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Lipoic acid improves hypertriglyceridemia by stimulating triacylglycerol clearance and downregulating liver triacylglycerol secretion

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Abstract

Elevated blood triacylglycerol (TG) is a significant contributing factor to the current epidemic of obesity-related health disorders, including type-2 diabetes, nonalcoholic fatty liver disease, and cardiovascular disease. The observation that mice lacking the enzyme *sn*-glycerol-3-phosphate acyltransferase are protected from insulin resistance suggests the possibility that the regulation of TG synthesis be a target for therapy. Five-week old Zucker Diabetic Fatty (ZDF) rats were fed a diet containing (*R*)- α -lipoic acid (LA, ~200 mg/kg body weight per day) for 5 weeks. LA offset the rise in blood and liver TG by inhibiting liver lipogenic gene expression (e.g. *sn*-glycerol-3-phosphate acyltransferase-1 and diacylglycerol O-acyltransferase-2), lowering hepatic TG secretion, and stimulating clearance of TG-rich lipoproteins. LA-induced TG lowering was not due to the anorectic properties of LA, as pair-fed rats developed hypertriglyceridemia. Livers from LA-treated rats exhibited elevated glycogen content, suggesting dietary carbohydrates were stored as glycogen rather than becoming lipogenic substrate. Although AMP-activated protein kinase (AMPK) reportedly mediates the metabolic effects of LA in rodents, no change in AMPK activity was observed, suggesting LA acted independently of this kinase. The hepatic expression of peroxisome proliferator activated receptor α (PPAR α) target genes involved in fatty acid β -oxidation was either unchanged or decreased with LA, indicating a different mode of action than for fibrate drugs. Given its strong safety record, LA may have potential clinical applications for the treatment or prevention of hypertriglyceridemia and diabetic dyslipidemia.

Keywords

triglyceride; VLDL; chylomicron; obesity; lipotoxicity; dyslipidemia; AMP-activated protein kinase

Introduction

Recent estimates for the incidence of hypertriglyceridemia (fasting plasma triacylglycerol (TG) levels over 150 mg/dl) is 30% of U.S. adults [1]. However, because hypertriglyceridemia is also associated with more pernicious dyslipidemia (e.g. cholesterolemia and lipoproteinemia) and the metabolic syndrome [2], its current prevalence and health consequences likely far exceed these estimates. Notably, hypertriglyceridemia exacerbates the risk for pancreatitis, liver and coronary artery disease [3–5], and intervention studies show that lowering blood TG decreases cardiovascular events and mortality rates [6,7]. Report that mice lacking *sn*-

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glycerol-3-phosphate acyltransferase (GPAT), the accepted rate-controlling enzyme in *de novo* TG synthesis pathway (Fig. 1), exhibit decreased blood and liver TG, and improved insulin sensitivity suggests the possibility that the regulation of TG synthesis be a target for therapy [8]. But to date no therapeutic strategy targets GPAT.

Conventional treatments against hypertriglyceridemia include weight loss and exercise, dietary supplementation with fish oil or niacin [9,10], and, when lifestyle changes have not been successful, drug intervention with fibrates alone or combined with statins [11,12]. However, adverse side effects associated with these drugs remain a concern. In addition, the potential for worsening glycemic control and elevating release of liver enzymes may limit the use of niacin in diabetic and obese patients [13,14].

These safety issues and recent findings in the area of obesity and diabetic dyslipidemia [15, 16] led us to examine the lipid-lowering properties of (*R*)- α -lipoic acid (LA). LA, a naturally occurring cofactor of mitochondrial α -ketoacid dehydrogenases, has long been used to improve whole-body glucose tolerance in diabetes [17–20]. Mechanistically, LA acts on skeletal muscle to stimulate the recruitment of glucose transport protein 4 (GLUT4) from its storage site in the Golgi to the sarcolemma, thus facilitating glucose uptake. In contrast, the role of LA in lipid metabolism is poorly understood. In animal studies, the administration of LA was associated with weight loss, especially body fat loss [15]. This effect was largely attributed to the appetite-suppressing properties of LA and to increased energy expenditure. Here we show that unlike fibrates, which primarily help metabolize existing TG, LA improves blood TG both by inhibiting the synthesis and secretion of TG from the liver and by enhancing the clearance of TG-rich lipoproteins. Moreover, the metabolic effects observed with LA were independent of its known anorectic properties in rodents [15]. This study shows that LA downregulates gene expression of key liver enzymes involved in *de novo* fatty acid and TG syntheses independently of caloric intake, AMP-activated protein kinase (AMPK), and peroxisome proliferator activated receptor α (PPAR α).

Materials and methods

Animals and diets

Obese male Zucker rats (ZDF/GmiCrl-*fa/fa*, 4-week old) were purchased from Charles River Laboratories (Wilmington, MA) and treated throughout in accordance with Institutional Animal Care and Use Committee (IACUC) approved guidelines. Upon arrival, rats were acclimated for a week in individual cages in a controlled environment (ambient temperature $22 \pm 2^\circ\text{C}$, 12:12-h light-dark cycle, lights on from 7 am until 7 pm) with free access to food (Teklad Rodent Diet 8604) and water. At 5 weeks of age, the rats were randomly assigned to one of the three dietary treatments for 5 weeks. A first group (designated as 'noLA') of 5 rats was fed Purina 5008 (Dyets Inc., Bethlehem, PA) *ad libitum*. A second group (designated as 'LA') of 5 rats was fed Purina 5008 containing 2.4 g LA (Viatris GmbH & Co. KG, Radebeul, Germany) per kg diet to satiation. Given that LA lowered the rats' appetite [15], a third group (6 rats which were designated as 'Pair-fed') was fed restricted amounts of Purina 5008 to match the food intake of LA-treated rats. Throughout the trial, two-day feeding rations were given between 1 and 3 pm, and scheduled tissue sampling (see below) took place on the next day so that rats had free access to food at least up to the time of sample collection. Because LA's anorectic effect on ZDF rats gradually weakens over time and ceases after 2 weeks of daily dietary supplementation, the 5-week feeding protocol also ensured that LA-fed and pair-fed rats were in the same nutritional state at the time of tissue harvesting. All animals were provided distilled water to drink throughout. Food and water intake, as well as body weight were recorded every other day. LA concentration in the diet was confirmed by HPLC analysis with coulometric detection [21]. In addition to ZDF rats, a group of 4 lean male Zucker rats

(HsdHlr:ZUCKER-*Lep^{fa}*, Harlan, Indianapolis, IN) were used to establish baseline triglyceridemia, glycemia, and insulinemia.

