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Reversal of obesity-induced hypertriglyceridemia by (*R*)-α-lipoic acid in ZDF (fa/fa) rats

Xiaohua Yi, Anjeza Pashaj, Mengna Xia, Régis Moreau *

Department of Nutrition and Health Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583, USA

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ABSTRACT

Controlling elevated blood triacylglycerol translates into substantial health benefits. The present study aimed to evaluate the triacylglycerol-lowering properties of (*R*)-α-lipoic acid (LA) once circulating triacylglycerol levels have become elevated, and identify the molecular targets of LA. Nine-week old male ZDF (fa/fa) rats were fed a chow diet supplemented with 3 g LA per kg diet or pair fed for two weeks (8 rats per treatment). We determined changes in blood triacylglycerol, insulin, non-esterified fatty acids, and ketone bodies concentrations. We analyzed the expression of genes and proteins involved in fatty acid and triacylglycerol metabolism in liver, epididymal fat, and skeletal muscle. Feeding LA to ZDF rats (a) corrected severe hypertriglyceridemia, (b) lowered abdominal fat mass, (c) raised circulating fibroblast growth factor-21 and *Fgf21* liver gene expression, (d) repressed lipogenic gene expression of ATP-citrate synthase (*Acly*), acetyl-coA carboxylase 1 (*Acaca*), fatty acid synthase (*Fasn*), *sn*-glycerol-3-phosphate acyltransferase 1 (*Gpam*), adiponutrin (*Pnpla3*) in the liver and adipose tissue, (e) decreased hepatic protein levels of ACC1/2, FASN and 5′-AMP-activated protein kinase catalytic subunit α (AMPKα), (f) did not change phospho-AMPKα/AMPKα and phospho-ACC/ACC ratios, (g) stimulated liver gene expression of PPARα target genes carnitine O-palmitoyltransferase 1β (*Cpt1b*) and acyl-CoA thioesterase 1 (*Acot1*) but not carnitine O-palmitoyltransferase 1α (*Cpt1a*). This is evidence that short-term LA feeding to obese rats reverses severe hypertriglyceridemia. FGF21 may mediate the beneficial metabolic effects of LA.

1. Introduction

Controlling blood lipids is a major public health challenge of our time, when over 1.4 billion adults worldwide are overweight or obese. Hypertriglyceridemia, defined as abnormally high fasting serum triacylglycerol (TG) levels (>150 mg/dl or >1.8 mM), is a major risk factor for coronary heart disease, liver disease, and type-2 diabetes that affects ~30% of U.S. adults [1,2]. Lowering blood TG translates into substantial cardiovascular benefits [3]. Conventional therapies for hypertriglyceridemia include dietary weight loss and exercise, dietary supplementation with fish oil or niacin, and drug intervention, which may include fibrates or combined therapy with statins when LDL-cholesterol is elevated. Although some improvement is noted with drug therapies [4–7], serious associated side effects remain a concern [5,8–10].

These impediments led us to examine the therapeutic potential of (*R*)-α-Lipoic acid (LA), a naturally occurring cofactor with lipid regulating properties. LA is synthesized enzymatically from octanoate in most prokaryotic and eukaryotic microorganisms as well as plant and animal mitochondria. Although *de novo* synthesis provides LA needed for its function of cofactor in mitochondria, it can also be absorbed from foods (leafy green vegetables and red meats) and dietary supplements. There is strong evidence that orally supplied LA elicits biochemical activities with potential therapeutic value against an array of pathophysiological conditions, including diabetic polyneuropathies, age-associated cardiovascular, cognitive, and neuromuscular deficits [11]. The TG-lowering properties of dietary LA have recently been recognized, first in laboratory animals [12–15] then in human [16,17].

