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STUDIES ON THE REGULATION OF FGF21 GENE EXPRESSION BY (R)-α-LIPOIC ACID: MECHANISTIC INSIGHT INTO THE LIPID LOWERING PROPERTIES OF A DITHIOL DIETARY MOLECULE

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by

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STUDIES ON THE REGULATION OF FGF21 GENE EXPRESSION BY (R)-α-LIPOIC ACID: MECHANISTIC INSIGHT INTO THE LIPID LOWERING PROPERTIES OF A DITHIOL DIETARY MOLECULE

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Controlling blood lipids is a major public health challenge of our time. The pleiotropic hormone-like polypeptide fibroblast growth factor 21 (FGF21) was recently recognized as a potent modulator of lipid and glucose metabolism and a promising treatment strategy for obesity related metabolic disorders, including dyslipidemia. A cost effective and practical alternative to the administration of recombinant FGF21 is to stimulate endogenous FGF21 production through diet. Our research identified (R)-α-lipoic acid (LA), a naturally occurring enzyme cofactor and dietary molecule found in green leafy vegetable and red meat, as an inducer of FGF21 expression. LA stimulated FGF21 production, demonstrated by a 3-fold increase in circulating FGF21, in the obese animal by upregulating hepatic *Fgf21* expression. Concomitantly, feeding LA to dyslipidemic obese rats corrected high blood triacylglycerol levels and lowered abdominal adiposity by stimulating liver gene expression of peroxisome proliferator-activated receptor α (PPARα) target genes involved in lipid disposal and conjointly decreasing de novo lipid synthesis as a two-sided complementary mechanism to mitigate lipid burden. Since the

metabolic effects of LA mimic those of FGF21, we posit that FGF21 mediates the beneficial phenotype evoked by LA and sought to determine how LA and its reduced form, (R)-α-dihydrolipoic acid (DHLA), induce the *Fgf21* promoter. To that end, we employed HepG2 hepatocellular carcinoma cells under fed and starved states and in the presence of LA and DHLA. Serum withdrawal markedly induced *FGF21* mRNA abundance from 24 to 48 hours after serum removal. Both LA and DHLA induced *FGF21* mRNA levels at 36 hours after serum removal when compared to vehicle control. To further determine the regulatory mechanism of *FGF21* transcription, we have begun to develop the FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) assay coupled to qPCR to isolate and identify active regulatory promoter regions. We designed and validated a collection of primers specific for human *FGF21* promoter that will allow the functional mapping of a 4,573-bp 5' UTR region.

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TABLE OF CONTENTS

Contents

Page

LIST OF TABLES AND FIGURES

Page

Chapter 1

Chapter 2

Chapter 3

CHAPTER 1

INTRODUCTION

Controlling blood lipids is a major public health challenge of our time, when over 100 million Americans are overweight or obese. The National Cholesterol Education Program considers hypertriglyceridemia (i.e. fasting blood triglyceride (TG) levels >150 mg/dl or >1.5 mM) to be an independent risk factor for coronary heart disease [1]. Lowering blood TG translates into substantial cardiovascular benefits. For instance, a 29% reduction in TG is associated with a 22% reduction in coronary heart disease mortality [2]. Hypertriglyceridemia is also a major risk factor for liver disease and type-2 diabetes [3]. Despite significant advances in treatment ~65 million Americans have hypertriglyceridemia.

Conventional therapy for hypertriglyceridemia includes 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 (Table 1.1). As the country is facing an obesity epidemic, the likelihood of controlling hypertriglyceridemia by weight loss – on an epidemiological scale – is proving elusive at best. Although some improvement is noted with drug therapies, serious associated side effects remain a concern. Thus, clinicians must administer lipid-lowering drugs carefully, especially in patients with renal and hepatic dysfunction, and in the elderly. This grim

picture means that an ever-growing number of hypertriglyceridemic patients will not be adequately treated for their lipid disorder and remain at elevated risk for cardiovascular disease, liver disease, and type-2 diabetes. There is, therefore, a critical need to identify therapeutic agents that would lessen hypertriglyceridemia and also be safe and relatively inexpensive.

Fibroblast growth factor 21 (FGF21)

The endocrine hormone fibroblast growth factor 21 (FGF21) is a pleiotropic hormone-like protein that encoded by human FGF21 gene. FGF21 was classified as a fibroblast growth factor based on its structure, since it contains a typical structure domain of FGF and shares 10–30% sequence identity with other FGFs, mostly FGF19 [4]. It has long been detected as a powerful modulator of lipid and glucose metabolism and a promising treatment strategy for obesity related metabolic disorders [5,6,7,8]. FGF21 is expressed in liver, pancreas, adipose and muscle tissue [9,10]. FGF21 functions through binding to cell-surface FGF receptor (FGFR)/β-Klotho complex in target tissues [11,12] (Fig. 1.1. & 1.2.). Induction of liver FGF21 expression is required for normal activation of lipid oxidation and TG catabolism [5,13]. Conversely, genetic deletion of FGF21 results in fatty liver and hypertriglyceridemia [13]. The administration of FGF21 to obese rats and mice increases fat utilization and energy expenditure, and reduces plasma TG, glucose, insulin, and hepatic TG [5,6,7].

The liver secretes TAG as very-low density lipoprotein (VLDL) from a cytosolic TAG pool whose availability limits VLDL assembly and stability (Fig. 1.3.). In the TAG

synthetic pathway glycerol-3-phosphate acyltransferase (GPAT) catalyzes the rate-limiting acylation of glycerol-3-phosphate (glycerol-3-P) with acyl-CoA generating lysophosphatidic acid (LPA). Subsequent reactions, catalyzed by LPA acyltransferase and diacylglycerol acyltransferase (DGAT), generate diacylglycerol and finally TAG. Newly synthesized TAG is either deposited in intracellular lipid vacuoles or exported in VLDL particles. Reduced hepatic TG administrated by FGF21 to obese rats and mice is accompanied by a decrease in lipogenic gene expression of GPAT1, DGAT2, ACC1, FAS, stearoyl-CoA desaturase-1, and fatty acid elongase-6, and stimulation of PPARα lipolytic target genes [6,7].

Recombinant FGF21 (rFGF21) is currently being investigated as therapeutic against high blood lipids and type-2 diabetes [14]. However, the high costs of producing rFGF21 and the mode of delivery by injection are important limitations to its wide therapeutic use. A cost effective and practical alternative is to stimulate endogenous FGF21 production through diet.

(R)-α-lipoic acid

(R)-α-Lipoic acid (LA), also known as 1,2-dithiolane-3-pentanoic acid or thioctic acid, is a naturally occurring dithiol compound found in green-leafy vegetables and red meats. LA is synthesized enzymatically from octanoic acid in most prokaryotic and eukaryotic microorganisms as well as plant and animal mitochondria [15]. It contains one chiral center and has both R- and S-enantiomeric forms (Fig. 1.4.). Beside synthesized, LA is also absorbed from dietary sources such as muscle meats, heart, kidney, and liver,

and to a lesser degree, fruits and vegetables [16]. However, the amounts of LA consumed in the traditional Western diet is not appreciable; therefore, dietary supplements are the primary sources of LA (typically ranging from 50 to 600 mg), and most positive health outcomes reported in the literature derived from LA supplements.

Growing evidences show that orally supplied LA elicits biochemical activities with potential therapeutic value against a host of pathophysiological conditions, including diabetic polyneuropathies, age-associated cardiovascular, cognitive, and neuromuscular deficits [15]. LA has been described as a potent biological antioxidant and a detoxification agent (Fig. 1.5.). Many cases indicate that LA was not directly exerting its anti-oxidation abilities but by modulating glutathione (GSH) via nuclear factor (erythroid-derived 2)-like 2 (transcription factor Nrf2), and then plays an important role for protection against ischemic damage [17]. Besides, LA/DHLA has also been reported to improve age-associated cardiovascular, cognitive, and neuromuscular deficits, and has been implicated as a modulator of various inflammatory signaling pathways [18-23]. This impressive array of cellular and molecular functions has piqued considerable interest among the lay public and the research community for the use of LA both as a nutritive supplement and as a pharmacotherapy.

Studies done by Diesel et al. showed that LA/DHLA mediates insulin pathway and glucose uptake by modulating the IR/PI3K/Akt pathway at different levels (Fig. 1.6.). The mediation of LA with the insulin-signaling cascade via different levels of regulatory components proved beneficial in animal studies due to the improvements made in skeletal muscle glucose uptake, whole-body glucose tolerance, and insulin resistance [26,27].

Beyond that, improvements in glucose handling were also detected in clinical trials both intravenously and orally with type 2 diabetics taking LA [27-29].

Recently, the TG-lowering properties of dietary LA have been recognized, first in laboratory animals [30,31] then in clinical trials [32,33]. Despite these reports, the mechanism by which LA regulates blood TG is not well known and the degree to which LA can improve severe hypertriglyceridemia poorly documented.