Tissue sampling

Weekly, food was withdrawn (at 8:00 am) 2 h prior to blood (~200 μ l) collection by tail clipping. Blood was collected in EDTA-coated tubes and plasma obtained by centrifugation at $12,000 \times g$ for 1 min and stored at -80°C . At 10 weeks of age, rats were anesthetized with pentobarbital i.p. injection (50 mg/kg body weight) and diethyl ether inhalation at 10:00 am, 2 h after food withdrawal. Skeletal muscle (soleus and vastus lateralis), liver, and perivisceral fat (epididymal + mesenteric + omental + retroperitoneal fat) were quickly removed, weighed, frozen in liquid N_2 , and stored at -80°C . Fresh tissue samples were also stored in RNAlater for mRNA determination by real-time PCR. Hepatosomatic index (I_H) and visceral adiposomatic index (I_{VA}) were calculated as (organ or tissue weight/body weight) $\times 100$.

Blood plasma analyses

Plasma glucose concentrations were determined using an automated HemoCue Glucose 201 analyzer (HemoCue Inc.) and insulin by ELISA (Linco Research, Inc.). Alanine aminotransferase activity was determined using a kit from Catamount. TG-rich lipoproteins (chylomicrons and VLDL) were separated by overlaying plasma with water and centrifuging at $16,000 \times g$ for 30 min at 4°C . This procedure removes chylomicrons, chylomicron remnants and some large VLDL leaving the majority of VLDL in the infranatant.

Lipid analyses

Blood lipid profiles were analyzed by TLC using Uniplate Silica Gel G plates (250 μm , Analtech, Inc.), chloroform:methanol:water (65:30:5) to separate polar lipids, and hexane:diethyl ether:acetic acid (80:20:1.5, by vol.) to further resolve neutral lipids. Lipids were visualized with iodine vapor and identified based on the migration distances of the standards (TLC 18-5, Nu-Chek Prep, Inc.). TG and cholesteryl esters were further authenticated following hydrolysis with microbial lipase (Sigma-Aldrich). Blood plasma TG was measured using the Serum Triglyceride Determination kit (Sigma-Aldrich). NEFA, total cholesterol, and HDL-cholesterol concentrations were determined enzymatically by using the NEFA-C kit, the Cholesterol E kit, and the HDL-Cholesterol E kit, respectively (Wako Chemicals). Livers were powdered under liquid N_2 and lipids extracted according to [22] in chloroform:methanol (2:1). Half the organic layer was used to determine total lipid content gravimetrically. The other half of the organic layer was transferred to a tube containing 0.2 g silicic acid to remove phospholipids and used to measure TG, total cholesterol, and NEFA as above.

Intravenous lipid tolerance test

Five-week old ZDF rats were randomly assigned to the 'LA' or 'Pair-fed' group and then given that particular diet for 5 weeks. Following an overnight starvation (16 h), 10-week old rats were anesthetized by i.p. injection of pentobarbital (90 mg/kg body weight). Under general anesthesia, a bolus of chylomicron-like lipid emulsion (Liposyn II 20%, Hospira, Inc.) was injected intravenously at a dose of 0.8 ml/kg body weight. Blood samples (0.1 ml) were obtained by tail bleeding before injection (-10 and -2 min) and at 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 100, 120, 140, and 160 min after injection, diluted to 0.3 ml in saline containing 1.8 mg EDTA. Blood plasma was obtained by centrifugation at $12,000 \times g$ for 1 min and stored at -80°C . Chylomicrons and their remnants were concentrated at the top of the plasma by centrifugation (at $16,000 \times g$ for 10 min at 4°C) and the non-chylomicron fraction was obtained by puncturing the bottom of the centrifuge tube. Whole plasma and plasma non-chylomicron

TG were measured using the Serum Triglyceride Determination kit (Sigma-Aldrich), and chylomicron-like TG calculated by subtracting non-chylomicron TG from whole plasma TG.

Liver glycogen analysis

Glycogen was measured according to the method of Keppler and Decker [23], which is based on glycogen hydrolysis with fungal glucoamylase and glucose determination with hexokinase and glucose-6-phosphate dehydrogenase. Liver samples were homogenized in ice-cold 0.6 M perchloric acid using a Potter-Elvehjem homogenizer. A 0.2-ml aliquot of homogenate (determination of total glucose) was mixed with 0.1 ml of 1 M KHCO_3 , 2 ml of glucoamylase solution (0.1% [w/v] in 0.2 M acetate buffer, pH 4.8) and incubated in a water bath set at 40°C for 2 h with shaking. After 2 h, amylase was inactivated with 1 ml of ice-cold 0.6 M perchloric acid and the sample was centrifuged at $10,000 \times g$ for 10 min at 4°C. Glucose was measured in the supernatant by using the Glucose Assay kit (GAHK-20, Sigma-Aldrich). Another 0.2-ml aliquot of homogenate (determination of non-glycogen glucose) was mixed with 0.1 ml of 1 M KHCO_3 , 2 ml of 0.2 M acetate buffer (pH 4.8) and 1 ml of ice-cold 0.6 M perchloric acid, vortexed, kept on ice for 10 min, and centrifuged at $10,000 \times g$ for 10 min at 4°C. Glucose was measured in the supernatant by using the Glucose Assay kit. Tissue glycogen was calculated as the difference between total glucose minus non-glycogen glucose and expressed as $\mu\text{mol glucosyl/g tissue}$.

