α -Linolenic acid suppresses cholesterol and triacylglycerol biosynthesis pathway by suppressing SREBP-2, SREBP-1a and -1c expression

Satoshi Fukumitsu · Myra O. Villareal ·
Shoko Onaga · Kazuhiko Aida · Junkyu Han ·
Hiroko Isoda

Received: 13 June 2012 / Accepted: 15 October 2012 / Published online: 9 November 2012
© Springer Science+Business Media Dordrecht 2012

Abstract α -Linolenic acid (ALA), a major fatty acid in flaxseed oil, has multiple functionalities such as anti-cardiovascular and anti-hypertensive activities. In this study, we investigated the effects of ALA on lipid metabolism and studied the possible mechanisms of its action in differentiated 3T3-L1 adipocytes using DNA microarray analysis. From a total of 34,325 genes in the DNA chip, 87 genes were down-regulated and 185 genes were up-regulated at least twofold in differentiated 3T3-L1 adipocyte cells treated with 300 μ M ALA for a week, 5–12 days after induction of cell differentiation, compared to ALA-untreated 3T3-L1 adipocytes (control). From the Reactome analysis results, eight lipid metabolism-related genes involved in cholesterol and triacylglycerol biosynthesis pathway and lipid transport were significantly down-regulated by ALA treatment. Furthermore, ALA significantly decreased the mRNA expressions of *sterol regulatory element binding protein (SREBP)-2*, *SREBP-1a*, *SREBP-1c* and *fatty acid synthase (FAS)* in 3T3-L1 adipocyte cells. On the other hand,

the average levels of the gene expressions of *carnitine palmitoyltransferase 1a (CPT-1a)* and *leptin* in 300 μ M ALA treatment were increased by 1.7- and 2.9-fold, respectively, followed by an increase in the intracellular ATP content. These results show that ALA is likely to inhibit cholesterol and fatty acid biosynthesis pathway by suppressing the expression of transcriptional factor SREBPs. Furthermore, ALA promotes fatty acid oxidation in 3T3-L1 adipocytes, thereby increasing its health benefits.

Keywords α -Linolenic acid · n-3 Polyunsaturated fatty acid · Adipocyte · Cholesterol biosynthesis · Triacylglycerol biosynthesis

Abbreviations

ALA	α -Linolenic acid
CPT-1a	Carnitine palmitoyltransferase 1a
PUFAs	Polyunsaturated fatty acids
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
SREBP	Sterol regulatory element binding protein
FAS	Fatty acid synthase
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
SRE	Sterol regulatory element

Introduction

Flax (*Linum usitatissimum*) is one of the richest sources of ALA (18:3n-1) (Cunnane et al. 1993;

S. Fukumitsu · K. Aida
Central Laboratory, Nippon Flour Mills Co., Ltd.,
Midorigaoka, Atsugi, Kanagawa, Japan

S. Fukumitsu · J. Han · H. Isoda (✉)
Graduate School of Life and Environmental Sciences,
University of Tsukuba, Tsukuba, Ibaraki, Japan
e-mail: isoda.hiroko.ga@u.tsukuba.ac.jp

M. O. Villareal · S. Onaga · J. Han · H. Isoda
Alliance for Research on North Africa (ARENA),
University of Tsukuba, Tsukuba, Ibaraki, Japan

Romans et al. 1995). Flaxseeds, which are an ingredient in multigrain breads and topping for breads, bagels and muffins in Europe, Canada and the USA, contain 37 % of its mass as oil of which 50 % is ALA (Cunnane et al. 1993; Romans et al. 1995). Flaxseeds are consumed daily and have been the focus of interest in the field of food and nutrition elements due to their potential health benefits.

ALA, one of the n-3 polyunsaturated fatty acids (PUFAs) which are the essential fatty acids in the human diet, is also converted to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Burdge 2006). Research showed that fish-oil intake lowers triacylglycerol content (Annuzzi et al. 1991; Roche and Gibney 2000). The triacylglycerol-lowering effect of fish oil has also been attributed to the EPA and DHA that are found in fish. Previous reports have shown that ALA in blood is inversely related to the risk of coronary heart disease in men, and ALA ingestion improved blood pressure in humans and rats (Djousse et al. 2003; Takeuchi et al. 2007; Sekine et al. 2007). Furthermore, with food culture in Japan becoming more westernized, metabolic syndrome has been gradually increasing, and prevention is needed. ALA consumption may contribute to the prevention of metabolic disorders such as obesity, dyslipidemias and diabetes, and the daily consumption of flaxseed oil containing ALA is considered advantageous.

