Effects of 5-aminolevulinic acid on a murine model of diet-induced obesity

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The effects of 5-aminolevulinic acid (5-ALA) on obesity were investigated using a murine model (diet-induced obese mice). Diet-induced obese mice were divided into 4 groups: a control group (C group), which was fed a high-fat diet; a low-5-ALA dose (10 mg/kg/day) group (10A group); a moderate-5-ALA dose (30 mg/kg/day) group (30A group); and a high-5-ALA dose (100 mg/kg/day) group (100A group). 5-ALA was administered by mixing the high fat diet for 8 weeks. Body weight increases in the 30A and 100A groups were significantly smaller compared with those of the C group. Body fat measurements by X-ray computed tomography indicated that the 100A group showed a tendency toward low visceral fat quantities during the final week of the study. Visceral fat weights in the 30A and 100A groups were slightly low. The levels of serum alanine aminotransferase (ALT) and total cholesterol (TC) in the 10A group was slightly low, whereas the 30A and 100A groups showed significantly lower ALT and TC values. Liver lipid concentration showed a dose-dependent decrease with ALA. Thus, in this diet-induced obese murine model, administration of 5-ALA had a significantly beneficial impact on the visceral fat, serum ALT and TC, and liver lipid concentration.

Key Words: 5-aminolevulinic acid, diet-induced obese mouse model, visceral fat, lipid metabolism, high fat diet

The amino acid 5-aminolevulinic acid (5-ALA) is synthesized by both animals and plants, and is a precursor of porphyrin. Porphyrin is a component of hemoprotein in hemoglobin in animals, whereas it is a component of chlorophyll in plants.[1,2] In recent years, 5-ALA has attracted attention as a result of its application in the diagnosis of malignant tumors and in photodynamic therapy (PDT) on the basis of the selective accumulation of protoporphyrin IX (PpIX), a metabolite of 5-ALA, in tumor cells following *in vivo* administration of 5-ALA.[3–6] Furthermore, it is thought that 5-ALA is involved in cytochrome production, which is crucial for energy production in cells, and that it plays a central role in energy production in animals. It has recently been reported that administration of 5-ALA led to an increase in cytochrome c oxidase (COX) activity in murine liver.[7] Therefore, it is suggested that 5-ALA may be an effective treatment against various diseases and aging. Obesity is considered to be a lifestyle disease resulting from overeating and lack of exercise. However, a decrease in the function of the mitochondria, which is responsible for cellular energy production, is considered to be an additional underlying cause.[8] Thus, it was thought that 5-ALA, which is involved in cytochrome protein production and plays a central role in energy production, may be effective for improving or preventing obesity-related diseases. Animal models for obesity include: ob/ob mice, which have an abnormality in the leptin gene; db/db mice, which have an abnormality in the leptin receptor gene;

and diet-induced obese animals.[9–11] It is considered that dietary obese animals have a disease mechanism similar to that of human obesity where the obesity is induced by consumption of high fat and high calorie diets. This study investigated the effects of 5-ALA on diet-induced obesity by administering 5-ALA to a diet-induced obesity (DIO) model, the C57BL/6J-DIO mouse.

Materials and Methods

Experimental animals. Eleven-week-old male C57BL/6J-DIO mice, which were prepared by feeding a high-fat diet (D12492 feed, Research Diets, Inc., New Brunswick, NJ) to 4-week-old male JAXC57BL/6J mice for 7 weeks, were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan). After arrival, mice were acclimated to the laboratory environment by maintenance on the same high-fat diet for 2 weeks and were divided into 4 groups of 6 mice according to body weight. Mice were housed in an animal laboratory with free access to the test diets and water. Temperature and humidity were set at $21 \pm 2°C$ and $55 \pm 15\%$, respectively, with a 12 h light/12 h dark cycle (light period: 7:00–19:00). This study was conducted according to the guidelines for care and use of laboratory animals of Meiji Co., Ltd. (Kanagawa, Japan).

Test materials and administration. Test materials included 5-ALA phosphate and sodium ferrous citrate, which were provided by SBI Pharma Co., Ltd. (Tokyo, Japan). The structural formula of 5-ALA is shown in Fig. 1. Test materials of 0.012%, 0.035%, and 0.12% (w/w) concentrations were orally administered by mixing with high-fat diets. The quantities of 5-ALA administered corresponded to 10, 30, and 100 mg/kg/day 5-ALA phosphate, respectively. Sodium ferrous citrate was added to food mixtures to make the molar ratio of 5-ALA phosphate to sodium ferrous citrate of 1:0.5. The high-fat diet (D12492) comprised 20% protein, 20% carbohydrate, and 60% fat.