Real-time PCR

Total RNA was extracted from liver or muscle tissues using the SV Total RNA Isolation System (Promega Corporation), and the first DNA strand synthesized using the RETROscript kit (Ambion Inc.). Real-time PCR was performed by using the DNA Engine Opticon 2 system (MJ Research, Inc.) and DyNAmo SYBR Green qPCR Kit (Finnzymes Oy) according to the manufacturer's instructions. Amplicon authenticity was confirmed by agarose gel electrophoresis and melt curve analysis. 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 threshold cycle (C_T) versus log amount of template. Since PCR efficiencies between target genes and housekeeping genes were comparable, unknown amounts of target in the sample were normalized by comparing the C_T values with the external standard, i.e. β -actin, cyclophilin A, and acidic ribosomal phosphoprotein P0 (36B4). Primer sequences used are shown in Table 1.

Nuclear protein isolation and immunoblotting

Hepatic total, cytoplasmic and nuclear proteins were isolated from frozen liver in the presence of protease inhibitors (P8340, Sigma-Aldrich), phosphatase inhibitors (P2850 and P5726, Sigma-Aldrich) and dithiothreitol. Proteins were subjected to reducing SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with lipoamide dehydrogenase (Rockland) and histone H3 (Upstate) antibodies to verify successful subcellular fractionation. Muscle tissue was homogenized in Hepes buffer containing 1% Triton X-100, 10% glycerol, dithiothreitol, protease and phosphatase inhibitors, and centrifuged at $12,000 \times g$ for 20 min at 4°C. Proteins from the supernatant were used for immunoblotting. Antibodies to SREBP-1 (2A4 and H-160) were obtained from Santa Cruz Biotechnology, Inc. Antibodies to ChREBP were obtained from Novus Biologicals, Inc. Antibodies to ACC, phospho-ACC (Ser 79), AMPK α (clone 23A3), and phospho-AMPK α (Thr 172, clone 40H9) were obtained from Cell Signaling Technology, Inc. Other antibodies used were: uncoupling protein 3 (UCP3, Calbiochem), α -tubulin (clone 6-11B-1, Sigma-Aldrich), and β -actin (clone AC-15, Sigma-Aldrich). Antibody binding was visualized using the ECL Western Blotting System (GE Healthcare Life Sciences) and band density quantified using an AlphaImager imaging system (Alpha Innotech Corporation).

Hepatic VLDL-TG secretion

A separate set of 5-week old ZDF rats was randomly assigned to the 'LA' or 'Pair-fed' treatment for 5 weeks. After overnight (16 h) starvation, 10-week old rats were anesthetized by i.p. injection of pentobarbital (90 mg/kg body weight) and blood collected by tail clipping 20 min prior to an i.v. injection of Triton WR-1339 (400 mg/kg body weight) to block VLDL clearance [24]. Blood was subsequently drawn at 30, 50, 70, 90, 110, and 130 min post injection via tail bleeding. VLDL-TG was measured using the Serum Triglyceride Determination kit (Sigma-Aldrich).

Statistical analysis

Statistical significance was determined by one-factor ANOVA followed by Student-Newman-Keuls multiple comparison test or unpaired Student *t*-test (Statview package, SAS Institute Inc.). Temporal changes in blood plasma TG, HDL-cholesterol and total cholesterol were analyzed by comparing Areas Under the Curve (AUC) generated by the trapezoidal rule. The Kruskal-Wallis or Mann-Whitney tests were used whenever a given data set showed variance heterogeneity. All statistical tests were performed to the 5% significance level.

Results

Lipoic acid improves blood TG independently of glycemia or insulinemia

Prior to its supplementation, LA levels in the chow (2.4 g/kg) were monitored by HPLC, which confirmed that animals received the intended LA dose from the diet. We estimate that ~40 mg LA/kg body weight per day was the effective amount of LA absorbed by the rats, assuming that ~20% of ingested LA was bioavailable [25]. Feeding LA to ZDF rats (~200 mg LA/kg body weight per day) prevented the lipemic appearance of blood plasma (Fig. 2A). Lipemia was caused by high blood plasma TG as judged by lipid TLC (Supplementary data, Fig. S1). Feeding LA mitigated the rise in plasma TG observed after 7 weeks of age, which otherwise reached 5.1 mM (or 451 mg/dl) at 9 weeks of age in rats fed the control diet devoid of LA (Fig. 2B). Because food intake and weight gain were decreased in rats fed LA versus nonsupplemented chow *ad libitum* (Table 2), a group of ZDF rats was pair fed the control diet. Pair-fed rats grew at the same rate as LA-treated rats (Table 2). But as opposed to LA-fed rats, animals pair-fed the control diet developed hypertriglyceridemia (4.5 mM at 9 weeks of age, Fig. 2B) and their plasma was as lipemic as the plasma of rats fed nonsupplemented chow *ad libitum* (data not shown), indicating that the TG-regulating properties of LA extend beyond its anorectic effect. Thus, use of a pair-fed group is undoubtedly a major strength of the study as a decrease in caloric intake can lower triglyceridemia. LA also significantly raised ($P < 0.02$) HDL-cholesterol with no marked changes to plasma total cholesterol (Fig. 2C, D) and non-esterified fatty acids (NEFA, Table 2).

Steps were taken to identify the TG-rich lipoproteins affected by LA. Blood TG remained elevated following an overnight fast (16 h) but were significantly improved (~50%, $P < 0.02$) in LA-fed versus pair-fed rats (Fig. 2E). Because TG in 16-h fasted plasma originate from VLDL, the data suggest that LA markedly affects hepatic TG metabolism.

