Biochemical and Biophysical Research Communications 455 (2014) 212–217

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Alpha lipoic acid induces hepatic fibroblast growth factor 21 expression via up-regulation of CREBH

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ARTICLE INFO

Article history:
Received 23 October 2014
Available online 5 November 2014

Keywords:
FGF21
Alpha lipoic acid
CREBH
Liver

ABSTRACT

Hepatic expression of fibroblast growth factor 21 (FGF21), one of the most promising therapeutic candidates for metabolic syndrome, is induced by multiple factors associated with fasting, including cyclic AMP response element-binding protein H (CREBH). Alpha lipoic acid (ALA), a naturally occurring thiol antioxidant, has been shown to induce metabolic changes that are similar to those induced by FGF21, including weight loss and increased energy expenditure. Here, we investigated the effect of ALA on hepatic FGF21 expression. ALA treatment enhanced CREBH and FGF21 mRNA expression and protein abundance in cultured hepatocytes. ALA increased FGF21 promoter activity by up-regulating CREBH expression and increasing CREBH binding to the FGF21 promoter, indicating that ALA up-regulates FGF21 at the transcriptional level. Moreover, inhibition of endogenous CREBH expression by siRNA attenuated ALA-induced FGF21 expression. Finally, treatment of mice with ALA enhanced fasting-induced up-regulation of CREBH and FGF21 in the liver and inhibited feeding-induced suppression of their expression. Consistently, ALA increased serum FGF21 levels in both fasted and fed mice. Collectively, these results indicate that ALA increases hepatic FGF21 expression via up-regulation of CREBH, identifying ALA as a novel positive regulator of FGF21.

1. Introduction

Fibroblast growth factor 21 (FGF21) belongs to a 'hormone-like' subfamily of FGFs that are deficient in heparin binding and can be released into the circulation [1]. FGF21 is an emerging therapeutic candidate for metabolic syndrome due to its favorable effects on energy homeostasis, lipid metabolism, and insulin sensitivity [2–4]. While peroxisome proliferator-activated receptor (PPAR) α has been regarded as a major transcription factor mediating fasting-induced hepatic FGF21 expression [5], there are a number of other transcription factors that are known to modulate hepatic FGF21 transcription, including Nur77 [6], cyclic AMP response element-binding protein H (CREBH) [7], retinoic acid receptor β [8], and retinoid-related orphan receptor α [9].

CREBH, an endoplasmic reticulum (ER) membrane-bound transcription factor, plays a critical role in fasting-induced hepatic FGF21 expression [7,10,11]. CREBH-deficient mice exhibited impaired fasting-induced expression of FGF21 [12]. CREBH interacts with PPARα, which acts as a transcriptional coactivator of CREBH for FGF21 expression [7]. In agreement with previous studies [12,13], our recent study showed that hepatic CREBH expression is nutritionally regulated, and is high during fasting and suppressed by feeding [14]. Furthermore, hepatic CREBH expression is induced by fenofibrate treatment, which is known to activate PPARα and induces FGF21 expression in the liver [14].

Alpha lipoic acid (ALA) is an essential cofactor for mitochondrial respiration [15]. It is often used to manage diabetic complications and has a diverse range of activities, including the capacity to promote regeneration of other antioxidants [16,17], repair oxidized proteins [18,19], chelate metal ions [20,21], and inhibit inflammation through the inhibition of NF-κβ [22,23]. ALA has also been shown to regulate glucose and lipid metabolism by modulating PPARα-regulated genes and key enzymes [24]. We previously demonstrated that ALA activates adenosine monophosphate (AMP)-activated protein kinase (AMPK) and reduces lipid accumulation in the livers of rodents fed a high-fat diet [25]; however, no study has examined the effects of ALA on CREBH expression and hepatic FGF21 expression. Therefore, this study was designed

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http://dx.doi.org/10.1016/j.bbrc.2014.10.147
0006-291X/

to evaluate whether ALA increases hepatic FGF21 expression via up-regulation of CREBH.

2. Materials and methods

2.1. Reagents and plasmids

ALA was provided by Viatris GmbH & Co. KG (Frankfurt, Germany). The CREBH antibody was purchased from Allele Biotech (San Diego, CA), the FGF21 antibody was purchased from Abcam (Cambridge, MA), and the actin antibody was purchased from Sigma (St. Louis, MO). cDNA encoding a constitutively active form of mouse CREBH was provided by Dr. Choi (Chonnam National University, Gwangju, Korea). Mouse FGF21 promoter reporter constructs were provided by Dr. Kliewer (University of Texas Southwestern Medical Center, Dallas, TX).