Metabolic syndrome has emerged as a worldwide health problem and is strongly associated with cholesterol and fatty acids biosynthesis (Alberti et al. 2005; Shimano 2001). Recent evidence suggests that both biosynthetic pathways can be controlled by a common family of transcription factors designated SREBP-1a, -1c and -2 (Shimano et al. 1996, 1997, 1999; Horton et al. 1998; Pai et al. 1998). Our previous report has shown that administration of flaxseed lignan to mice significantly reduced high-fat diet-induced visceral and liver fat accumulation, blood total cholesterol and triacylglycerol. Furthermore, flaxseed lignan also suppressed *SREBP-1c* mRNA level and its target gene, *FAS* mRNA (Fukumitsu et al. 2008).

One widely used model for studying the effects of dietary lipids and metabolic factors on adipocyte is the mouse 3T3-L1 adipocyte cell line, which was derived from disaggregated mouse embryos and selected based on the propensity of these cells to differentiate into adipocytes in culture (Green and Meuth 1974; Nishimura et al. 2004). Cell culture models of adipocytes

faithfully express many genes that are markers of adipocytes in vivo (Rosen et al. 1999). Furthermore, studies using cell culture models showed that cultured differentiated adipocytes remain in their differentiated state which is not observed in studies using animal models.

Except for ALA, the n-3 PUFAs have been well-studied for their effect on the regulation of gene expression specifically on the regulation of adipocytes differentiation and the regulation of *stearoyl-CoA desaturase 1* mRNA expression. And although there are already reports on ALA-rich flaxseed oil's effect on hepatic cholesterol metabolism in rat (Clarke et al. 2002; Madsen et al. 2005; Sessler et al. 1996; Vijaimohan et al. 2006), the molecular mechanism and genome-wide information using DNA microarray analysis of the effect of ALA on 3T3-L1 adipocytes has not yet been investigated.

The purpose of this study is to reveal the lipid metabolism-related effects of ALA, specifically on cholesterol and triacylglycerol biosynthesis and fatty acid oxidation, and to elucidate the molecular mechanisms of the inhibitory effects of ALA treatment on 3T3-L1 adipocyte cells based on DNA microarray and real-time PCR data.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Life Technologies Japan Ltd. (Tokyo, Japan). Penicillin–streptomycin was purchased from Sigma-Aldrich Co. (Tokyo, Japan). ALA was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cells and cell culture

Mouse 3T3-L1 preadipocytes were purchased from the Japan Health Sciences Foundation (Tokyo, Japan). 3T3-L1 preadipocytes were cultured and the differentiation into adipocytes was quantified using the Adipogenesis Assay Kit (Cayman Chemical Co., Ann Arbor, USA). Briefly, cells were cultured in DMEM containing 10 % FBS in the presence of 1 % penicillin–streptomycin mixture and incubated at 37 °C in a humidified atmosphere of 5 % CO₂. At

2 days post confluence (day 0), cells were induced to differentiate with adipocyte conversion cocktail containing 10 % FBS, 0.5 mM methylisobutylxanthine, 1 μ M dexamethasone and 1 μ g/ml insulin. At 3 days after the induction (day 3), cells were maintained in and refed every 2 days with medium containing 10 % FBS and 1 μ g/ml insulin. Related studies in published articles report a rate of differentiation of about 90 % at around 5 days following induction of differentiation, wherein cells display the characteristic lipid-filled adipocyte phenotype (Harmon and Harp 2001; Horton et al. 1998). In this study, we performed the methods referred to these publications and the same trend of about 90 % cell differentiation was observed (data not shown). 3T3-L1 adipocytes were treated with 300 μ M ALA from day 5 to day 12 after induction of differentiation. Control cells (undifferentiated and differentiated cells) were incubated without addition of ALA. Cell viability was spectrophotometrically determined using WST-1 reagent (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions (Nissen et al. 1997).

Microarray analyses

Sample preparation was carried out in accordance with the Affymetrix GeneChip 3' IVT Express Kit user's manual. Briefly, total RNA was reverse transcribed into double stranded cDNA, and biotin labeled aRNA was generated using the 3' IVT Express Labeling Kit (Affymetrix, Santa Clara, CA, USA). After fragmentation, biotin-labeled aRNA was hybridized to the Affymetrix mouse 430 PM Array strips (Affymetrix) containing probes for 45,123 mouse genes for 16 h at 45 °C in the Hybridization Station (Affymetrix). Hybridized arrays were washed and stained in GeneAtlas Fluidics Station and GeneAtlas Instrument Control Software using the Hybridization, Wash and Stain Kit (Affymetrix). The chips were then scanned using the Affymetrix GeneAtlas Imaging Station to obtain the mRNA expressions data of 34,324 genes from mouse genome. Microarray data were generated by using two independent samples from 3T3-L1 cells. Primary data analysis was carried out using the softwares Partek Express (Ryoka Systems Inc., Tokyo, Japan) which is a stand-alone software and Reactome which is a software of a knowledge base of biologic pathway and processes (Vastrik et al. 2007; Croft et al. 2011).

