for any of the animals. At the level of incorporation in the diet (3 g/kg), LA is considered safe in rats (10), including ZDF rats (7). Based on an average daily feed intake of 20 g per rat and average initial body weight of 303 g per rat, LA intake amounted to 200 mg/kg body wt per day during the trial. Blood plasma TG levels predictably increased from 5.9 to 7.0 mM during the 2-wk trial in paired-treatment control rats. In contrast, feeding LA to ZDF rats reversed the progression of hypertriglyceridemia by decreasing blood TG from 5.0 to 2.0 mM (P < 0.005, n = 4) in the same time span, i.e., a 70% decrease vs. pair-fed controls.

Differential gene expression induced by LA in liver. Among the 22,517 genes constituting the rat reference genome (build 3.4 Aug 2013 updates, Ref. 25), the mean number of genes transcribed (≥1 hit) in the liver was 14,861 (66% of the total genes in the transcriptome) for pair-fed rats and 15,142 (67% of the total genes in the transcriptome) for LA-fed rats (Supplemental Data). Of the 14,861 genes expressed in untreated livers, 14,383 (97%) were expressed in LA-treated livers. Of the 22,517 genes constituting the rat reference genome (build 3.4 Aug 2013 updates, Ref. 25), the mean number of genes transcribed (≥1 hit) in the liver was 14,861 (66% of the total genes in the transcriptome) for pair-fed rats and 15,142 (67% of the total genes in the transcriptome) for LA-fed rats (Supplemental Data). Of the 14,861 genes expressed in untreated livers, 14,383 (97%) were expressed in LA-treated livers. Among DEGs, a total of 110 genes (78 up- and 32 downregulated genes by LA vs. pair feeding, Fig. 1A and Supplemental Data), met the selection criteria: 1) FDR adjusted P value ≤ 0.05 (q value) and 2) minimum of twofold difference in normalized read counts between treatments. The genomic distribution of these genes did not reveal chromosomal clusters of highly or weakly expressed genes (Fig. 1B). We validated six liver transcripts predicted to change significantly in the RNA-Seq dataset (representing three upregulated and three downregulated genes) by qRT-PCR. The analysis confirmed all predicted transcriptional changes (Fig. 1C). Of the 110 identified liver genes, 22 did not have biological process GO annotation to support the GO analysis and thus were eliminated from the analysis. Functional annotation clustering using the MATLAB clustergram function revealed distinct gene networks were affected by feeding LA. The top-level GO categories included immune response, response to organic substance, long-chain fatty acid metabolic process, organic acid metabolic process, and lipid metabolic process (Table 3).

Table 1. qRT-PCR primers used to confirm gene expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ → 3’)</th>
<th>Product Size, bp</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
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<tr>
<td>Acox2</td>
<td>AGGAAGATCTGAGCTGAGATG</td>
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<tr>
<td>Acx2</td>
<td>AGCTGCGAGCTGAGATG</td>
<td>134</td>
<td>NM_001034951.1</td>
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<tr>
<td>Crat</td>
<td>CGCTAGATCTGAGATG</td>
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<td>NM_001000405.2</td>
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<tr>
<td>Aacs</td>
<td>AGAGAGTCGCTGAGATG</td>
<td>134</td>
<td>NM_023104.1</td>
</tr>
<tr>
<td>Acly</td>
<td>AGCTGAGATCTGAGATG</td>
<td>160</td>
<td>NM_021987.2</td>
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<tr>
<td>Ppia</td>
<td>ATGGATGATCTGAGATG</td>
<td>113</td>
<td>NR_058887.2</td>
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<tr>
<td>Irf7</td>
<td>AATGGATGATCTGAGATG</td>
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<td>Mx1</td>
<td>ATGGATGATCTGAGATG</td>
<td>166</td>
<td>NM_173096.3</td>
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<td>Slf3</td>
<td>TTGCTGAGATCTGAGATG</td>
<td>144</td>
<td>NM_053687.1</td>
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<tr>
<td>Acaca</td>
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<td>298</td>
<td>NM_022193.1</td>
</tr>
<tr>
<td>SerpinA12</td>
<td>CGCGATGATCTGAGATG</td>
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<tr>
<td>Ppia</td>
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<td>140</td>
<td>NM_017101.1</td>
</tr>
<tr>
<td>Rplp0</td>
<td>AAGAGAGATCTGAGATG</td>
<td>161</td>
<td>NM_022402.2</td>
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</tbody>
</table>

Sequence is shown as sense primer followed by antisense primer.