2.2. Cell culture

Mouse hepatocyte AML-12 cells were cultured in 5% CO_2 at 37 °C in DMEM/F12 medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, insulin–transferrin–selenium (ITS; Gibco-BRL), dexamethasone (40 ng/ml; Sigma, St. Louis, MO) and antibiotics (Gibco-BRL). Equal numbers of cells (5×10^5/60 mm culture dish) were seeded onto tissue culture plates. When cell confluence reached approximately 80%, the cells were subjected to serum starvation for 24 h in medium containing 0.5% fetal bovine serum and were treated as indicated below. Cells were infected with Ad-CREBH (CREBH-expressing adenovirus; see below) in serum-free medium for 2 h. The medium was then replaced with medium containing 0.5% fetal bovine serum for 24 h. Cells were subsequently processed for the isolation of RNA or protein, as described below.

2.3. Generation of recombinant adenoviruses

CREBH was inserted into the HindIII/XhoI sites of the shuttle vector pAdTrack-CMV. The vectors were then electroporated into BJ5183 cells that contained the adenoviral vector Adeasy to generate recombinant adenoviral plasmids. Recombinant plasmids were amplified in HEK-293 cells and purified by CsCl (Sigma) gradient centrifugation. Viral preparations were collected and desalted, and titers were determined using Adeno-X Rapid titer (BD Bioscience, San Jose, CA) according to the manufacturer's instructions. The efficiency of adenoviral infection was assessed using a recombinant adenovirus encoding CREBH fused to green fluorescent protein (GFP; data not shown).

2.4. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed, as described previously [14]. In brief, treated AML-12 cells were fixed with 1% formaldehyde, washed with ice-cold phosphate-buffered saline, and then harvested. The soluble chromatin was subjected to immunoprecipitation with an anti-CREBH antibody. Precipitated DNA was amplified by reverse transcriptase (RT)-PCR using primers specific for the promoter regions of FGF21. The primers used for PCR were as follows: CREBHRE site proximal region, 5′-TCCTAGAAATCCAAACTCGGCCCC-3′ and 5′-AGCTGTAT CAGCAGT GTGTAAGAG-3′; distal region, 5′-ACCAGAAGAGGGAGT CAGATC TTG-3′ and 5′-CAGGCAAAAAGAATTCGCTTGCAC-3′.

2.5. Quantitative real-time RT-PCR

Total RNA was isolated from tissue extracts using Trizol reagent (Invitrogen/Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. cDNA was synthesized from 5 μg of total RNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). Quantitative real-time RT-PCR was performed with the Power SYBR Green PCR Master Mix Kit (Applied Biosystems/Life Technologies, Warrington, UK) and the Step One real-time PCR system (Applied Biosystems, Foster City, CA) using the following program: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primer sets were designed using AB StepOne software (v2.1) based on sequences obtained from GenBank. The primers used for PCR were as follows: CREBH, 5′-TGGATCCGCTAACGTTGCA-3′ and 5′-GCCCCTCGCCTTG CTT-3′; FGF21, 5′-GTACCTCTACACAGATGACGACCAA-3′ and 5′-CG CCTACCACTGTTCCATCCT-3′; and GAPDH, 5′-GAAGGGTGGAGCCAA AAG-3′ and 5′-GCTGACAATCTTGAGTGAGTTG-3′. The relative levels of each mRNA were normalized to *Gapdh* mRNA expression.

2.6. Western blot analysis

Cell lysates were prepared using IPH lysis buffer [50 mmol/l Tris (pH8.0), 150 mmol/l NaCl, 5 mmol/l EDTA, 0.1 mmol/l phenylmethylsulfonyl fluoride (PMSF), and 0.5% NP-40] containing protease inhibitor cocktail (Sigma) and dithiothreitol. Cells were incubated on ice for 30 min, after which the lysates were subjected to centrifugation at 12,000 rpm for 10 min. Supernatants were collected and quantified using the Bio-Rad protein assay (Bio-Rad, Richmond, CA). Cell lysates were resolved by SDS–PAGE and transferred to a PVDF membrane (Millipore Corporation, Bedford, MA). The membrane was incubated in blocking buffer, followed by incubation with an anti-CREBH or FGF21 antibody, as indicated. Membranes were washed and incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive proteins were visualized by chemiluminescence according to the manufacturer's instructions (UVITec, Cambridge, UK). The membrane was stripped and re-probed with an anti-actin antibody to confirm equal protein loading.