Despite these reports, the mechanism by which LA regulates blood TG is not known and the degree to which LA can improve severe hypertriglyceridemia not well documented. We showed previously that LA supplementation prevented the rapid rise in blood plasma VLDL-TG that occurs between week 7 and week 9 of age in ZDF rats [14]. In the present study, we sought to determine the extent to which short-term dietary supplementation with LA affects overt hypertriglyceridemia, and to identify the molecular targets of LA. Results show that feeding LA to ZDF rats reversed hypertriglyceridemia, lowered abdominal fat mass, repressed genes of long-chain fatty acid and glycerolipid synthesis in the liver and adipose tissue, upregulated the production of fibroblast growth factor 21 (FGF21), and upregulated specific PPARα target genes involved in long- and medium-chain fatty acyl ester metabolism.

* Corresponding author. Fax: +1 402 472 1587.
E-mail address: rmoreau2@unl.edu (R. Moreau).

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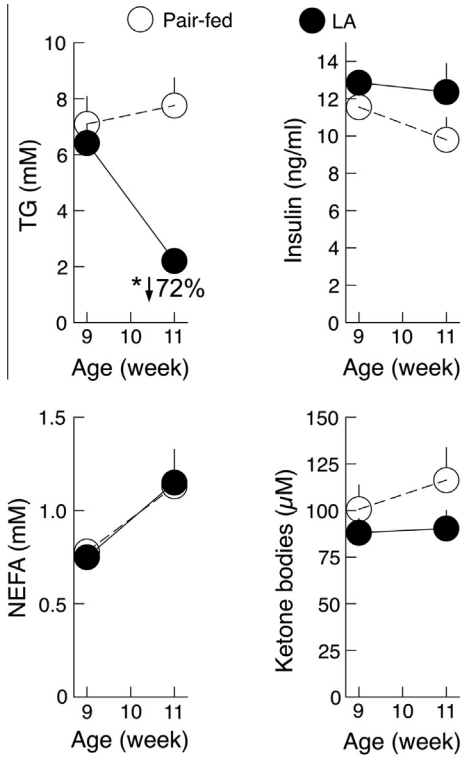

Fig. 1. Time-courses of blood plasma TG (*$P < 0.001$), insulin, non-esterified fatty acids (NEFA), and ketone bodies (3-β-hydroxybutyrate + acetoacetate) in 3-h fasted ZDF rats fed ± LA ($n = 8$).

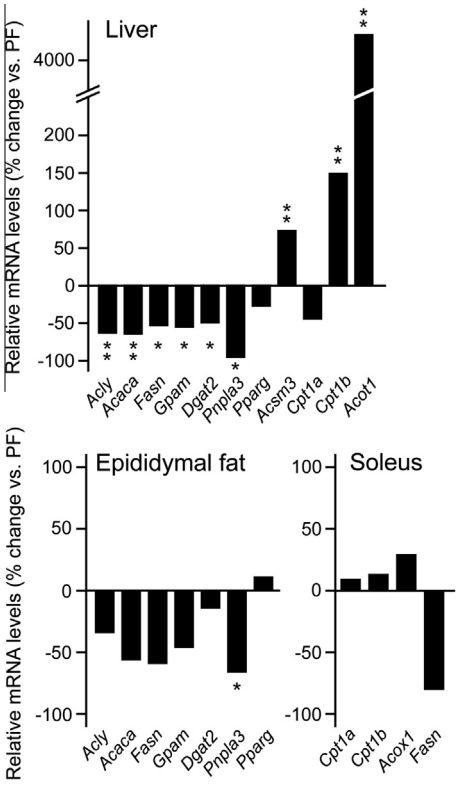

Fig. 2. Transcriptional changes induced by LA. mRNA levels (expressed as % of control pair-fed group, $n = 8$) of selected genes in liver, epididymal fat, and soleus muscle. *$P < 0.05$, **$P < 0.02$. PF, pair-fed group.

Table 1
Body weight, weight gain, food intake, adipose and liver weights in ZDF rats at the end of the 2-week feeding trial.