Quantitative real-time PCR

Total RNA was extracted using Isogen reagent (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions. RNA (1 μ g) was reverse transcribed using a PrimeScript RT reagent Kit (TaKaRa, Shiga, Japan), and amplified using SYBR Premix EX Taq (TaKaRa), according to the manufacturer's protocol in a Rotor-Gene 6000. Sequences of primers used for quantitative real-time PCR were as follows: *SREBP-1c*, 5'-atcgccgcggaagctgtcgggtagcgtc-3' and 5'-actgtctgtgtgtgatgagctggagcat-3'; *SREBP-1a*, 5'-tagtccgaagccgggtggcgccggcgccat-3' and 5'-gatgtcgttcaaaaccgtgtgtgtccagttc-3'; *SREBP-2*, 5'-cacaatatcattgaaagcgctaccgggtcc-3' and 5'-ttttctgatggccagcttcagcaccatg-3'; *FAS*, 5'-tgggttctagccagcagagt-3' and 5'-accaccagagaccgttatgc-3'; *CPT-1a*, 5'-ccaggctacagtgggacatt-3' and 5'-gaacttgcccatgtccttgc-3'; *leptin*, 5'-tgacacaaaaccctcatca-3' and 5'-tcattggctatctcagcac-3'; *18 s rRNA*, 5'-cgcggttctatttgttggt-3' and 5'-tcgtcttcgaaactccgact-3'. The relative amount of each transcript was normalized to the amount of *18 s rRNA* in the same cDNA sample. The thermal cycler (QIAGEN, Tokyo, Japan) conditions were as follows: 2 min at 50 °C and then 10 min at 95 °C, followed by two-step PCR for 40 cycles at 95 °C for 15 s followed by 60 °C for 1 min.

ATP assay

ATP was extracted from 3T3-L1 adipocytes and preadipocytes, which were treated with or without ALA, seeded in 96-well plates using the Kinshiro ATP extraction system kit (LL-100-2; Toyo Ink, Tokyo, Japan) and measured with the Kinshiro ATP luminescence kit (LL-100-1; Toyo Ink) according to the manufacturer's instructions. ATP levels were read by a Perkin-Elmer Wallac 1,420 luminometer (Wellesley, MA, USA). Protein concentration was determined by the method of Lowry using bovine serum albumin as a standard (Lowry et al. 1951).

Statistical analyses

Statistical analyses of the results of the cell viability assay, real-time PCR analysis and ATP assay were performed using SPSS for Windows, version 14.0 J (SPSS Japan Inc, Tokyo, Japan). Data are presented as mean $\pm$ SD values. The homogeneity of variances and means were determined using the Levene test and 1-way analysis of variance (ANOVA), respectively.

Differences between groups were examined for statistical significance using Tukey's HSD test, or the Games-Howell post hoc test (where variance was unequal). $P < 0.05$ was considered significant.

The scanning data of DNA microarray were analyzed by the software Partek Express (Ryoka Systems Inc., Tokyo, Japan) which estimates gene significance using Analysis of Variance (ANOVA).

Results and discussion

Cytotoxicity of ALA on 3T3-L1 adipocytes

The preliminary test of cell cytotoxicity (WST-1 assay) was performed to determine which ALA concentration to use. Then, we confirmed the concentration of 300 μM ALA showed the highest activity on 3T3-L1 adipogenesis without cell cytotoxicity (data not shown). From these findings, we decided to use 300 μM ALA for the DNA microarray analysis. As shown in Fig. 1, results of cell cytotoxicity showed that there were no statistically significant differences between the differentiated 3T3-L1 adipocytes treated with or without 300 μM ALA. In this study, we found that treatment with 300 μM ALA caused the lipid droplet size in 3T3-L1 adipocytes to become smaller compared with the control. Treatment with ALA, therefore, has a lipid size reduction effect but cannot cause the degradation or the formation of lipid droplets in adipocytes throughout the treatment period. Therefore, we believe that 300 μM ALA used in this study is the physiological concentration that can help maintaining healthy functions in lipid metabolism. Although flaxseed oil containing ALA cannot be used in a similar manner as a prescribed medicine to treat severe lifestyle-related diseases, regular consumption of 300 μM ALA (approximately 0.5 g/day as flaxseed oil) as food may help in improving the overall health of an individual.