2.7. In vitro transient transfections and reporter assays

AML-12 cells were plated at a density of 1×10^5 cells per well in a 24-well plate and incubated for 24 h. The promoter reporter constructs (300 ng/well) were transiently transfected using Lipofectamine Plus Transfection Reagent (Invitrogen/Life Technologies), and β-galactosidase plasmids were co-transfected as an internal control. Cells were transfected for 5 h, washed to remove plasmid, and then maintained in conditioned medium. Cells were harvested 24 h after transfection and assayed for luciferase and β-galactosidase activity. Cell lysates (20 μL per sample) were analyzed using a luciferase assay kit according to the manufacturer's instructions (Promega, Madison, WI). Luciferase activity was detected using a SIRUS Luminometer (Berthold, Pforzheim, Germany) and was normalized to β-galactosidase activity.

2.8. Animal experiments

All animal procedures were approved by the local ethics committee and carried out in accordance with institutional guidelines for animal research. Male 8-week-old C57BL/6N mice were purchased from Koatech (Kyunggi-Do, Korea) and divided into two groups: fasting and feeding. Animals were fasted for 24 h with free access to water or fasted for 24 h and re-fed during the following 12 h. Fasted and fed mice received an intraperitoneal injection of ALA (100 mg/kg/day) or saline for 7 days. ALA-treated mice were

divided into two groups, namely, fasting and feeding, following the protocol described above. After 7 days, the animals were sacrificed for the collection of blood and liver samples.

2.9. Analysis of blood samples

Serum FGF21 levels were determined using a Quantikine mouse FGF21 ELISA kit (USCN Life Science, Wuhan, China). Serum β-hydroxybutyrate levels were determined using colorimetric β-hydroxybutyrate ELISA kits (USCN Life Science).

2.10. Statistical analysis

Data were evaluated using ANOVA followed by a post hoc least significant difference test and expressed as the mean ± SEM. Values of $P < 0.05$ were considered statistically significant. All experiments were repeated independently three or more times.

3. Results

3.1. ALA increased CREBH and FGF21 expression in cultured hepatocytes

First, we examined whether ALA increases CREBH and FGF21 expression in cultured hepatocytes. CREBH mRNA expression was slightly increased at 0.5 h and peaked at 12 h after ALA treatment (Fig. 1A), and its protein levels gradually increased from 0.5 h to 24 h after ALA treatment (Fig. 1B). FGF21 mRNA (Fig. 1A) and protein levels (Fig. 1B) increased starting at 1 h after ALA treatment, and continued to increase until 24 h after treatment. To confirm the association between CREBH and FGF21 expression, we examined the effect of adenovirus-mediated overexpression of CREBH (Ad-CREBH) on FGF21 production in cultured hepatocytes. As expected, Ad-CREBH increased FGF21 mRNA and protein levels in AML-12 cells (Fig. 1C and D).

3.2. CREBH is required for ALA to stimulate FGF21 expression in cultured hepatocytes

To ascertain whether the induction of FGF21 expression by ALA is mediated by CREBH, AML-12 cells in which endogenous CREBH was down-regulated by transfection with CREBH-specific siRNA were treated with ALA. siRNA-mediated knockdown of CREBH attenuated the ALA-induced increase in FGF21 expression (Fig. 2A). The protein abundance of these genes was further confirmed by Western blot analysis (Fig. 2B).

3.3. ALA activates FGF21 promoter activity and induces CREBH binding to the FGF21 promoter

To investigate the mechanism by which ALA increases FGF21 production, AML-12 cells were transiently transfected with a human FGF21 promoter reporter construct. ALA increased FGF21 promoter luciferase activity in a dose-dependent manner (Fig. 3A). To determine whether ALA induces CREBH binding to the FGF21 promoter, a ChIP assay was performed using AML-12 cells. CREBH was recruited to the proximal region of the FGF21 promoter, which contains putative CREBH-binding sites (Fig. 3B). These data collectively indicate that ALA increases FGF21 production at the transcriptional level by up-regulating CREBH expression and increasing its binding to the FGF21 promoter.