Table 2. Morphometric parameters, food intake, and 3 h fasted blood plasma TG levels in ZDF rats fed ± LA for 2 wk

<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>Initial body weight, g</th>
<th>Final body weight, g</th>
<th>Weight gain, %</th>
<th>Cumulative food intake, g/kg body wt</th>
<th>Abdominal adipose mass, g/rat</th>
<th>Liver weight, g/rat</th>
<th>Initial plasma TG, mM</th>
<th>Final plasma TG, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair-fed</td>
<td>306 ± 4</td>
<td>357 ± 11</td>
<td>17 ± 3</td>
<td>993 ± 40</td>
<td>17.0 ± 0.3</td>
<td>16.4 ± 1.3</td>
<td>5.9 ± 0.5</td>
<td>7.0 ± 0.8</td>
</tr>
<tr>
<td>LA</td>
<td>300 ± 5</td>
<td>338 ± 14</td>
<td>13 ± 3</td>
<td>1,005 ± 44</td>
<td>15.1 ± 0.4</td>
<td>16.4 ± 0.7</td>
<td>5.0 ± 0.8</td>
<td>2.0 ± 0.2</td>
</tr>
</tbody>
</table>

Data are shown as means ± SE of 4 rats/group. Weight gain (% of initial body wt) = [(final body wt − initial body wt)/initial body wt] × 100. TG, triacylglycerol; ZDF, Zucker diabetic fatty; LA, R-α-lipoic acid; Ia, abdominal adiposomax index; Ih, hepatosomatic index. *P < 0.013, **P < 0.005 vs. pair-fed group.
A: differential gene expression analysis revealed 110 liver differentially expressed genes (DEG) between LA-fed (LL) and pair-fed animals (LP) \((n = 4\) livers per group). FPKM values (fragments per kilobase exon per million fragments mapped) of genes are used for each of the replicates in LL and LP to perform a hierarchical clustering with Euclidean distance metric and average linkage on both the replicates and the FPKM expression values of DEGs via the MATLAB clustergram function. For coloring purposes the FPKM values are further standardized per gene such that the mean is 0 with a standard deviation of 1, such that red shows high(er) expression and green shows low(er) expression compared with the mean.

B: genomic distribution of DEGs in liver (LA vs. pair-fed treatment). The graph depicts the chromosomal location of all significantly upregulated and downregulated genes in rat liver when LL and LP are compared. Transcripts were not assigned to chromosome Y since this chromosome is excluded from the rat reference genome (RGSC 3.4).

C: confirmatory analysis of liver and epididymal fat gene expression by qRT-PCR. Individual gene expression was determined by qRT-PCR as described under MATERIALS AND METHODS and expressed as fold change (FC) in log base 2 vs. pair-fed controls and graphed alongside FC derived from RNA-Seq analysis. All data met the two-FC cutoff (absolute log2 \(\geq 1\)) vs. pair-fed control \((P < 0.05, n = 4\) rats/group). D: Venn diagram depicting unique (nonoverlapping circles) and shared (overlapping circles) sets of DEGs between LA-fed and pair-fed rats. We obtained 110 and 10 annotated DEGs for liver and adipose (epididymal fat) tissue.
Response, response to biotic stimulus, response to stress and reflective of changes in immune response and lipid metabolism in liver and epididymal fat transcriptomes were primarily due to the rat strain, feeding schedule, or sampling time. The reason for this discrepancy is not entirely clear; it may be, however, is the absence of DEG related to circadian rhythm. A departure from this report, in this respect, our data are in good agreement with an earlier report carried out in lean rats (12). A departure from this report, however, is the absence of DEG related to circadian rhythm. The reason for this discrepancy is not entirely clear; it may be due to the rat strain, feeding schedule, or sampling time. LA-induced expression of interferon-related genes is one of the original results of the present study. Four out of the nine DEG in epididymal fat were related to interferon-mediated immune response, namely Ifi27l2b, Mx1, Mx2, and Irf7. These four genes were concomitantly upregulated in liver alongside genes implicated in inflammatory response (Gbp5, Cxcl9, and Cxcl10) and GSH-dependent detoxification (Gstp1 and Gsta5) (Supplemental Data).