DNA microarray analysis of the effect of ALA on the global gene expression in adipocytes

To elucidate the molecular mechanism controlled by ALA, we investigated the change in gene expression of the adipocytes treated with or without 300 μM ALA for 1 week, 5–12 days after induction of differentiation, using the DNA microarray analysis. The gene

Fig. 1 Cytotoxic effect of α -linolenic acid (ALA) on 3T3-L1 adipocytes as determined by WST-1 assay. The percent cell viability was calculated relative to the undifferentiated 3T3-L1 cells. $n = 3$ replicates from 2 independent experiments. Error bars represent the mean $\pm$ S.D. * $P < 0.05$ versus differentiated 3T3-L1 cells, ** $P < 0.01$ versus differentiated 3T3-L1 cells, Undiff, undifferentiated cells; Diff, differentiated cells

expression profile of the 300 μM ALA-treated and -untreated differentiated 3T3-L1 adipocyte (control) cells, obtained using microarray with 45,123 probe sets, show that 21,516 genes were up-regulated, while 23,611 genes were down-regulated. We then identified the genes with significant change in gene expressions (decreased or increased expressions by >2.0 -fold), and results showed that 87 transcripts were down-regulated and 185 up-regulated with a significant change (>2.0 -fold, $P < 0.05$), compared to the differentiated 3T3-L1 adipocytes (control) (data not shown).

DNA microarray analysis revealed a down-regulation in the gene expressions of metabolism-related genes after ALA treatment

Genes that were significantly expressed (>2.0 -fold) were subjected to the *overrepresentation analysis* using the Reactome software and the results identified the enriched function-related gene groups (Table 1). Table 1 shows a summary of the metabolism-related genes down-regulated (>2.0 -fold) by ALA treatment determined using DNA microarray analysis softwares Partek express and Reactome. Ten genes (*SC5D*, *TM7SF2*, *CYP51*, *HMGCS1*, *SQLE*, *ACSL3*, *ABCA1*, *ACSS2*, *ADH1* and *SULT1A1*), statistically overrepresented in the

metabolism pathway by following 300 μ M ALA treatment, were mapped to their relevant pathways in Table 1. The fold changes (down regulation over 2.0-fold) from the ratio of values of the 300 μ M ALA-treated 3T3-L1 adipocytes to the differentiated 3T3-L1 adipocytes (control) are also shown in table (Table 1). A previous report has shown that the saturated free fatty acid such as palmitic acid can induce insulin resistance and obesity in 3T3-L1 adipocytes (Van Epps-Fung et al. 1997; Yi et al. 2008). On the other hand, PUFAs, such as EPA and DHA, decreased the adipose tissue mass and suppressed the development of obesity in rodents (Madsen et al. 2005). In the present study, ALA affected and suppressed genes that are involved in the following pathways: cholesterol biosynthesis, triacylglycerol biosynthesis, lipid transport, phase I-functionalization of compounds and phase II conjugation (Table 1). *SC5D*, *TM7SF2*, *CYP51*, *HMGCS1* and *SQLE* genes play an active role in cholesterol biosynthesis, from the step wherein it is utilized to synthesize acetyl-CoA until cholesterol is produced (Sugawara et al. 2001; Bennati et al. 2006; Rozman and Waterman 1998; Ferno et al. 2006). The protein encoded by *ACSL3* gene activates fatty acids to synthesize acyl-CoA. This means that the suppression of the expression of *ACSL3* will significantly decrease the hepatic de novo fatty acid synthesis (Bu et al. 2009). Moreover, *ACSS2* which encodes for a cytosolic enzyme that catalyzes the conversion of acetic acid to acetyl-CoA was also down-regulated. *ACSS2* gene expression is regulated by transcription factors SREBPs that activate genes required for the synthesis of cholesterol and unsaturated fatty acids (Luong et al. 2000). Another gene that was down-regulated was *ABCA1* gene which encodes for a protein that serves as cholesterol transporter that is widely expressed throughout the body and the deficiency of which reduces lipid efflux to exogenous apoE (Hirsch-Reinshagen et al. 2004). In this study, it appears that the down-regulation of *ABCA1* by ALA treatment was caused by the reduction in the intracellular cholesterol level. This indicates that treatment with ALA in differentiated 3T3-L1 adipocytes caused a down-regulation of the gene expression of the genes involved in cholesterol and triacylglycerol biosynthesis pathways. In addition, *ADH1* and *SULT1A1* genes which are involved in xenobiotic metabolism (Gemma et al. 2006; Senggunprai et al. 2009) were down-regulated by ALA (Table 1). *ADH1* and *SULT1A1* genes expressions have been reported to be changed by alcohol and drug

administration, and the changes in the expression of these genes lead to detoxification (Senggunprai et al. 2009; Gemma et al. 2006). In particular, the protein encoded by *ADH* gene is able to metabolize and detoxify ethanol and a wide variety of substances, including other aliphatic alcohols, hydroxysteroids and lipid peroxidation products (Gemma et al. 2006). Furthermore, the protein encoded by *SULT1A1* gene catalyzes transfer of a sulfonyl group from 3'-phosphoadenosine 5'-phosphosulfate to an acceptor substrate compound containing either an hydroxyl or amino group (Senggunprai et al. 2009). These mechanisms are important to protect our biological bodies against various xenobiotics including environmental chemicals, therapeutic drugs and dietary compounds (Singh and Michael 2009; Senggunprai et al. 2009; Gemma et al. 2006).