3.4. ALA increases CREBH and FGF21 expression in the livers of both fasted and re-fed mice

Finally, the effect of ALA on hepatic CREBH and FGF21 expression was examined *in vivo*. As shown in Fig. 4A, CREBH and FGF21 mRNA levels were up-regulated by fasting, and intraperitoneal (IP) injection of ALA significantly increased their expression further. In addition, ALA treatment partially, but significantly, reversed the feeding-induced suppression of CREBH and FGF21.

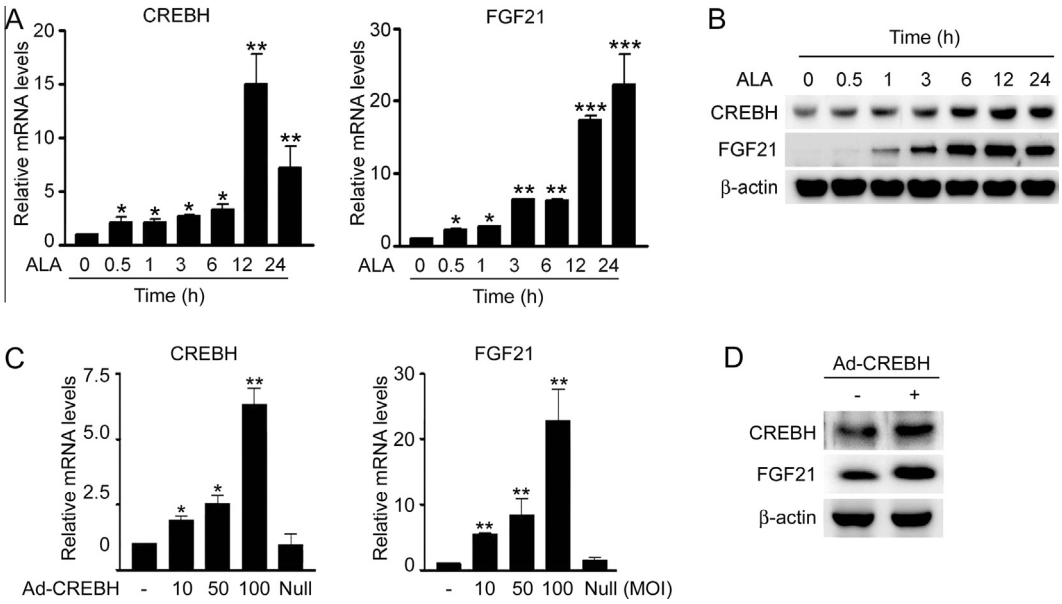

Fig. 1. Effect of alpha lipoic acid (ALA) on cyclic AMP response element-binding protein H (CREBH) and fibroblast growth factor 21 (FGF21) expression in cultured hepatocytes. (A) Representative real-time reverse transcriptase (RT)-PCR analysis of the effect of ALA on CREBH and FGF21 mRNA expression. AML-12 cells were incubated with ALA (1 mM) for the indicated times. Data represent the means ± SE of three independent measurements. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, vs. control. (B) Representative Western blot analysis of the effect of ALA on CREBH and FGF21 protein expression in AML-12 cells. (C) Representative real-time RT-PCR analysis of the effect of adenovirus-mediated overexpression of CREBH (Ad-CREBH) on FGF21 mRNA levels. Data represent the means ± SE of three independent measurements. $^{*}P < 0.01$; $^{**}P < 0.001$, vs. control. (D) Representative Western blot analysis of the effect of Ad-CREBH on FGF21 protein levels in AML-12 cells.