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Table 1.1. Therapies for managing dyslipidemia

Therapy	TG	LDL-C	HDL-C	Possible side effects
Fibrates	↓ 40-60%	↑ 5-30%	↑ 15-25%	Myopathy, rhabdomyolysis, elevated liver enzymes
Statins	↓ 20-40%	↓ 18-55%	↑ 5-15%	Myopathy, rhabdomyolysis, elevated liver enzymes
Niacin	↓ 30-50%	↓ 5-25%	↑ 20-30%	Flushing, worsening glycemia, elevated liver enzymes
Fish oil	↓ 30-50%	↑ 5-10%	↑ 5-10%	Fishy aftertaste, gastrointestinal upset

TG, triglyceride; LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol

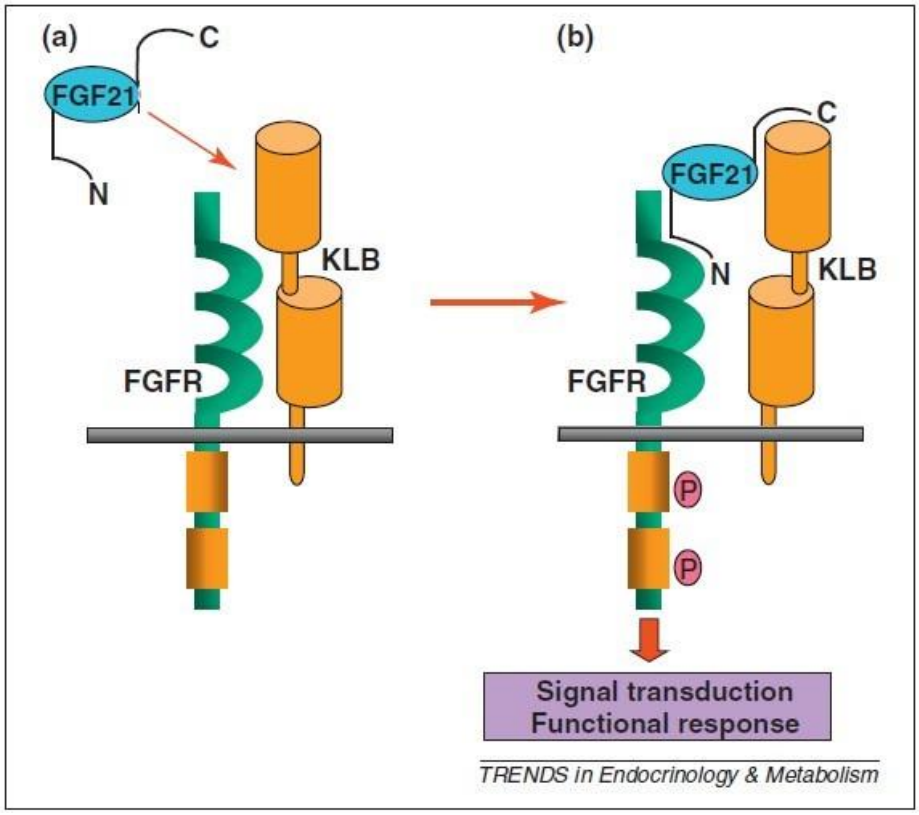

Figure 1.1. The mechanism of FGF21 receptor activation. (a). The FGFR/KLB receptor complex is silent without FGF21. (b). FGF21 binds to KLB via its C-terminus, then causes a conformational change in FGFR or FGF21 or both, which later allow FGF21 binds to FGFR via its N-terminus. The binding of FGF21 with FGFR/KLB receptor complex will trigger a following downstream signaling and a series of cellular responses. [Figure source: Kharitonenkov, Larsen et al. 2010].

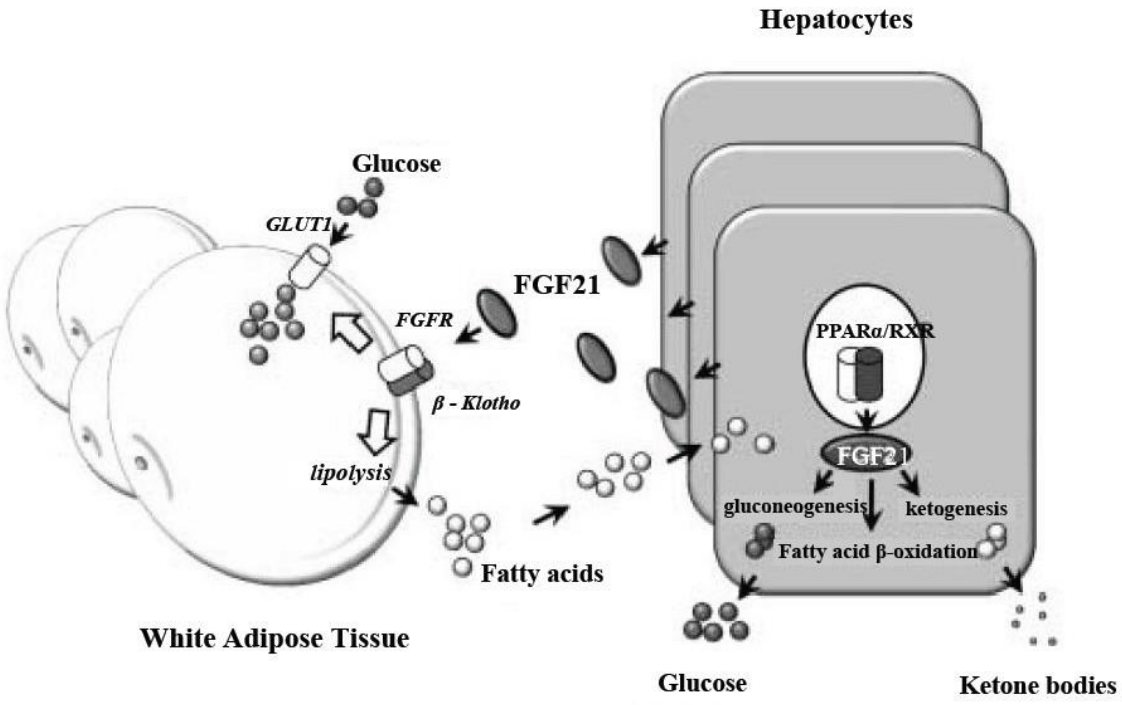

Figure 1.2. Effects of FGF21 on hepatocytes and adipocytes in animal models. a). FGF21 binds to FGFR/KLB receptor complex while β-Klotho stimulating glucose uptake in differentiated adipocytes through GLUT1 induction. b). Fasting stimulates both FGF21 gene expression in hepatocytes and the circulating levels of FGF21 protein which can improve the lipolysis in adipocytes and ketogenesis and gluconeogenesis in liver directly induced by PPARα. [Figure modified from: P Iglesias, et al. 2012].

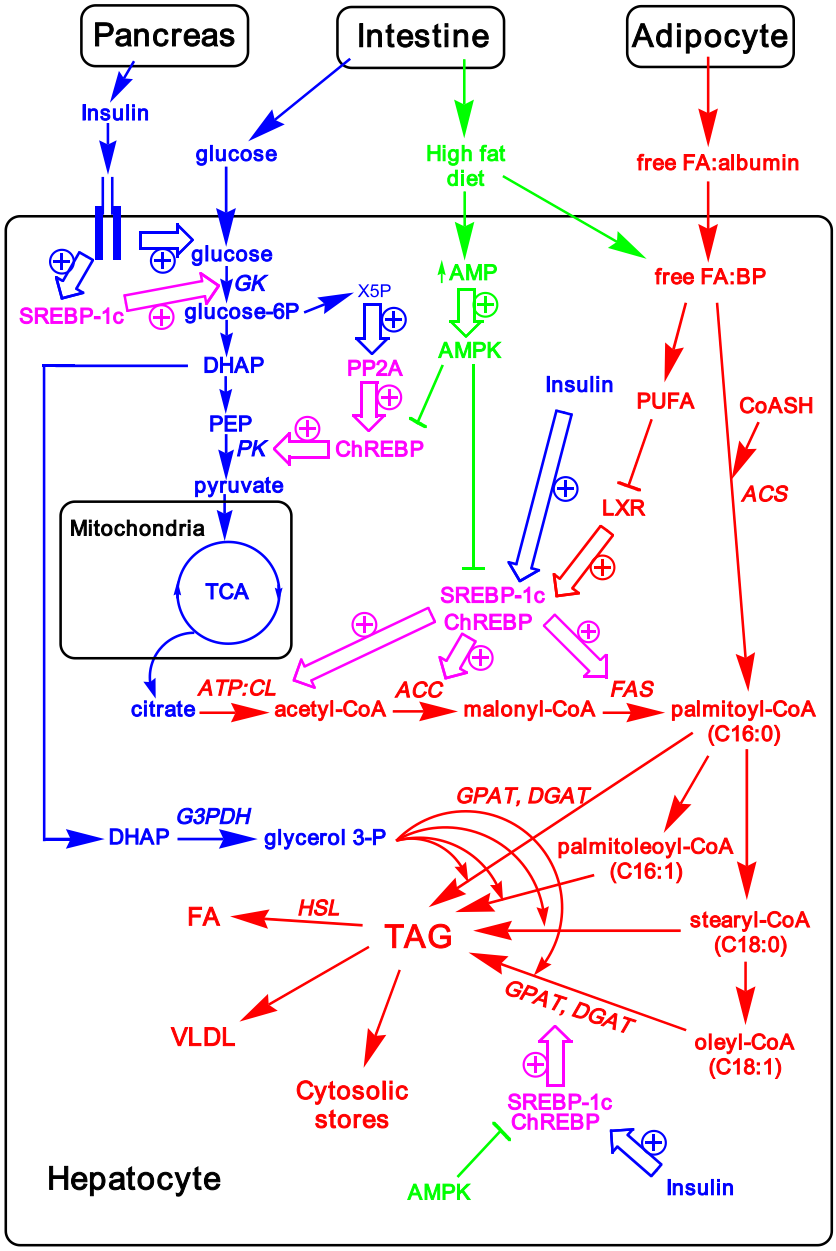

Figure 1.3. Liver triglyceride (TAG) synthesis. ACC, acetyl-CoA carboxylase; ACS, Acyl-CoA synthase; AMPK, AMP-activated protein kinase; ATP:CL, ATP:citrate lyase; ChREBP, carbohydrate responsive element binding protein; DGAT, diacylglycerol O-acyltransferase; FA:BP, fatty acid binding protein; FAS, fatty acid synthase; GK, glucokinase; G3PDH, glycerol-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; HSL, hormone sensitive lipase; LXR, liver X receptor; PK, pyruvate kinase; PP2A, protein phosphatase 2A; PUFA, polyunsaturated fatty acid; SREBP-1c, sterol regulatory element binding protein-1c; VLDL, very low density lipoprotein; X5P, xylulose-5-phosphate.