Methods. C57BL/6J-DIO mice were divided into 4 groups as follows: a control group (C group), which was fed a high-fat diet;
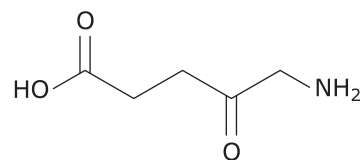

Fig. 1. The structure of 5-ALA.

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doi: 10.3164/jcbn.13-58
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J. Clin. Biochem. Nutr. | September 2015 | vol. 57 | no. 2 | 145–150

a low 5-ALA dose group (10A group); a moderate 5-ALA dose group (30A group); and a high 5-ALA dose group (100A group). The 10A, 30A, and 100A groups were fed *ad libitum* high-fat diets, containing 0.012%, 0.035%, and 0.12% (w/w) 5-ALA, respectively, for 8 weeks. The amount of 5-ALA administered to each group in the form of 5-ALA phosphate was equivalent to 10, 30, and 100 mg/kg/day. Body weights and food intake were measured twice weekly during the study. Body fat was measured in each mouse under isoflurane anesthesia using a LaThetaTM100® X-ray computed tomography (CT) system (Hitachi Aloka Medical, Ltd., Tokyo, Japan). Measurements were obtained before 5-ALA containing diet, and again at 25 and 49 days. After 8 weeks, blood samples were collected under anesthesia, and autopsies were conducted. Liver, pancreas, spleen, gastrocnemius muscle, mesentery, epididymis, and retroperitoneum adipose tissues were removed. Serum was separated from blood samples, and alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), triglyceride (TG), total protein (TP), and albumin (ALB) concentrations were measured using a biochemical auto analyzer (FUJI Dri-Chem 3500®; FujiFilm Co., Ltd., Kanagawa, Japan). TC and TG concentrations were also measured in the liver to determine lipid contents according to the method developed by Forch et al.[12] In brief, lipids were extracted from the liver using a solution containing chloroform and methanol at a 2:1 ratio and measured using triglyceride and cholesterol E-Tests from Wako (Wako Pure Chem. Ind., Ltd., Tokyo, Japan). Total RNA was isolated from frozen liver samples using an RNeasy kit (QIAGEN, Tokyo, Japan). First standard cDNAs were synthesized using a Prime Script® RT reagent kit (TAKARA BIO, Shiga, Japan), and real-time PCR was performed using gene-specific primers and SYBR green dye (SYBR® Premix Ex Taq™; TAKARA BIO) in a Thermal Cycler Real Time System (TAKARA BIO), according to the manufacturer's instructions. Primer pairs for UCP2 (uncoupling protein 2), LPL (lipo protein lipase), and FAS (fatty acid synthase) from TAKARA BIO were used to amplify corresponding transcripts. Relative mRNA expression was calculated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control. Table 1 shows the sequences of the gene specific PCR primers. Expression ratios from the C group were arbitrarily set at 1.

Statistical analysis. Means ± SD were calculated for each group. Multiple comparison tests were conducted using Stat Light statistical software, which was developed by Yukms Co., Ltd. (Tokyo, Japan). Parametric Dunnett's tests were performed when equal variance was identified by Bartlett's tests, and non-parametric Dunnett's tests were performed for data with unequal variances.

Results

Body weight and energy intake. Final body weights and weight gain during the study were low in the 30A and 100A groups, when dietary 5-ALA was administered as 5-ALA phosphate to DIO mice for 8 weeks. Furthermore, final body weights in the 30A group were significantly lower than those in the C group, and final body weights in the 100A group tended to be lower than in the C group ($p = 0.074$; Table 2). These data were reflected by lower weight gain in 30A and 100A groups compared with the C group (Table 2). However, energy intake did not differ between treatment groups (Table 2).