	Dietary treatment	
	Pair-fed	LA
Initial body weight (g)	320 ± 6	318 ± 7
Final body weight (g)	369 ± 9	358 ± 10
Weight gain (%)	15 ± 2	12 ± 2
Cumulative food intake (g/kg body weight)	967 ± 27	963 ± 30
Abdominal adipose weight (g/rat)	17.7 ± 0.6	15.3 ± 0.2*
I_{AA} (%)	4.8 ± 0.1	4.3 ± 0.1*
Liver weight (g/rat)	17.6 ± 0.8	17.7 ± 0.7
I_H (%)	4.7 ± 0.1	4.9 ± 0.1

Weight gain (% of initial body weight) = [(final body weight − initial body weight)/initial body weight] × 100. I_{AA}, abdominal adiposomatic index; I_H, hepatosomatic index. Data are shown as the mean ± SE of 8 rats/group.
* $P < 0.05$ compared with the pair-fed group.

2. Materials and methods

2.1. Animals and diets

Obese male Zucker rats (ZDF/GmiCrl-*fa/fa*, 7-week old) were purchased from Charles River Laboratories and handled throughout in accordance with Institutional Animal Care and Use Committee approved guidelines. The feeding study was designed as an intervention trial where LA (3 g/kg diet, MAK Wood) is administered in the diet after blood TG have become elevated. At this level of supplementation we estimate LA intake to approximate 200 mg/kg body weight per day. Upon arrival, rats were acclimated for two weeks 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) and water. At 9 weeks of age, the rats were randomly assigned to one of two treatments (Purina 5008 + LA or Purina 5008 pair-feeding) for two weeks. Throughout the trial, animals were given two-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.

2.2. Blood plasma analyses

Three-hour fasted blood was collected at the beginning and end of the trial in EDTA-coated tubes and plasma obtained by centrifugation at 12,000×*g* for 1 min and stored at −80 °C. Plasma TG was measured using the Serum Triglyceride Determination kit (Sigma–Aldrich). NEFA and ketone bodies (3-β-hydroxybutyrate + acetoacetate) were determined enzymatically by using commercial kits (Wako Diagnostics). Insulin and FGF21 were measured by ELISA (Millipore).

2.3. Tissue sampling

At 11 weeks of age, rats were anesthetized with inhalant isoflurane and pancreas, liver, abdominal fat (epididymal + mesenteric + omental + retroperitoneal fat), small intestine, and skeletal muscle (soleus and gastrocnemius) were quickly removed, weighed, frozen in liquid N_2, and stored at –80 °C.

2.4. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolation from tissue using TRIzol and treated with DNase I. RNA integrity was confirmed on an Agilent Bioanalyzer 2100. First strand cDNA was synthesized with oligo(dT) and random primers using BioRad iScript. qRT-PCR was performed on