In the postprandial state (2 h after a meal), TG are associated with chylomicrons, chylomicron remnants, and VLDL. Separation of the chylomicron and VLDL fractions from 2-h fasted plasma revealed that LA-mediated TG lowering was mainly confined to the chylomicron fraction (Fig. 3A). To evaluate whether chylomicron clearance contributed to the TG-lowering effect of LA, intravenous lipid tolerance test was conducted using chylomicron-like particles. As compared with pair-fed rats, LA-fed rats exhibited a significant decrease in post-injection TG Area Under the Curve (~42%, $P < 0.007$, Fig. 3B). Thus, these findings indicate that LA stimulates the postprandial clearance of chylomicron-TG.

The aforementioned effects of LA on blood lipids were observed in prediabetic ZDF rats. Glycemia was not significantly different between experimental groups (Table 2), nor was glycemia markedly elevated compared to age-matched lean Zucker rats (147 ± 14 mg glucose/dl). Typically, obese male ZDF (*fa/fa*) rats given free access to Purina 5008 are severely diabetic (500–600 mg glucose/dl) by the age of 14 weeks, and show a steady increase in blood glucose from the age of 8 weeks. Slight departure from this feeding protocol, due to the transport and acclimation of the animals to our facilities, can delay the onset of hyperglycemia and best explain why the rats were prediabetic at end of the trial. With regard to insulinemia, all ZDF rats irrespective of the treatment were hyperinsulinemic (Table 2) showing plasma insulin 11-fold higher than age-matched lean Zucker rats (1.1 ± 0.1 ng insulin/ml). These data indicate that feeding LA improves severe hypertriglyceridemic conditions under hyperinsulinemia prior to the animals becoming fully diabetic.

Lipoic acid lowers fat deposits, decreases liver TG, and increases liver glycogen

LA-fed ZDF rats possessed ~27% less perivisceral fat and their livers were ~36% heavier by weight than pair-fed rats (Table 2). While a reduction in body fat could be construed as a benefit of LA, an enlarged liver may indicate adverse health consequences, including liver steatosis. However, the LA-induced increase in liver size was not accompanied by higher plasma alanine aminotransferase activity (20.2 ± 4.6 U/l), indicating the absence of liver injury from LA (Table 2). Quantitation of hepatic lipid levels definitively excluded liver steatosis. Indeed, feeding LA significantly lowered both total lipid and TG levels in livers of ZDF rats (Table 3). Moreover, dietary LA did not significantly affect liver total cholesterol and NEFA levels. In contrast, LA-fed rats exhibited elevated liver glycogen (+27%) versus pair-fed rats, and glycogen content ($\mu\text{mol/g}$ liver) was positively correlated ($P = 0.013$, $r = 0.603$) with liver weight (g/rat). These results suggest that LA mediates storage of excess dietary energy as glycogen rather than TG (Table 3). Knowing that hepatocellular glycogen retains significant amounts of water [26], increased glycogen content may in part account for liver weight gain.

Lipoic acid downregulates liver lipogenic gene expression and decreases VLDL secretion

To determine the molecular targets involved in TG lowering, expression of enzymes involved in hepatic TG metabolism were monitored in ZDF rats fed $\pm$ LA. Results show that liver gene expression of two key enzymes of TG synthesis, i.e. *sn*-glycerol-3-phosphate acyltransferase-1 (GPAT-1), and diacylglycerol O-acyltransferase-2 (DGAT-2), were significantly decreased by 81 and 56%, respectively, in LA-treated versus pair-fed rats (Fig. 4A). The specificity of this LA-mediated effect was demonstrated when mRNA levels of diacylglycerol O-acyltransferase-1 (DGAT-1), an enzyme sharing little homology with DGAT-2, were unchanged by LA. Moreover, feeding LA to ZDF rats decreased the mRNA levels of acetyl-CoA carboxylase-1 (ACC-1), ACC-2, and fatty acid synthase (FAS) by 66, 52, and 74%, respectively, compared to pair-fed animals (Fig. 4A). These data, combined with the results showing lower liver TG content (Table 3) and decreased VLDL-TG secretion (-31% , $P < 0.03$, Fig. 4B, C), strongly suggest LA improves hypertriglyceridemia by inhibiting hepatic *de novo* TG synthesis and VLDL production. To our knowledge, this is the first study to identify GPAT-1 and DGAT-2 as molecular targets of LA-mediated TG lowering.

The downregulation of lipogenic genes by lipoic acid is associated with an inhibition of ChREBP

We assessed whether known transcription factors of lipogenic genes are involved in mediating the effect of LA. ChREBP and SREBP-1c are transcription factors that induce ACC, FAS, GPAT-1, and DGAT-2 gene expression [27–30] or these genes contain putative DNA-binding sites in the promoter region. We reasoned that LA may improve hypertriglyceridemia by negatively regulating ChREBP and/or SREBP-1c. Experiments were undertaken to measure

protein content and transcriptional activity of ChREBP and SREBP-1c in livers of ZDF rats fed $\pm$ LA. LA decreased total hepatic and cytoplasmic levels of ChREBP and SREBP-1c (Fig. 5A, B). Moreover, nuclear ChREBP was decreased in LA-treated livers, whereas nuclear SREBP-1c was not significantly affected (Fig. 5C). These results correlated with the repression of liver-type pyruvate kinase gene expression, a gene under the control of ChREBP, and not SREBP-1c (Fig. 5D). In contrast, the hepatic expression of SREBP-1c (Fig. 5D), which is auto-regulated, was unaffected by LA. This is consistent with a selective repression of ChREBP synthesis and activity by LA independent of caloric intake.