Of particular interest in uncovering the mechanism by which LA regulates lipid metabolism and corrects hypertriglyceridemia is the observed significant decrease of genes encoding patatin-like phospholipase domain-containing protein 3 (Pnpla3, FC = 3.5370, Supplemental Data), patatin-like phospholipase domain-containing protein 5 (Pnpla5, FC = 2.5240), elongation of very-long chain fatty acids protein 6 (Elov6, FC = 1.6783), ATP-citrate synthase (Acly, FC = 1.6015), glycerol-3-phosphate acyltransferase 1 (Gpam, FC = 1.2317), and acetoacetyl-CoA synthetase (Aacs, FC = 1.2045).

Pnpla3 encodes a membrane-associated multifunctional enzyme, which is expressed in adipose and nonadipose tissues and has both TG lipase and acylglycerol O-acyltransferase activities (23). Association studies have consistently linked organic substances, lipid metabolic process, and organic acid metabolic process. Of these ontological categories, lipid metabolic process was the only category downregulated by LA. In this regard, our data are in good agreement with an earlier report carried out in lean rats (12). A departure from this report, however, is the absence of DEG related to circadian rhythm. The reason for this discrepancy is not entirely clear; it may be due to the rat strain, feeding schedule, or sampling time. LA-induced expression of interferon-related genes is one of the original results of the present study. Four out of the nine DEG in epididymal fat were related to interferon-mediated immune response, namely Ifi27l2b, Mx1, Mx2, and Irf7. These four genes were concomitantly upregulated in liver alongside genes implicated in inflammatory response (Gbp5, Cxcl9, and Cxcl10) and GSH-dependent detoxification (Gstp1 and Gsta5) (Supplemental Data).

Differential gene expression induced by LA in epididymal fat pad. In the epididymal fat, the mean number of genes transcribed was 16,405 (73% of the total genes in the transcriptome) for pair-fed rats and 16,337 (73% of the total genes in the transcriptome) for LA-fed rats. Of the 16,405 genes expressed in untreated epididymal fat, 15,789 (96%) were expressed in LA-treated epididymal fat. A total of 10 DEGs (7 up- and 3 downregulated genes by LA vs. pair feeding, Supplemental Data) remained after we filtered data out using the same stringent criteria applied to liver transcriptome. The mRNA levels of three upregulated and three downregulated genes were quantified by qRT-PCR to validate RNA-Seq data.