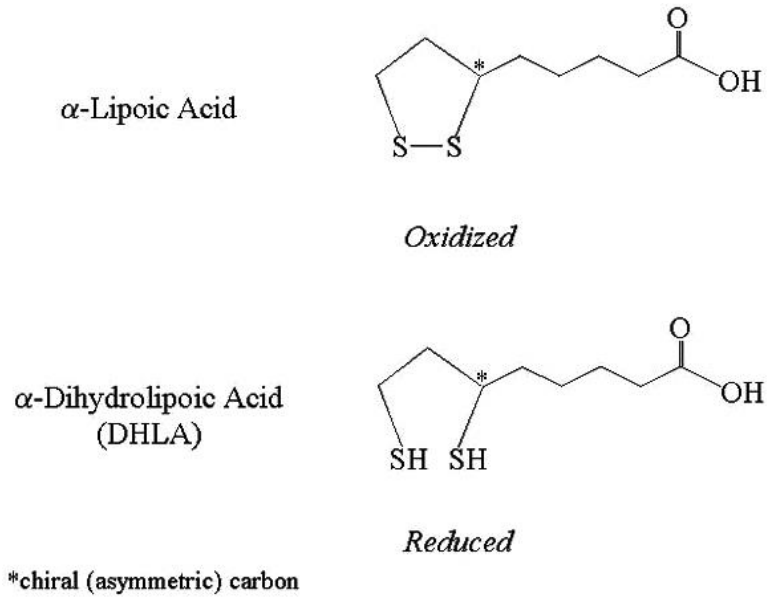

Figure 1.4. LA and its reduced form, DHLA. DHLA is naturally generated in the body after the consumption of LA. The presence of an asymmetric carbon yields two enantiomers, R-LA (R-DHLA) and S-LA (S-DHLA), during organic synthesis. Only the naturally occurring R-LA (R-DHLA) enantiomer is bound covalently to the epsilon amino group of lysine residues of mitochondrial multienzyme complexes.

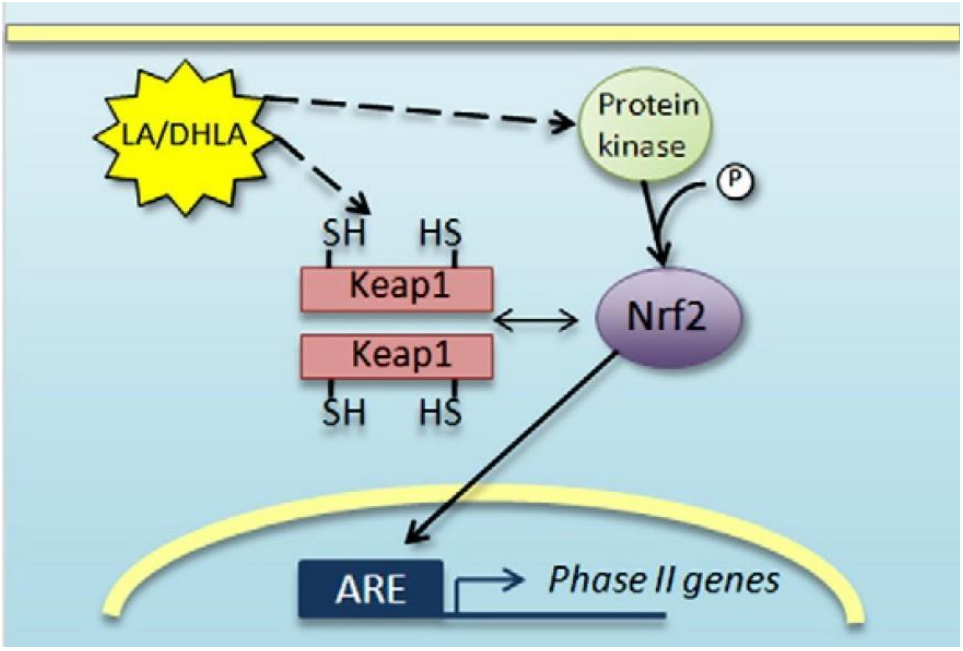

Figure 1.5. Proposed action of LA for induction of Phase II genes through Nrf2-mediated transcription. LA may oxidize critical thiols on the Keap1 dimer to halt Nrf2 degradation, and to prevent Keap1 from binding newly synthesized Nrf2. LA may also activate protein kinase signaling pathways that cause phosphorylation of Nrf2 on Ser40. This is the event that allows it to dissociate from Keap1 [24, 25]. Nrf2 can then localize to the nucleus and bind to the ARE, promoting transcription of genes for the Phase II detoxification response. [Figure source: K.P. Shay et al. et al. 2009].

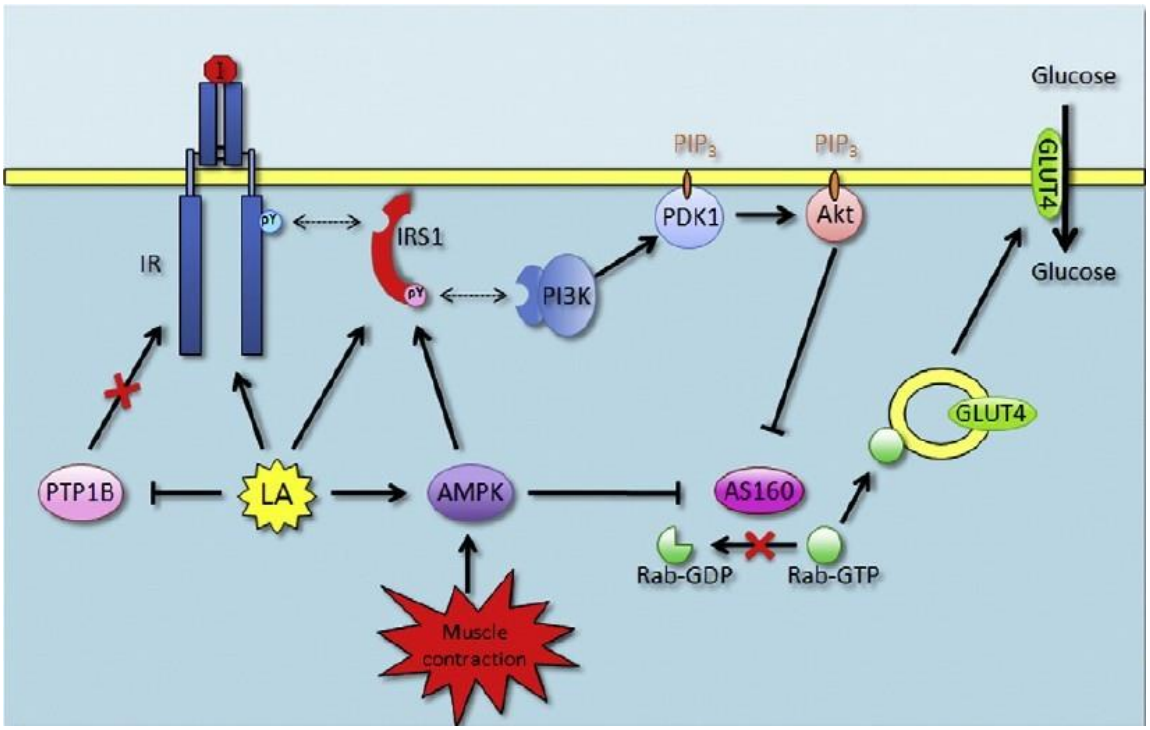

Figure 1.6. **Role of lipoic acid in IR/PI3K/Akt-dependent activation of glucose uptake in skeletal muscle.** Diesel et al. IR: insulin receptor. PTPB1: protein tyrosine phosphatase B1. IRS1: insulin receptor substrate 1. AMPK: AMP-activated protein kinase. PIP3: PtdIns-3, 4, 5-P3. PDK1: PtdIns-dependent kinase 1. Rab-GAP: GTPase-activating protein. [Figure source: K.P. Shay et al. et al. 2009].

CHAPTER 2

REVERSAL OF OBESITY-INDUCED HYPERTRIGLYCERIDEMIA BY (R)-α-LIPOIC ACID IN ZDF (fa/fa) RATS

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.

MATERIALS AND METHODS

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 has 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.

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).

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.

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 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 lM 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 Table 2.1.

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.

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 (a-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 3,30-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.

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.

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. 2.1). 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 2.2). LA lowered abdominal fat mass (–2.4 g/rat, $P < 0.05$) and abdominal adiposomatic index (–0.5%, $P < 0.05$) (Table 2.2). 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 lM; LA, 90 ± 10 lM). 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. 2.2). In liver, feeding LA significantly repressed expression of Acly (–60%), Acaca (–61%), Fasn (–50%), Gpam (–52%), Dgat2 (–46%), and Pnpla3 (–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. 2.3A). FASN abundance was decreased by LA (although not significantly) in epididymal fat and gastrocnemius muscle (Fig. 2.3B,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. 2.2). 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 (C4 to C11 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 (C12 to C20 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 CPT1a) 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. 2.3). 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. 2.4A). This observation coincided with the upregulation of liver Fgf21 expression (+174%, $P < 0.03$, Fig. 2.4B). 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. 2.4C). 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 the beneficial phenotype evoked by LA.

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Table 2.1. qRT-PCR primers used to quantify gene expression

Gene	Sequence (5'→3') [a]	Product size (bp)	GenBank [b]
Acly	ATGCCCCAAGATTCAGTCCC CCACTGAAGGCTCATCTCGG	160	NM_016987.2
Acaca	GTTACTCGTTTTGGGGGAAA CAGCCCACACTGCCTGTA	298	NM_022193
Fasn	GTGGAAGACACTGGCTCGAA TGGTACACTTTCCCGCTCAC	195	NM_017332.1
Gpam	CGTGGGAAGGTGTTGCTATT CAGCAATTGCCTCTTGGACT	248	NM_017274
Dgat2	AACACGCCCAAGAAAGGTG AGGGCAGATGCCTCCAGAC	303	NM_001012345
Pnpla3	GTCGGATGAGAGCATCTGG TAATGGGCAGGAGGTTGCAG	113	XR_085887.2
Pparg	GACACAGACAAAACATCAGTGG CTGTGTCAACCATGGTAATTTCTTG	156	NM_013124.3
Acsm3	CCAGCTGTGGGAAGCAGTAT ACTCCAGATGGATTTGGTGGTT	180	NM_033231.1
Cpt1a	CCACAAATTACGTGAGTGACTGGTGG CCAGAAGACGAATGGGTTTGAGTTCC	213	NM_031559
Cpt1b	GCTCATTTCCGGGACAAAGGCAAG TTGGAGGTCTTGTTCCTTATGGGAC	163	NM_013200
Acot1	GGCCACCCTGAGGTAAAAGGA TGGTTTCTCAGGATAGTCACAGG	194	NM_031315.1
Acox1	CAGCCCTACTGTGACTTCCA ACCTGGGCGTATTTCATCAG	279	NM_017340.2
Fgf21	CCTGGAGCTCAAAGCCTTGA AAACTGCAGGCCTCAGGATC	129	NM_130752.1
Ppia	CGAGCTGTTTGCAGACAAAG GTGTGAAGTCACCACCCTGG	140	NM_017101.1
Rplp0	CTAGAGGGTGTCCGCAATGT AGGAAGGCCTTGACCTTTTC	161	NM_022402

[a] Shown as sense primer followed by antisense primer.