Lipoic acid does not stimulate liver and muscle AMPK

AMPK was proposed to mediate some of LA's metabolic effects in rodents, in part, by stimulating the flux of long-chain acyl-CoA through carnitine palmitoyltransferase 1 (CPT1) and β -oxidation versus TG and long-chain fatty acid syntheses [31,32]. Moreover, AMPK represses ChREBP-dependent lipogenic gene expression by phosphorylation of ChREBP, which inactivates its DNA-binding activity [33]. Thus, we hypothesized that LA lowered TG synthesis by stimulating AMPK. Hence, experiments were undertaken to measure AMPK α and its phosphorylation state (Thr 172) in the liver and skeletal muscle of ZDF rats fed the experimental diets (Fig. 6). In addition, the phosphorylation state (Ser 79) of ACC was determined as a surrogate measure for direct AMPK activity. Results show that liver and soleus AMPK α content, and AMPK α and ACC phosphorylation states remained unchanged among the treatments. Liver ACC level was significantly decreased by LA (Fig. 6A) thus corroborating the marked decline in gene expression (Fig. 4A). These data suggest that LA acted independently of liver and muscle AMPK.

Lipoic acid does not stimulate liver fatty acid β -oxidation

To determine whether fatty acid β -oxidation contributed to the lipid-lowering properties of LA, mRNA levels of liver and skeletal muscle PPAR α and PPAR α target genes involved in mitochondrial and peroxisomal fatty acid β -oxidation were measured by real-time PCR (Fig. 7). Comparisons between LA-fed and pair-fed rats indicate that hepatic expression of PPAR α and CPT1 α was unchanged, whereas liver transcripts of acyl-CoA oxidase-1 (ACO-1) and enoyl-CoA hydratase-3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (BIFEZ) were decreased with LA (Fig. 7A). However, in vastus lateralis muscle the gene expression of ACO-1 was significantly elevated in LA-treated rats compared to pair-fed controls (Fig. 7B). Collectively, these data indicate that liver fatty acid β -oxidation is not part of LA-induced lipid lowering, whereas β -oxidation may be stimulated by LA in skeletal muscle.

Discussion

When a carbohydrate-rich meal is absorbed, glycemia rises and initiates secretion of insulin from β -cells of the pancreas. Within seconds, the elevated plasma insulin levels induce the transcription of glycolytic and lipogenic genes. When absorbed calories exceed energy expenditure, lipids will be deposited in the liver and adipose tissue, and if left unchecked will lead to obesity and hypertriglyceridemia. Fasting triglyceridemia reflects the balance between the secretion of TG-rich VLDL by the liver and their removal from the circulation by peripheral tissues. Our data indicate that LA lessens severe hypertriglyceridemia in ZDF rats, in part, by inhibiting hepatic *de novo* TG synthesis and VLDL-TG secretion. Our data also indicate that feeding LA both lowers chylomicron remnants 2 h after a meal and stimulates the clearance of chylomicron-like TG following intravenous administration. Thus, this study defines a rationale that LA enhances lipase-catalyzed clearance of TG-rich lipoproteins. However, it is noteworthy that overproduction of TG-rich VLDL by the liver is primarily responsible for hypertriglyceridemia in this genetically obese animal model [34]. Hence, the role of LA on hepatic TG metabolism is viewed as key to the TG-lowering properties of LA.

Although the TG-lowering effect of LA has been inferred before [16,31,35,36], the mechanism involved is completely unknown. Our data fill this gap and show that dietary LA downregulates the expression of key liver enzymes of TG synthesis pathway, decreases liver glycerolipid content, and in turn, lowers hepatic VLDL-TG secretion. The liver secretes TG as VLDL from a cytosolic TG pool with the availability of core lipids being a limiting factor for VLDL assembly and stability. Two key enzymes in TG synthesis are GPAT and DGAT. GPAT catalyzes the initial rate-limiting acylation of *sn*-glycerol-3-phosphate with fatty acyl-CoA. Although liver GPAT-1 is only one of four isoforms [37], it contributes 30 to 50% of the total liver activity [38]. Unlike microsomal GPATs, GPAT-1 is up-regulated transcriptionally by reinitiating a high-carbohydrate, fat-free diet and by insulin [39]. This supports an important role for GPAT-1 in *de novo* TG synthesis. DGAT catalyzes the final acylation step to yield TG. Two mammalian *Dgat* genes belonging to different gene families have been identified [40,41]. Although DGAT-1 and -2 catalyze similar reactions, only DGAT-2 has a critical role in hepatic TG synthesis and VLDL production; DGAT-1 participates in cholesterol ester biosynthesis [42]. Our observation that LA downregulated the expression of liver *Dgat2*, but not that of *Dgat1*, suggests a specific inhibition of glycerolipid synthesis.

The long-term transcriptional regulation of hepatic lipogenic genes is primarily under the control of SREBP-1c and ChREBP, two members of the bHLH/LZ superfamily of transcription factors. While LA decreased the cytoplasmic abundance of both SREBP-1c and ChREBP, only the nuclear content of ChREBP was decreased in LA-treated livers. These data suggest that ChREBP, but not SREBP-1c, was associated with the transcriptional downregulation of liver lipogenic genes initiated by LA. Supporting this notion is the observation that the gene expression of liver-type pyruvate kinase (a ChREBP target gene), and not SREBP-1c (a SREBP-1c target gene), was downregulated by LA. The potential role of ChREBP in regulating TG synthesis was previously documented in studies showing that inhibition of ChREBP with RNA interference in *ob/ob* mice decreased plasma and liver TG, and lowered liver GPAT-1 mRNA while SREBP-1c was unaffected [43]. Furthermore, the repressed gene expression of lipogenic enzymes, decreased adipose mass, and occurrence of glycogen-rich liver observed in LA-treated ZDF rats are reminiscent of the phenotype exhibited by ChREBP^{-/-} mice [44].