Table 3. GO categories significantly affected in liver of ZDF rats fed ± LA

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Process Description</th>
<th>LA Regulation</th>
<th>P Value</th>
<th>q Value</th>
<th>Gene Count</th>
<th>Total Count</th>
<th>Gene Symbol</th>
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</thead>
<tbody>
<tr>
<td>GO:0002252</td>
<td>immune effector process</td>
<td>up</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>259</td>
<td>Cxcl10, Cxcl9, Ifit3, Mx1, Mx2, Oas1a, Oas1b, Oasl, Oasl, Rsad2</td>
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<tr>
<td>GO:0009607</td>
<td>response to biotic stimulus</td>
<td>up</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td>634</td>
<td>Abcc3, Alpl, Cmpk2, Cxcl10, Cxcl9, Ednra, Gsp1, Ifi27l2b, Mx1, Mx2, Oasl, Oasl, Plac8, Mpa2l, Rsad2, Irf7</td>
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<tr>
<td>GO:0006955</td>
<td>immune response</td>
<td>up</td>
<td>0</td>
<td>0.0001</td>
<td>14</td>
<td>530</td>
<td>Cxcl10, Cxcl9, Isig5, Gbp5, Irf7, Mx1, Mx2, Oasl, Oasl, Oasl, Oasl, Oasl, Plac8, Mpa2l, Rsad2, Vnn1</td>
</tr>
<tr>
<td>GO:0006950</td>
<td>response to stress</td>
<td>up</td>
<td>0</td>
<td>0.0017</td>
<td>25</td>
<td>2158</td>
<td>Acot2, App, Cxcl10, Cxcl9, Dmbt1, Ednra, Fam129a, Isig5, Gbp5, Gsp1, Mx1, Mx2, Mx2, Oasl, Oasl, Oasl, Oasl, Oasl, Plac8, Mpa2l, Rsad2, Trib3, Vnn1, Zfp354a</td>
</tr>
<tr>
<td>GO:0010033</td>
<td>response to organic substance</td>
<td>up</td>
<td>0</td>
<td>0.0017</td>
<td>23</td>
<td>1864</td>
<td>Abcc3, Aco2, Alpl, Cmpk2, Cxcl10, Cyp4a2, Dmbt1, Ednra, Fgf21, Isig5, Gbp5, Gsp1, H19, Ifi27l2b, Mat2a, Mx2, Oasl, Oasl, Mpa2l, Serpina7, Trib3, Usp18, Zfp354a</td>
</tr>
<tr>
<td>GO:0001676</td>
<td>long-chain fatty acid metabolic process</td>
<td>up</td>
<td>0</td>
<td>0.0031</td>
<td>6</td>
<td>100</td>
<td>Acot1, Aco2, Acsf2, Crat, Crys11, Cyp4a2</td>
</tr>
<tr>
<td>GO:0006082</td>
<td>organic acid metabolic process</td>
<td>up</td>
<td>0</td>
<td>0.0093</td>
<td>13</td>
<td>740</td>
<td>Acot1, Aco2, Acsf2, Crat, Crys11, Cyp4a2, Ddah1, Gsp1, Hdc, Mat2a, Phgdh, Rdhd16, Vnn1</td>
</tr>
<tr>
<td>GO:0023052</td>
<td>response to hormone stimulus</td>
<td>up</td>
<td>0</td>
<td>0.0149</td>
<td>9</td>
<td>5178</td>
<td>App, Cxcl10, Ednra, Fgf21, Mt2a, Rab30, Trib3, Unc5b, Vnn1</td>
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<td>GO:0032094</td>
<td>lipid metabolic process</td>
<td>down</td>
<td>0</td>
<td>0.0172</td>
<td>5</td>
<td>107</td>
<td>A2m, Aco2, Acsf2, Cyp4a2, Dmbt1, Gsp1, H19, Mpa2l, Serpina7, Trib3, Usp18, Zfp354a</td>
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<tr>
<td>GO:0006629</td>
<td>pentose biosynthetic process</td>
<td>down</td>
<td>0</td>
<td>0.0190</td>
<td>9</td>
<td>813</td>
<td>Aacs, Acly, Aco2, Cxcl9, Dmbt1, Gsp1, H19, Mpa2l, Serpina7, Trib3, Usp18, Zfp354a</td>
</tr>
<tr>
<td>GO:0019322</td>
<td>regulation of multiorganism process</td>
<td>down</td>
<td>0</td>
<td>0.0275</td>
<td>2</td>
<td>2</td>
<td>G6pd, Loc100360180</td>
</tr>
<tr>
<td>GO:0043900</td>
<td>response to lipopolysaccharide</td>
<td>up</td>
<td>0.0001</td>
<td>0.0299</td>
<td>6</td>
<td>155</td>
<td>Isig5, Mx2, Oasl, Oasl, Oasl, Oasl, Rsad2</td>
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<tr>
<td>GO:0032496</td>
<td>response to steroid hormone stimulus</td>
<td>up</td>
<td>0.0001</td>
<td>0.0356</td>
<td>8</td>
<td>318</td>
<td>Abcc3, Alpl, Cmpk2, Cxcl10, Ednra, Gsp1, H19, Mpa2l</td>
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<tr>
<td>GO:0048545</td>
<td>response to steroid hormone stimulus</td>
<td>up</td>
<td>0.0001</td>
<td>0.0403</td>
<td>11</td>
<td>630</td>
<td>Abcc3, Aco2, Alpl, Cyp4a2, Dmbt1, Ednra, Gsp1, H19, Mpa2l, Mat2a, Serpina7</td>
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</tbody>
</table>