[b] GenBank accession number.

Table 2.2. 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.

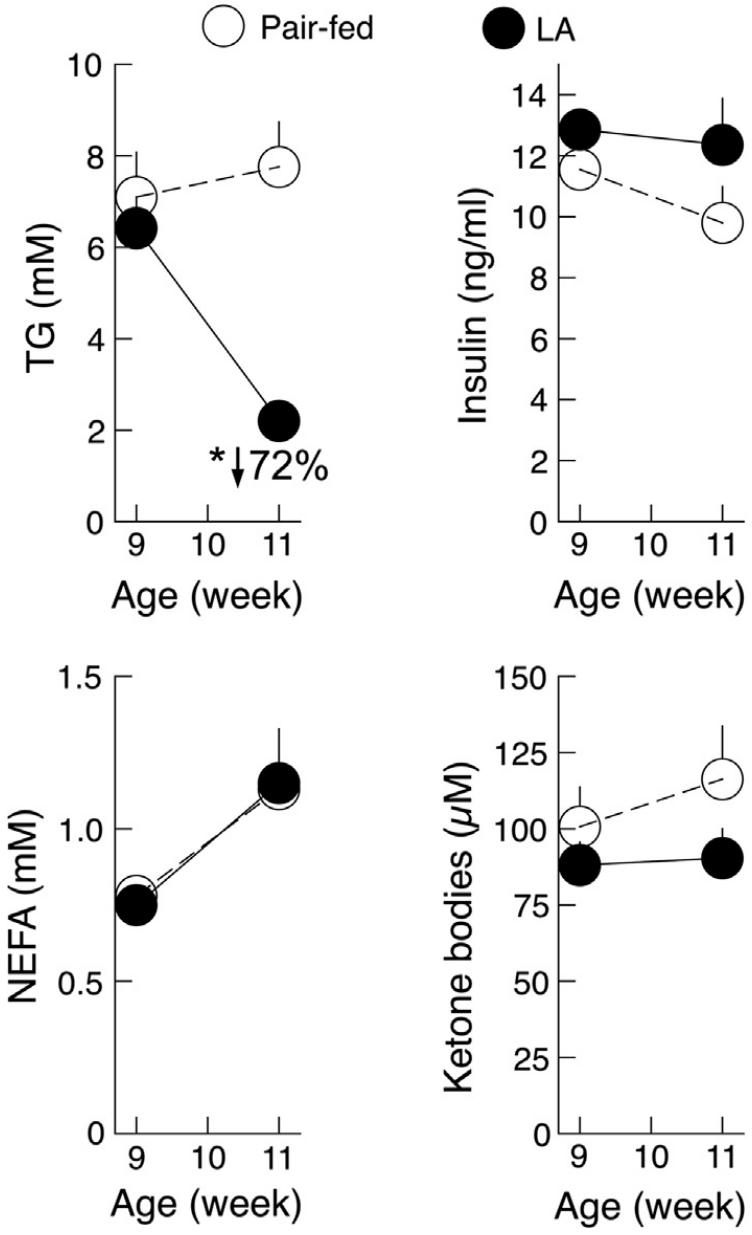

Figure 2.1. Time-courses of blood plasma TG (*P<0.001), insulin, non-esterified fatty acids (NEFA), and ketone bodies (3-β-hydroxybutyrate + acetoacetate) in 3-h fasted ZDF rats fed ± LA (n = 8).

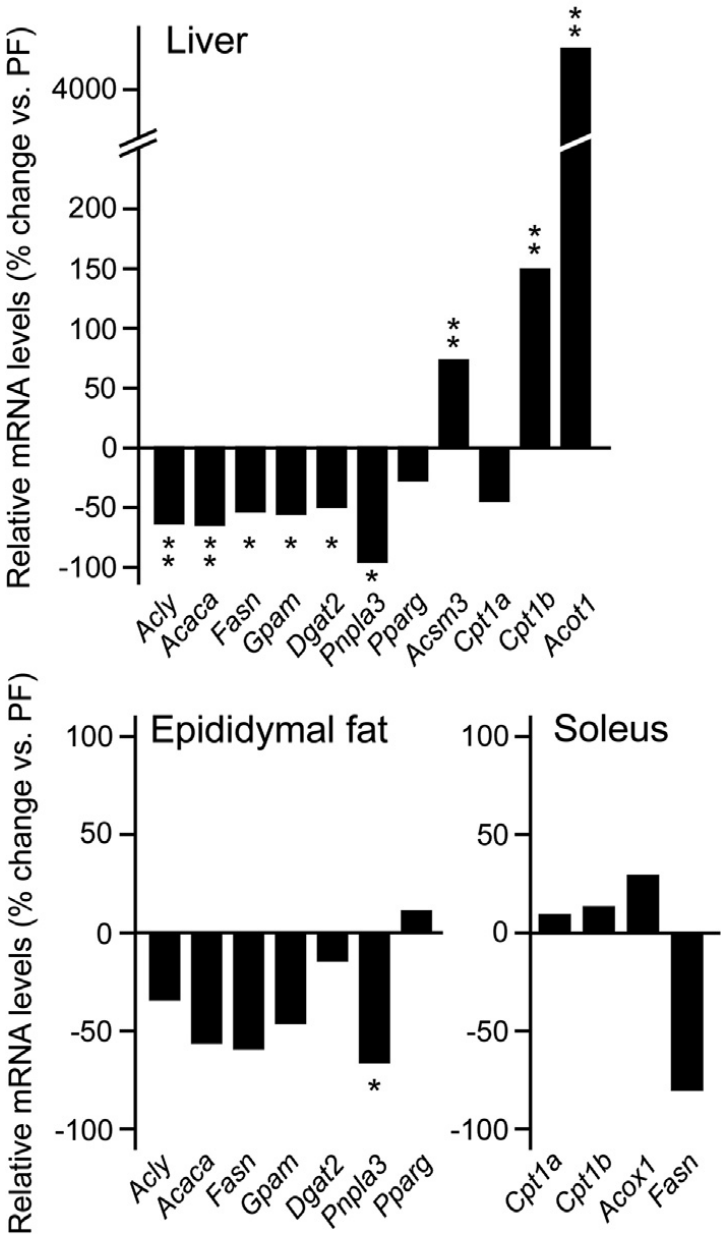

Figure 2.2. Transcriptional changes induced by LA. mRNA levels (expressed as % of control pair-fed group, n = 8) of selected genes in liver, epididymal fat, and soleus muscle. *P < 0.05,**P < 0.02. PF, pair-fed group.

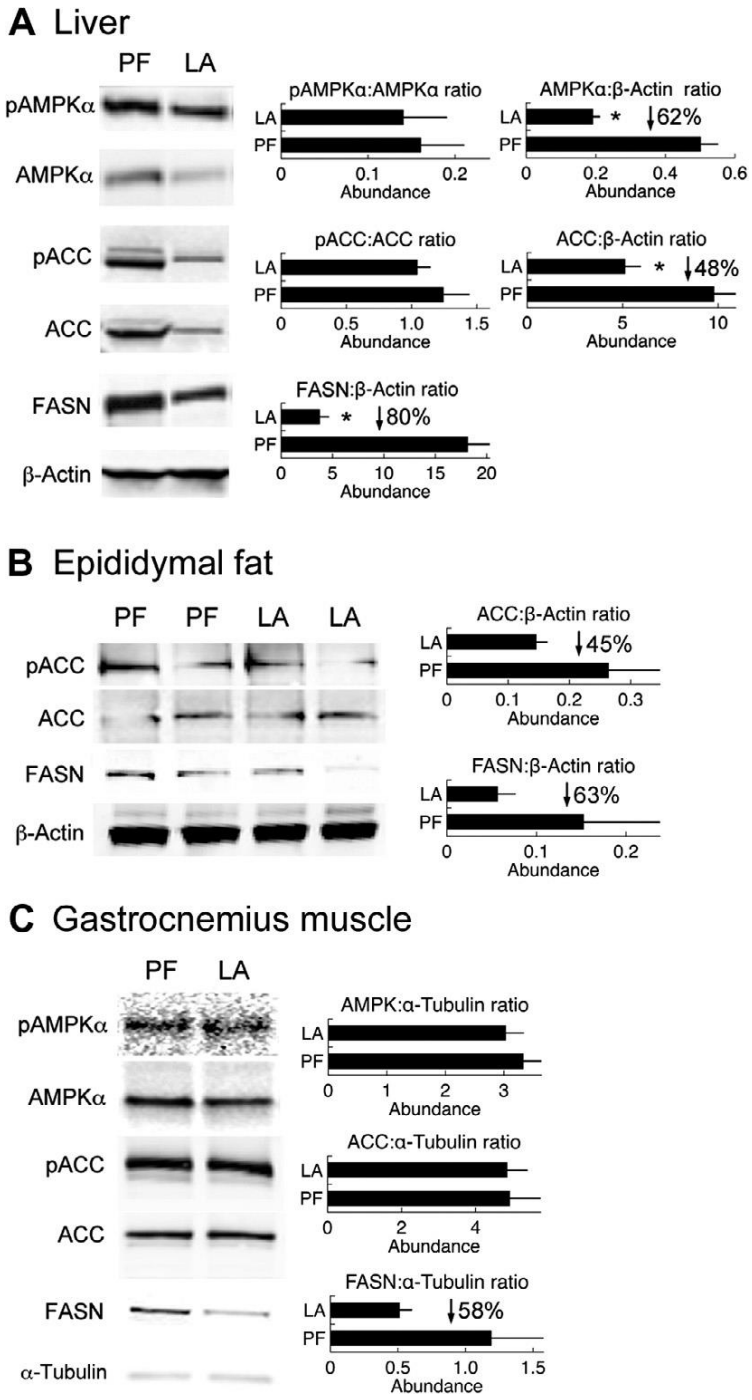

Figure 2.3. Lipoic acid represses FASN and ACC independently of AMPK. Immunoblots and densitometry depicting the tissue contents of phosphorylated AMPKα (pAMPKα, Thr 172), total AMPKα (sum of AMPKα1 and AMPKα2), phosphorylated ACC (pACC, Ser 79), total ACC (ACC1/2), FASN, and β-actin or α-tubulin as 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.