Effect of ALA on the mRNA expressions of lipid metabolism-related factors

Results of the DNA microarray analyses clarified that ALA has a regulatory effect on cholesterol and fatty acid biosynthesis pathway. In this study, we focused on the lipid metabolism-related effects of ALA, specifically on cholesterol and triacylglycerol biosynthesis. To confirm these results, the expressions of the genes of the transcription factor SREBPs regulating the expressions of the lipid metabolism-related genes were investigated using real-time PCR.

SREBPs have been established as lipid synthetic transcription factors especially for cholesterol and fatty acid synthesis (Shimano 2001, 2002). There are three known SREBP isoforms designated as SREBP-1a, SREBP-1c and SREBP-2 (Horton et al. 1998). In vivo studies results suggest that SREBP-1 is more active for lipogenic genes while SREBP-2 is more specific to cholesterogenic genes (Shimano et al. 1996, 1997, 1999; Pai et al. 1998; Horton et al. 1998). In addition, SREBP-1c increases the expression of genes involved in the fatty acid synthetic pathway, such as *FAS* (Shimano 2001). Furthermore, it has been reported that *SC5D* gene is regulated by SREBPs because the promoter activity of *SC5D* was inhibited by oxysterol which mediates the regulation of SREBPs (Sugawara et al. 2001). It has been also reported that the binding of SREBP-2 to the sterol regulatory element (SRE) of *TM7SF2* produced approximately 26-fold promoter activation, whereas mutation of the SRE motif caused a dramatic decrease of

Table 1 Summary of the metabolism-related genes down-regulated (>twofold) by ALA treatment determined using DNA microarray

Function	Accession number	Symbol	Gene name	Fold change ^a 300 μ M ALA
Cholesterol biosynthesis	NM_172769	Sc5d	Sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (<i>S. cerevisiae</i>)	−2.63
	NM_028454	Tm7sf2	Transmembrane 7 superfamily member 2	−2.31
	NM_020010	Cyp51	Cytochrome P450, family 51	−2.14
	NM_145942	Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	−2.07
	NM_009270	Sqle	Squalene epoxidase	−2.03
Triacylglyceride biosynthesis	NM_001033606	Acs13	Acyl-CoA synthetase long-chain family member 3	−2.41
	NM_019811	Acss2	Acyl-CoA synthetase short-chain family member 2	−2.22
Lipid transport	NM_013454	Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	−3.21
Phase I—functionalization of compounds	NM_007409	Adh1	Alcohol dehydrogenase 1 (class I)	−2.27
Phase II conjugation	NM_133670	Sult1a1	Sulfotransferase family 1A, phenol-preferring, member 1	−2.25

^a Fold change data were calculated from the ratio of 300 μ M ALA-treated 3T3-L1 adipocyte to the untreated 3T3-L1 adipocyte

transactivation by SREBP-2 (Schiavoni et al. 2010). Researchers reported that *CYP51* and *SQLE* genes contained a potential copy of consensus SRE sequence which could bind to SREBPs, and *HMGCS1*, *ACSL3* and *ACSS2* genes are known as SREBP-controlled gene (Ferno et al. 2006; Luong et al. 2000; Nagai et al. 2002; Rozman 2000). As shown in Fig. 2a, real-time PCR results showed that the *SREBP-1a*, *SREBP-2*, *SREBP-1c* and *FAS* mRNA levels in the 300 μ M ALA-treated 3T3-L1 adipocytes were significantly decreased by 0.79-fold, 0.59-fold, 0.43-fold and 0.58-fold, respectively, compared to the differentiated 3T3-L1 adipocytes.

Additionally, *Cpt-1a* and *leptin* are lipid metabolism-related enzymes and fatty acid oxidation-related enzymes genes. In this study, the average mRNA expression levels of *CPT-1a* and *Leptin* in the 300 μ M ALA-treated adipocytes were increased by 1.7-fold and 2.9-fold, respectively, compared to differentiated 3T3-L1 adipocytes (Fig. 2b). *Cpt-1a* is responsible for early stage differentiation in adipocytes which is necessary for the fatty acid oxidation of long-chain fatty acids in mitochondria (Tsuruoka et al. 2005). A

Fig. 2 Effect of α -linolenic acid (ALA) on the mRNA expressions of lipid metabolism-related genes. Relative mRNA expression levels were quantified by real-time PCR with the values normalized against the 18 s rRNA expression ($n = 3$ replicates from 3 independent experiments). Error bars represent the mean $\pm$ S.D. ** $P < 0.01$ versus differentiated 3T3-L1 cells