Listed Gene Ontology (GO) terms are sorted by q value in ascending order.
NAFLD with a nonsynonymous single nucleotide polymorphism in *Pnpla3* (14, 34, 37), which enhances hepatic lipid synthesis because of a gain of function (39). Yet deletion of *Pnpla3* in mice does not cause hepatosteatosis or metabolic syndrome (3, 9). Although not yet fully characterized, the enzyme encoded by *Pnpla5* is understood to have lipid hydrolase activity. *Pnpla3* and *Pnpla5* are located on rat chromosome 7 and separated by a short 36,104 bp noncoding region. This close proximity may explain co-regulation of these genes. The LA-mediated downregulation of *Pnpla3* and *Pnpla5* may in fact reflect events secondary to the overall abatement of neutral lipid stores.

Elov6 encodes a condensing enzyme that catalyzes the elongation of C12-C16 long chain saturated fatty acids and, to a lesser extent, C18:0 and those with a low desaturation degree in the endoplasmic reticulum. Elov6 expression is positively correlated with severity of hepatosteatosis and liver injury in nonalcoholic steatohepatitis (28). Downregulation of Elov6 as mediated by LA may reflect the diminished availability of saturated fatty acid substrates secondarily to repression of *Acly* and *Acaca/Acacb* (liver *Acaca*, FC = −0.9838; adipose *Acaca*, FC = −1.1741; liver *Acacb*, FC = −0.9239). *Acly* encodes ATP-citrate synthase, the primary enzyme responsible for the synthesis of cytosolic acetyl-CoA in many tissues, while the enzymes acetyl-CoA carboxylase 1 and 2, encoded by *Acaca* and *Acacb* respectively, convert acetyl-CoA into malonyl-CoA. In turn, malonyl-CoA is substrate of de novo lipogenesis and elongation of very-long chain fatty acids protein 6. Thus, the combined downregulation of *Acly*, *Acaca/Acacb*, and *Elov6* is consistent with the inhibition of de novo lipogenesis in liver and abdominal adipose tissue and decreased supply of long-chain fatty acyl-CoA to GPAT1. GPAT1, encoded by *Gpam*, localizes to the mitochondria outer membrane where it esterifies acyl-group from acyl-CoA to the sn-1 position of glycerol-3-phosphate, the first and rate-determining step in glycerolipid biosynthesis. Moreover, the downregulation of *Aacs*, which encodes acetoacetyl-CoA synthetase, is congruent with the observed decrease in abdominal adiposity. Indeed, by catalyzing the conversion of acetoacetate to acetyl-CoA in the cytosol, acetoacetyl-CoA synthetase utilizes ketone bodies for fatty acid synthesis during adipogenesis.

In the present study, dietary supplementation with LA induced liver gene expression of enzymes involved in the bioactivation and catabolism of fatty acyls of varying chain length, including acyl-CoA thioesterase 1 (*Acot1*, FC = 7.6099; Supplemental Data), acyl-CoA thioesterase 2 (*Acot2*, FC = 2.0916), acyl-CoA synthetase family member 2 (*Acscf2*, FC = 1.8848), and carnitine O-acetyltransferase (*Crat*, FC = 1.2075).