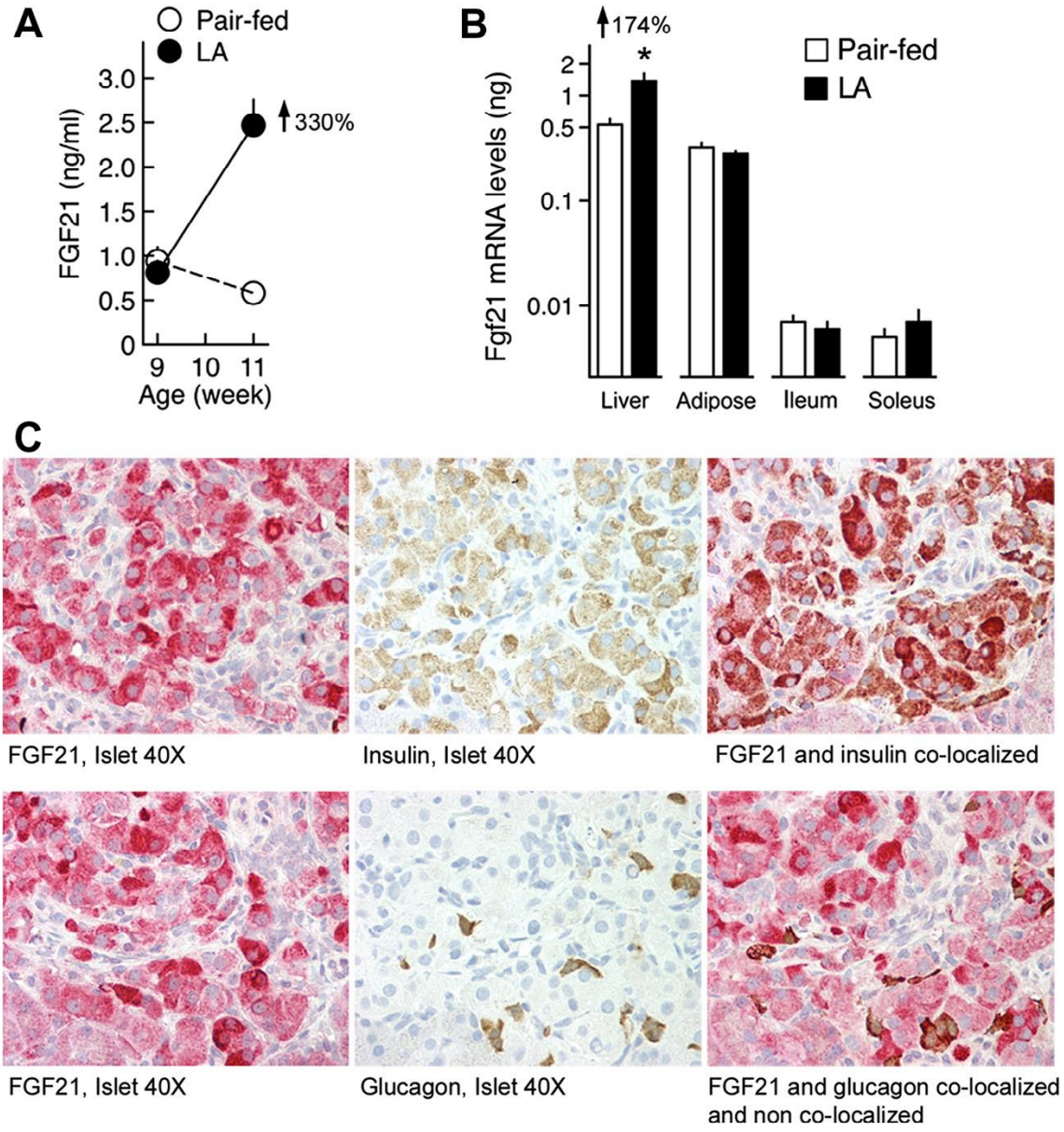

Figure 2.4. Lipoic acid induces FGF21 production. (A) Temporal changes in blood plasma FGF21 showing a 330% increase in LA-fed animals at the end of the trial ($P < 0.0002$, $n = 8$). (B) Relative Fgf21 mRNA levels in 125 ng total RNA of each tissue, and normalized to housekeeping gene Ppia. Data represent 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 insulinpositive β-cells. Data is representative of two rats fed LA.

CHAPTER 3

ELUCIDATE THE MECHANISM BY WHICH (R)-α-LIPOIC ACID UP-REGULATES FGF21 GENE EXPRESSION

INTRODUCTION

In Chapter 2, we report that naturally occurring dietary compound (R)-α-lipoic acid (LA) stimulates liver *Fgf21* expression (+174%) and blood FGF21 levels (+330%) and replicates the adaptive metabolic response to fasting supported by FGF21 *in vivo*. In particular, LA reversed hypertriglyceridemia, lowered abdominal fat mass, repressed genes of long-chain fatty acid and glycerolipid synthesis in the liver and adipose tissue, and upregulated specific PPARα target genes involved in long- and medium-chain fatty acyl ester metabolism [1]. The potential impact of this work is that LA may prove to be a safe and affordable means to stimulate FGF21 production.

Based on this research, we hypothesize that FGF21 mediates the lipid lowering properties of LA (Fig. 3.1). Through this original work, we seek to shift current clinical practice by building a case for LA as a dietary inducer of endogenous FGF21 and a non-prescription means to treat FGF21-responsive metabolic disorders. Elucidating the novel mechanism by which LA lowers TG will stimulate interest in its therapeutic use and justify further research on potential additive or synergistic effects of combined therapy with LA in lipid metabolic disorder.

We now seek to determine the precise mechanism of induction of the *FGF21* promoter by this dietary molecule. It means to identify the regions of the promoter that respond to LA and DHLA [2]. Since *FGF21* may be regulated via an alternative distal promoter P1 (retrieved from the Transcriptional Regulatory Element Database, TRED), proximal promoter P2 may not account for full *FGF21* inducibility and justifies the survey of a ~5,000-bp genomic DNA region that includes *FGF21* distal promoter and spans clusters of acetylated histones and DNase hypersensitivity sites (Fig. 3.6). To this end, we have developed the FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) assay to isolate regulatory regions that overlap to a large degree with DNase I hypersensitivity regions and identify nucleosome-depleted regions of chromatin by qPCR [3,4,5].

With this molecular tool, we plan to interrogate human *FGF21* promoter regulation from HepG2 hepatocellular carcinoma cells under fed and starved states and in the presence of LA and DHLA. Our results will provide new insights into how nutrient-responsive transcription factor binding sites regulate *FGF21*.

MATERIALS AND METHODS

HepG2 cells

HepG2 cells were cultured in DMEM (5.5 mM glucose) supplemented with 10% fetal bovine serum (FBS) and 0.25X antibiotics/antimycotics to 40-50% confluence and used thereafter for the experiments. We conducted two experiments. Experiment 1

evaluated the inducibility of FGF21 upon serum withdrawal as an attempt to replicate FGF21 induction seen *in vivo* during starvation. To that end, HepG2 cells were cultured in the absence or presence of FBS as described in Figure 3.2 (Experiment 1). HepG2 cells continued to proliferate and maintain their morphology in absence of FBS. Serum withdrawal also eliminated potential confounding factors brought about by constituents found in FBS (e.g. growth factors, cytokines) with which the compounds of interest (dietary dithiols) may have unintended interactions. In Experiment 2, HepG2 cells were grown in the presence of LA (50 μM), DHLA (50 μM), or DMSO control for up to 48 hours in the absence of FBS, according to the protocol described in Figure 3.2. The final concentration of DMSO was 0.1%, a concentration that is widely accepted as being inert to cells, including HepG2 cells.

Abundance of *FGF21* mRNA by quantitative Real-Time PCR (qRT-PCR)

HepG2 total RNA was extracted at the 0, 12, 24, 36 and 48-hour time points by using BioRad Aurum kit, DNase I treated and quantified by NanoDrop. RNA integrity was confirmed by using Agilent Bioanalyzer 2100. First strand cDNA was synthesized with oligo(dT) and random primers using BioRad iScript. qRT-PCR was performed by using a BioRad CFX96 and SYBR Green supermix according to the manufacturer's instructions. *FGF21* mRNA was normalized to housekeeping gene cyclophilin A (*PPIA*). Primer sequences are shown in Table 3.1.

FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements)-qPCR

The FAIRE assay is used to isolate active regulatory DNA loci through the identification of nucleosome-depleted region ('open chromatin'), which is less efficiently cross-linked to protein and will segregate to aqueous phase during phenol/chloroform extraction. The purified input control HepG2 DNA and FAIRE DNA will later be detected by qPCR. Input control DNA and FAIRE DNA are all produced from HepG2 cells according to the FAIRE method in Figure 3.3. For our purpose, HepG2 DNA will be isolated at the 48-hour time point in Experiment 1 and at the 36-hour time point in Experiment 2. Mapping the response of the *FGF21* promoter (chromosome 19) requires the design and validation of a collection of primer pairs whose qPCR products cover the ~5,000-bp region of interest. To that end, 25 primers pairs specific for human *FGF21* promoter (PPs, overlapping between two adjacent PPs) were designed and validated.

Statistical analysis

Statistical significance was determined with the unpaired two-tailed Student t-test with Welch's correction (Experiment 1), or the Kruskal-Wallis test followed by Dunn's multiple comparison test (Experiment 2). All statistical tests were performed to the 5% significance level.

RESULTS AND DISCUSSION

Effect of serum starvation on *FGF21* transcription in HepG2 cells

Serum withdrawal induced a marked increase in *FGF21* mRNA abundance (Fig. 3.4). The induction was significant from 24 hours (+5,530% *vs*. 0 hour) to 48 hours (+12,460% *vs*. 0 hour) after serum removal. In contrast, the reintroduction of serum (10% FBS) at 24 hours reversed the induction and brought *FGF21* mRNA levels back down to pre-experimental concentrations. Housekeeping gene, *PPIA*, was not markedly affected by serum manipulation and remained highly expressed at all time points. The absence of serum for 48 hours did not stop HepG2 cells from proliferating. Cell confluence was not noticeably different among +/- serum treatments.