AMPK did not take part in TG lowering seen in LA-fed ZDF rats. Although AMPK was reportedly activated by LA in rodent tissues [31,45] and linked to the decrease in blood and muscle TG, we found no change in the phosphorylation state of AMPK α or its downstream target, ACC, in the livers and soleus muscles of ZDF rats. This disparity between studies is likely attributed to the choice of animal model. AMPK activity is diminished in the liver and muscle tissues of ZDF rats [46]. Since leptin stimulates AMPK [47,48] and ZDF rats are leptin resistant, the possibility exists that ZDF rats lack an important stimulatory pathway for AMPK. It further suggests that AMPK activation by LA requires a functional leptin signaling, or alternatively, a higher LA dose than the one provided in the present study. However, since feeding LA to leptin resistant ZDF rats reproduced the outcome of activated AMPK (i.e. downregulation of fatty acid esterification to form TG), an alternative regulatory pathway independent of a leptin/AMPK axis must exist, which based on our gene expression data relies on the transcriptional repression of ACC-1, FAS, GPAT-1 and DGAT-2.

If, as we postulate, feeding LA prevented dietary substrates from entering the TG synthetic pathway, then LA-fed rats must handle a surfeit of lipogenic substrates when compared to pair-fed rats. Regarding the handling of dietary fat by LA-treated rats, gene expression of PPAR α and PPAR α target genes involved in fatty acid β -oxidation revealed that hepatic β -oxidation did not contribute to LA action. However, based on the gene expression of acyl-CoA oxidase-1 in vastus lateralis, fatty acid β -oxidation in skeletal muscle is regarded as a possible route of fat elimination in LA-fed rats. Regarding the handling of dietary carbohydrates in the liver,

glucose is either stored as glycogen, converted to pyruvate, or undergoes incomplete glycolysis to dihydroxyacetone phosphate, which produces *sn*-glycerol-3-phosphate (i.e., a precursor of glycerolipids). Our observation that glycogen content is increased in the liver of LA-fed ZDF rats concomitantly with the repressed gene expression of liver-type pyruvate kinase supports the view that LA induces dietary carbohydrates to be stored as glycogen while inhibiting liver glycolysis. Moreover, the well-recognized stimulatory role of LA on glucose uptake in skeletal muscle [18] could indirectly be important to the regulation of liver TG by metabolizing or storing dietary carbohydrates in muscle.

Although known for its role in glucose uptake [17,49], the TG-lowering properties of LA were unrecognized until recently. The present study contributes to the growing body of knowledge by showing that LA improves hypertriglyceridemia in ZDF rats by downregulating hepatic GPAT-1 and DGAT-2 gene expression, inhibiting liver TG secretion as VLDL, and stimulating clearance of TG-rich lipoproteins. Moreover, where previous reports provided evidence that LA lowers triglyceridemia, the lack of pair feeding or difference in growth rate between LA-treated and control groups cast doubt on the true reason for the observed effects. By including a pair-fed group in the study design we show here that the effect of LA is independent of calorie intake. Thus LA is not just an anorectic compound but also has a true metabolic effect on triglyceridemia. Our data are the first to identify the pathway and potential mechanism by which LA inhibits hepatic TG synthesis in an AMPK-independent manner. Furthermore, our observation that LA does not stimulate PPAR α -dependent genes strongly suggests that LA acts via a mechanism that is distinct from fibrate drugs. Thus, we believe that a novel means of controlling triglyceridemia in this animal model has been revealed. Given its strong safety record [50] LA may have therapeutic applications for the treatment or prevention of hypertriglyceridemia and diabetic dyslipidemia in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

ACC, acetyl-CoA carboxylase
 ACO-1, acyl-CoA oxidase-1
 AMPK, AMP-activated protein kinase
 BIFEZ, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme
 ChREBP, carbohydrate responsive element binding protein
 CPT1, carnitine palmitoyltransferase 1
 Cyclo A, cyclophilin A
 DGAT, diacylglycerol O-acyltransferase
 FAS, fatty acid synthase
 GPAT-1, *sn*-glycerol-3-phosphate acyltransferase-1
 LA, (*R*)- α -lipoic acid
 L-PK, liver-type pyruvate kinase
 NEFA, non-esterified fatty acids
 PPAR α , peroxisome proliferator activated receptor α

SREBP-1c, sterol regulatory element binding protein-1c
 TG, triacylglycerol
 VLDL, very low-density lipoprotein
 ZDF, Zucker Diabetic Fatty
 36B4, acidic ribosomal phosphoprotein P0

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Fig. 1. Hepatic triglyceride synthesis. Mitochondrial and microsomal *sn*-glycerol-3-phosphate acyltransferases (mtGPAT and msGPAT, respectively) catalyze the acylation of *sn*-glycerol-3-phosphate (glycerol-3-P) with acyl-coenzyme A (acyl-CoA) generating lysophosphatidic acid (LPA), which is the rate-controlling step in *de novo* triglyceride (TG) synthesis. Subsequent reactions are catalyzed by LPA acyltransferase (LPAAT) generating phosphatidic acid (PA), PA phosphatase (PAP) and diacylglycerol O-acyltransferase (DGAT) generating diacylglycerol (DAG) and finally TG. Newly synthesized TG is either deposited in intracellular lipid vacuoles or exported in very low-density lipoprotein (VLDL) particles. Triglyceride hydrolase (TGH), a glycoprotein distributed to the endoplasmic reticulum particularly in

regions surrounding mitochondria helps mobilize TG stored in lipid droplets prior to incorporation in VLDL particles by catalyzing the initial lipolysis followed by re-esterification by DGAT.

Fig. 2.

Lipoic acid improves hypertriglyceridemia in ZDF rats. **(A)** Appearance of 2-h fasted blood plasma from 10-week old ZDF rats fed $\pm$ LA for 5 weeks. Blood plasma of ZDF rats pair fed the control diet was as lipemic as the plasma of rats fed the control diet *ad libitum* (group 'noLA') (data not shown). Time-courses of blood plasma TG **(B)**, HDL-cholesterol **(C)**, and total cholesterol **(D)** in 2-h fasted ZDF rats fed $\pm$ LA and lean Zucker rats fed chow. Data are shown as the mean $\pm$ SEM for 5–6 ZDF rats/group and 4 lean Zucker rats. Lower case letters denote statistical analysis, where the Area Under the Curves (AUC) not sharing a common letter are significantly different (TG and HDL-cholesterol, $P < 0.02$). Error bars may lie within the symbols. **(E)** Overnight (16 h)-fasted blood plasma TG (VLDL-TG) in ZDF rats fed with

LA or pair-fed the control diet for 5 weeks ($n = 5$ rats/group). Data represent the mean $\pm$ SEM ($*P < 0.02$).