Body fat analysis using X-ray CT. Fig. 2 shows quantities of total, visceral, and subcutaneous fat measured using X-ray CT. No differences in fat quantities were observed between the C and 5-ALA-treated groups before the start of the study, and visceral and subcutaneous fat gradually increased from the start of the study in all groups. Weight changes in C and 10A groups were similar, whereas those in the 30A and 100A groups increased more slowly. After 49 days, quantities of total and visceral fat tended to be lower in the 100A group compared with the C group, with p values of 0.064 and 0.077, respectively.

Fat, organ, and muscle weights. Mesenteric, epididymal, and retroperitoneal fat weights were measured after maintenance on 5-ALA containing diet for 8 weeks. Visceral fat weights were determined as the sum of mesenteric, epididymal, and retroperitoneal fat weights (Fig. 3). Visceral fat weights were slightly but insignificantly lower in 30A and 100A groups than in the C group. The mesenteric, epididymal, and retroperitoneal fat weights in the 30A and 100A groups were also slightly lower than in the C group.

The weights of major organs (liver, pancreas, and spleen) and muscle (gastrocnemius muscle) were measured after maintenance

**Table 1.** Primers sets for real-time PCR analysis

	GenBank accession no.	Primer sequence (5' to 3')	Size (bp)
UCP2	NM_011671	F: GCAAGCATGTGTATGGCACAGTAA	110
		R: AAATGTGGGCCTTCGGTCAG	
LPL	NM_008509	F: CCCACAAGTGTAGTCGTCATTCAA	109
		R: GCAAGGGCTAACATTCCAGCA	
FAS	NM_007988	F: TGGAGACACCCTGGTCTGTGA	77
		R: TGGCAAATGCCACACGGTA	
GAPDH	NM_008084	F: TGTGTCCGTCGTGGATCTGA	150
		R: TTGCTGTTGAAGTCGCAGGAG	

F, forward; R, reverse; UCP2, uncoupling protein 2; LPL, lipoprotein lipase; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphatedehydrogenase.

Table 2. Body weights and energy intake in mice fed 5-ALA containing diets

	Group			
	C	10A	30A	100A
Body weight (g)				
Initial	33.1 ± 1.7	33.1 ± 1.9	33.2 ± 1.7	33.1 ± 1.8
Final	42.1 ± 2.9	41.7 ± 2.6	38.6 ± 2.2*	38.9 ± 1.4
Gain	9.0 ± 1.9	8.6 ± 1.3	5.4 ± 2.5*	5.7 ± 0.5**
Energy intake (kcal)	749.4 ± 61	736.0 ± 53	749.4 ± 73	720.4 ± 25

Values are mean ± SD, n = 5–6. *p<0.05, **p<0.01 vs C group.
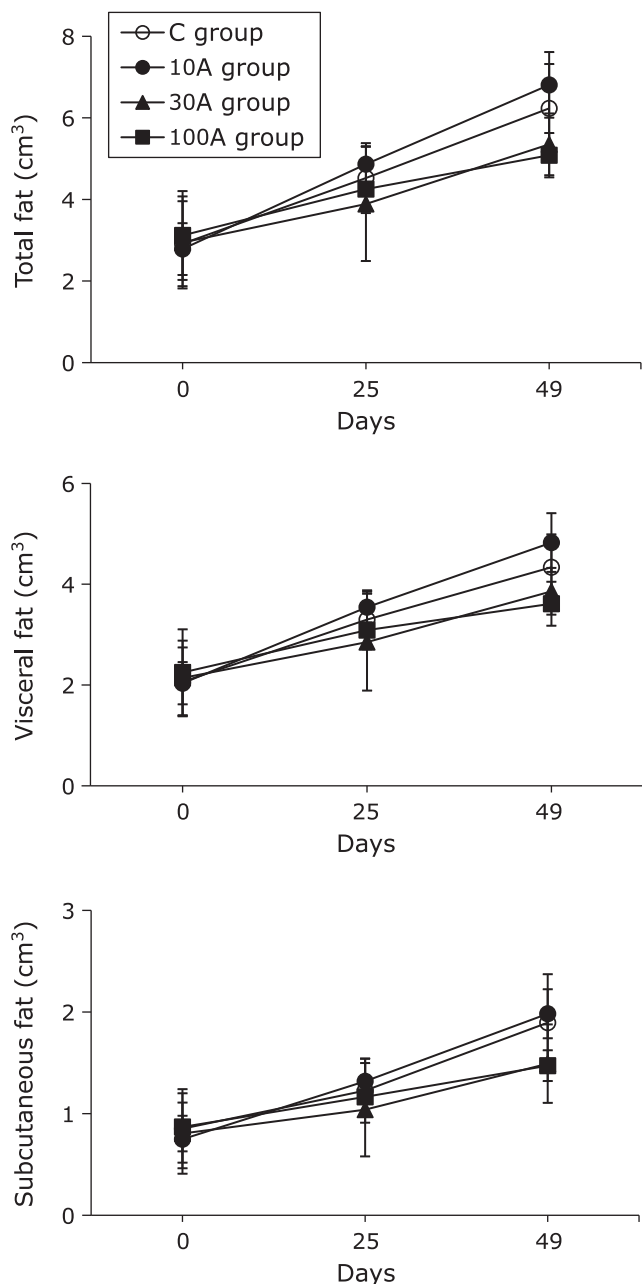
Fig. 2. Changes in relative quantities of total (the sum of the visceral and subcutaneous fat), visceral, and subcutaneous fat in 5-ALA-treated mice were measured using X-ray CT. Body fat was measured before and at 25 and 49 days of 5-ALA containing diet. Values are mean ± SD, n = 5–6.