Effect of LA and DHLA on *FGF21* transcription in serum starved HepG2 cells

Both LA and DHLA induced *FGF21* mRNA levels when compared to vehicle control (Fig. 3.5). DHLA-mediated induction (+900%, $P<0.019$) of *FGF21 vs*. DMSO control was stronger than that of LA (+670%, $P<0.024$) at 36 hours. FGF21 induction by the dithiols was not significant at 48 hours, suggesting the induction was transient. *FGF21* mRNA levels in DMSO controls at 36 hours were markedly different than those at 48 hours. The reason for this change is not entirely clear, it may be linked to the transient effect of the dithiols. Housekeeping gene, *PPIA*, was not markedly affected by the treatment and remained highly expressed at all time points.

Design and validation of FAIRE-qPCR primer pairs

The sequences of the 25 primer pairs are shown in Table 3.2. The authenticity of the

qPCR products generated with primers was confirmed by melt curve analysis and agarose gel electrophoresis. qPCR efficiencies were assessed with 5-fold serial dilutions of HepG2 DNA and plotting quantification cycle (Cq) versus log amount of template. Efficiency curves (EC %) were between 89% and 114% (Fig. 3.7). The 25 qPCR products covered 4,573 bp of FGF21 promoter (Fig. 3.8) with some overlap.

Taken together, our findings in HepG2 cells treated with LA and DHLA reproduced the induction of hepatic FGF21 gene expression seen in LA-fed animals. Our data indicating that DHLA induced FGF21 mRNA levels with higher potency than LA suggest that DHLA may the relevant form of α-lipoic acid in this process. Experimental evidence supports this view. Indeed, studies have shown that cells and tissues enzymatically convert LA into DHLA, and the conversion to DHLA is critical to support biological effects [6]. Attempts to enrich cells or mitochondria with exogenous LA using a triphenylphosphonium-conjugated lipoyl derivative showed that although the lipoyl conjugate accumulated in cells and mitochondria, it was not protective against a range of stressors as a consequence of its poor enzymatic reduction to dihydrolipoyl conjugate caused by steric hindrance. LA was protective after reduction to DHLA, a reaction catalyzed by thioredoxin reductase and glutathione reductase in the cytosol, and dihydrolipoyl dehydrogenase in mitochondria.

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[6] S.E. Brown, M.F. Ross, A. Sanjuan-Pla, A.R Manas,. R.A. Smith, M.P. Murphy, Targeting lipoic acid to mitochondria: synthesis and characterization of a triphenylphosphonium-conjugated alpha-lipoyl derivative. Free radical biology & medicine 42 (2007) 1766-1780

Table 3.1. Primer sequences for determining *FGF21* coding mRNA by qRT-PCR

Human gene	Primer	Sequence (5'→3') [a]	Tm [b]
FGF21	F1	GGGAGTCAAGACATCCAGGT	60.0°C
	R1	GGCTTCGGACTGGTAAACAT	60.0°C
PPIA	F1	TTCATCTGCACTGCCAAGAC	56.2°C to 60.7°C
	R1	TGTCCACAGTCAGCAATGGT	56.2°C to 60.7°C

[a] Shown as sense primer followed by antisense primer.

[b] Appropriate annealing temperature.

Table 3.2. Sequences of the 25 primer pairs designed for FAIRE-qPCR assay

PPs	Sequence (5'→3') [a]	Product size (bp)	qPCR protocol [b]
PP1	GTGGCACATGTCTGTGGTCT CGAGATTCCTTCAGGAAAGGT	193	isothermic qPCR
PP2	ATAGCACCGCTGACTGCAC ACCTCTGTGGTGGATTTTGC	216	isothermic qPCR
PP3	CTTCCACCAGCCTGTTTCTC GGACCCAGGAACAACTCCTT	244	TD PCR
PP4	GAGGTGGCGGAGATTCCTA AAAAGCGGACTGTGGATCTG	162	isothermic qPCR
PP5	CAGATCCACAGTCCGCTTTT CTCCACCTTCCAGCCATC	230	TD PCR
PP6	GATGGCTGGAAGGTGGAG TGGCACCGGAAGAGAGAG	280	TD PCR
PP7	GGTGCCAGGGCTTAGAGTC AGCCTGGCAAACAGGTTCT	159	TD PCR
PP8	GGGATGGAGAACCTGTTTGC GCAGTGCAGGTCTGGAAAGT	207	TD PCR
PP9	CCTTTTGGGGAATTCTAGGG GCCTTGGACCAGAGAAAATG	203	isothermic qPCR
PP10	GCATTTTCTCTGGTCCAAGG GGTCTCTGCGTCTTGATGGT	207	isothermic qPCR
PP11	CTACCATCAAGACGCAGAGACC TGCAGAGAAGAAAACGCTGACA	218	isothermic qPCR
PP12	GCAGAAAGAGAAGGCAGCAT ACACCTTCTCCTGCCTCAC	286	TD PCR
PP13/14	TGAGGCAGGAGAATGGTGTG AGTCATCCTGCTGTGCTGTG	238	TD PCR
PP15	ACCATTGAAGCACAGCACAG TTCTGTCACACCGGCATAAG	213	isothermic qPCR
PP16	TCGTGGTCTGCTGAGTAACG AACAACCGGTGGGTATAGGG	167	TD PCR

PP17	TATACCCACCGGTTGTTTCC AATGGGAGGGTCAGTCTGTG	238	isothermic qPCR
PP18	AATCCTTCCCCACAGACTGA GGGAGCCTGGACTCCTAAGT	193	isothermic qPCR
PP19	CCAGACCCTTCTGCTCACAC AGGGAGCTTTGGTCTGGACT	230	TD PCR
PP20	AGGAGTCCAGACCAAAGCTC ACCCAGAGGGAAGACAGGTT	247	isothermic qPCR
PP21	AGCCAACCTGTCTTCCCTCT CAACCTCATCTGTCCCTGCT	218	isothermic qPCR
PP22	ATGTCCAGGGTCTGAGCATC ATGCAGATCACGCAGAACAA	232	isothermic qPCR
PP23	TCTTGTTCTGCGTGATCTGC TGTAACCTGGGGTCCTTTCA	228	isothermic qPCR
PP24	ACCCCAGGTTACATCATCCA CAGCTGACAGAACACCCTTG	177	isothermic qPCR
PP25	TCTGTCAGCTGAGGATCCAG GCACAGAAACCCACAGTCCT	203	isothermic qPCR
PP26	GGACTGTGGGTTTCTGTGCT ATCCTCCCTGATCTCCAGGT	165	isothermic qPCR

[a] Shown as sense primer followed by antisense primer.

[b] qPCR protocol employed for the validation of primer pairs. TD qPCR, Touch-down qPCR.

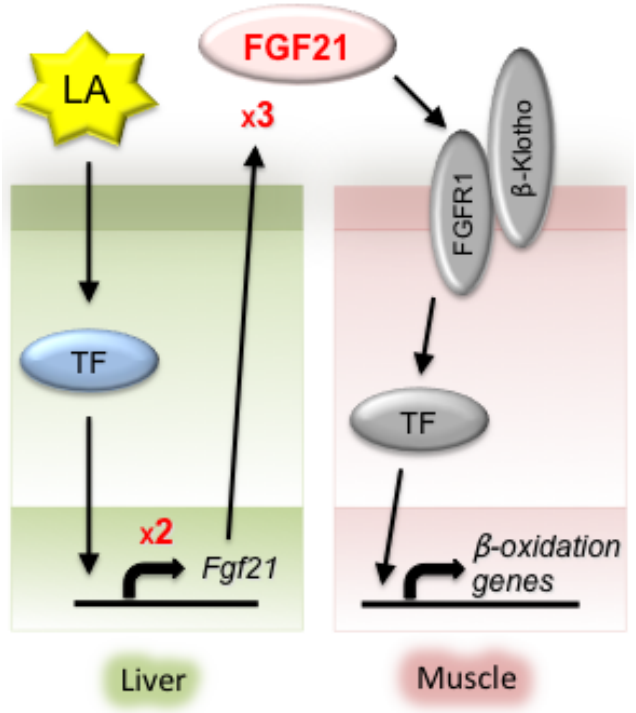
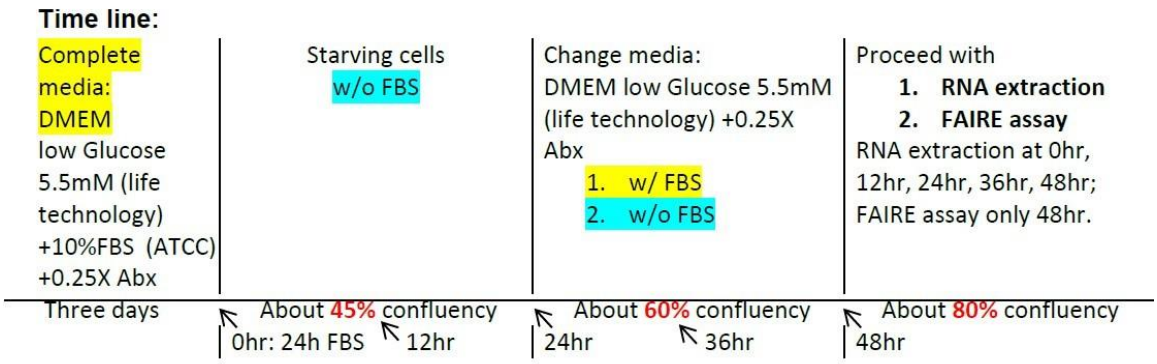


Figure 3.1. Hypothesis that fibroblast growth factor-21 (FGF21) is the missing link in LA-mediated disposal of fat in target tissues. TF = transcription factor.