Fig. 3.

Lipoic acid stimulates clearance of chylomicron-like particles. (A) TG content associated with chylomicrons (white column) or VLDL (black column) in 2-h fasted blood plasma of ZDF rats fed $\pm$ LA ($n = 4-5$ rats/group). Data represent the mean $\pm$ SEM. Values not sharing a common letter within plasma fraction are significantly different ($P < 0.05$). (B) Intravenous lipid tolerance test carried out in 16-h fasted ZDF rats fed $\pm$ LA for 5 weeks. A bolus of Liposyn II 20% (0.8 ml/kg body weight) was injected at $t = 0$ min. Plasma chylomicron-like TG was calculated by subtracting non-chylomicron TG from whole plasma TG. Insert, the Area Under the Curve (AUC, corrected for baseline TG) is significantly different between LA-fed and pair-fed rats (-42% , $*P < 0.007$, $n = 7$ rats/group).

Fig. 4. Lipoic acid represses the hepatic expression of lipogenic genes and decreases in vivo hepatic secretion of VLDL-TG. **(A)** Liver mRNA levels of enzymes involved in TG synthesis; GPAT-1, DGAT-1, and DGAT-2, and *de novo* fatty acid synthesis; ACC-1, ACC-2, and FAS. Livers were obtained from 10-week old ZDF rats fed $\pm$ LA for 5 weeks. mRNA levels were quantified by real-time PCR as described under "Materials and methods" and expressed as % of control group 'noLA' (mean $\pm$ SEM for 5 rats/group). Values not sharing a common letter are significantly different (GPAT-1, DGAT-2, and ACC-1, $P < 0.001$; DGAT-1, $P < 0.002$; ACC-2, $P < 0.02$; FAS, $P < 0.03$). **(B)** Blood plasma VLDL-TG concentrations prior ($t = -20$ min) and following ($t = 30, 50, 70, 90, 100$, and 130 min) i.v. administration of Triton WR-1339

(n = 5 rats/group, mean $\pm$ SEM.). ZDF rats were purchased at 4 weeks of age, acclimated for a week, then fed LA or pair-fed the control diet for 5 weeks. Experiments debuted after an overnight (16 h) fast. Note that fasting tryglyceridemia (t = -20 min) was significantly decreased in LA-fed versus pair-fed rats (* P < 0.02) and remained significantly lower throughout the experiment. (C) Hepatic VLDL-TG secretion rate was decreased (-31%, * P < 0.03, n = 5 rats/group) in LA-fed rats versus pair-fed rats. To determine VLDL-TG secretion rate (μ M/min), curves were fitted by linear least-squares regression using a coefficient of determination (r^2) > 0.97. Only data collected within the first 90 min post Triton WR-1339 injection were included in the regression analysis.

Fig. 5.

Effects of lipioic acid on the hepatic content and activity of ChREBP and SREBP-1c. **(A)** ChREBP and precursor SREBP-1c content in total liver extract were measured by immunoblotting as described under “Materials and methods”. **(B)** ChREBP and precursor SREBP-1c content in hepatic cytoplasmic fraction (immunoblotting). **(C)** ChREBP and mature SREBP-1c content in hepatic nuclear fraction (immunoblotting). **(D)** Transcriptional activity of ChREBP toward liver-type pyruvate kinase, and SREBP-1c toward its own gene. Gene expression was determined by real-time PCR as described under “Materials and methods” and expressed as % of control group ‘noLA’. Data are shown as the mean + SEM for 5 rats/group (real-time PCR) or 4 rats/group (immunoblotting). β -Actin was used as a loading control of liver and cytoplasmic proteins, and histone H3 as a loading control of nuclear proteins. Values not sharing a common letter are significantly different ($P < 0.05$).

Fig. 6.

Lipoic acid does not stimulate liver or skeletal muscle AMPK. Immunoblots and densitometry depicting the liver (**A**) and soleus muscle (**B**) contents of phosphorylated AMPK α (pAMPK α , Thr 172), total AMPK α (sum of AMPK α 1 and AMPK α 2), phosphorylated ACC (pACC, Ser 79), and total ACC (sum of ACC-1 and ACC-2). Uncoupling protein 3 (UCP3) content was determined as a mitochondrial protein loading control. β -Actin was used as an overall loading control of liver proteins, and α -tubulin as an overall loading control of muscle proteins. Data represent the mean $\pm$ SEM for 4 rats/group. Values not sharing a common letter are significantly different ($P < 0.05$).

Fig. 7.

Effects of lipoic acid on the mRNA levels of PPAR α and PPAR α target genes in liver and skeletal muscle. Gene expression was determined by real-time PCR as described under "Materials and methods" and expressed as % of control group 'noLA'. **(A)** Liver mRNA levels of PPAR α , CPT1 α , ACO-1, and BIFEZ. Values not sharing of common letter are significantly different (PPAR α and CPT1 α , $P < 0.001$; ACO-1, $P < 0.003$; BIFEZ, $P \leq 0.01$). **(B)** Vastus lateralis mRNA levels of PPAR α , CPT1 β , ACO-1, and BIFEZ. Values not sharing of common letter are significantly different (ACO-1, $P < 0.05$). Liver and muscle data represent the mean + SEM for 5 rats/group.