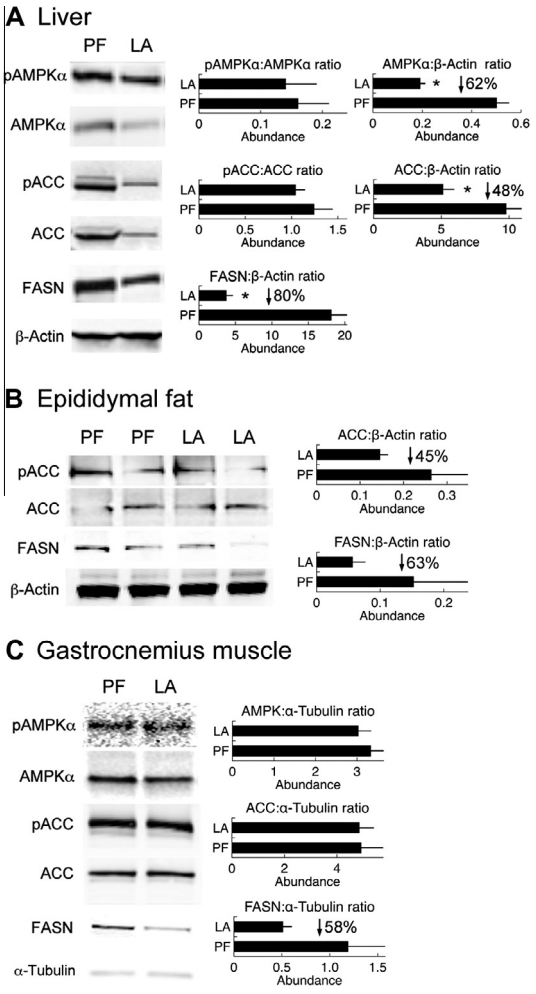

Fig. 3. Lipoic acid represses FASN and ACC independently of AMPK. Immunoblots and densitometry depicting the tissue contents of phosphorylated AMPKα (pAMPKα, Thr 172), total AMPKα (sum of AMPKα1 and AMPKα2), phosphorylated ACC (pACC, Ser 79), total ACC (ACC1/2), FASN, and β-actin or α-tubulin as loading control. *Denotes statistical significance ($n = 8$); liver AMPKα, $P < 0.0002$; liver ACC, $P < 0.007$; liver FASN, $P < 0.0002$. PF, pair-fed group.

a BioRad CFX96 using SYBR Green supermix. Amplicon authenticity was confirmed by melt curve analysis and agarose gel electrophoresis. PCR efficiencies were assessed with serial dilutions of the template (0.001–100 ng cDNA/reaction) and 0.3 μM of each primer, and plotting quantification cycle (Cq) 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 60 S acidic ribosomal protein P0 (*Rplp0*). Primer sequences are shown in Supplementary Table S1.

2.5. Western blotting

Liver, skeletal muscle and adipose tissues were homogenized as previously described [14]. Commercial antibodies to FASN (BD Biosciences), ACC, phospho-ACC (Ser 79), AMPKα (clone 23A3), and phospho-AMPKα (Thr 172, clone 40H9) from Cell Signaling, α-tubulin (clone 6–11B-1, Sigma–Aldrich), and β-actin (clone AC-15, Sigma–Aldrich) were used. Antibody binding was visualized using LiCOR IR Dye secondary antibodies and Odyssey scanner.

2.6. Immunohistochemistry

Immunohistochemistry experiments were carried out to determine which endocrine cells of the pancreas produce FGF21. To that end, anti-FGF21 (LifeSpan Biosciences, LS-B5864), anti-glucagon (α-cells, Dako, A0565), and anti-pre/proinsulin (β-cells, Dako, A0564) antibodies and formalin-fixed, paraffin-embedded pancreas specimens of ZDF rat were used. Antibody to FGF21 was detected with anti-rabbit secondary (Vector, BA-1000) and alkaline phosphatase staining kits (Vector AK-5000; Vector SK-5100), which produced a fuchsia- or red-colored deposit. Antibody to glucagon and antibody to insulin were stained using a horseradish peroxidase-based detection system (Dako LSAB+, K0690) and 3,3′-diaminobenzidine chromogen substrate (Dako DAB+, K3468), which produced a brown precipitate. Tissues were also stained with a positive control antibody (Factor VIII) to ensure that the tissue antigens were preserved and accessible for analysis. The negative control consisted of performing the entire immunohistochemistry procedure on adjacent sections in the absence of primary antibody. Slides were imaged with a DVC 1310C digital camera coupled to a Nikon microscope.

2.7. Statistical analysis

Statistical significance was determined by unpaired two-tailed Student *t*-test with Welch's correction. All statistical tests were performed to the 5% significance level.

3. Results and discussion

The present study demonstrates that dietary supplementation with LA for two weeks ameliorates whole-body lipid status in ZDF rats, a model of obesity and severe hypertriglyceridemia. Using this model we showed previously that LA supplementation prevented the rapid rise in blood plasma VLDL-TG that predictably occurs between 7 and 9 weeks of age [14]. In the present study, LA not only stopped the progression of hypertriglyceridemia, but also normalized blood TG (Fig. 1). This situation is relevant to the human situation where individuals seek treatment after hypertriglyceridemia is diagnosed.