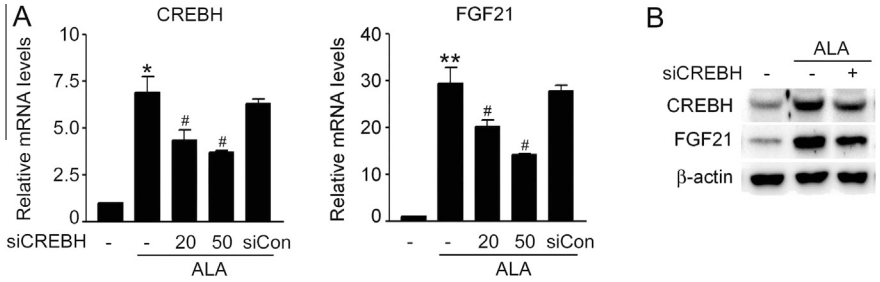

Fig. 2. Effect of siRNA-mediated knockdown of CREBH on ALA-induced FGF21 expression. (A) Representative real-time RT-PCR analysis of the effect of siRNA-mediated knockdown of CREBH on FGF21 mRNA expression in ALA-treated cells. AML-12 cells were infected with the indicated doses (nM) of siCREBH or siControl (50nM) for 24 h, and then treated with ALA (1 mM). Data represent the means ± SE of three independent measurements. $^{*}P < 0.01$; $^{*}P < 0.001$, vs. control; $^{\#}P < 0.05$ vs. ALA treatment alone. (B) Representative Western blot analysis of the effect of siRNA-mediated knockdown of CREBH on FGF21 mRNA expression in ALA-treated cells.

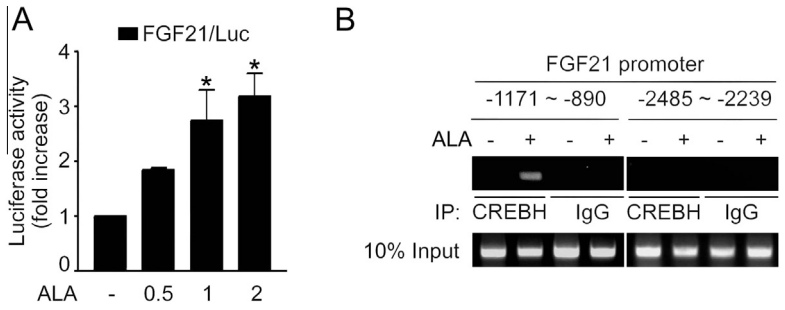

Fig. 3. Effect of ALA on FGF21 promoter activity and CREBH binding to the FGF21 promoter. (A) The effect of ALA on FGF21 promoter activity. AML-12 cells were transfected with an FGF21 promoter reporter construct (300 ng/well) and then incubated with the indicated dose (mM) of ALA for 24 h. Data represent the means ± SE of three independent measurements. $^{*}P < 0.05$ vs. ALA treatment alone. (B) Chromatin immunoprecipitation was performed using an anti-CREBH antibody. AML-12 cells were infected with Ad-CREBH or Ad-Null (MOI = 100) for 24 h.