Fig. 3 Effect of α -linolenic acid (ALA) on the intracellular ATP levels in 3T3-L1 cells. Starting at 5 days after the induction of 3T3-L1 cells differentiation, cells were treated with or without 300 μ M ALA for 1 week. ATP levels were measured using the Kinshiro ATP luminescence kit and protein concentration was determined by the method of Lowry. $n = 3$ replicates from 2 independent experiments. Error bars represent the mean $\pm$ S.D. $**P < 0.01$ versus differentiated 3T3-L1 cells

previous report has shown that C75, an α -methylene- γ -butyrolactone, interferes with the binding of malonyl-CoA to the active site of Fas enzyme, inhibits Fas and activates Cpt-1 α to alter ATP content (Ronnett et al. 2006) and Cpt-1 α induced leptin and decreased fatty acid synthesis (Wang et al. 1999). As shown in Fig. 3, the 300 μ M ALA-treated cells significantly increased the intracellular ATP content in 3T3-L1 adipocytes compared to the differentiated 3T3-L1 adipocytes.

Previous studies have shown that dietary intake of ALA is associated with a decreased risk of cardiovascular diseases in human and plasma cholesterol and triacylglycerol-lowering effects of ALA rich flaxseed oil in high-fat diet fed rat (Vijaimohan et al. 2006; Djousse et al. 2003). However, the effects of ALA on the signal transduction of cholesterol and triacylglycerol biosynthesis in adipocytes are not clearly understood. As shown in Fig. 4, these results show that the expression of *SREBPs* (*SREBP-1c*, *SREBP-1a*, *SREBP-2*) was significantly decreased by 300 μ M ALA treatment in differentiated 3T3-L1 adipocytes.

Fig. 4 Schematic representation of the cascade of reactions involving the cholesterol and triacylglycerol biosynthesis-related genes and fatty acid oxidation in 3T3-L1 adipocytes following α -linolenic acid (ALA) treatment. Black arrows indicate down-regulated expression ($>$ twofold) determined using DNA microarray analysis while gray arrows indicate the changes in lipid metabolism-related gene expressions determined using real-time PCR. G3P, glycerol-3-phosphate; TAG, Triacylglycerol

As a result, we considered that ALA suppresses the expression of genes related to the de novo cholesterol and triacylglycerol biosynthesis (*SC5D*, *TM7SF2*, *CYP51*, *HMGCS1*, *SQLE*, *ACSL3*, *ABCA1*, *ACSS2*) based on DNA microarray analysis. However, further studies to investigate the direct regulatory effect of SREBPs on lipid synthesis by using knockdown and knockout SREBPs gene expression will be needed. In addition, ALA enhanced the expression of genes related to the fatty acid oxidation (*CPT-1a*, *leptin*) in differentiated 3T3-L1 adipocytes based on real-time PCR analysis. Further experimental and clinical investigations will also be needed to ascertain the beneficial effects of ALA on health.

In conclusion, this study presents ALA, one of the n-3 PUFAs, as beneficial for the improvement of lipid-metabolism by specifically targeting cholesterol, triacylglycerol biosynthesis and fatty acid oxidation in differentiated 3T3-L1 adipocytes, the molecular mechanisms of which was elucidated using DNA microarray and real-time PCR analyses. These results will add another dimension to our understanding of the molecular mechanism of the effect of ALA on the differentiated 3T3-L1 adipocyte and will be helpful in understanding the potent application of ALA contained in flaxseed oil, as a food that can help preventing metabolic and cardiovascular diseases. This is the first report that clarifies the molecular mechanisms related to cholesterol and triacylglycerol biosynthesis pathway in 3T3-L1 adipocytes by ALA treatment.

Acknowledgments We are grateful to all the members of the Isoda Laboratory (University of Tsukuba, Ibaraki, Japan) and Central Laboratory (Nippon Flour Mills Co., Ltd., Kanagawa, Japan) for their scientific input and contributions.