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 1). LA lowered abdominal fat mass (−2.4 g/rat, $P < 0.05$) and abdominal adiposomatic index (−0.5%, $P < 0.05$) (Table 1). At the end of the trial, all ZDF rats regardless of treatment were hyperinsulinemic (10–12 ng insulin/ml). Dietary LA did not alter blood plasma NEFA concentrations, which rose from 0.8 mM to 1.1 mM, suggesting active lipolysis in the adipose tissue prevailed. It also suggests that the uptake by peripheral tissues of NEFA released from fat deposits was not stimulated by LA. Plasma ketone bodies were not significantly different among treatment groups at the end of the trial (Pair-fed, 116 ± 18 μM; LA, 90 ± 10 μM). As ketone bodies rise in the blood during fasting when glucose is not readily available, the present data indicate that LA did not stimulate ketogenesis and both groups of animals displayed comparable degree of fasting.

Since the diet used in the study is low in fat and high in carbohydrates, lipogenesis from carbohydrates in the setting of overnutrition is stimulated in ZDF rats. Hence, the changes observed in lipid status are explained primarily by postprandial changes to the conversion rate of carbohydrates to fat. To ascertain the molecular targets of LA, gene expression of enzymes involved in *de novo* fatty acid and TG syntheses was determined (Fig. 2). In liver, feeding LA significantly repressed expression of *Acly* (−60%), *Acaca* (−61%), *Fasn* (−50%), *Gpam* (−52%), *Dgat2* (−46%), and *Pnpla3*