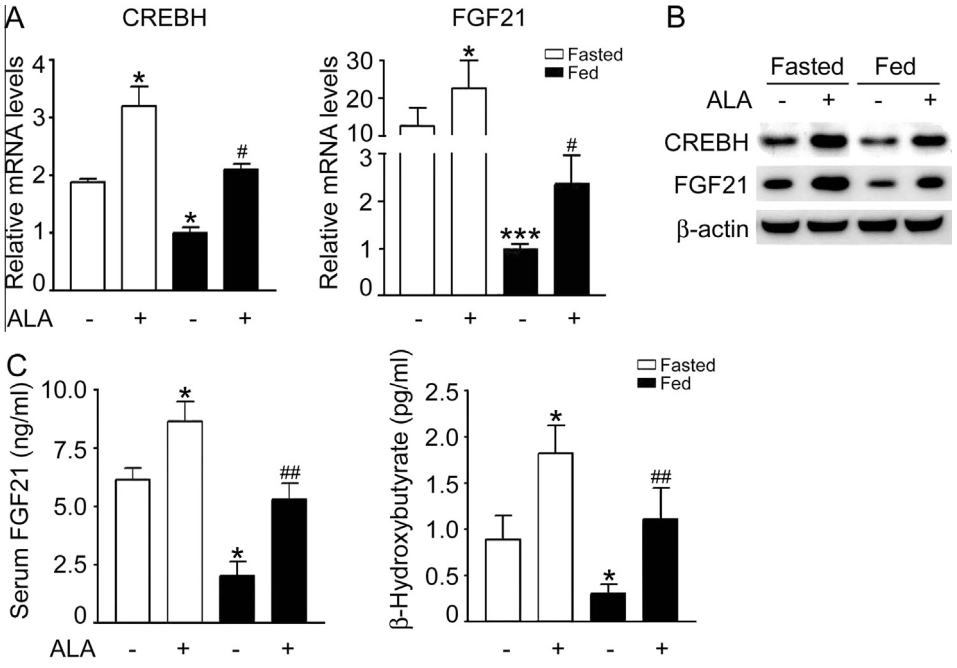

Fig. 4. Effect of ALA on hepatic CREBH and FGF21 expression in mice. (A) Representative real-time RT-PCR analysis and (B) representative Western blot analysis of the effect of ALA on CREBH and FGF21 expression *in vivo*. Male 8-week-old C57BL/6 mice received intraperitoneal injections of ALA (100 mg/kg) for 1 week and were then subjected to fasting and re-feeding. (C) Analysis of serum FGF21 and β-hydroxybutyrate levels in fasted/fed mice treated with ALA. Data represent the means ± SE of three independent measurements ($n = 4$ in each group). $^{*}P < 0.05$; $^{***}P < 0.001$, vs. fed mice without ALA. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$, vs. fasted mice without ALA.

In agreement with the mRNA expression data, the protein levels of CREBH and FGF12 were also up-regulated by ALA treatment both in fasted and fed mice (Fig. 4B). Moreover, ALA treatment increased serum levels of FGF21 and beta-hydroxybutyrate in both fasted and fed mice (Fig. 4C).

4. Discussion

It is well known that FGF21 plays a pivotal role in improving glucose and lipid metabolism [3,26,27]. Indeed, recent studies suggest that recombinant FGF21 or an FGF21 analogue could be a novel therapeutic strategy for the treatment of several components of metabolic syndrome, including diabetes, dyslipidemia, and obesity [28–30]. In this respect, it is natural that FGF21-based therapy for metabolic syndrome has become an active area of research interest. In addition to the administration of exogenous FGF21, it would be valuable to identify pharmacological agents that increase endogenous FGF21 production. Along these lines, several recent studies have reported that fenofibrate increases endogenous FGF21 levels through up-regulation of hepatic PPARα [5,31,32]. Fenofibrate is known to increase hepatic fat oxidation, decrease hepatic *de novo* lipogenesis, and improve insulin sensitivity [31,33]. These data suggest that the beneficial effects of fenofibrate on lipid metabolism are, at least in part, mediated by FGF21 induction [31,32]. Furthermore, a widely used anti-diabetic agent, metformin, which has beneficial effects on glucose and lipid metabolism induced FGF21 production through both AMPK-dependent [34] and independent [35] mechanisms. Here, we identify another positive pharmacological regulator of FGF21 production. In this study, we found that ALA increased FGF21 production through up-regulation of the fasting-induced transcription factor CREBH.

Although ALA is known primarily as an antioxidant, it has the potential to regulate energy homeostasis in both central and peripheral tissues [36,37]. ALA regulates many signaling pathways and modulates transcription and enzymatic activities related to energy expenditure, oxidative stress, and inflammation [38]. In rodents, ALA stimulates fat oxidation in the muscle and inhibits lipid synthesis in the liver through both AMPK-dependent and independent pathways [37,39]. Consistent with animal studies, a recent clinical study showed that ALA reduces body weight and improves multiple metabolic parameters [40–42]. The present study showed that ALA treatment of cultured hepatocytes enhances the expression of FGF21 and CREBH, which are also induced by fasting. Furthermore, knockdown of endogenous CREBH abolished ALA-induced FGF21 expression. Indeed, ALA treatment enhanced FGF21 and CREBH expression in fasted livers and partially blunted feeding-induced suppression of their expression. ALA-treated mice also showed significantly increased levels of circulating FGF21.

In conclusion, our data showed that ALA induced hepatic FGF21 expression via up-regulation of CREBH, suggesting ALA as a novel positive regulator of FGF21 production. In addition, these data suggest that the ability of ALA to improve metabolic parameters appears to be mediated, at least partially, by increased hepatic FGF21 expression.

Acknowledgments

This work was supported by grants from the National Research Foundation of Korea (2012R1A2A2A01043867 and 2012R1A2A1A03670452), which is funded by the Ministry of Science, ICT and Future Planning, a grant from the Korea Health Technology R&D Project of the Ministry of Health and Welfare of the Republic of Korea (A111345) and a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI14C1234)

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