Table 1

Real-time PCR primers used to quantify gene expression

Name	Sequence (5'→3') ^a	Product size (bp)	GenBank ^b
GPAT-1	CGTGGGAAGGTGTTGCTATT CAGCAATTGCCCTCTTGGACT	248	NM_017274
DGAT-1	GAGACCGCGAGTTCTACAGG TCAGGGGAATGCTCACTAGG	204	XM_579557
DGAT-2	AACACGCCCAAGAAAGGTG AGGGCAGATGCCTCCAGAC	303	NM_001012345
ACC-1	GTTACTCGTTTTGGGGGAAA CAGCCCACTGCCTGTA	298	NM_022193
ACC-2	TTGGAGGCAACAGGGTTATC TAGAAAAGCAATCCCGTGCT	356	NM_053922
FAS	GCCTCACTCCGAGGAACAAACA ^c CCCGGCATTGAGAATAGTGGCA ^c	436	NM_017332
SREBP-1c	GGAGCCATGGATTGCACATT ^c AGAGAGGAGCCCAGAGAAGC	179	XM_213329
L-PK	CGTTCAGCCAGCTTCTATC CCTGTCACCAATCACCAG	236	NM_012624
PPAR α	CTCGGAGGGCTCTGTCATC ACTGTGCTACGCCAGCTTT	230	NM_013196
CPT1 α	CCACAAATTACGTGAGTGACTGGTGG CCAGAAGACGAATGGGTTTGAGTTCC	213	NM_031559
CPT1 β	GCTCATTTCCGGGACAAAGGCAAG TTGGAGGTCTTGTTCCTTATGGGAC	163	NM_013200
ACO-1	CAGCCCTACTGTGACTTCCA ACCTGGGCGTATTTTCATCAG	279	NM_017340
BIFEZ	TGGGCTGTCACTATCGGATT GCGGGGTTCTATGGGTTTAT	269	NM_133606
β -Actin	CCTTCCTCCTGGGTATGGAATCC GAGCAATGATCTTGATCTTCATGGTG	205	NM_031144
Cyclo A	CGAGCTGTTTGCAGACAAAG AGCCACTCAGCTTGGCAGT	300	NM_017101
36B4	CTAGAGGGTGTCCGCAATGT AGGAAGGCCTTGACCTTTTC	161	NM_022402

^a Shown as sense primer followed by antisense primer.^b GenBank accession number.^c Primers from S.R. Commerford, L. Peng, J.J. Dube, R.M. O'Doherty, In vivo regulation of SREBP-1c in skeletal muscle: effects of nutritional status, glucose, insulin, and leptin, Am. J. Physiol. Regul. Integr. Comp. Physiol. 287 (2004) R218–227.

Table 2

Body weight, weight gain, food intake, adipose and liver weights, blood plasma NEFA, glucose, insulin, and alanine aminotransferase (ALT) activity in ZDF rats at the end of the feeding trial.

	noLA	Dietary treatment LA	Pair-fed
Initial body weight (g)	134 ± 8	135 ± 7	132 ± 5
Final body weight (g)	322 ± 8 ^a	268 ± 4 ^b	261 ± 9 ^b
Weight gain (%)	143 ± 10 ^a	101 ± 9 ^b	98 ± 2 ^b
Cumulative food intake (g/rat)	778 ± 16 ^a	605 ± 18 ^b	654 ± 13 ^b
Visceral adipose weight (g/rat)	16.3 ± 0.4 ^a	11.4 ± 0.4 ^b	15.6 ± 0.3 ^a
<i>I</i> _{VA} (%)	5.0 ± 0.1 ^b	4.3 ± 0.1 ^c	5.6 ± 0.1 ^a
Liver weight (g/rat)	13.8 ± 1.2 ^a	14.0 ± 0.4 ^a	10.3 ± 0.5 ^b
<i>I</i> _H (%)	4.2 ± 0.2 ^b	5.2 ± 0.1 ^a	3.7 ± 0.1 ^b
Plasma NEFA (mM)	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.2
Plasma glucose (mg/dl)	167 ± 11	184 ± 34	149 ± 11
Plasma insulin (ng/ml)	12.5 ± 1.3	12.0 ± 1.3	11.7 ± 1.7
Plasma ALT (U/l)	20.2 ± 10.9	20.2 ± 4.6	17.3 ± 1.7

Weight gain (% of initial body weight) = [(final body weight – initial body weight)/initial body weight] × 100. *I*_{VA}, visceral adiposomatic index; *I*_H, hepatosomatic index. Data are shown as the mean ± SEM of 5–6 rats/group. Values not sharing a common superscript within rows are significantly different ($P < 0.05$).

Table 3

Dietary LA lowers liver TG concentration in ZDF rats. Livers were obtained at the end of the 5-week feeding trial 2 h after the last meal and assayed for total lipids, TG, total cholesterol, NEFA, and glycogen content.

Analyte	noLA	Dietary treatment LA	Pair-fed
Total lipids (mg/g liver)	24.5 ± 0.8 ^a	17.6 ± 1.2 ^b	23.9 ± 0.8 ^a
	338.8 ± 28.3 ^a	248.0 ± 21.1 ^b	245.2 ± 12.5 ^b
TG (μmol/g liver)	32.1 ± 2.6 ^a	23.8 ± 2.2 ^b	36.4 ± 2.2 ^a
	454.5 ± 72.3	331.8 ± 28.0	371.8 ± 18.5
Total cholesterol (μmol/g liver)	4.8 ± 0.3 ^a	4.6 ± 0.1 ^{ab}	4.0 ± 0.1 ^b
	67.0 ± 9.7 ^a	63.9 ± 1.6 ^{ab}	41.7 ± 2.2 ^b
NEFA (μmol/g liver)	0.7 ± 0.1	0.8 ± 0.1	1.0 ± 0.1
	10.0 ± 1.8	11.0 ± 1.1	9.7 ± 1.1
Glycogen (as glucosyl) (μmol/g liver)	201.7 ± 21.7	221.0 ± 27.5	174.1 ± 22.1
	2.86 ± 0.53 ^{ab}	3.14 ± 0.48 ^a	1.81 ± 0.26 ^b

Data are shown as the mean ± SEM of 5–6 rats/group. Values not sharing a common superscript within rows are significantly different ($P < 0.05$).