on 5-ALA containing diet for 8 weeks (Table 3). No differences in major organ or gastrocnemius muscle were observed between 5-ALA-treatments and C group.

Biochemical examination of blood. Data from analyses of serum biochemical markers after maintenance on 5-ALA containing diet for 8 weeks are listed in Table 4. ALT levels were slightly lowered in the 10A group; they were significantly lower in the 30A and 100A groups compared with the C group. AST levels were also slightly but insignificantly decreased in the 5-ALA treatment groups. TC levels were lower in all three 5-ALA treatment groups compared with the C group, but were only significantly lower in the 30A group. No significant differences

in TG and ALB levels were observed among groups. However, TP levels were significantly lower in the 30A and 100A groups compared with the C group.

Liver lipid contents. Liver lipid, TC, and TG concentrations were determined and expressed as mg/g liver (Table 5). Liver lipid, TC, and TG concentrations tended to be reduced with increasing 5-ALA-treatments, and TC concentrations in the 30A group were significantly lower than in the C group.

Hepatic gene expression. To investigate the molecular mechanisms underlying the effects of 5-ALA on hepatic lipid metabolism *in vivo*, we examined the expression of lipid metabolism-related genes in the liver using real-time RT-PCR. As shown in Fig. 4, mRNA expression of the lipogenic enzyme FAS tended to be lower in all 5-ALA treatment groups than in the C group and was significantly lower in the 100A group. mRNA expression of the downstream target genes of fatty acid oxidation UCP2 and LPL tended to be higher in 5-ALA treatment groups than in the C group. However, these effects were insignificant.

Discussion

Obesity is major risk factor for diabetes and cardiovascular disease, and most cases of obesity are related to lifestyles factors such as overeating and sedentary behaviors. However, decreased mitochondrial energy metabolism is also believed to be an underlying cause.[8] The amino acid 5-ALA is universally present in animal and plant cells and is a precursor of the hemoprotein and chlorophyll component porphyrin.[1,2] Moreover, 5-ALA is involved in the production of heme cytochrome enzymes that play central roles in mitochondrial energy production. Therefore, we hypothesized that 5-ALA may decrease or prevent obesity by stimulating mitochondrial respiration. This is the first study to investigate the effects of 5-ALA in a murine model of DIO.

Several murine models are available for studies of obesity, including the ob/ob mouse that possesses a mutation in the leptin gene, the db/db mouse that possesses a mutation in the leptin receptor gene, and the DIO model.[9–11] High-fat diets increase energy intake in humans and rodents,[13] although some mouse strains, such as AKR/J, C57BL/6J, DBA/2J, and A/J, are more susceptible to obesity than others.[14] In particular, body weights of male C57BL/6J mice are readily affected by the diet,[13,15,16] whereas female C57BL/6J mice show resistance to the influence of diet on body weight.[17,18] In the present study, the effects of 5-ALA were investigated in a male murine model of DIO, which demonstrates similar pathogenesis to obese humans who consume high-fat or high-calorie diets. Previous reports show significantly increased body and fat weights in male C57BL/6 mice fed high-fat diet.[19] In addition, obesity was established in C57BL/6J-DIO mice by feeding them with a high-fat diet (60% fat) immediately after weaning, providing a suitable murine model of DIO.