References

- Alberti KG, Zimmet P, Shaw J (2005) The metabolic syndrome—a new worldwide definition. *Lancet* 366:1059–1062
- Annuzzi G, Rivellese A, Capaldo B, Di Marino L, Iovine C, Marotta G, Riccardi G (1991) A controlled study on the effects of n-3 fatty acids on lipid and glucose metabolism in non-insulin-dependent diabetic patients. *Atherosclerosis* 87:65–73
- Bennati AM, Castelli M, Della Fazio MA, Beccari T, Caruso D, Servillo G, Roberti R (2006) Sterol dependent regulation of human TM7SF2 gene expression: role of the encoded 3beta-hydroxysterol Delta14-reductase in human cholesterol biosynthesis. *Biochim Biophys Acta* 1761:677–685
- Bu SY, Mashek MT, Mashek DG (2009) Suppression of long chain acyl-CoA synthetase 3 decreases hepatic de novo fatty acid synthesis through decreased transcriptional activity. *J Biol Chem* 284:30474–30483
- Burdge GC (2006) Metabolism of alpha-linolenic acid in humans. *Prostaglandins Leukot Essent Fatty Acids* 75:161–168
- Clarke SD, Gasperikova D, Nelson C, Lapillonne A, Heird WC (2002) Fatty acid regulation of gene expression: a genomic explanation for the benefits of the mediterranean diet. *Ann N Y Acad Sci* 967:283–298
- Croft D, O’Kelly G, Wu G, Haw R, Gillespie M, Matthews L, Caudy M, Garapati P, Gopinath G, Jassal B, Jupe S, Kalatskaya I, Mahajan S, May B, Ndegwa N, Schmidt E, Shamovsky V, Yung C, Birney E, Hermjakob H, D’Eustachio P, Stein L (2011) Reactome: a database of reactions, pathways and biological processes. *Nucl Acids Res* 39 (Database issue):D691–D697
- Cunnane SC, Ganguli S, Menard C, Liede AC, Hamadeh MJ, Chen ZY, Wolever TM, Jenkins DJ (1993) High alpha-linolenic acid flaxseed (*Linum usitatissimum*): some nutritional properties in humans. *Br J Nutr* 69:443–453
- Djousse L, Hunt SC, Arnett DK, Province MA, Eckfeldt JH, Ellison RC (2003) Dietary linolenic acid is inversely associated with plasma triacylglycerol: the National Heart, Lung, and Blood Institute Family Heart Study. *Am J Clin Nutr* 78:1098–1102
- Ferno J, Skrede S, Vik-Mo AO, Havik B, Steen VM (2006) Drug-induced activation of SREBP-controlled lipogenic gene expression in CNS-related cell lines: marked differences between various antipsychotic drugs. *BMC Neurosci* 7:69
- Fukumitsu S, Aida K, Ueno N, Ozawa S, Takahashi Y, Kobori M (2008) Flaxseed lignan attenuates high-fat diet-induced fat accumulation and induces adiponectin expression in mice. *Br J Nutr* 100:669–676
- Gemma S, Vichi S, Testai E (2006) Individual susceptibility and alcohol effects: biochemical and genetic aspects. *Ann I Super Sanita* 42:8–16
- Green H, Meuth M (1974) An established pre-adipose cell line and its differentiation in culture. *Cell* 3:127–133
- Harmon AW, Harp JB (2001) Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis. *Am J Physiol Cell Physiol* 280:C807–C813
- Hirsch-Reinshagen V, Zhou S, Burgess BL, Bernier L, McIsaac SA, Chan JY, Tansley GH, Cohn JS, Hayden MR, Wellington CL (2004) Deficiency of ABCA1 impairs apolipoprotein E metabolism in brain. *J Biol Chem* 279:41197–41207
- Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, Shimano H (1998) Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *J Clin Invest* 101:2331–2339
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
- Luong A, Hannah VC, Brown MS, Goldstein JL (2000) Molecular characterization of human acetyl-CoA synthetase, an enzyme regulated by sterol regulatory element-binding proteins. *J Biol Chem* 275:26458–26466