The enzymes encoded by *Acot1* and *Acot2* catalyze the hydrolysis of long-chain acyl-CoAs (C12 to C20 in chain length) to the free fatty acid and coenzyme A in the cytosol and the mitochondrion, respectively. Consequently, they have the potential to regulate intracellular fatty acyl-CoA-to-free fatty acid ratio. Liver *Acot1* expression was markedly stimulated upon treatment with PPAR-α agonist Wy-14643 (11), suggesting ACOT1 participates in lipid catabolism. Likewise, *Acot2*, which is constitutively expressed in heart and brown fat, can be strongly induced in liver by PPAR-α agonist clofibrate (18). Mechanistically, free fatty acids may translocate to the nucleus bound to fatty acid binding proteins where they serve as ligands of nuclear receptors, PPAR and HNF4-α (45). Thus, by converting acyl-CoA to free fatty acids, ACOT1/2 may influence gene expression and stimulate lipid catabolism via feed-forward mechanism. Furthermore, ACOT1/2 may play a role in alternative oxidative pathway by channeling free fatty acids toward ω-oxidation. Although ω-oxidation is a minor pathway of hepatic fatty acid oxidation compared with β-oxidation, it becomes increasingly relevant in periods of fatty acid overload (17). ω-Oxidation occurs in the endoplasmic reticulum instead of mitochondria. The dicarboxylic acids formed by this pathway are activated to the CoA esters in the endoplasmic reticulum and fully oxidized via peroxisomal β-oxidation.

The protein encoded by *Acscf2* is partially characterized as a mitochondrial acyl-CoA synthetase that catalyzes the formation of fatty acyl-CoA from ATP, CoA, and medium-chain fatty acids. This reaction may serve two important functions that mitigate liver steatosis: first, the bioactivation of medium-chain fatty acids for β-oxidation, and second, the production of hexanoyl-CoA and octanoyl-CoA, which repress the transcription of lipogenic genes *Acaca* and *Fasn* (16, 35).

The enzyme encoded by *Crat* resides mainly in the mitochondria where it catalyzes a reversible reaction between acetyl-CoA and acetylcarnitine, thereby contributing to maintaining the CoA/acytetyl-CoA balance in the cell (31, 32). Unlike its acetyl-CoA precursor, acetylcarnitine can traverse cellular membranes and is recovered in blood and urine (29). Through this efflux mechanism CrAT relieves mitochondria of a surplus of acetyl-CoA, thus of the allosteric inhibition of pyruvate dehydrogenase by acetyl-CoA. Moreover, CrAT itself has a putative role in gene expression whereby increased CrAT activity is expected to lower acetyl-CoA levels available for histone acetylation.

LA upregulates genes encoding enzymes (*Acot1*, *Acot2*, *Acscf2*, and *Crat*) involved in the metabolism of short-, medium-, and long-chain fatty acids with the anticipated functional consequences of stimulating mitochondrial fatty acid catabolism, relieving mitochondria of the excess of potentially pernicious fatty acyl metabolites and generating fatty acyl signaling molecules.

On the basis of the present examination of liver and abdominal fat transcriptomes in obese rats with hypertriglyceridemia, the defined number of DEG associated with normalization of blood TG levels provides insight into tissue regulatory networks of potential clinical importance. Our findings suggest that LA represses pathways of de novo fatty acid and neutral glycerolipid production both in liver and epididymal fat while stimulating atypical pathways of fatty acid disposal as a two-pronged mechanism for mitigating TG overload.

ACKNOWLEDGMENTS

We recognize the technical assistance of University of Nebraska-Lincoln’s Center for Biotechnology and Genomic Research Core Facility in carrying out next-generation sequencing and Life Sciences Annex staff for animal handling and basic care.

GRANTS

This study was supported in part by University of Nebraska-Lincoln ARD Hatch Act, Nebraska Tobacco Settlement Funds, Layman Seed Award, University of Nebraska-Lincoln Faculty Seed Grant, and Nebraska Research Initiative.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
AUTHOR CONTRIBUTIONS


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