No differences in total energy intake were observed between 5-ALA-treated and C groups when 5-ALA was mixed into the diets of C57BL/6J-DIO mice for 8 weeks. However, whereas weight gains and serum TP levels were significantly lower in the 30A and 100A groups than in the C group, serum ALB levels were unaffected, indicating insignificant nutritional effects of 5-ALA in the 30A and 100A groups. Body fat measurements using X-ray CT indicated that visceral fat quantities in the 100A group tended to be lower in the final week of the study and that fat quantities in the 30A and 100A groups were slightly reduced at the time of autopsy. As shown in Table 2 and Fig. 3, there was no statistically significant difference, but the body weight gain seemed to be dependent on fat accumulation. On the other hand, because a change in the muscle mass due to 5-ALA treatment was not noted, we hypothesized that the weight loss reflected the amount of fat. Therefore, it was assumed that the amount of weight gain and fat accumulation were reduced by 5-ALA treatment. These results suggest that body weight gain and fat accumulation might
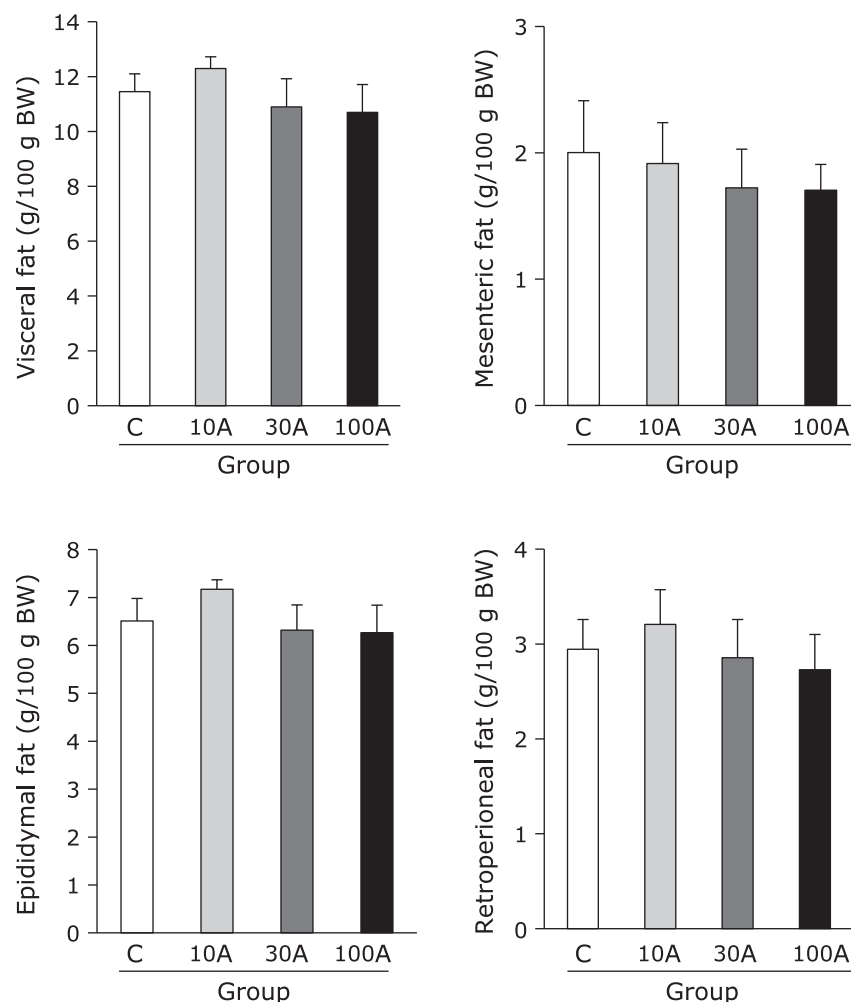
Fig. 3. Mesenteric, epididymal, retroperitoneal, and visceral fat (the sum of the mesenteric, epididymal, and retroperitoneal fat) weight were measured in mice that were maintained on 5-ALA containing diet for 8 weeks. Values are mean ± SD, n = 5–6.