Experimental 1: Effect of serum starvation on *FGF21* transcription in HepG2 cells



Experiment 2: Effect of LA & DHLA on *FGF21* transcription in serum-free HepG2 cells

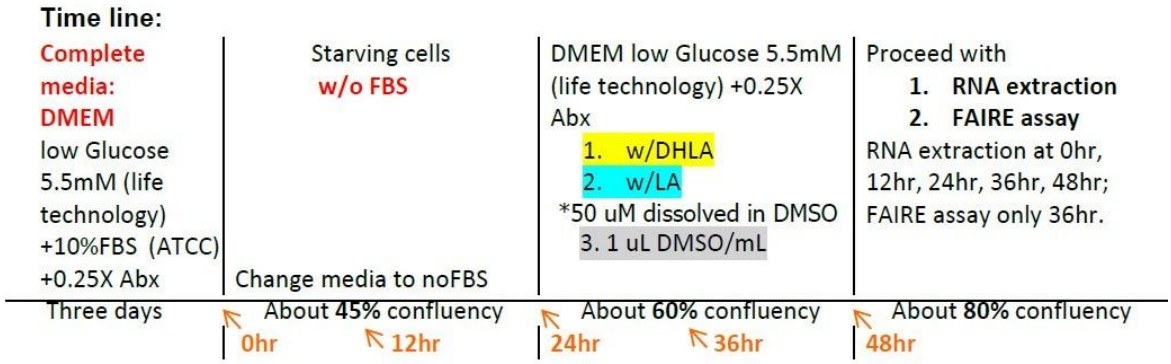


Figure 3.2. Experimental design of HepG2 cell studies

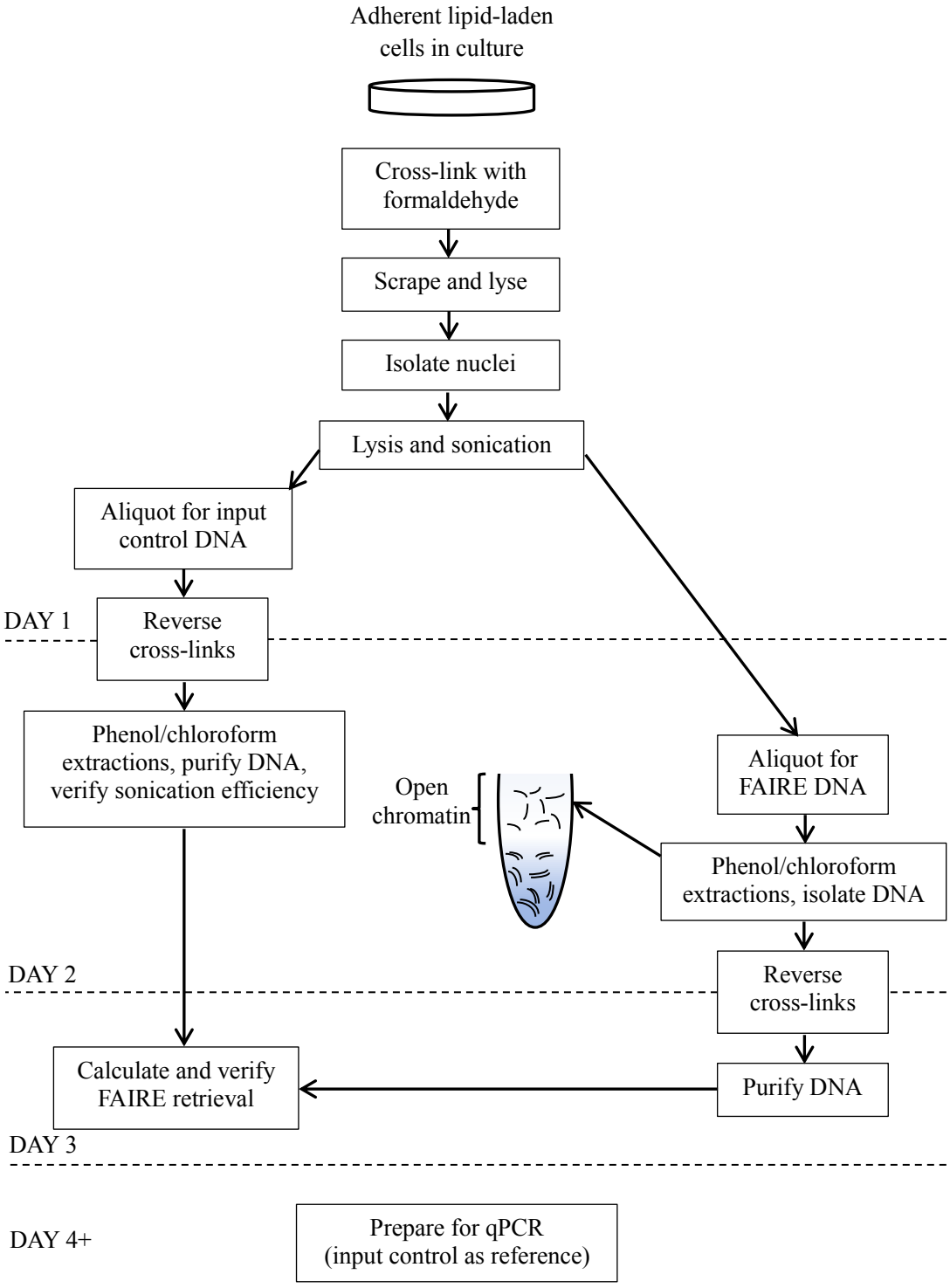


Figure 3.3. FAIRE assay protocol. [Adapted from Simon et al. 2012].

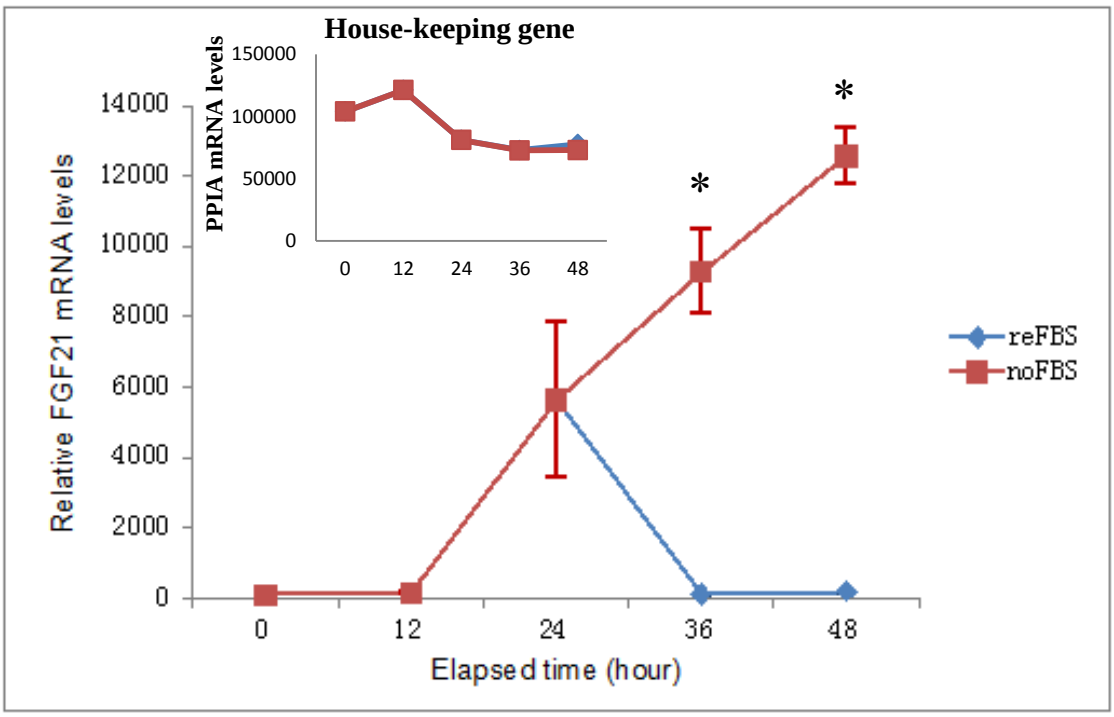


Figure 3.4. Time-course of *FGF21* mRNA abundance as affected by the presence or absence of serum (Experiment 1). reFBS indicates that FBS (10% final concentration) was reintroduced at 24 hours. Serum withdrawal strongly induced *FGF21* mRNA steady-state levels at 24, 36 and 48 hours. The reintroduction of serum blocked *FGF21* induction at the 36 hours (*P < 0.0007) and 48 hours (*P < 0.0001) time points (n=6).

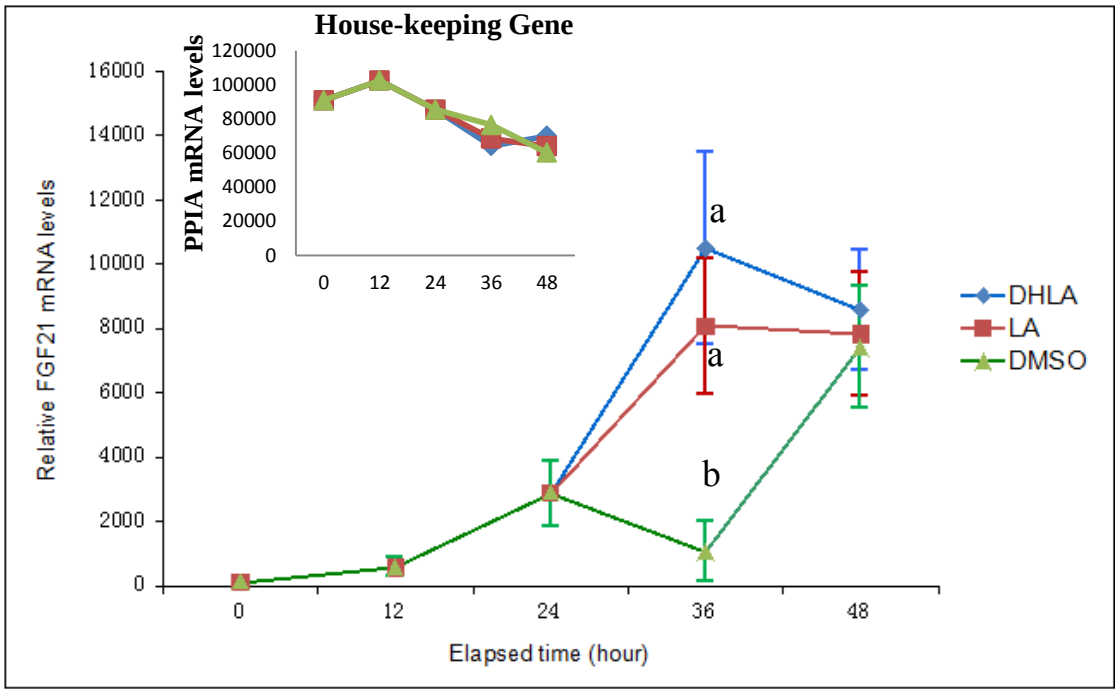


Figure 3.5. Time-course of *FGF21* mRNA abundance as affected by LA and DHLA (Experiment 2). DHLA (+900%, P<0.019, n=6) and to a lesser extent LA (+670%, P<0.024, n=6) significantly increased *FGF21* mRNA levels at 36 hours *vs*. DMSO control. Data not sharing a common letter at a given time point are significantly different. Cells were grown in the absence of serum from the 0-hour time point. DMSO, LA (50 μM) or DHLA (50 μM) were added at 24 hours.