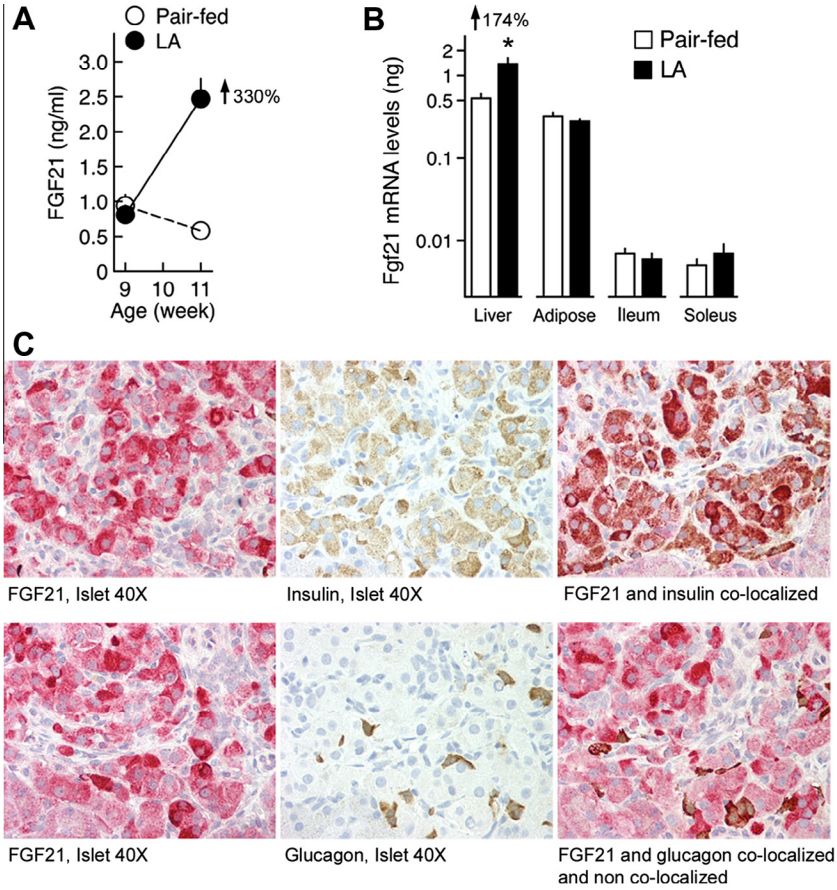

Fig. 4. Lipoic acid induces FGF21 production. (**A**) Temporal changes in blood plasma FGF21 showing a 330% increase in LA-fed animals at the end of the trial ($P < 0.0002$, $n = 8$). (**B**) Relative *Fgf21* mRNA levels in 125 ng total RNA of each tissue, and normalized to housekeeping gene *Ppia*. Data represent the mean + SEM for 8 rats/group for liver (*$P < 0.03$), adipose, and soleus muscle; 4 rats/group for ileum. (**C**) Immunochemical detection of FGF21 in pancreatic islets showing strong co-localization within insulin-positive β-cells. Data is representative of two rats fed LA.

(–92%). Similarly, in epididymal fat pad, mRNA levels of *Acly* (–38%), *Acaca* (–54%), *Fasn* (–57%), *Gpam* (–44%), and *Pnpla3* (–64%) were repressed or trended down in LA-fed animals. Decreases in liver *Acaca* and *Fasn* expression translated into significant changes in protein levels of ACC (–48%, $P < 0.007$) and FASN (–80%, $P < 0.0002$) (Fig. 3A). FASN abundance was decreased by LA (although not significantly) in epididymal fat and gastrocnemius muscle (Fig. 3B,C). The downregulation of *Acly*, *Acaca*, and *Fasn* in the liver and adipose tissue of LA-fed animals is an illustration of repressed carbohydrate-to-fat conversion. Operating simultaneously to lower blood TG, we showed that LA enhances the clearance of TG-rich chylomicron-like particles [14]. The role of the liver vs. non-hepatic tissues in TG clearance is consequential as approximately one third of dietary fatty acids absorbed by the small intestine and assembled in chylomicrons is taken up by the liver [18].

In addition to regulating fatty acid *de novo* synthesis and esterification to glycerolipids, our data indicate that LA upregulated the gene expression of liver *Acsm3*, *Cpt1b*, and *Acot1* (Fig. 2). Through these enzymes, LA has the potential to modulate cellular concentrations of medium- and long-chain fatty acids and their acyl-CoA metabolites. ACSM3 catalyzes the formation of medium-chain fatty acyl-CoA (C_4 to C_{11} in chain length) in mitochondria. This reaction serves two important functions that oppose steatosis, (i) the bioactivation of medium-chain fatty acids into intermediates of β-oxidation, (ii) the production of hexanoyl-CoA and octanoyl-CoA, which repress the transcription of lipogenic genes *Acaca* and *Fasn* [19,20]. ACOT1 is a cytosolic enzyme that hydrolyzes long-chain acyl-CoA (C_{12} to C_{20} in chain length) to the free fatty acid and CoA. Liver *Acot1* expression was markedly stimulated upon treatment with PPARα agonist Wy-14643 [21], suggesting ACOT1 participates in lipid disposal. Free fatty acids generated in the cytosol by ACOT1 associate with fatty acid binding proteins (FABPs) and translocate to the nucleus where they are ligands of nuclear receptors, PPARs and HNF4α among others [22]. Thus, by converting acyl-CoA to free fatty acids, ACOT1 may influence gene transcription. Although dietary LA induced liver *Cpt1b* expression, the absence of *Cpt1a* induction strongly suggests that the flux of long-chain fatty acyl-CoA through mitochondrial β-oxidation was not stimulated by LA. One explanation may be that β-oxidation is already elevated in ZDF rats [23] thus further enhancement cannot be achieved. Exacerbation of mitochondrial β-oxidation to maximum capacity is perceived as detrimental in the obese and diabetic as incompletely oxidized fatty acids that are being generated contribute to insulin resistance [24]. In contrast, approaches to relieve mitochondria, such as through the depletion of malonyl-CoA decarboxylase (an enzyme that promotes β-oxidation by relieving malonyl-CoA-mediated inhibition of CPT1α) have been shown to restore insulin sensitivity [25].