Table 3. Organ and muscle weights in mice fed 5-ALA containing diet

	Group			
	C	10A	30A	100A
Organ weight				
Liver (g/100 g body weight)	3.48 ± 0.32	3.29 ± 0.21	3.36 ± 0.24	3.35 ± 0.12
Pancreas (g/100 g body weight)	0.80 ± 0.03	0.78 ± 0.05	0.83 ± 0.09	0.83 ± 0.09
Spleen (g/100 g body weight)	0.19 ± 0.02	0.18 ± 0.01	0.20 ± 0.03	0.19 ± 0.02
Muscle weight				
Muscle (g/100 g body weight)	0.79 ± 0.07	0.78 ± 0.04	0.82 ± 0.05	0.83 ± 0.06

Values are mean ± SD, n = 5–6.

**Table 4.** Biochemical analysis of serum in mice fed 5-ALA containing diet

	Group			
	C	10A	30A	100A
AST (U/L)	52.2 ± 21.7	42.5 ± 6.1	45.5 ± 8.2	47.2 ± 6.3
ALT (U/L)	31.7 ± 11.9	24.0 ± 3.8	18.0 ± 4.6*	17.3 ± 6.6**
Total-cholesterol (mg/dl)	211.5 ± 21.0	188.2 ± 33.6	173.7 ± 12.7*	181.2 ± 10.6
Triglyceride (mg/dl)	56.2 ± 21.5	43.8 ± 13.0	64.2 ± 20.5	49.2 ± 16.2
Total-protein (g/dl)	4.9 ± 0.2	4.9 ± 0.2	4.6 ± 0.2*	4.5 ± 0.2**
Albumin (g/dl)	2.4 ± 0.1	2.3 ± 0.2	2.4 ± 0.2	2.4 ± 0.2

Values are mean ± SD, n = 5–6. *$p<0.05$, **$p<0.01$ vs C group. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Table 5. Liver lipid concentrations in mice fed 5-ALA containing diet

	Group			
	C	10A	30A	100A
Lipid (mg/g liver)	107.0 ± 34.0	95.8 ± 14.3	78.2 ± 7.7	77.6 ± 6.3
Total-cholesterol (mg/g liver)	5.68 ± 2.54	4.53 ± 1.24	3.11 ± 0.33*	3.47 ± 0.55
Triglyceride (mg/g liver)	73.3 ± 34.0	62.2 ± 19.4	40.9 ± 7.4	42.2 ± 7.3

Values are mean $\pm$ SD, $n = 5$–6. *$p < 0.05$ vs C group.
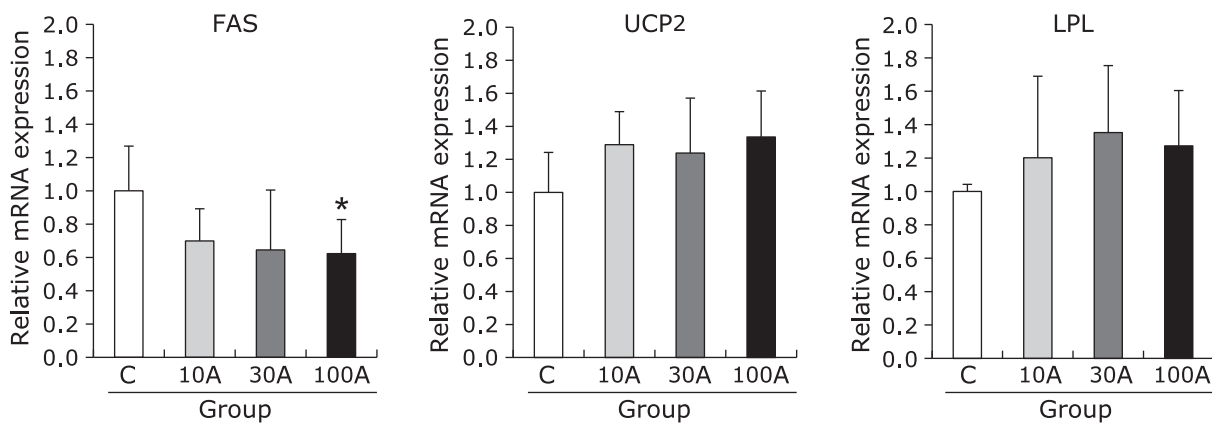
Fig. 4. Hepatic gene expression in mice after maintenance on 5-ALA containing diet for 8 weeks. Values are mean $\pm$ SD, $n = 5$–6; *$p < 0.05$ vs the C group. Expression ratios relative to the housekeeping gene GAPDH for the C group were arbitrarily set at 1. FAS, fatty acid synthase; UCP2, uncoupling protein 2; LPL, lipoprotein lipase.

be reduced by 5-ALA treatments in this murine model of DIO.