- Madsen L, Petersen RK, Kristiansen K (2005) Regulation of adipocyte differentiation and function by polyunsaturated fatty acids. *Biochim Biophys Acta* 1740:266–286
- Nagai M, Sakakibara J, Nakamura Y, Gejyo F, Ono T (2002) SREBP-2 and NF-Y are involved in the transcriptional regulation of squalene epoxidase. *Biochem Biophys Res Commun* 295:74–80
- Nishimura K, Hatano Y, Setoyama T, Tsumagari H, Miyashita K, Lu S, Jisaka M, Nagaya T, Yokota K (2004) Control of life cycle of mouse adipogenic 3T3-L1 cells by dietary lipids and metabolic factors. *Appl Biochem Biotechnol* 118:97–114
- Nissen E, Pauli G, Vollenbroich D (1997) WST-1 assay—a simple colorimetric method for virus titration. *In Vitro Cell Dev Biol Anim* 33:28–29
- Pai JT, Guryev O, Brown MS, Goldstein JL (1998) Differential stimulation of cholesterol and unsaturated fatty acid biosynthesis in cells expressing individual nuclear sterol regulatory element-binding proteins. *J Biol Chem* 273:26138–26148
- Roche HM, Gibney MJ (2000) Effect of long-chain n-3 polyunsaturated fatty acids on fasting and postprandial triacylglycerol metabolism. *Am J Clin Nutr* 71:232S–237S
- Romans JR, Johnson RC, Wulf DM, Libal GW, Costello WJ (1995) Effects of ground flaxseed in swine diets on pig performance and on physical and sensory characteristics and omega-3 fatty acid content of pork: I. Dietary level of flaxseed. *J Anim Sci* 73:1982–1986
- Ronnett GV, Kleman AM, Kim EK, Landree LE, Tu Y (2006) Fatty acid metabolism, the central nervous system, and feeding. *Obes (Silver Spring)* 14:201S–207S
- Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, Milstone DS, Spiegelman BM, Mortensen RM (1999) PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol Cell* 4:611–617
- Rozman D (2000) Lanosterol 14alpha-demethylase (CYP51)—a cholesterol biosynthetic enzyme involved in production of meiosis activating sterols in oocytes and testis—a minireview. *Pflugers Arch* 439:R56–R57
- Rozman D, Waterman MR (1998) Lanosterol 14alpha-demethylase (CYP51) and spermatogenesis. *Drug Metab Dispos* 26:1199–1201
- Schiavoni G, Bennati AM, Castelli M, Fazio MA, Beccari T, Servillo G, Roberti R (2010) Activation of TM7SF2 promoter by SREBP-2 depends on a new sterol regulatory element, a GC-box, and an inverted CCAAT-box. *Biochim Biophys Acta* 1801:587–592. doi:10.1016/j.bbalip.2010.01.013
- Sekine S, Sasanuki S, Aoyama T, Takeuchi H (2007) Lowering systolic blood pressure and increases in vasodilator levels in SHR with oral alpha-linolenic acid administration. *J Oleo Sci* 56:341–345
- Senggunprai L, Yoshinari K, Yamazoe Y (2009) Inhibitory effects of kynurenic acid, a tryptophan metabolite, and its derivatives on cytosolic sulfotransferases. *Biochem J* 422:455–462
- Sessler AM, Kaur N, Palta JP, Ntambi JM (1996) Regulation of stearoyl-CoA desaturase 1 mRNA stability by polyunsaturated fatty acids in 3T3-L1 adipocytes. *J Biol Chem* 271:29854–29858
- Shimano H (2001) Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. *Prog Lipid Res* 40:439–452
- Shimano H (2002) Sterol regulatory element-binding protein family as global regulators of lipid synthetic genes in energy metabolism. *Vitam Horm* 65:167–194
- Shimano H, Horton JD, Hammer RE, Shimomura I, Brown MS, Goldstein JL (1996) Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *J Clin Invest* 98:1575–1584
- Shimano H, Horton JD, Shimomura I, Hammer RE, Brown MS, Goldstein JL (1997) Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells. *J Clin Invest* 99:846–854
- Shimano H, Yahagi N, Amemiya-Kudo M, Hasty AH, Osuga J, Tamura Y, Shionoiri F, Iizuka Y, Ohashi K, Harada K, Gotoda T, Ishibashi S, Yamada N (1999) Sterol regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes. *J Biol Chem* 274:35832–35839
- Singh MS, Michael M (2009) Role of xenobiotic metabolic enzymes in cancer epidemiology. *Methods Mol Biol* 472:243–264
- Sugawara T, Fujimoto Y, Ishibashi T (2001) Molecular cloning and structural analysis of human sterol C5 desaturase. *Biochim Biophys Acta* 1533:277–284
- Takeuchi H, Sakurai C, Noda R, Sekine S, Murano Y, Wanaka K, Kasai M, Watanabe S, Aoyama T, Kondo K (2007) Antihypertensive effect and safety of dietary alpha-linolenic acid in subjects with high-normal blood pressure and mild hypertension. *J Oleo Sci* 56:347–360
- Tsuruoka N, Kidokoro A, Matsumoto I, Abe K, Kiso Y (2005) Modulating effect of sesamin, a functional lignan in sesame seeds, on the transcription levels of lipid- and alcohol-metabolizing enzymes in rat liver: a DNA microarray study. *Biosci Biotechnol Biochem* 69:179–188
- Van Epps-Fung M, Williford J, Wells A, Hardy RW (1997) Fatty acid-induced insulin resistance in adipocytes. *Endocrinology* 138:4338–4345
- Vastrik I, D'Eustachio P, Schmidt E, Gopinath G, Croft D, de Bono B, Gillespie M, Jassal B, Lewis S, Matthews L, Wu G, Birney E, Stein L (2007) Reactome: a knowledge base of biologic pathways and processes. *Genome Biol* 8:R39
- Vijaimohan K, Jainu M, Sabitha KE, Subramaniam S, Anandhan C, Shyamala Devi CS (2006) Beneficial effects of alpha linolenic acid rich flaxseed oil on growth performance and hepatic cholesterol metabolism in high fat diet fed rats. *Life Sci* 79:448–454
- Wang MY, Lee Y, Unger RH (1999) Novel form of lipolysis induced by leptin. *J Biol Chem* 274:17541–17544
- Yi P, Lu FE, Xu LJ, Chen G, Dong H, Wang KF (2008) Berberine reverses free-fatty-acid-induced insulin resistance in 3T3-L1 adipocytes through targeting IKKbeta. *World J Gastroenterol* 14:876–883