AMPK did not participate in LA's TG lowering. Although AMPK was reportedly activated by LA in rodent tissues [26,27] and linked to the decrease in blood and muscle TG, LA feeding to ZDF rats decreased liver AMPKα content (–62%, $P < 0.0002$) and failed to alter AMPKα phosphorylation (Fig. 3). AMPK activity, assessed by the phosphorylation state of downstream target ACC (Ser79), did not change with LA. Timmers et al. reported that LA supplementation could prevent lipid accumulation associated with feeding a high-

fat diet in Wistar rats independently of AMPK [28]. Disparity between studies is also attributed to the choice of animal model. AMPK activity is diminished in liver and muscle of ZDF rats [29], due in part to the absence of leptin-dependent stimulation of AMPK [30,31]. Since feeding LA to leptin-resistant ZDF rats mimicked some of the metabolic consequences of AMPK (i.e. downregulation of fatty acid esterification to form TG), an alternative mechanism independent of AMPK exists in ZDF rats.

The hormone-like protein FGF21 modulates lipid and glucose metabolism with potential therapeutic benefit for obesity-related metabolic disorders [32–35]. We report that LA, a naturally occurring micronutrient incorporated to the diet at a concentration comparable to LA intake considered safe in humans [11,36,37], upregulates FGF21 production. Blood plasma FGF21 increased 330% in LA-fed rats vs. pair-fed controls ($P < 0.0002$, Fig. 4A). This observation coincided with the upregulation of liver *Fgf21* expression (+174%, $P < 0.03$, Fig. 4B). When compared to the liver, *Fgf21* expression was 2 to 5-fold lower in epididymal fat, and ~50-fold lower in distal ileum and soleus muscle. We did not observe inducement of *Fgf21* by LA in adipose, distal ileum, or soleus, suggesting tissue specificity that may stem from LA hepatic metabolism. The quality of pancreatic RNA did not permit quantification of *Fgf21* mRNA in this organ. However, FGF21 was detected immunologically in the endocrine pancreas of ZDF rats. Double labeling with pre/proinsulin and glucagon resulted in co-localized staining within insulin-producing β-cells (Fig. 4C). Weaker co-localized staining was focally present with FGF21 in glucagon-positive α-cells, but individual cells also showed single monochromatic staining for FGF21 or glucagon. The most intensely stained FGF21-positive cells did not show significant co-localized staining with glucagon. Mechanism of LA-induced FGF21 expression is not yet known. Li et al. showed that sodium butyrate administered intraperitoneally to mice (500 mg/kg body weight) augmented *Fgf21* expression and circulating levels in a PPARα-dependent manner [38]. Induction of liver *Fgf21* expression is required for normal activation of lipid oxidation and TG catabolism [32,39]. Conversely, genetic deletion of *Fgf21* results in hepatosteatosis and hypertriglyceridemia [39]. The administration of recombinant FGF21 to obese rats and mice increases fat utilization and energy expenditure, and reduces plasma TG, glucose, insulin, and hepatic TG [32–34]. Reduced hepatic TG is accompanied by a decrease in lipogenic gene expression of *Acaca*, *Fasn*, *Gpam*, *Dgat2*, stearoyl-CoA desaturase-1, and fatty acid elongase-6, and stimulation of PPARα target genes [33,34]. Strikingly, feeding LA to lean and obese rats replicates these metabolic effects [14,40].

Taken together, our findings suggest that LA increases pathways of fatty acid oxidation while conjointly decreasing pathways of *de novo* production as a two-sided complementary mechanism for how LA feeding decreases TG burden. The study shows that LA upregulates FGF21 production in the obese by inducing hepatic *Fgf21* expression. FGF21 expression in liver and serum may mediate to the beneficial phenotype evoked by LA.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.063.

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