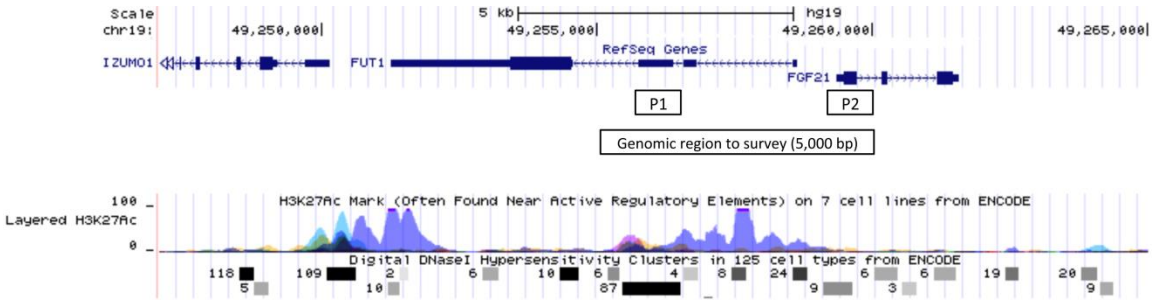


Figure 3.6. Human *FGF21* gene and promoter structure, and positioning of promoter P1 and P2, the ~5,000-bp region to survey, and acetylated histone marks found near active regulatory elements.

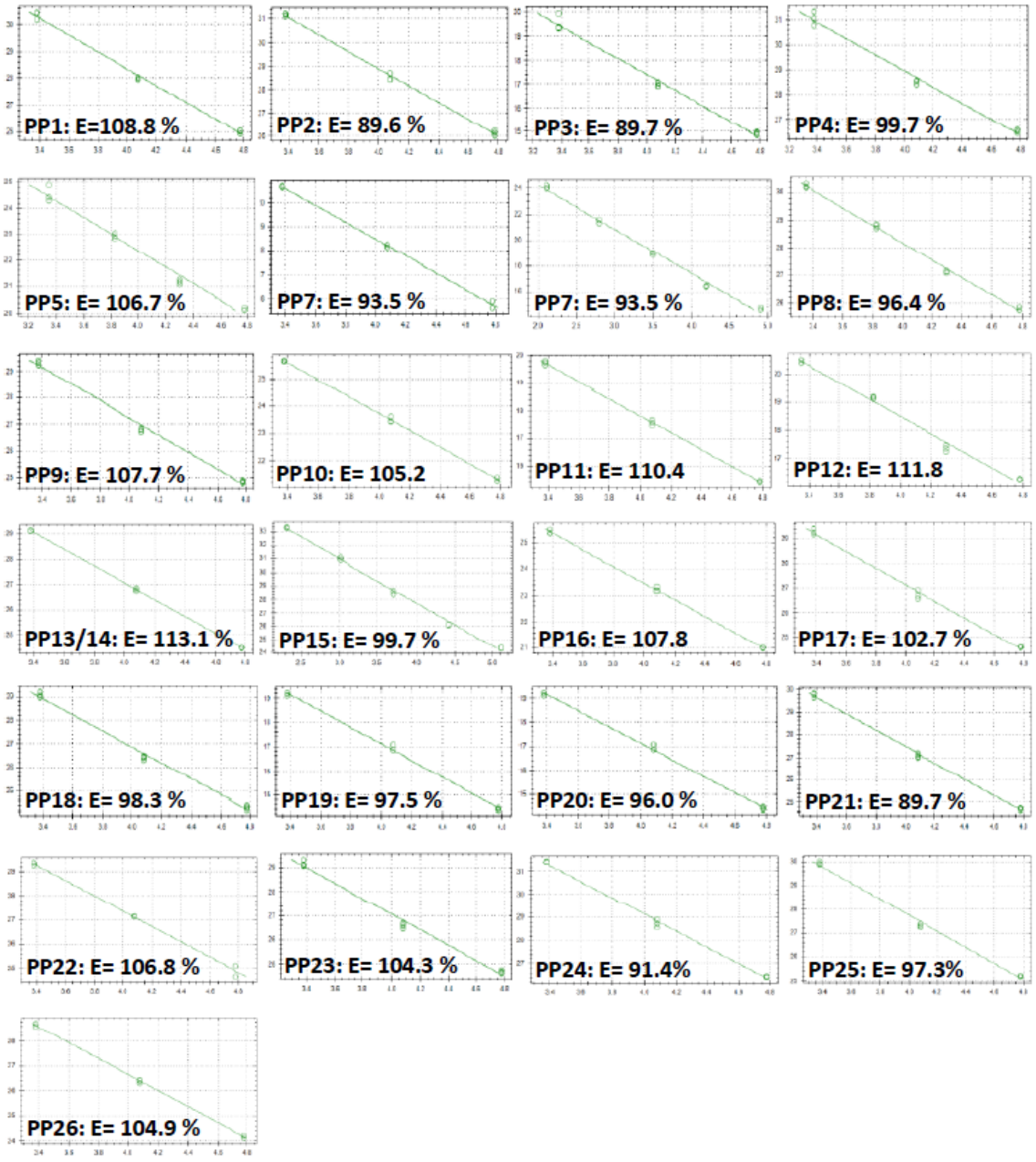


Figure 3. 7. The qPCR efficiency curves (EC %) of the 25 primer pairs. Horizontal axis: Log Starting Quantity; Vertical axis: Cq value.

Figure 3. 8. Sequence (4,573 bp) of the human *FGF21* 5' UTR (chromosome 19).

Starts @ 49,255,156

Ends @ 49,259,728

AGAGAGGCTGGAGGGTC = start of predicted promoter P1

AGGTTGGCCCACGGCCA = start of predicted promoter P2

CTGTCAGCTGAGGAT…GCCGCCGAAA = 385 bases of Exon 1 of human *FGF21*

TGAGATCATGCCATTACACTCCAGCCTGGGCAACAAGAGTGAAACTCCGTCTC
CAAAAAAAAATAAATAAAATTAGCTTGGCATGGTGGCACATGTCTGTGGTCTC
AGCTACACCGGATGCTAAGGCGGGAGGATCCCCGGAGCTCACAATGAGCCGC
GATAGCACCGCTGACTGCACTCCAGCTTGCGCGACAGAGAGGGACCCTGTCTT
AAAAAAAAAAAAAAAAAAAAAAAAGAAAGTGGTCCAGGTTCCTACACCTT
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AGAGGTGGTGATGGGAAACGATAGTTCAAGTAAAACAAGAGAGGCTGGAGG
GTCAGGAGCGGAAGGAGCATCTTGGTTCCTGGAGGAGTAATGGCTGGGGAGG
TGGCGGAGATTCCTAGGGCCTTAGGAAGACCTGGAGAAGAGGTTGGGGGTGC
ACCTCCTGGTTCTGAAGGAGTTGTTCCTGGGTCCCAGGAACAACTGAGTGGG
GCAGTCCCCACTTACCCGAGCTGCTTGCAGGTGGCAGATCCACAGTCCGCTTT
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CHAPTER 4

CONCLUSION

Recent research work in metabolism and nutrition has shed new light on the ability of FGF21 to improve metabolic disorders related to glucose and lipid homeostasis and on the inducibility of the FGF21 gene by bioactive food compounds [1]. Research into the metabolic effects and molecular mechanism of action of α-lipoic acid as a relevant natural inducer of FGF21 production has been pursued in this thesis. Our research shows that (R)-α-lipoic acid stimulated liver FGF21 gene expression (+175%) and blood FGF21 levels (+330%) and replicated the adaptive metabolic response to fasting supported by FGF21 *in vivo*. Further studies of the regulation of the FGF21 promoter will have implications both in basic research and in clinical setting.

In recent years, there has been a significant push toward the development of new therapeutics that target lipid risk factors other than hypercholesterolemia, such as hypertriglyceridemia. There is a growing interest in the therapeutic potential of α-lipoic acid against dyslipidemia. Seven clinical trials on the topic were published over the previous three years out of a total of 17 clinical trials on α-lipoic acid. Careful review of these studies indicates α-lipoic acid (600 mg/day) safely lowers blood triacylglycerol once triacylglycerol have become elevated [2-8]. As of now, however, no clinical trial with α-lipoic acid in hypertriglyceridemic subjects has been conducted in the United

States. Before clinical trials with α-lipoic acid can be initiated, the molecular targets and cellular signaling of α-lipoic acid will need to be uncovered.

The molecular mechanism of α-lipoic acid proposed herein supports a shift from the current paradigm that α-lipoic acid acts as an antioxidant to the new concept that α-lipoic acid regulates gene transcription. Although historically recognized as an antioxidant able to reduce various oxidized targets (including other antioxidants), α-lipoic acid does not act as a traditional antioxidant here. At the doses ingested from a supplemented diet and because it is very rapidly metabolized, α-lipoic acid does not accumulate to sufficient cellular levels (low μM) to have a lasting impact similar to that of traditional antioxidants (e.g., ascorbate or tocopherol) [9, 10]. Rather, α-lipoic acid transiently stimulates cell signaling and transcription pathways relevant to lipid homeostasis [11]. Experimental evidence collected in recent years, including our own, support a new yet definable mechanism of α-lipoic acid action that ultimately results in its universal lipid-lowering effect.

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