Blood analyses showed lower serum ALT and TC in 5-ALA-treated mice. Moreover, liver lipid concentrations were lower in the 5-ALA-treated groups than in the C group. Studies on gene analysis of the liver and COX[7] revealed that because 5-ALA is involved in the production of heme cytochrome enzyme by 5-ALA treatment, β oxidation of fatty acid in the liver increased and their metabolism improved. Therefore, it might be considered that there was some relation to the lipid metabolism in the liver. More detailed mechanism of TC level decrease by 5-ALA treatment is unclear, so further studies should be needed.

In this study, expression of UCP 2 and LPL, which are involved in fatty acid oxidation and are located downstream of liver fat metabolism genes, were insignificantly increased, and the lipid synthesis gene FAS was significantly suppressed by dietary 5-ALA. These data might suggest that 5-ALA possibly influence liver lipid metabolism. The association between expression of FAS and decrease in visceral fat will need further examination in future. In agreement, Ogura et al.[7] reported a 1.5-fold increase in mitochondrial COX activity and increased ATP in the liver following oral administration of 5-ALA to mice for 15 weeks. These data indicate that 5-ALA may induce aerobic energy metabolism by regulating COX activity, leading to suppression of visceral fat accumulation.

UCP enzymes are mitochondrial proton channels, comprising six transmembrane domains. UCP1, 2, and 3 are specifically expressed in brown fat tissue, white fat tissue, and skeletal muscle, respectively, and their putative roles in energy consumption and metabolism have led to their consideration as target molecules for anti-obesity drugs.[20] Shimamura et al.[21] reported that UCP1 mRNA and protein levels in brown fat tissue increased in a concentration-dependent manner in response to dietary consumption of 5-ALA for 14 days. Furthermore, oxygen consumption increased under the same experimental conditions, suggesting that 5-ALA induces UCP1 in brown fat tissue, and may lead to increased fatty acid

oxidation and energy consumption. Although gene expression analyses of fat tissues were not performed in this study, UCP expression in fat tissues will be an important focus of future studies.

Owing to use as a photosensitizing precursor during clinical photodynamic diagnoses and therapies, mechanisms of 5-ALA adsorption, distribution, and metabolism are well characterized.[22,23] Higashikawa et al.[24] recently showed that simultaneous consumption of 5-ALA and iron improved fasting and postprandial glucose levels in mildly hyperglycemic subjects without appreciable side effects. Thus, 5-ALA is an essential molecule in human and animals and may be associated with various metabolic disorders.

In contrast, Sadler et al.[25] assessed mitochondrial function in high-fat diet fed mice, showing decreased tricarboxylic acid (TCA) cycle function in muscles and increased expression of proteins involved in oxidative phosphorylation and lipid metabolism after 2 weeks. However, after 16 weeks, TCA cycle, β oxidation, and respiratory chain activities were the same or higher than in mice fed a regular diet. Thus, high-fat diets may not have direct effects on mitochondrial function. In addition, insulin resistance, which is considered to be the primary cause of decreased glucose tolerance among elderly people, is related to decreased mitochondrial activity, although this can be corrected with exercise.[26] Comparisons of sedentary and endurance-trained adults and elderly subjects showed that decreased mitochondrial fatty acid oxidation did not result from aging, but could be attributed to decreased activity levels.[27] Therefore, a detailed investigation of the effects of differing activity levels and 5-ALA on high fat diet-induced obesity in DIO mice will be of particular interest.

In conclusion, the present data indicate that 5-ALA administration for 8 weeks reduces visceral fat and produces beneficial hepatic effects in a murine model of DIO.

Conflict of Interest

M. Koganei, Y. Saitou, T. Yamaji and T. Takahashi are employees of Meiji Co., Ltd. K. Tsuchiya, F. Abe and T. Tanaka are employees of SBI Pharma Co., Ltd. I. Horinouchi and Y. Izumi are employees of Biomaterial in Tokyo Co., Ltd.

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